Chromosomal Instability and Aging

Basic Science and Clinical Implications

edited by
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To those who have helped me along the way: my parents, Kay and Toshi, my teachers, and my husband, Rusty.

And to Mr. William T. Comfort, Jr., and the John A. Hartford Foundation, whose kindness and interest in this project were a constant source of inspiration.

—FMH

To our many hardworking and imaginative trainees and young colleagues, especially FMH. They will serve as our "cultural germ lines" to carry forward an unbroken lineage of scientific progress.

—SMW and GMM

Foreword

Most of us avoid thinking about the established fact that we are mortal. We know that essentially all organisms, including humans, must ultimately succumb to death, but until we face that immediate reality, we choose to ignore it. Of course, there are the hallmarks of aging that we know so well: graying and loss of hair, wrinkling skin, stiff and painful joints, loss of short-term memory (but often an amazing retention of long-term memory!), cataracts, hearing loss, diabetes, slow wound healing, cardiovascular failure, appearance of tumors, etc.—the list goes on and on. Most if not all of these clinical manifestations are consequences, not causes of aging. Wrinkling skin is caused by many years of sunlight exposure, but wrinkles are not fatal. Nor does gray hair contribute to our ultimate demise.

Are these symptoms of aging programmed through some sort of molecular clock that is set as the embryo develops, or are they the inevitable consequence of the cumulative wear and tear on our genomes, as we face a plethora of environmental chemicals and radiation that damage our DNA? If DNA damage is responsible, then what is the contribution from the reactive oxygen species that are generated in our metabolizing cells and that also cause genomic damage? We don't yet have the definitive answers, but the full armamentarium of modern molecular biology has now been recruited to address these questions and others in laboratories throughout the world.

Much of the current excitement in the field of aging has been captured in this comprehensive volume, which features chapters prepared by scientists at the cutting edge of research on the relationships between genomic instability and aging. A thorough treatment is provided of the human hereditary syndromes that express phenomena of aging, including those that cause premature death. A number of chapters deal with the role of chromosomal telomere shortening as a contributor to aging. Cell senescence and its validity as a model for aging are critically evaluated. Important systems for studying aging are described with their special features that may or may not be relevant models for human aging, including yeast, roundworms, fruit flies, and rodents. Although the editors have cautioned that this volume is not intended to be encyclopedic, it clearly provides a valuable and stimulating reference for anyone wishing to learn about current research in this fascinating field. Furthermore, the chapters are generally quite accessible to the nonspecialist as the various model systems are introduced.

The most prominent human hereditary disease that exhibits a premature aging phenotype is Werner syndrome. It is provocative that the gene now known to be responsible for Werner syndrome is one of five human homologs of the *recQ* gene from the bacterium *Escherichia coli*. The *recQ* gene was originally discovered in a search for genes responsible for the loss in viability that accompanies thymine starvation in bacteria. Thus, seemingly esoteric revelations from the study of these single-cell organisms (which do not age!) may give us important clues to the mechanisms of aging in humans. It is curious that defects in only one of those five *recQ* homologs result in premature human aging, although deficiencies in at least three of them predispose to cancer. What could be the connection?

While cancer incidence is a conspicuous feature of the senescent phenotype in mammals, it is not really a stage in the normal aging process. It results in shortened life spans for many people, but it surely does not impact the maximum life span. However, we might ask whether some of the same phenomena that lead to genomic instability and eventual cancer could participate in other processes leading to the termination of life. In that sense the maximum life span might indeed be a consequence of accumulating genomic instability that eventually becomes incompatible with life.

There are two fundamental issues in relation to aging and the termination of life: (1) maximum life span and (2) health during the aging years. If it were our choice, how long should we be able to live? And would we wish that all humans on the planet should be able to live that long, or just you and me? And would we wish to live an "extra" 50 years, if we would likely be blind or otherwise incapacitated for the final 40 of those years? For many of us the practical question is how to ensure that our terminal years are more comfortable and rewarding in good health, rather than how to extend the human life span. Of

course, most of us would like to live to an age that approaches the maximum life span, whatever that is.

Finally, there does indeed appear to be a significant hereditary component to life span, so if you desire a long life then you should be very careful in choosing your parents.

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Preface

Understanding the biological basis of aging has fascinated people throughout recorded history, and is one of the great remaining scientific questions. The question has never been more important than now, as we anticipate the impact that a rapidly growing older population will have on the social, political, and medical landscape over the next 50 years. There is increasing evidence that aging involves damage to the genome, and it is certainly the case that such damage explains much of the coupling of most cancers to aging. This volume brings together expert reviews on issues related to the role of chromosomal instability in the modulation of life span and health span.

The primary aim of this book is to provide the scientific community with a current treatise on the cellular and molecular bases of aging and chromosomal instability in human diseases and model organisms. We intend this book for students, scientists, and physicians interested in the biology of aging and human genetics, and for those studying genomic instability in the fields of biochemistry, genetics, therapeutic radiology, oncology, and pathology. The realization that aging could be studied by using the methods of modern molecular biology and genetics has led to an explosion of knowledge in the field. Indeed, one of the difficulties of beginning a career in aging research has been how widely scattered the information is, with relevant publications appearing in numerous and diverse scientific journals. In this sense, the biology of aging is a "supraspecialty" encompassing many other fields, rather than a narrow subspecialty. This text will pro-

vide readers with a background for understanding a wide range of the most important work, and a context for future discoveries.

This book is not intended to be encyclopedic, nor the final word on the subjects presented. Progress in research on chromosomal instability and aging continues at a remarkable pace, and we apologize for the inevitable lapses between this publication and the most current literature. This book is well referenced through the beginning of 2002, and we are extremely grateful to our contributors and colleagues who made sure that the latest possible information was included within the time constraints of the publishing process. Fortunately, the Internet has emerged as a means of rapid dissemination of scientific information. One of us (GMM) has been crucial in launching a site devoted to aging research. At present, the site (http://sageke.sciencemag.org) is freely available.

We have assembled a group of leading investigators to contribute to this book. Their individual scientific contributions have been remarkable, and it is a pleasure to acknowledge our indebtedness to them. Their generosity in contributing to this enterprise has been inspiring. We thank Professor Phil Hanawalt for graciously agreeing to write the Foreword for this volume. We also wish to thank our colleagues who anonymously reviewed and commented on the chapters. Their collective expertise and individual thoughtful criticisms and suggestions are greatly appreciated. The responsibility for any errors or inaccuracies that remain, however, lies with us.

We received special assistance from many individuals throughout the writing and editing of this book. We owe a special debt of gratitude to Mr. William T. Comfort, Jr., whose generosity and timely support made this book indescribably easier to produce. We also gratefully acknowledge the support and encouragement of the John A. Hartford Foundation, especially by Mr. James F. O'Sullivan.

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Fuki M. Hisama
Sherman M. Weissman
George M. Martin

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1
Introduction

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I. INTRODUCTION

A useful approach to the introduction of any book about the biology of aging (after first clarifying the essential terminology) is to consider three traditional questions that have always driven biogerontological research. In keeping with that philosophy, we first shall give a brief summary of what can be referred to as *senescent phenotypes* (*What* is aging?). We will conclude that there is a remarkable range of senescent phenotypes that impact physiological functions at all levels of analysis and in all body systems. Next, we will consider what is surely the most fundamental of all gerontological questions (*Why* do we age?). The evolutionary biological theory of why aging occurs remains by far the most satisfying explanation, although there have been certain challenges to that idea. Finally, we will very briefly introduce the third—and by far the most difficult—question, one to which the modern tools of molecular biology and genetics have only recently begun to be successfully applied (*How* do we age?).

There is little doubt that genomic instability—the theme of this book—is a major pathway toward senescent phenotypes, particularly of the numerous neoplastic proliferations that emerge during the last half of the life spans of mammalian species. The mitochondrial genome and the nuclear genome are important targets of such instability. A recent surprise is the evidence that mitochondrial dysfunction also may participate in the pathogenesis of disorders of proliferative homeostasis, probably including atrophies as well as hyperplasias, as they are among the cell's generators of signals to implement apoptosis (1,2). The equations that ensure a healthy, steady state of cell numbers within our various tissues include factors for the determination of cell death as well as cell birth. Mitochondria are important generators of signals that lead cells to commit suicide; the alterations

of such signals by dysfunctional mitochondria may thus alter the balance of these equations.

These components of DNA damage are not likely to be the only pathways modulating life span and the rates of development of senescent phenotypes. Several other classes of gene action can contribute to the pathobiology of aging, including those that modulate posttranslational alterations to proteins, such as glycations (3).

II. DEFINITIONS

In this book, as in most of the literature that deals with the biology of aging, the term *aging* is used more or less synonymously with the term *senescing* or *senescence*. These terms are meant to encapsulate the slow, insidious, and progressive declines in structure and function of an organism after it has attained sexual maturity and the adult phenotype. As such, it is distinct from what happens in development. Gene action in development, however, is clearly of great significance for what happens in the later half of the life span. Let us consider the metaphor of a protein-synthesizing factory to describe a living organism. The life span of that factory depends on how well it is constructed and how well it is maintained after construction has been completed. The latter involves a variety of quality control mechanisms to maintain macromolecular integrity and proliferative homeostasis.

Not all alterations that occur in old organisms are deleterious. Some are compensatory—adaptive responses to specific types of declines in structure and function. An example is the Starling phenomenon—the increased end-diastolic filling to maintain cardiac output in many old people (4). Such compensation has been referred to as *sageing* (5). But these compensations eventually fail, allowing the full emergence of senescent phenotypes.

Rates of aging are typically measured by the speed at which the probability of organismal death increases as a function of postmaturational age. These are exponential functions and often are referred to as Gompertz curves, named after the 19th century actuary who first described this relationship. Recent studies of the life tables of very large numbers of fruit flies, medflies, roundworms, and people, however, have shown that those rates appear to slow in extremely aged individuals (6). The underlying mechanisms are not yet understood.

Genetic loci that play major roles in the modulation of life span and senescent phenotypes have been referred to as *gerontogenes* (7). This term is becoming well entrenched in the gerontological literature, but it is perhaps an unfortunate choice, as its literal interpretation is that these are genes whose primary functions are to lead directly to senescence. As we shall see in Sec. V. Why Do We Age?, such an interpretation is not consistent with evolutionary biological explanations of the nature of aging. The term *gerontogens* has been coined to refer to

putative *environmental* agents that have the potential to accelerate features of senescence (8). We have mutagens, carcinogens, and teratogens, so why not gerontogens? The best candidate for a "global gerontogen" (one that can essentially advance all features of senescence and thus shorten the life span) is gluttony! We shall learn more about the role of calories in modulating life span in Sec. VI. How Do We Age?

III. HERITABILITY AND CHANCE IN AGING

Like all phenotypes, the variable features that clinicians and pathologists observe in older human subjects, and that biologists observe in their aging experimental organisms, result from gene–environment and gene–gene interactions. Although some consider chance events as part of the contributions to the environmental variance, the role of poorly defined stochastic elements in the modulation of life span and the rates of development of senescent features deserves special emphasis (9). Gerontologists who work with genetically identical organisms raised under rigorously controlled environments regularly observe evidence of this in their life table determinations. A particularly compelling example is the determination of the distribution of survival for a cohort of genetically identical *Caenorhabditis elegans* worms grown in suspension cultures with a chemically defined medium (10). One must invoke stochastic events to explain the substantial variation in longevities observed in such experiments. For our own species, twin studies have given heritability estimates of only around 25% (11); this is a reflection of the importance of both environmental and stochastic elements in determining the variance of that phenotype and, by inference, in the rates of development of life-shortening senescent phenotypes. There is, however, a strong rationale behind our preoccupation with genetic approaches to our subject, a preoccupation reflected in this book. A genetic analysis has the potential, of course, to get at first principles. Strictly biochemical and physiological approaches typically reveal a plethora of alterations in the tissues of old organisms, many of which could turn out to be epiphenomena.

IV. WHAT IS AGING?

The phenotypic characterizations of aging in such important model organisms as *Drosophila melanogaster* and *C. elegans* are surprisingly superficial. Laboratory animal models of aging have been extremely valuable, but, with the outstanding exception of research by evolutionary biologists (who use genetically heterogeneous wild-type stock), represent highly inbred stocks developed in artificial laboratory environments. Thus, they may present a biased picture of the pathobiology of aging in any given species.

By far the most comprehensive picture of the results of the aging process is given by studies in our own species, mostly by physicians and pathologists. Although space does not permit a thorough review of those findings, let it suffice to say that physiological and pathological alterations can be found in each of the body systems and in all of the organs. At the molecular level, posttranslational alterations in long-lived proteins have been particularly well documented. Among these alterations, glycation is thought to be particularly important (3). Oxidative adducts have been found to accumulate in DNA, particularly in mitochondrial DNA (12). Lipids are also subject to peroxidative change, perhaps contributing to the accumulations of lipofuscin pigments around the nuclei of a number of cell types. Cross-sectional and longitudinal studies have documented declines in a variety of physiological functions, with the most interesting differences being among individuals revealed by longitudinal studies (13). A plethora of pathologies emerge during the latter decades of the human life span, including atrophies (often accompanied by interstitial fibrosis and fatty infiltrates of parenchymal tissues), hyperplasias, benign and malignant neoplasias (especially those of epithelial origins), several types of arteriosclerosis, osteoporosis, osteoarthritis, ocular cataracts, type 2 diabetes mellitus, loss of subcutaneous fat in the extremities, and hypogonadism. Almost half of all urban-dwelling East Bostonians over the age of 85 years have been found to suffer from probable dementia of the Alzheimer type (14). Other dementing disorders, such as frontal–temporal and Lewy body dementias, also begin to increase with age. Fortunately, a type of dementia due to multiple small strokes and known as *multi-infarct dementia* is much less common today, since age-related increases in blood pressure (common in developed societies) have been brought under control with antihypertensive medications. Some argue that the observed monotonic rate of loss of dopaminergic neurons is likely to result in Parkinson's disease in all of us, were we to live long enough to permit its phenotypic expression. Peripheral neuropathies also become rather prevalent in older people. There are declines in the ability to smell and to hear. Glaucoma or retinal degeneration may lead to blindness. But any child can recognize old from young people merely by observing the wrinkling of skin, the graying and the thinning of hair, and the slowing of movements. The last, incidentally, seems to be a universal feature of virtually all aging animals.

V. WHY DO WE AGE?

The evolution of life history strategies is driven by the ecologies in which species evolve. Consider, for example, a population of field mice facing its usual formidable array of predators plus an array of infectious agents, periods of drought and food deprivation, and the ever-present danger of a serious accident. How many such mice are likely to survive even one harsh winter, let alone two such winters? Very few indeed, if any, would survive for more than one year. Thus, al-

leles that only reach some phenotypic level of expression after one year are quite unlikely to contribute to the gene pool of the next generation. These could be good or bad alleles, it makes no difference. The vast bulk of the inherited alleles are those contributed by the much younger, actively reproducing members of the population. The result is a life history strategy that is selected for rapid development and early fecundity. There is no need for selection for elaborate and energetically expensive biochemical means to protect the somatic tissues during a period of years—that energy is best used for the production of progeny. Given the emergence of ecologies with much lower hazard functions, however, there is the potential for natural selection to result in a much different life history strategy—one characterized by slower development, fewer progeny over longer periods of time, and substantial increases in life span that likely would have required enhanced mechanisms for the protection of macromolecular integrity and the maintenance of physiological homeostasis for longer periods of time. These concepts have received strong support from laboratory experiments (15) and from investigations in the field (16). Like all scientific theories, however, this one should also be subject to challenge. I have mentioned above, for example, the peculiar observation that mortality rates actually decrease at very advanced ages for many organisms, including humans. This is not predicted by evolutionary theory, and current explanations are still controversial. There is also still some uncertainty concerning the extent to which grandparents may have contributed to the reproductive fitness of grandchildren among remote ancestors. A recent field study of baboons and prides of lions provides no such evidence of grandmaternal contributions to such fitness in these contemporary populations of those particular mammalian species (17).

One can conclude that the evolutionary theory of why we age is alive and well (18). There is as yet no compelling evidence that aging evolved because "it was good for the species to rid itself of older individuals." Aging, unlike development, does not result from sequential, determinative alterations in gene expression selected to *produce* aging. Instead, aging may be regarded as a *by-product* or as an *epiphenomenon* of gene action that was selected for an entirely different purpose—namely, to enhance reproductive fitness. Evolutionary theory teaches us, however, that life span is plastic. Given the chance, natural selection has shown that nature could do a much better job at keeping our DNA and proteins and proliferating cells in peak working condition for extended periods of time than it has, say, at prolonging the life span of the lowly field mouse. Perhaps we scientists can learn the secrets of these differences!

VI. HOW DO WE AGE?

Now comes the hard part, trying to unravel the underlying fundamental processes of aging. Here we are faced with a fundamental dilemma. Evolutionary theory in-

vokes the possibility of gene actions involving a variety of different genetic loci. One class of actions is known as *antagonistic pleiotropy*, by which alleles, selected for positive effects early in the life span, come to have negative effects late in the life span (19). There are potentially a large number of such loci and thus a large number of common polymorphisms that can influence life span. We might refer to these as leading to "public" modulations of aging. A second idea is that we all carry some number of constitutional mutations that have escaped the forces of natural selection. Although individually rare, there are a great variety of such possibilities. We might refer to these as leading to "private" modulations of aging (20). When viewed collectively, these various genetic modulations provide for a complex array of potential mechanisms of aging. The dilemma, however, results from the extremely well-documented observation in a number of species that the simple intervention of partial caloric restriction can lead to a substantial increase in maximum life span (21). Such a result is consistent with the view that there cannot be a very large number of independent mechanisms of aging. This is supported by the surprising observation that allelic variants leading to increased life span in such diverse organisms as *C. elegans* and *D. melanogaster* affect the same neuroendocrine-mediated pathway involving members of the insulin-like growth factor family of gene products (22). Moreover, these experiments may provide an explanation for the coupling of reproductive behavior with modulation of life span. However, downstream effector mechanisms in those pathways remain to be elucidated. They may address, in part, the free radical theory of aging, which invokes reactive oxygen species as the agents inducing macromolecular damage. The extent to which these results with nematodes and fruit flies, whose somatic cells (with the exception of those within the germ line) are all postreplicative, can be applied to the aging of mammals remains to be determined. As mentioned above, the loss of proliferative homeostasis, including the emergence of cancer, is a conspicuous component of the senescent phenotype in mammals and is not observed in nematodes and flies. Moreover, there can be no doubt that the theme of this book is highly germane to the pathogenesis of the striking coupling between cancer and aging.

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2

Overview of Chromosomal Instability and Aging Mechanisms

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I. INTRODUCTION

DNA encodes information essential for cellular maintenance and survival. Over a lifetime, DNA is subjected to continual attack by external sources of damage, as well as damage from endogenous sources or errors during DNA replication. The resulting genomic instability would be overwhelming except for the presence of DNA repair systems. Some of the consequences of unrepaired DNA injury include cell cycle arrest, programmed cell death, or apoptosis, genetic diseases in offspring, or the development of cancer. Indeed, one of the striking characteristics of aging in many organisms is an increased risk of cancer. Age has been called "the most potent of all carcinogens" (1). Cancer is now understood to be a genetic disease resulting from the accumulation of a series of mutational events; tumor progression results from clonal selection and evolution of tumor cells (2,3). The underlying genetic instability implicit in this process suggests a close link between the study of DNA damage and repair on the one hand and cancer on the other. In addition, several Mendelian chromosomal instability disorders are caused by mutations in genes involved in DNA metabolism and repair, and are accompanied by many, although not all, of the features of accelerated aging. These conditions are therefore called *segmental progeroid syndromes* (4).

For the purposes of this book, we define chromosomal instability as persistent, if not permanent, changes affecting DNA structure or chromatin structure. Here, we present an elementary overview of the types of DNA damage. We will

introduce the concept of the DNA repair network and the cellular response to genetic damage. We will describe additional types of chromosomal instability such as telomere shortening and mitochondrial DNA damage. We will describe the importance of human diseases and animal models in terms of their ability to illuminate the relationship between chromosomal instability and aging.

II. DNA DAMAGE AND REPAIR

The spontaneous mutation rate is low and differs among species. Among DNA microbes, the average spontaneous mutation rate per base pair varies approximately 100,000-fold from 10^{-10} to 10^{-5}. In the human body, containing approximately 10^{14} cells, the mutation rate has been estimated to be 2×10^{-7} per gene per cell division (5). The mutation rate at specific loci has been studied in vivo in humans. For example, one assay tests the frequency of T cells that can grow in the presence of 6-thioguanine. These cells therefore have mutations altering the expression of the hypoxanthine phosphoribosyltransferase gene (HPRT). The frequency of HPRT mutations ranges from 3×10^{-6} to 10×10^{-6} (6). In kidney epithelial cells, the HPRT mutant frequencies are 20-fold higher than those found in T cells, possibly reflecting tissue-specific differences in mutation accumulation, differences in mutant frequency in vivo compared with cultured cells, or selection against HPRT deficiency in T cells.

The sources of DNA damage are protean. Environmental agents may act as mutagens, thus increasing the likelihood of the occurrence of mutations. Known agents in this category include ultraviolet (UV) light (the major source being exposure to sunlight), ionizing radiation, cigarette smoke, and various carcinogens such as asbestos and possible dietary factors. Whereas exposure to environmental factors frequently can be reduced or minimized by behavioral modification, other sources of DNA alteration are unavoidable: errors during DNA replication (which occur with each cell division), damage from by-products of normal cellular metabolism (including reactive oxygen species—superoxide anions, hydroxyol radicals, and hydrogen peroxide—derived from oxidative respiration), and products of lipid peroxidation. Other weak mutagens, such as thermally promoted hydrolysis of nucleotide residues by water, may occur under physiological conditions. Deamination results in base substitutions, such as the substitution of hypoxanthine for adenine or thymine for 5-methylcytosine. Therefore, even in nondividing cell populations, DNA damage occurs. Organisms have evolved a variety of mechanisms to compensate for these otherwise high mutation rates. These repair pathways have been studied in detail in a variety of model systems, which space does not allow us to discuss here. A very brief overview of the major repair pathways is presented here as a basis for understanding the relevant diseases in subsequent chapters. A recent comprehensive list of 130 human DNA repair–

related genes in humans has been reported based on searches of the draft sequence of the human genome (7). The reader is also referred to an outstanding summary and thorough discussion of the topic of DNA repair and mutagenesis by Friedberg and colleagues (8).

A. Nucleotide Excision Repair

The function of nucleotide excision repair (NER) is to remove a wide variety of bulky adducts, such as pyrimidine dimers, typically caused by exogenous agents, such as UV light (9,10). The effect of these lesions is to distort the double helix and interfere with replication and transcription. A schematic representation of NER is presented in Figure 1 in Chapter 17. First, the DNA lesion is recognized by a protein complex. Second, unwinding of DNA around the site of damage is accomplished by genetic xeroderma pigmentosum complementation group D (XPD) and XPB helicase activities of the TFIIH transcription factor; the undamaged strand binds replication protein A. Third, endonucleases XPG, ERCC1, and XPF cleave the damaged strand, effectively removing a 24–32 base region containing the damaged DNA. Fourth, the empty gap is filled in by the usual DNA replication machinery, and the ends are sealed by DNA ligase.

B. Base Excision Repair

Base excision repair (BER) recognizes, excises, and replaces DNA bases damaged by endogenous oxidation, methylation, deamination, and hydrolytic processes (11,12). One of several related glycosylases, each specialized for a particular type of base modification, releases the damaged base from the sugar–phosphate backbone of the DNA molecule. This is followed by cleavage at the abasic site by APE1 endonuclease, with a minor contribution from APE2 endonuclease. DNApolβ fills in the one-nucleotide gap, followed by a sealing of the nick by XRCC1–ligase 3, although there are at least four adenosine triphosphate (ATP)–dependent DNA ligases.

In addition, single-strand DNA breaks also may be dealt with by enzymes from the BER pathway. Poly(ADP-ribose) polymerase enzymes, XRCC1, and polynucleotide kinase participate in binding the single-strand breaks and initiation of repair of a single-strand break.

C. Mismatch Repair

Mismatch repair (MMR) removes nucleotides that have been mispaired by DNA polymerases during DNA replication and small insertions/deletions resulting from errors during replication of repetitive sequences or during recombination (13–15). Homologs of the bacterial *mutS* and *mutL* genes encode proteins that identify sub-

strates for mismatch repair. Different proteins recognize specific types of mismatches; others are used during meiotic recombination. The targeting of the strand for excision may depend on recognition of contact of the newly synthesized strand based with the replication complex. Additionally, factors important in DNA replication that also function in excision of the mismatch and resynthesis process include polδ, polε, replication protein A, and others.

D. Double-Strand Breaks

Double-strand breaks (DSBs) result from exposure to ionizing radiation, x-rays, from enzymatic cleavage, or during replication of a single-strand break. DSBs are especially problematic because of the absence of a normal strand to serve as a template. Arrest of the cell cycle to facilitate repair of the DSB is mediated by p53. DNA damage-response proteins, including ataxia–telangiectasia mutated (ATM) and ataxia–telangiectasia related (ATR), are recruited. There are two logically alternate pathways for double-strand break repair, homologous recombination or nonhomologous end joining, which are reviewed elsewhere (16,17).

In homologous recombination, $5' \rightarrow 3'$ exonuclease activity results in a single-strand overhang that will facilitate a homology search. Replication A protein (RPA) along with RAD51-related proteins are assembled, and the homologous sequence on the second, intact chromosome, is identified. By a complex process of strand pairing, formation of a Holliday junction structure, branch migration, and resolution of the Holliday junction by resolvases, the DNA break is repaired. In the alternate pathway, the broken ends of the DNA are recognized by the Ku70/Ku80 complex together with DNA dependent–protein kinase, and the break is repaired by the action of XRCC4–ligase 4. In nonhomologous end joining, there is no template to guide the repair, and the result is less precise, with the loss or gain of a few nucleotides.

III. GENOME MAINTENANCE WITH AGING

What are the effects on the process of genome maintenance when taking into consideration the dimension of time? In humans (as reviewed in Chapter 7, an increase in aneuploidy and structural chromosomal aberrations occurs in peripheral blood lymphocytes from older subjects compared with younger subjects. The frequency of mutations in the hypoxanthine phosphoribosyltransferase (HPRT) gene, as measured by the frequency of T cells that can grow in the presence of 6-thioguanine, is significantly lower in newborns than in adults (18,19). In mice, an increasing level of chromosomal aberrations in many organs is seen with increasing age, and there are tissue-specific differences in the observed mutational rate (liver has a higher mutation rate than brain) and in the spectrum of mutations

(20,21). There is also evidence for an age-related decline in specific DNA repair mechanisms. In cultured skin fibroblasts and lymphoblastoid cell lines from normal donors ranging in age from infancy to 80 years, there is an age-associated decrease in the repair of ultraviolet (UV)–induced DNA damage and a concomitant decrease in DNA repair proteins (22,23).

IV. CELLULAR SENESCENCE

As an alternative to the death of a cell and its removal, organisms have developed a separate process for preventing cell growth. This process is termed cellular senescence, because it was first observed by passage of cells in culture, but it can be rapidly induced by other agents (as reviewed in Chapter 3 in which recent data on the signals that trigger senescence, the characteristics of the cellular senescent phenotype, and the implications for development of cancer and aging in mammals are discussed). The extent to which cell senescence occurs in vivo and its role in organismal aging is a topic of current debate and discussion.

V. TELOMERES

Telomeres consist of nucleoprotein complexes containing repetitive DNA sequences that cap the ends of linear chromosomes. The primary role of telomeres is to protect chromosomal ends from being recognized as a double-stranded DNA break and inappropriately activating DNA repair pathways. Most somatic tissues lack telomerase activity, and DNA polymerase is unable to replicate the extreme end of the chromosome; hence, the telomeres undergo progressive shortening with each cell division. Telomere length is maintained by a specialized reverse transcriptase termed *telomerase*. In approximately 90% of tumors, telomerase is reactivated during the process of cell immortalization. Thus, telomerase may be an important link between aging and cancer. There are several findings that suggest a link between telomere shortening and aging. First, normal somatic cells have a finite life span, and telomeres shorten as a function of age and in culture and perhaps in vivo (24). Therefore, it has been suggested that telomere shortening is a cellular "clock" that counts the number of cell divisions, and that signals growth arrest, or replicative senescence, in primary cells. To test whether telomere length signals cell senescence, several research groups have forced telomerase expression in primary cells and have tested the effects on telomere length and cell senescence. These studies have demonstrated that expression of telomerase can extend the replicative life span of primary cells beyond the normal limit.

Wright and Shay address the telomere hypothesis as an elegant way to unify the concepts of cancer development and replicative aging (Chapter 4). However,

telomere shortening cannot be considered a universal explanation for aging. An inverse relationship between telomere length and donor age has been found by some investigators (25), but others have not found a significant correlation between donor age and replicative life span, which presumably reflects telomere length (26). The telomeres of mouse and rat cells are several times longer than those of human cells, and Shay and Wright (27), among others, have suggested that human cells depend on counting cell divisions whereas rodent cells do not. This is because the longer life span of humans requires additional cellular mechanisms (i.e., replicative senescence) to guard against malignant transformation. The late-generation telomerase-deficient mice presented in Chapter 22 demonstrate some, but not all features typical of premature aging.

In Chapter 5, Stansel and Griffith describe the specialized telomere-associated proteins that contribute to telomere structure and the regulation of its length. They have also demonstrated isolation of a unique structure—telomeric duplex loops (t loops) in chromosomes from humans, mice, and *Oxytricha nova*—that gives new insights into telomere biology. Telomerase-deficient mice have been generated, and they enable direct study of the effects of the enzyme deficiency on viability, fertility, cellular proliferation, immune function, and tumor formation (Chapter 22). More recently, a new role for Ku has been discovered at the telomere in yeast and mammals. Ku is a nuclear protein complex that has been intensively studied because of its critical role in double-strand break repair and during V(D)J recombination. In Chapter 6, Gilley and Chen emphasize that the nuclear microenvironment is dynamic and may determine which function Ku serves in a particular context.

VI. EPIGENETIC MECHANISMS

Epigenetics is the study of changes in gene function that do not arise from a change in the DNA sequence. An epigenetic hypothesis was proposed several decades before any mechanisms had been elucidated to explain certain observed phenomena (28). An example of an observable epigenetic effect includes the regulation of X inactivation in mammals. Females have two X chromosomes; if both were active, they would have twice the amount of X-encoded proteins as normal males. To solve the problem of "dosage compensation" during female embryogenesis, one of the two X chromosomes is silenced per cell and is observed as the well-known Barr body. An RNA termed XIST is expressed from one X chromosome (either the maternal or paternal chromosome) per cell during early development, binds to it, and results in modification of its chromatin structure and subsequent inactivation.

Another example of an epigenetic effect is imprinting. Imprinted genes display parent-of-origin effects in the offspring. Typically, of the two parental copies of a gene, one copy is silenced in the progeny. In 1991, researchers identified the

first imprinted genes; since then, several dozen have been identified (29–31). Approximately half are active only when inherited from the mother (the paternal copy of the gene is silenced), and half are active only when inherited from the father (the maternal gene is silenced). How the paternal or maternal copy of the gene is tagged for selective silencing is influenced by several factors. The enzymatic addition of methyl (CH_3) groups to DNA is an important factor.

In eukaryotes, DNA is compacted by its association with histone and nonhistone proteins in a polymer called *chromatin*. DNA wraps around an octamer of core histone proteins, forming a beadlike structure called the nucleosome, the basic unit of chromatin. Chromatin structure plays a central role in regulating the transition between transcriptionally active and transcriptionally silent states. Additional layers of control include enzymes that add acetyl groups to histone (acetylases) or remove them (deacetylases), which lead, respectively, to opening or closing of the chromatin, thereby modifying the access of transcription factors and altering gene expression. Additional histone modifications, such as methylation and interactions with other proteins, appear to provide a "fine-tuning" mechanism to regulate biological functions, including cell cycle progression and chromosomal instability (32). In Chapter 8, Howard contemplates the instability in higher order chromatin that accompanies aging, its potential mechanisms, and its effects. It is becoming increasingly clear that an understanding of genes and the genome must include the role of epigenetics and the epigenome.

VII. MITOCHONDRIA, OXIDATIVE STRESS, AND CHROMOSOMAL INSTABILITY

Mitochondria are double-membrane subcellular organelles that have many functions, including oxidative phosphorylation resulting in the production of energy in the form of ATP, the metabolism of fatty acids through beta-oxidation, and import and export of metabolites for further cellular processing (33). Many mitochondrial processes are essential to life; however, these same processes produce deleterious by-products that contribute to cellular oxidative stress and aspects of aging. A relatively large percentage of cellular oxygen consumption takes place in a compartment—namely, mitochondria—comprising a small percentage of the total cell volume. Since oxidative damage is a major culprit for aging in higher organisms, many studies of aging have focused on mitochondrial function and oxidative damage to mitochondria.

A. Mitochondrial Genetics

Mitochondria contain their own DNA (mitochondrial DNA), which is separate from the DNA contained in the cell nucleus. Unlike nuclear DNA, which is in-

herited from both parents, mitochondrial DNA (mtDNA) is inherited exclusively from the mother's ovum. The mitochondrial genome (16,569 bp) is tiny compared with the nuclear genome (3×10^9 bp). The mitochondrial genome encodes 24 ribosomal RNAs, 22 transfer RNAs, and 13 genes coding for subunits of the respiratory chain. The remaining mitochondrial proteins are encoded by nuclear genes. These characteristics result in some unusual genetic and clinical features.

1. Heteroplasmy

Because there are hundreds of mitochondria per cell, when a mutation arises in mtDNA, that mitochondrion and its descendants contain the mutation, but they coexist with other, normal mitochondria—a situation termed *heteroplasmy*. When the cell divides, the mitochondria are partitioned between the daughter cells, which may receive varying percentages of normal and mutant mitochondria.

2. Threshold Expression

Different organs in the human body have different requirements for mitochondrial energy metabolism. It is generally agreed that the brain, heart, and skeletal muscle have the highest energy demands followed by the kidneys, endocrine system, and liver (34). The phenotypic expression of a pathological mtDNA mutation will be determined by the relative proportion of wild-type versus mutant mitochondria in a given tissue. The percentage of mutant mtDNAs needed to impair energy metabolism enough to cause organ dysfunction is called the *threshold effect* (35).

3. Mitochondrial Mutation Rate

The mutation rate in mitochondrial DNA is significantly higher than that observed in nuclear genes. The accumulation of sequence polymorphisms has been estimated to occur 17 times faster in mtDNA than in nuclear DNA (36,37). This has been attributed to the generation of free radicals from oxidative phosphorylation (which causes DNA damage) as well as to the paucity of DNA repair mechanisms in mitochondria as compared with the nucleus (38).

B. Mitochondria, Aging, and Human Disease

Age-related declines in activities of enzymes of oxidative phosphorylation, a critical mitochondrial process that produces energy in the form of adenosine triphosphate, have been shown in several tissues including brain and skeletal muscle. Mutations accumulate in mitochondrial DNA with age in somatic tissues (39). In one study, a common 5-kb deletion has been shown to increase 10,000-fold from young to old individuals, with regional differences between the basal ganglia, cerebellum, and cortex (40). Other studies have shown extensive rearrangements

of mitochondrial DNA in skeletal muscle from older compared with younger subjects as well as base substitutions (41,42).

A likely cause of somatic mtDNA mutations is damage from reactive oxygen species (ROS), by-products of normal oxidative metabolism. Superoxide is the major ROS produced by mitochondria. It is converted to hydrogen peroxide by the group of enzymes known as *superoxide dismutases*. Hydrogen peroxide in turn may be converted to another free radical, the hydroxyl radical, by the Fenton reaction and damage not only DNA but also proteins and lipids (43).

In 1988, Harding and colleagues (44) and Wallace et al. (45) reported the first mitochondrial mutations causing human disease. Since then, many other mitochondrial diseases caused by mitochondrial point mutations or multiple mitochondrial deletions have been identified (46). There are a number of typical presentations of mitochondrial disease in humans, including (1) Kearns–Sayre syndrome, characterized by ptosis, ophthalmoplegia, retinitis pigmentosa, hearing loss, cardiac conduction defects, short stature, and elevated cerebrospinal fluid protein; (2) mitochondrial encephalopathy with lactic acidosis and strokes (MELAS); (3) myoclonic epilepsy with ragged red fibers (MERRF); (4) Leber hereditary optic neuropathy (LHON) with sudden unilateral or bilateral painless central visual loss; and (5) Leigh syndrome, or subacute necrotizing encephalomyopathy. The pleiotropic manifestations of mitochondrial disease may include, for example, diabetes mellitus, hearing loss, bone marrow aplasia, and dystonia. The presence of a clinical mitochondrial disorder may be suggested by plasma and cerebrospinal fluid lactate, pyruvate, plasma amino acids, urine organic acids, carnitine, and other routine clinical studies such as magnetic resonance imaging of the brain. More specialized and specific testing to support the diagnosis of a mitochondrial disease includes the finding of ragged red fibers on Gomori trichrome staining of a muscle biopsy, biochemical assays of the activity of the mitochondrial oxidative phosphorylation enzymes in a muscle biopsy or a fibroblast sample, or by molecular analysis of mitochondrial DNA for deletions or point mutations.

The identification of nuclear-encoded mitochondrial genes is yielding new insights into the interaction of both genomes in mitochondrial function. Some of the nuclear genes shown to influence mitochondrial function include frataxin (47), adenine nucleotide transporter 1 (ANT1) (48), TWINKLE (49), and SURF1 (50). An area of active investigation and debate is the relationship of mitochondrial dysfunction to common, sporadic, age-related neurodegenerative diseases such as Alzheimer's disease (AD) and Parkinson's disease (PD). AD affects memory, judgment, and other higher cognitive functions. The neuropathological hallmarks of AD include neurofibrillary tangles and β amyloid containing plaques. Oxidative damage in AD has been reported (51), and reduced cytochrome oxidase (COX) activity has been described in AD brains (52,53). PD presents with bradykinesia, rigidity, and tremor in the sixth to eighth decades. Pathologically, there is

selective loss of dopaminergic neurons in the substania nigra pars compacta and Lewy bodies. In 1-methyl-4-phenyl-1,2,3,6 tetrahydropyridine (MPTP) model of Parkinsonism in humans and other primates, complex I of the mitochondrial respiratory chain is inhibited (54,55). Whether mitochondrial dysfunction causes or merely accompanies AD, PD, and other common age-related neurodegenerative diseases remains to be determined. The most definitive answers are likely to come from studies in a variety of animal models as discussed in Chapter 23.

VIII. DISEASES OF DNA REPAIR

Remarkable progress has been made in identifying genes causing hereditary forms of cancer and chromosomal instability disorders. Many of these genes have turned out to have a role in DNA repair. These findings have elucidated the relationship of DNA repair genes and their effect at the level of the cell, organs, and in the whole organism.

These diseases have in common one or more of the following features: hypersensitivity to DNA-damaging agents, an increase over basal mutation rates, increased risk of specific cancer types, segmental progeroid features, developmental abnormalities, growth deficiency, or neurodegeneration. A brief overview of this important group of diseases is summarized in Table 1 and is discussed in detail in chapters to follow.

IX. MODEL SYSTEMS

Although an understanding of human aging and development of new therapies to prevent or delay age-associated declines in function are important goals of research in aging, humans have a particular drawback as research subjects. The long human life span makes evaluating the effect of any intervention on life span a lengthy process at best. Results might take 20 years or more to be apparent. However, aging is a universal process. Other organisms demonstrate reproducible, age-related declines in performance, learning, and locomotion. Therefore, at least some of the fundamental mechanisms of aging should be observable in organisms such as yeast, fruit flies (*Drosophila melanogaster*), and the roundworm (*Caenorhabditis elegans*). These model organisms are relatively inexpensive to maintain, and they enable experiments with hundreds or thousands of subjects and many different genotypes to be performed in a short time. The ability to create and screen for mutations, along with a relatively short life span, make these organisms particularly powerful models for identifying mutations that prolong life span and pinpointing genes whose normal function is to limit life span.

Table 1 Human Progeroid and Chromosomal Instability Syndromes

Syndrome	Inheritance	Age of onset	Clinical features	Cancer predisposition	Affected gene	Cellular changes	Chapter no.
Werner syndrome	AR[1]	Teens, 20s	Prematurely graying hair, cataracts, arteriosclerosis, muscle atrophy, type 2 diabetes mellitus	Sarcomas	WRN (recQ helicase family member)	↓Replicative life span, ↑mutation rate, chromosomal rearrangements	9
Bloom syndrome	AR[1]	1–2 years	Sun-sensitive facial erythema, malignancy, type 2 diabetes mellitus	Leukemia, lymphoma, solid tumors	BLM (a recQ helicase family member)	↑Sister chromatid exchange	10
Rothmund-Thomson syndrome	AR[1]	Birth	Poikiloderma, telangiectasia, prematurely graying hair, cataracts, photosensitivity	Osteosarcoma, squamous cell carcinoma	RECQL4	Conflicting reports of sensitivity to UV or gamma ray irradiation, and chromosomal instability	11
Hutchinson-Gilford progeria	Sporadic (new autosomal dominant mutation)	1–2 years	↓Subcutaneous fat, alopecia, osteoporosis, atherosclerosis	No	Unknown	Altered expression profile of cell cycle control and chromosomal processing proteins	12

(continues)

Table 1 *Continued.*

Syndrome	Inheritance	Age of onset	Clinical features	Cancer predisposition	Affected gene	Cellular changes	Chapter no.
Ataxia-telangiectasia	AR[1]	1–3 years	Neurodegeneration, immunodeficiency, malignancy	Leukemia, lymphoma, sarcoma	ATM	↑Ionizing radiation sensitivity, defective cell cycle checkpoint arrest	13
Nijmegen breakage	AR[1]	Birth	Growth retardation, immunodeficiency	Lymphoma	NBS1	Chromosomal breakage ↑ ionizing radiation sensitivity, cell cycle checkpoint deficiency	14
Dyskeratosis congenita syndrome	Majority XLR[3], some AD[2], AR[1]	5–15 years	Reticulate skin pigmentation, tongue leukoplakia, bone marrow failure, prematurely graying hair, malignancy, immunodeficiency	Hodgkin lymphoma, squamous cell carcinoma of skin or mucosa	DKC1 (pseudouridine synthase)	Chromosomal aberrations, defective double-strand break response/ repair	15

Fanconi anemia	AR[1]	Birth–10 years	Developmental abnormalities, radial ray defects, endocrinopathies, malignancy	Leukemia, squamous cell carcinoma of female genital tract and oral cavity	FANCA, FANCC FANCD2 FANCE FANCF FANCG BRCA2	Chromosomal aberrations, DNA repair deficiencies	16
Cockayne syndrome	AR[1]	2–4 years	Growth deficiency, cachexia, progeroid appearance, hearing loss, cataracts, photosensitivity, neurodegeneration	No	CS-A, CS-B, some cases XPB, XPD, XPG	↑UV sensitivity	—
Xeroderma pigmentosum	AR[1]	1–2 years	Skin photosensitivity, early frecklelike lesions, keratitis, mental retardation, ataxia	Early-onset skin cancer basal cell, squamous cell, malignant melanoma	XPA, XPB, XPC, XPD, XPE, XPF, XPG, XPV	↑UV sensitivity defective nucleotide excision repair, or translesion synthesis	17
Down syndrome	Usually sporadic	Birth	Developmental delay, cardiac or gastrointestinal malformations, Alzheimer-type neuropathology, malignancy	Leukemia	Trisomy 21	Impaired cell-mediated immunity	18

[1]AR = autosomal recessive, [2]AD = autosomal dominant, [3]XLR = x-linked recessive

As Marciniak presents in Chapter 19, certain aspects of aging may be modeled in a simple unicellular eukaryote, baker's yeast (*Saccharomyces cerevisiae*), and discoveries of evolutionarily conserved pathways affecting life span in yeast have been confirmed in more complex organisms such as *C. elegans*. In some cases, as for the *Sir2* gene that silences gene expression, the mechanism by which it mediates life span extension appears to differ in yeast and worm. Some aspects of aging, however, are likely to be found only in multicellular organisms.

Hartman, Ishii, and Johnson review the findings from long-lived and short-lived genetic variants of the roundworm, *C. elegans* in Chapter 20. An emerging theme from the study of dozens of distinct mutations is the relationship of longevity to increased stress resistance and conversely shortened life span with decreased stress resistance.

Tower's Chapter 21 on aging, somatic maintenance, and genomic stability in *D. melanogaster* describes the research using a variety of methods, including genetic selection of populations, single-gene mutations, and overexpression of transgenes to determine effects on life span. He argues that with the exception of transcription coupled repair, most of the major pathways of DNA repair are represented in *Drosophila*, and that the potential role of the relevant genes in regulating aging and genomic instability is an exciting area of exploration.

One of the disadvantages of using invertebrate models systems to study aging, however, is that it is difficult to study age-associated pathology or to study organ-specific pathological or biochemical changes. In this respect, the mouse as a model system is intermediate between simpler organisms and humans. Mice live longer and are more expensive to maintain compared with yeast, worms, or flies. Genetic modifications are more difficult to achieve in mice than in invertebrates, but mice offer an advantage in that we have an extensive basic understanding of their physiology, behavior, and pathology, which frequently closely resembles the same situations in humans.

A number of mouse mutants have been reported, including the Ames dwarf and the Snell dwarf, which are now recognized to be homozygous for autosomal recessive mutations, resulting in deficiencies of growth hormone, prolactin, thyroid-stimulating hormone, small size (30%) of their normal littermates' weight, and a 50% increase in life span (reviewed in Ref. 56). Laron dwarf mice, deficient for the growth hormone receptor/binding protein (57) have a more restricted hormonal defect, and also are long-lived. Ablation of $p66^{shc}$ expression in mice results in increased oxidative stress and increased life span (58). Understanding how these different models may or may not relate to each other in the regulation of life span is not yet understood. In studies of aging, in general, studying animal models with short life spans is not as useful as studying long-lived strains, because a wide variety of pathologies unrelated to the aging process can result in premature death. However, certain carefully selected mutant mice with short life spans and a premature aging phenotype also may yield new and valuable insights into human

aging. The klotho mouse (named for the Greek goddess who spins the thread of life) expresses a syndrome that includes arteriosclerosis, skin atrophy, osteoporosis, and pulmonary emphysema (59). The gene shares similarity with beta-glucosidase enzymes, and further investigation has revealed the unusual metabolic profile of low glucose levels in blood, decreased insulin production, and increased insulin sensitivity (60). Another mouse model with increased activity of the tumor suppressor p53 demonstrates some features of premature aging, including osteoporosis, muscle atrophy, and diminished wound healing. It does not demonstrate brain atrophy or cataracts and has a marked reduction in the age-related development of tumors (61). A disruption in the balance between aging and cancer resistance may be at work (62). As discussed in some of the examples above, gene-targeting techniques have made it possible to knock out the function of a specific gene, and thus to study the effects of the missing gene on multiple organ systems and also to study aging in the context of the whole animal. Consequently, the use of knockout and transgenic mouse models has become a particularly powerful approach to study aging and will revolutionize our understanding of the pathophysiology of age-related diseases. Specific mouse models presented in multiple chapters in this volume are the telomerase knockout mouse, the Ku knockout mouse, the adenine nucleotide transporter 1 (ANT1) knockout mouse, and the superoxide 2 (SOD2) knockout mouse. Perhaps most exciting of all is the potential to test the efficacy of new therapies in these models. In Chapter 23, Hinerfeld and Melov report studies testing synthetic antioxidants in a variety of animal models of oxidative stress with promising results.

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3
Cellular Senescence

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I. INTRODUCTION

Cell division is essential for the survival of multicellular organisms that contain renewable tissues. However, cell division also puts organisms at risk for hyperproliferative diseases, the most dangerous of which is cancer. Genomes are continually damaged by environmental insults, oxidative metabolism, and, in dividing cells, errors in DNA replication and mitosis. Depending on the level and type of damage, cells may attempt repair, irreversibly withdraw from the cell cycle, or die. In the dividing cells of renewable tissues, the major risk from genomic damage is that of somatic mutation. If a somatic mutation confers a proliferative or survival advantage to cells, or causes the genome to become unstable (and thus hypermutable), the stage is set for neoplastic transformation and the development of cancer. Cancer is, of course, a major age-associated disease (1–3). Cancer cells almost invariably harbor chromosomal aberrations, which are very likely necessary for the evolution of increasingly malignant phenotypes (3–5) (Fig. 1).

Complex organisms have evolved at least two cellular mechanisms to suppress the proliferation (used here interchangeably with "growth") of cells at risk for neoplastic transformation: apoptosis (programmed cell death) and cellular senescence (or the senescence response). Apoptosis causes controlled nonlytic cell death in response to cellular damage or physiological signals. Cancer cells frequently acquire mutations that make them less sensitive or insensitive to apoptotic signals or their execution (6,7). The senescence response, by contrast, irreversibly arrests growth when cells experience potentially oncogenic events. In addition, senescent cells acquire changes in function. Cellular senescence is a major barrier that cells must overcome to progress to full-blown malignancy (8–11).

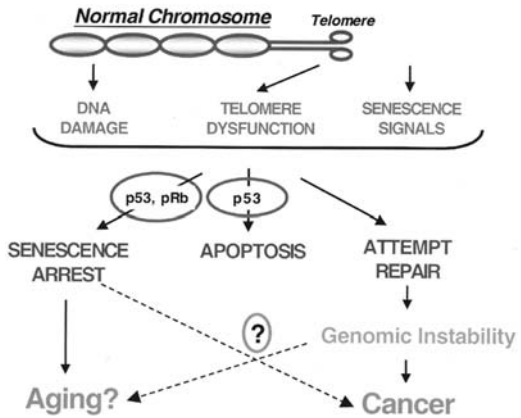

Figure 1 Cellular senescence can be induced by chromosomal damage, telomere dysfunction, or other events that put cells at risk for neoplastic transformation. In general, normal cells (with intact p53 and pRB pathways) respond to potentially oncogenic events by undergoing cellular senescence, which may contribute to certain aging phenotypes, including, possibly, late-life cancers. If only the p53 pathway is intact, DNA damage or telomere dysfunction generally promotes p53-mediated cell death. If neither the p53 nor the pRB pathway is intact, cells may survive with genomic rearrangements and instability, which can lead to cancer and possibly other aging phenotypes.

Both apoptosis and cellular senescence suppress tumorigenesis in mammals. However, whereas apoptosis kills and eliminates potential cancer cells, cellular senescence permanently arrests their growth.

Recent findings have shed new light on the causes of cellular senescence, the complexity of the senescent phenotype, and the potential consequences of cellular senescence for the development of cancer and aging phenotypes in mammalian organisms.

II. CAUSES OF CELLULAR SENESCENCE

Cellular senescence was first formally described as the process that limits the proliferation of human cells in culture (12,13). This process—referred to here as *replicative senescence*—is now known to be driven by the progressive shortening of telomeres (14–18). In recent years, it has become clear that cells arrest growth with a senescent phenotype in response to a variety of stimuli, most, if not all, of which have the potential to cause preneoplastic or neoplastic transformation (see Fig. 1). These stimuli include dysfunctional telomeres, DNA damage, disrupted chromatin structures, the expression of certain oncogenes, and supraphysiological

mitogenic signals (19–26). Several lines of evidence strongly support the idea that, at least in mammals, cellular senescence constitutes a fail-safe mechanism to prevent the growth of cells at risk for neoplastic transformation (1,8,10,11,27–29).

A. Telomeric Dysfunction

Telomeres are the DNA–protein structures at the ends of linear chromosomes that enable cells to distinguish a chromosome end from a double-strand break (DSB) in the genome. DNA DSBs are potentially catastrophic lesions. If not repaired, DSBs can lead to extensive DNA degradation. Unchecked chromosomal degradation inevitably results in loss of genetic information and cell death. Even if repaired, DSBs can lead to loss of heterozygosity (if repaired by homologous recombination) or chromosomal deletions, rearrangements, or translocations (if repaired by nonhomologous end joining). Thus, without the distinctive telomeric structure, chromosome ends are at risk for degradation, recombination, or random fusion by cellular DNA repair systems. Telomeric fusions are particularly dangerous because they frequently lead to chromosomal instability (30–34).

Owing to the biochemistry of DNA replication and the absence or insufficient activity of telomerase, mammalian telomeres shorten by 50–200 bp with each S phase of the cell cycle (14). Telomerase is the reverse transcriptase that can add telomeric sequences to DNA ends de novo (31,35). Robust expression of telomerase is generally restricted to the germ line and early embryonic cells, particularly in humans (36–38). Human somatic cells senesce when the average telomere length (or, more accurately, the average terminal restriction fragment length) reaches 4–7 kb (reduced from 15–20 kb in the germ line) (39). Ectopic expression of telomerase prevents telomeric shortening and replicative senescence in several types of human cells (e.g., fibroblasts, retinal epithelial cells, and endothelial cells) (18,40,41). However, telomerase does not prevent human fibroblasts from undergoing a senescence response to oncogenic RAS (42), nor does it prevent certain telomerase-positive tumor cells from undergoing a senescence response to DNA-damaging agents (43). Moreover, cells from laboratory mice have substantially longer telomeres than human cells, yet senesce after many fewer divisions, with much longer telomeres, and often despite expression of (endogenous) telomerase. It has been suggested that mouse cells senesce in culture because they acquire telomere-independent damage inflicted by the culture conditions (44–45), possibly the high oxygen in which most mammalian cells are cultured (46). Whatever the case, telomerase can prevent telomere-dependent (replicative) senescence but not telomere-independent (cellular) senescence.

Recent findings suggest that cellular senescence is triggered by loss of the telomeric structure rather than telomeric shortening per se (34,47). Telomeric structure, and hence telomeric function, can be disrupted when the telomeres become critically short or experience damage from endogenous or exogenous

sources. The telomeric structure also can be lost when there are changes in the expression, regulation, or structure of certain telomere-associated proteins. Mammalian telomeres are now known to end in a large loop, termed the *telomeric t loop*, the precise structure of which is not yet known (48). Telomeric t loop formation is critically dependent on the telomere binding protein TRF2 (48) and is likely facilitated by other telomere-associated proteins, such as TRF1 and TIN2 (48,49). Disruption of TRF2 function (by expressing a dominant negative TRF2 mutant) causes immortal human tumor cells to die and normal human cells to undergo senescence (50). Interestingly, normal human cells can proliferate with subsenescent telomere lengths, providing telomerase is ectopically expressed (51). Many telomerase-positive tumor cells also proliferate indefinitely with very short telomeres. These findings suggest that telomerase may act preferentially on the shortest telomeres. The mechanisms by which dysfunctional telomeres signal cells to undergo cellular senescence or cell death are poorly understood.

B. DNA Damage

Certain types and/or levels of damage to genomic DNA can cause normal mammalian cells to undergo a senescence arrest. This damage may derive from endogenous or exogenous sources and includes DNA base and sugar modifications as well as single- or double-strand breaks in the DNA (19,20,24). Because telomeric DNA appears to be repaired less efficiently than other parts of the genome (52,53), generalized genomic damage may cause telomeres to shorten (26,54) and/or malfunction. However, some types of damage—for example, oxidative damage caused by exposure to hydrogen peroxide—appear to induce the senescence response independent of an effect on telomeres (55). Moreover, deficiencies in any one of a number of components that participate in DNA repair can cause premature replicative senescence or a hypersensitive senescence response to DNA-damaging agents. Mammalian organisms (generally humans or mice) that are genetically deficient in DNA repair frequently are cancer prone and, in some cases, develop—prematurely—selected aging phenotypes (56–60).

The most striking example of the relationship between DNA damage and repair, cellular senescence, genomic instability, and aging is the Werner syndrome (WS). WS is caused by a deficiency in *WRN* (61), which encodes a DNA helicase (62) and exonuclease (63,64). WRN almost certainly participates in one or more major DNA repair pathway that is important for maintaining genomic stability (65–69). Cells derived from donors with WS undergo premature replicative senescence in culture (70), and donors with WS develop prematurely several phenotypes associated with aging, including cancer (57).

The senescence response to DNA damage may prevent the fixation of potentially oncogenic mutations in the genome. In addition, the senescence response prevents the proliferation of cells that harbor potentially cancer-causing mutations

and, in particular, the proliferation of cells with lesions that can lead to chromosomal instability.

C. Chromatin Perturbations

Agents that decondense or open chromatin structure, such as histone deacetylase inhibitors (21) or thymidine analogues (71), can cause cells to arrest growth with a senescent phenotype. The senescence arrest generally occurs within a few cell cycles, and is irreversible even after the agents are withdrawn.

Chromatin structure has long been thought to play a role in telomere-dependent and telomere-independent senescence (72,73). In the case of telomere-dependent senescence, the telomere is thought to condense the chromatin of neighboring genes, making them inaccessible to transcription factors. As telomeres shorten, their effect on nearby chromatin is thought to weaken, thus allowing previously silenced genes to be expressed. This phenomenon, known as the *telomere position effect*, is well documented in yeast and was recently shown to occur in human cells (74). In the case of telomere-independent senescence, the need to open heterochromatin during each S phase, or when DNA damage is repaired, is thought to make heterochromatin intrinsically unstable. Thus, the probability of heterochromatic domains being lost or becoming partially decondensed very likely increases with each cell division or instance of DNA repair. Again, this would permit the expression of previously silenced genes. Some heterochromatic domains may be more unstable that others. In either case, the expression of silenced genes is presumed to lead to the senescent phenotype. The identity of these genes is not yet known.

Heterochromatin also can suppress inappropriate recombination, and thus contribute to overall genomic stability. The best example of how heterochromatin, suppression of genomic instability, replicative senescence, and aging are interrelated is illustrated by the SIR proteins. SIR proteins mediate heterochromatin formation at silenced mating-type and other loci in the yeast *Saccharomyces cerevisiae* (75). The replicative life span of this single-celled organism is limited in large measure by the accumulation of extrachromosomal circles of rDNA (76). These circles are an indication of genomic instability, as they are produced by inappropriate recombination within the highly repetitive rDNA locus. Loss of SIR3/4 or SIR2 function shortens the life span of yeast, whereas overexpression of SIR2 extends yeast life span and suppresses the formation of extrachromosomal rDNA circles (77). Thus, heterochromatinization by SIR2 suppresses genomic instability (presumably by limiting the access of recombination proteins) and promotes the longevity of yeast cells. SIR2 is a NAD-dependent histone deacetylase (78), which raises the possibility that it modulates chromatin structure in response to the energy status of cells (79,80). Recently, overexpression of the *Caenorhabditis elegans* SIR2 ortholog was shown to extend the life span of this

multicellular nematode (81). Although is not yet clear whether *C. elegans* SIR2 mediates heterochromatin formation or suppresses genomic instability in nematodes, it is intriguing that SIR2 positively influences the life span of two evolutionarily distant organisms. Even more intriguing, mammalian SIR2 orthologs recently have been shown to deacetylate the p53 protein (82,83), an important tumor suppressor whose activity is essential for the senescence response of mammalian cells (11,84–87). Acetylation of p53 activates its activity as a transcription factor, and thus deacetylation by SIR2 suppresses p53 activity (82,83). It is not yet known whether overexpression of mammalian SIR2 has an effect on life span, but SIR2-overexpressing mammals might be cancer prone owing to reduced p53 activity.

D. Oncogenes/Mitogenic Signals

In recent years, it has become apparent that strong mitogenic stimuli also can induce normal mammalian cells to express a senescentlike phenotype. This phenomenon was first described for overexpression of the oncogenic (mutated) form of RAS-HA in rodent or human cells (22,88). Oncogenic RAS was known to transform immortal human and rodent cells and facilitate their conversion to tumorigenicity. However, in normal cells, overexpression of the RAS oncoprotein induced a senescence growth arrest. This senescence response was not induced if mutations or the expression of viral oncogenes inactivated the p53 and pRb tumor suppressor pathways. Consistent with this result, activated forms of two downstream effectors of RAS function—the RAF and MEK signaling protein kinases—also induce a senescence response when overexpressed in normal mammalian cells (23,25,89). In addition, overexpression of E2F1, a transcription factor important for regulating cell cycle progression into the S phase, elicits a senescent response in normal human fibroblasts (90). The E2F1-induced senescence response is also p53 dependent and is specifically mediated by the p14 tumor suppressor gene. Thus, when faced with certain supraphysiological mitogenic signals, which have the potential to cause neoplastic growth, normal cells permanently arrest growth with a senescent phenotype.

III. SENESCENT PHENOTYPE

The senescence response entails many changes in cellular phenotype, such that senescent cells are strikingly distinct from their nonsenescent or presenescent counterparts. The senescence-acquired traits constitute a profile of characteristics that collectively are termed the *senescent phenotype* (1,27). The fundamental features of the senescent phenotype are an irreversible arrest of cell proliferation and cell type–specific changes in differentiated cell functions. In addition, in some cell types, cellular senescence causes resistance to signals that induce apoptotic cell

death. Much more is known about the mechanisms responsible for the growth arrest than what might be responsible for the resistance to apoptosis or altered functions.

A. Growth Arrest

One of the defining characteristics of senescent cells is their stable and essentially irreversible arrest of cell proliferation. Senescent cells arrest growth with a G1 DNA content. Once this growth arrest occurs, physiological mitogens cannot stimulate senescent cells to enter the S phase of the cell cycle (27,28,91,92). This failure to initiate DNA replication in response to mitogens is not caused by a *general* breakdown in growth factor signal transduction, although selected signal transduction components are altered in senescent cells (93–96). Rather the immediate cause of the growth arrest appears to be the repression of a subset of growth-stimulatory genes and the upregulation of a subset of growth-inhibitory genes. The senescence-associated changes in growth regulatory gene expression have been most clearly established for cultured human fibroblasts, in which replicative senescence and the mitogen responsiveness of many genes have been extensively characterized.

In senescent human fibroblasts, many genes, including at least three protooncogenes (*JUN*, *MYC*, and *RAS-Ha*), remain mitogen inducible (97–99). However, at least three mitogen-inducible, growth-stimulatory genes become repressed in senescent fibroblasts (*FOS*, *E2F1*, and *ID*) (99–101). Repression of these genes very likely causes or contributes to the senescence growth arrest, because restoration of *FOS*, *E2F1*, and *ID* expression in varying combinations can at least partially restore the ability of senescent cells to initiate DNA synthesis (102–104).

In addition to the repression of growth-stimulatory genes, senescent cells overexpress at least two potent growth-inhibitory proteins. These proteins are the p16 and p21 CDKIs (inhibitors of cyclin-dependent protein kinases) (105–110). Both p16 and p21 are important regulators of the pRB and p53 tumor suppressor pathways, respectively, and both are critical for establishing and maintaining the senescence growth arrest (11,84,85,111). Moreover, ectopic overexpression of p16 or p21 causes human fibroblasts to arrest growth with a senescent phenotype (112,113). These findings suggest that overexpression of p21 and p16 may act upstream of the repression of *FOS*, *E2F*, and other genes, and can at least partly, if not completely, explain the senescence-associated growth arrest (84,85,114).

B. Resistance to Apoptosis

For several cell types, senescent cells are substantially more resistant than presenescent cells to dying in response to apoptotic stimuli. These cell types include human fibroblasts, T lymphocytes, and certain epithelial cells, such as keratinocytes

(115–117). Very little is known about the mechanisms that are responsible for senescent cells' ability to resist apoptotic death. However, the resistance to apoptosis may explain why senescent cells accumulate in some tissues in vivo (118–122). That senescent cells are resistant to apoptosis underscores the distinction between cell death and cell senescence (123).

C. Functional Changes

In addition to the arrest of cell proliferation and resistance to apoptosis, cellular senescence entails selected changes in differentiated cellular functions.

Some of the phenotypic changes that are characteristic of senescent cells are common to most, if not all, cell types. These changes include an enlarged cell size, an increase in lysosome biogenesis, and a decrease in the rates of protein synthesis and degradation (27,91,92,124). In addition, most senescent cells express a neutral beta-galactosidase, termed the *senescence-associated beta-galactosidase* (118). The function of this enzyme is unknown, but, because it can be detected by a simple histochemical reaction, it is a useful marker for the senescent phenotype.

Senescent cells also display changes in the expression or regulation of cell type–specific genes. For example, replicative senescence of human adrenocortical epithelial cells causes a selective loss in the ability to induce 17α-hydroxylase, a key enzyme in cortisol biosynthesis (125). Senescent human dermal fibroblasts, by contrast, show a marked increase in expression of collagenase and stromelysin (metalloproteinases that degrade extracellular matrix proteins) and reduced expression of TIMP1 and 3 (tissue inhibitors of matrix metalloproteinases) (126,127). On the other hand, when human endothelial cells undergo senescence in culture, there is a marked increase in the expression of interleukin-1α and the cell-specific adhesion molecule I-CAM (128,129). There is also a striking decrease in the expression of thymosin-β-10 (122). Interestingly, endothelial cells that simultaneously express the senescence-associated beta-galactosidase and lack expression of thymosin-β-10 (presumptive senescent endothelial cells) have been identified at the sites of atherosclerotic lesions in tissue samples from adult human donors (122). Very little is known about the molecular basis for the phenotypic changes that occur when cells undergo a senescence response. However, because many of the genes that are overexpressed by senescent cells encode secreted factors, it has been proposed that senescent cells can, in principle, disrupt local tissue integrity and thus contribute to age-related pathology (130–131).

IV. CONTROL OF CELLULAR SENESCENCE

Although little is known about how the senescence response is initiated once cells experience a potentially oncogenic event, it is clear that tumor suppressor

genes control the response directly or indirectly. The most critical of these senescence-regulating tumor suppressor genes are those that encode the p53 and pRB proteins (11,84,85,132). These proteins lie at the heart of two critical tumor suppressor pathways, each composed of several upstream regulators and downstream effectors.

The p53 protein is a transcriptional activator and repressor whose activity is regulated primarily by the balance between synthesis and degradation and by posttranslational modifications such as phosphorylation and acetylation. It controls the expression of numerous genes, many of which cause transient cell cycle arrest, cellular senescence, or apoptosis in response to DNA damage and other stimuli. One of these genes encodes the p21 CDKI that is highly expressed by senescent cells (105,133,134). p53 also interacts with, and regulates, a number of proteins that participate in DNA repair, including the WRN helicase/exonuclease (66,86,97,135–139). pRB also stimulates or represses transcription, but does so indirectly. It interacts with other transcription factors, such as E2F and SP1. It also recruits chromatin-remodeling proteins to the promoters of a variety of genes that control cell cycle progression and differentiation (140–143). Like p53, pRB is regulated primarily by posttranslational modification, principally phosphorylation by the cyclin-dependent protein kinases. Thus, the p16 CDKI that is highly expressed by senescent cells acts upstream of pRB by regulating its phosphorylation (109). The p53 and pRB pathways are not independent. Rather they interact at multiple levels (84–85,144–149).

The p53 and pRB pathways are essential for cells to establish, as well as maintain, the senescence growth arrest (11,15,27,28,84–86) (see Fig. 1). Thus, potential cancer cells must lose p53 and/or pRB function to overcome the proliferative barrier imposed by cellular senescence. Moreover, together, the p53 and pRB pathways are the most commonly lost functions in mammalian cancers (141,149–152). This loss can occur by mutation or epigenetic silencing of one or more key components of the p53 or pRB pathways.

V. CELLULAR SENESCENCE AND CANCER

Much of the evidence that links cellular senescence and tumor suppression derives from studies of cells in culture. Nonetheless, there is substantial supporting evidence from studies of intact organisms. Perhaps the best evidence derives from mice in which genes encoding p53 or INK4a proteins have been inactivated in the germ line. The INK4a locus encodes the p16 CDKI, as well as p14/ARF, a protein that is derived from an alternative reading frame and indirectly regulates p53 stability (145,146,153). Cells derived from animals that lack a functional INK4a locus fail to senesce in response to multiple stimuli. In all cases, the animals develop cancer at an early age (154). Similarly, p53 -/- mice are composed of cells that re-

sist or fail to respond to senescence signals, and these animals are highly cancer prone (155–157). By contrast, a genetic manipulation that causes mammary epithelial cells to undergo premature replicative senescence suppresses the development of breast cancer in young mice exposed to the mouse mammary tumor virus (158). Human cells are markedly more resistant to neoplastic transformation than mouse cells. Nonetheless, in humans and mice, mutations that disrupt the senescence response generally lead to increased cancer incidence. One example is cells from humans with the Li-Fraumeni syndrome, a hereditary cancer-prone syndrome caused by mutations in p53 (159,160). These cells immortalize at a frequency that is well above the vanishingly low immortalization frequency of normal fibroblasts (161). Thus, there is increasing evidence that cellular senescence constitutes an important defense against the development of cancer in mammals, including humans.

Hanahan and Weinberg have proposed that cells must acquire a defined number of traits for a malignant tumor to form: unregulated growth (hypersensitivity to positive signals, resistance to inhibitory signals), resistance to apoptosis, unlimited replicative potential, angiogenesis, invasion, and metastasis (9). Clearly, a large number of mutations are required for cells to acquire these traits. It is not surprising, then, that most cancers show signs of genomic instability, which accelerates the acquisition of oncogenic mutations (4,5,162) (see Fig. 1). The most important function of cellular senescence may be to prevent the growth of cells at risk for developing chromosomal instability. Perhaps the greatest risks to genomic stability are telomere dysfunction and direct DNA damage, particularly DSBs (3,163–165). Both, of course, are potent inducers of the senescence response (19,24,47,166–168).

VI. CELLULAR SENESCENCE AND AGING

As noted earlier, the senescence response entails not only an irreversible arrest of cell proliferation but also selected changes in cell-type–specific functions. Moreover, the senescent phenotype frequently entails the secretion of factors that can alter the integrity, function, and proliferative homeostasis of tissues (1,169). This senescence-associated secretory phenotype is particularly striking in fibroblasts. Fibroblasts are major components and regulators of the stroma, which provides the supportive and instructive foundation for most epithelial organs. Senescent fibroblasts secrete extracellular matrix components, matrix-degrading enzymes, inflammatory cytokines, and growth factors. Thus, cellular senescence, particularly factors secreted by senescent stromal cells, may contribute to the decline in tissue function and integrity that is a hallmark of aging (see Fig. 1).

At first glance, the idea that cellular senescence (which very likely evolved to protect mammalian organisms from cancer) might also contribute to aging may

seem paradoxical. However, it is consistent with the evolutionary hypothesis of antagonistic pleiotropy. This hypothesis predicts that the same traits that were selected to optimize fitness in young adult organisms can have unselected deleterious effects in aged organisms (170). The growth arrest associated with cellular senescence may be the selected trait, which suppresses tumorigenesis in young organisms. By contrast, the altered functions of senescent cells may be unselected traits that can have deleterious effects. Presumably, these deleterious effects are negligible in young tissues where senescent cells are rare. However, as organisms age, senescent cells accumulate (118–122). It is possible, then, that as senescent cells accumulate, their altered functions, particularly their secretory phenotype, compromise the physiology and integrity of tissues.

This idea can be extended to understand the relationship between aging and cancer. We and others have proposed that senescent cells also might contribute to the exponential rise in cancer that occurs with age in many mammalian species (3,130,171). The secretory phenotype of senescent cells can, in principle, disrupt the tissue microenvironment, which is crucial for suppressing the growth and progression of cells with oncogenic mutations (172–173). Thus, damage, telomeric dysfunction, or errors in mitogenic signaling may cause senescent cells to accumulate, but their influence may become significant and deleterious only later in life when they reach sufficient numbers. Simultaneously, it is well established that mutations, including potentially oncogenic mutations, accumulate with age (174–177). Thus, the probability that senescent cells and cells with oncogenic mutations occur in close proximity very likely also increases with age. When this occurs, senescent cells may create a microenvironment that promotes the proliferation and neoplastic progression of the mutant cells. Our recent results indicate that senescent human fibroblasts can indeed stimulate the growth and tumorigenic progression of preneoplastic, but not normal, epithelial cells (178). Moreover, much of this growth-promoting activity could be attributed to the secretory phenotype of the senescent fibroblasts. Thus, despite protecting from cancer in young adults, cellular senescence may facilitate the development of cancer in aged organisms (see Fig. 1).

A number of hereditary syndromes have now been identified that cause genomic instability, susceptibility to cancer, and, in many cases, the premature development of a subset of aging phenotypes in mammals. These include the Werner and Bloom syndromes, which are caused by mutations or disruptions in the genes encoding the WRN and BLM DNA helicases (81,179), respectively. They also include naturally occurring mutations in humans and genetically engineered mutations in mice that inactivate components of DNA repair pathways. Examples include the Cockayne syndrome in humans and germ line deletion of the corresponding genes (which encode the CS-A or CS-B proteins) in mice (59) (both of which compromise nucleotide excision repair) and germ line deletion of the gene encoding Ku86 in mice (which compromises repair of DSBs by nonho-

mologous end joining) (58). It is tempting to speculate that the DNA repair defects that result from these genetic changes have two effects. First, they may increase the incidence of genomically unstable cells owing to improper or incomplete repair, thereby increasing the incidence of cancer. Second, they may increase the incidence of senescent cells owing to the accumulation of DNA damage, which might increase cancer susceptibility and the development of aging phenotypes.

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4

Telomeric Shortening and Replicative Aging

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I. INTRODUCTION

Replicative aging was first described 40 years ago, when Hayflick (1) reported that normal human lung fibroblasts did not divide indefinitely like cancer cells but rather exhibited a limited ability to proliferate in culture. This paper contradicted the prevailing concept that all cells were immortal, and it was met with great skepticism at the time. Most of the criticism centered on the artificial nature of the in vitro culture environment and the likelihood that inadequate culture conditions were preventing the long-term proliferative capacity of the cells (see Refs. 2 and 3 for recent summaries of these views). As we shall discuss below, this criticism is valid in a variety of culture systems, so that we now must reevaluate some of the data that we had thought reflected replicative aging. However, the identification of telomeric shortening as the mitotic clock that counts cell divisions, the demonstration that telomeres shorten in vivo with donor age, the proof that telomerase can prevent telomeric shortening and immortalize cells, and the observation that most cancer cells have escaped these controls by upregulating/reactivating telomerase have all combined firmly to establish replicative aging as a legitimate in vivo biological process. This chapter will review the development of our current concepts of the mechanisms regulating replicative aging and discuss some of the outstanding issues facing the field.

II. M1/M2 MODEL

The two-stage model of cellular senescence, in which M1 (mortality stage 1) and M2 (mortality stage 2) represent independent mechanisms limiting the proliferative capacity of normal cells, was derived to explain the behavior of human cells transfected with an inducible simian virus 40 (SV40) large T antigen (4). Various viral oncoproteins (T antigen from SV40, E6/E7 from the high-risk strains of human papilloma viruses, and E1A/E1B from adenovirus type 5) can extend the cultured life span of normal human fibroblasts significantly, but do not directly immortalize the cells. Rather than entering a period of prolonged quiescence, as do normal cells at the limit of their proliferative capacity, cells expressing such proteins enter crisis. An ongoing process of cell division and apoptosis characterizes crisis, so that the population size initially ceases to increase and eventually declines to the point that the culture is frequently lost. Occasionally, as a very rare event, an immortal cell emerges from crisis. An interesting behavior was observed when IMR90 human embryonic lung fibroblasts were immortalized using a dexamethasone-inducible large T antigen. The immortal cells stopped dividing when T antigen was deinduced by the removal of dexamethasone; this suggested that the continued expression of T antigen was necessary for the maintenance of the immortal state. However, the cells also stopped dividing when T antigen was deinduced during the period of extended life span prior to crisis when the cells were not yet immortal.

The model we proposed to explain these observations postulates that senescence initially is caused by the first of two independent mechanisms, mortality stage 1 or the M1 mechanism. Although early studies suggested that the M1 mechanism required the actions of p53 and pRB (4–6), recent observations have raised the possibility that the involvement of the RB pathway reflects a stress response to inadequate culture conditions (see Sec. V), and that p53 is the important cellular sensor of M1. Tumor virus proteins like SV40 T antigen bind to both p53 and pRB and block their activity, thus bypassing M1 and extending the life span of the entire population of cells until an independent second mechanism, M2, is activated. M2 causes crisis, and is not directly affected by the presence of T antigen or similar viral proteins. If a critical M2 gene becomes inactivated, the cells can escape crisis and become immortal. However, these immortal cells have a perfectly functional M1 mechanism, which was never mutated but simply blocked by the presence of T antigen. Consequently, if T antigen is removed any time after the M1 mechanism has been induced, the cells stop dividing due to the actions of M1-activated p53 and pRB.

III. TELOMERE SHORTENING

The ends of vertebrate chromosomes are capped with many kilobases of hexameric TTAGGG sequences and are called *telomeres*. Telomeres fulfill many differ-

ent functions. The two most relevant to replicative aging are to buffer the effects of the inability of the replication machinery to copy the very ends of linear DNA (the"end-replication problem") and to protect the ends of chromosomes from being recognized as broken DNA needing repair. The lagging strand of semiconservative replication is synthesized as a series of Okazaki fragments, each requiring a new RNA primer that is then extended by DNA polymerase. Because there is no DNA beyond the end of the chromosome that can be used as a template for making another RNA primer, the normal replication complex is unable to fill in the gap between the last priming event and the end of the chromosome. The lagging strand is thus shorter than its parental template, leaving a 3′ overhang. The leading strand is expected to go all the way to the end of the chromosome, but there are processing events that generate a 3′ overhang in its daughter duplex as well (7,8). Telomeric binding factors, such as TRF2, are important for preventing the cell from recognizing the ends of the chromosomes as being damaged DNA (9). Cells that express the ribonucleoprotein telomerase are able to add TTAGGG sequences to the ends of the chromosomes using the complementary sequence present in the telomerase integral RNA (hTR for the human enzyme) as a template for the reverse-transcriptase activity of the catalytic subunit (hTERT for the human enzyme). This enzyme is thus able to compensate for the end-replication problem and prevent telomeric shortening. Although the integral RNA component hTERT is present in virtually all cells, telomerase is turned off in most human tissues during development (10) due to the repression of the catalytic subunit hTERT (11), and as a consequence, telomeric shortening begins.

The understanding of the molecular mechanisms underlying replicative aging was significantly advanced by demonstrations that telomeric shortening occurred both in vivo and in culture as a function of population doublings (12–15). Although a few earlier reports had speculated about possible roles of telomeric shortening (e.g., see Refs. 16 and 17), a series of reports in the 1990s directly addressed the issue and established that telomeres shortened in tissues as a function of donor age (e.g., see Refs. 12, 13, and 18–21) and in culture as a function of the number of cell divisions (e.g., see Refs. 14, 19, 22, and 23). The hypothesis that telomeric shortening limited the proliferative capacity of normal cells (14,24,25) was strengthened by the contrast of normal versus cancer cells. Replicative aging has been thought to be a brake against the formation of cancer since at least the early 1970s. Cancer cells need to accumulate many mutations to become malignant, and it probably requires at least 20–30 doublings for a cell that acquires one mutation to grow to a large enough population size for the next mutation to occur. Normal cells would thus reach senescence before acquiring enough mutations to become tumorigenic. If telomeric shortening caused replicative aging, then immortal cancer cells would need a mechanism to prevent telomeric shortening. Telomerase activity was first demonstrated in HeLa cells in 1989 (26), and then in cells immortalized in culture (27) and ascites cells from ovarian carcinoma patients (28). The

development of a polymerase chain reaction (PCR)–based telomerase assay that could demonstrate activity in small tissue samples permitted the widespread demonstration that 85–90% of all cancers expressed telomerase (29,30). These developments provided substantial correlations between telomeric shortening and replicative aging versus telomerase expression and immortalization, but direct proof was lacking. Furthermore, one needed to rationalize telomeric shortening with the observations underlying the two-stage model of replicative aging.

IV. TELOMERES AND DIRECT PROOF OF THEIR REGULATION OF M1/M2

Telomeric shortening was incorporated as the mechanism driving the two-stage model by postulating that shortening would be the mitotic clock that counts cell divisions. The M1 mechanism would be induced not when perhaps one or a few telomeres became "too short," but when there were still some telomeric repeats remaining. Later in the chapter, several models for this induction will be considered. If the M1 mechanism were blocked by agents such as T antigen, then the cells could continue dividing. Telomerase is not induced by T antigen, and telomeres continue to shorten during the period of extended life span prior to crisis (27,91). The M2 mechanism would represent terminal telomeric shortening when there were so few repeats remaining that the direct consequences of the lack of repeats (e.g., end-to-end chromosome fusions, potential detachment of the telomeres from the nuclear matrix) would cause cells to stop dividing and eventually die. Escape from M2 would represent the inactivation of the pathway by which telomerase is repressed. The reexpression of telomerase (27) then would permit the cell to add telomeric repeats to the denuded chromosome ends, maintain telomere length, and become immortal.

Proof that telomere length regulated M2 was obtained in 1996. T antigen expressing immortal cells with short telomeres were treated with a nonspecific agent that caused a few kilobases of telomeric elongation. After this treatment was stopped, the parental or the experimentally elongated cells were fused to young normal fibroblasts that had telomeres much longer than in either of the immortal cells. Telomerase was repressed in the hybrids. The hybrids between the normal cells and the immortal cells with experimentally elongated telomeres divided many more times than did the hybrid between the normal cells and the parental immortal cell, proving that short telomeres limited the proliferative capacity of the hybrids (31). Because T antigen was expressed in the hybrids and would transdominantly block M1, these experiments established that telomeric shortening caused M2. This conclusion was confirmed in later experiments showing that telomerase expression prevented crisis in T antigen expressing–cells (32).

The cloning of the cDNA for the catalytic subunit of human telomerase (hTERT) in 1997 (33,34) provided the ability directly to test whether or not telomeric shortening also caused the induction of M1. Telomerase activity was restored in normal diploid fibroblasts following the introduction of an exogenous hTERT (35). The cells contained the factors needed to recruit telomerase to their telomeres, so that telomere length was maintained or elongated. The demonstration that blocking telomeric shortening prevented replicative aging established telomeres as the mitotic clock that caused M1. These immortalized cells remained diploid, continued to exhibit normal checkpoint controls, could be growth arrested by the overexpression of oncogenes such as H-*ras*, and did not form tumors (36,37). At least three additional functions (H-*ras* to activate autonomous cell division, SV40 large T antigen to block pRB and p53 checkpoint/apoptotic mechanisms, and SV40 small t antigen to provide additional phosphatase activity) were required before these immortal cells could form tumors (38,38a).

V. "TELOMERE-INDEPENDENT" MECHANISMS OF GROWTH ARREST

Many reports have appeared suggesting a telomere-independent mechanism of cellular senescence is present in epidermal keratinocytes (39,40) and in mammary (39), adenoid (41), thyroid (42), and prostate (43) epithelial cells. The investigators who reported these findings found that inactivation of the p16/pRB pathway (by methylation of the p16 gene or by expression of viral oncogenes such as the human papilloma virus protein E7) was required before telomerase could immortalize these epithelial cells. Many of these epithelial cells were grown in a chemically defined medium in which the proliferative life span of 10–20 doublings is much less than the approximately 50 doublings that are seen when keratinocytes are grown on feeder layers (44). We have shown that keratinocytes can be immortalized by telomerase alone without inactivating p16 when grown in the more hospitable environment provided by feeder layers (which produce additional growth factors, extracellular matrix and/or epithelial/mesenchymal interactions). Similarly, mammary epithelial cells fail to exhibit the p16-dependent "mortality stage 0" (45) when grown on feeder layers (45a). Skin fibroblasts expressing telomerase, which growth arrest after 25 doublings in a chemically defined medium supplemented with 0.25% serum, resume growth and are immortal when transferred back to their normal medium containing 10% serum. Collectively, these results (46) demonstrate that culture conditions that superficially appear adequate can nonetheless fail to support the long-term growth of normal cells. Although the possibility that telomere-independent mechanisms for counting cell divisions exist cannot be excluded, all of the evidence to date for these alternatives can be explained by inadequacies of the culture environment.

There is significant evidence demonstrating that telomeric shortening reflects important in vivo biology. This evidence includes the observations that telomeres shorten in vivo as a function of donor age (e.g., see ref. 13), that in vitro life span due to telomeric shortening is inversely proportional to donor age (14,23), that cells from patients with premature aging syndromes have a decreased proliferative capacity in vitro that is either due to shorter initial telomere lengths—such as progeria (23) or increased rates of shortening, including the Werner syndrome (47) and ataxia telangiectasia (47)—that telomerase can prevent telomeric shortening and immortalize cells (35), and that most cancer cells express telomerase (29). Given the evidence that inadequate conditions can prevent the long-term cultivation of many cell types, it is now necessary to establish reasonable evidence that the same process is occurring in vivo before something is described as a telomere-independent mechanism of replicative aging.

One report claimed that replicative life span does not decrease with donor age (48). This study has been widely misinterpreted because of a failure to appreciate several important points. First, the modern concept of replicative aging is based on cell divisions, not on chronological time. The investigators who made this report examined skin biopsies taken from the inner surface of the upper arm. The biopsy site was chosen to minimize the effects of damage due to sun exposure. By doing so, they chose a protected area in which the stimulus to fibroblast cell turnover is also likely to be minimal. Second, in vitro life span is a poor measure of the fraction of cells with short telomeres in vivo. It only takes three divisions for young cells comprising 12.5% of the population to overgrow any old cells (12.5% divides to give 25%, which divides to give 50%, which divides to give 100%). Since the scatter in cellular life span is typically at least ± 5 doublings, one only observes differences if the whole population in culture were sufficiently uniformly aged so that few cells with long telomeres remained. Choosing a protected area with little cell turnover would minimize the ability to observe the behavior of those cells that had divided multiple times in vivo. The evidence that telomeric shortening regulates cultured life span is compelling, and the evidence that many tissues exhibit telomeric shortening in vivo is also well established. The demonstration that a reduced cultured life span is not observed in areas with low cell turnover may be true but is misleading, and in no way detracts from the potential importance of replicative aging to human biology.

VI. MOUSE CULTURE-INDUCED GROWTH ARREST

After 15–20 divisions in culture, mouse cells undergo what has been called *cellular senescence*. Studies using the telomerase-RNA knockout mouse (49) have shown that this growth arrest is independent of telomere length. If telomeric shortening is not inducing rodent cellular senescence, then what is? A core concept of

modern theories of aging is that longer-lived species have evolved more efficient maintenance and repair capacities to better protect themselves against the damage that occurs with the passage of time. It is known that mouse and rat cells repair DNA damage much less efficiently than human cells (50), and are much more sensitive to a variety of agents that produce oxidative stress (51). There is considerable evidence that the usual culture environment induces DNA damage in mouse cells. The transcriptional activity of p53 increases 10- to 40-fold during the first three passages of normal mouse embryo fibroblasts (52). Furthermore, proliferation stops after only three to four doublings in cells from mice containing defects in a large variety of DNA repair functions—specifically Ku80 (53), ATM (54), BrCA2 (55), and XPG (56). Since these animals are viable, their cells must be able to divide many more than four times in vivo. There are now several examples in which rodent cells do not exhibit a premature growth arrest. Mouse cells remain diploid and are immortal if cultured in a special medium (57), as are rat Schwann cells and rat oligodendroglial precursor cells (58,59). We thus believe that the growth arrest observed in mouse cells represents the same general phenomenon of a stress-induced checkpoint response to an inadequate culture environment as discussed above regarding epithelial cells (46,60–62).

One factor contributing to the confusion over mouse cellular senescence has been the use of colony formation as a surrogate for proliferative capacity. There have been many studies showing a decrease in colony formation in mice as a function of donor age (e.g., see refs. 63 and 64), and this was used as evidence for replicative aging in mouse cells. Although we believe that replicative aging may be one component of organismal aging (a proposition that has not yet been firmly established), many other processes (e.g., oxidative damage, DNA damage, mitochondrial deletions, protein cross linking) also are occurring in vivo. Microarray analysis of human fibroblasts as a function of donor age suggests that damage-response mechanisms are being activated with organismal aging (65). Decreased colony formation has long been used to assay toxicity induced by different agents. The accumulation of damage (from whatever cause) should produce a decreased cloning efficiency as a function of donor age independent of any mechanism capable of counting cell divisions. The evidence linking mouse culture-induced growth arrest to in vivo aging is thus based on data that do not distinguish between damage and proliferative aging.

Replicative aging is thought to limit the number of available cell divisions, and thus to act as a brake against the accumulation of the multiple mutations needed for a cell to become malignant. A 70-kg man who lives for 80 years has to be 14,000—140,000 times more resistant to cancer than a 0.2-kg rat (or a 20-g mouse) that lives for 2 years (e.g., [70 kg $\div$ 0.2 kg] $\times$ [80 years $\div$ 2 years] = 14,000). We believe that conventional mutation/tumor-protection mechanisms (e.g., DNA repair, immune surveillance, protection against oxidative damage) are adequate to protect against tumor formation during the short life of small organ-

isms, and that replicative aging evolved to provide the manyfold increased protection needed by larger and longer lived species (60,62). Although one cannot prove the absence of a phenomenon, at present, there is no evidence that replicative aging exists in rat and mouse cells.

VII. WHAT DOES "CELLULAR SENESCENCE" MEAN?

The term *cellular senescence* has been indiscriminately applied to almost any condition in which irreversible growth arrest occurs. Biochemical markers associated with both human and mouse growth arrest reflect the accumulation of negative growth regulators (e.g., $p16^{INK4a}$, $p21^{Cip1}$), and factors (such as the senescence-associated (SA)–β galactosidase) that are induced during stress responses. These so-called "senescence-associated markers" are very nonspecific and can be produced by DNA damage and other insults, including hydrogen peroxide (66), genotoxic drugs like bleomycin or etoposide (67), overexpression of oncogenes such as H-*ras* V12 (68), or checkpoint factors like $p16^{INK4a}$ (69). Any stimulus producing DNA damage would be expected to mimic many aspects of the senescent phenotype, assuming M1 is produced by a too short telomere(s) that is no longer masked from the DNA repair apparatus and thus produces a DNA damage response. What is the justification for using the term *senescence*? A heart attack at age 99 might legitimately be described as being related to human aging, whereas it is clear that a heart attack at age 35 represents a different phenomenon. In both cases, the overt phenotype is organismal death due to cardiac arrest. By the same token, the DNA damage signal from short telomeres or bleomycin treatment lead to the same phenotype of irreversible growth arrest, but this does not imply that they should be lumped together as the same phenomenon. The term *cellular senescence* carries with it clear semantic connotations that the process being described is thought to have some relevance to aging. We believe that the term *cellular senescence* should be reserved for situations in which the evidence for this is at least tenable. Because of the widespread misuse of the term *cellular senescence*, we are using the phrase *replicative aging* to describe the process by which the counting of cell divisions is used to limit the proliferation of normal cells.

VIII. IMR90 AND OXYGEN: WHAT IS THE ROLE OF P16?

Oxidative damage is the most widely studied mechanism for organismal aging (e.g., see Refs. 51, 70, and 71), and many studies have related oxidative damage to replicative aging of cultured fibroblasts. Atamna et al. (72) found that reactive oxygen species rise during the last 15 doublings prior to senescence in IMR90 lung fibroblasts, and that an extra 20 doublings can be obtained by including spe-

cific antioxidants in the medium. Von Zglinicki et al. have shown that 40% oxygen can induce single-strand breaks in the G-rich strand of telomeres (73), and that these breaks are repaired much less efficiently than those elsewhere in the genome (74). They also found that antioxidants can reduce the rate of telomeric shortening during the last 15 doublings and prolong cellular life span (73), and they have proposed that progressive oxidative damage, rather than cell division, is the major mechanism driving telomeric shortening in cultured cells. This conclusion is inconsistent with the observation that the extension of life span of IMR90 fibroblasts obtained by growing cells in 1% oxygen is independent of the length of treatment (75): The same extra 10–15 doublings are observed for cells grown in 1% oxygen for virtually their entire life span as for cells transferred to 1% oxygen just before they stop growing. Furthermore, oxidative damage to telomeres produces random breaks and does not affect the size of the G-rich 3′ overhang (73). However, the rate of telomeric loss in different cultured cell types is proportional to the size of the G-rich 3′ overhang (8), which argues strongly that events at the end of the chromosome (the end-replication problem plus processing mechanisms) are the most important determinant of the rate of telomeric shortening.

There is, nonetheless, evidence that oxidative damage can contribute to "inadequate growth conditions." In contrast to the behavior of the many varieties of skin fibroblasts we have examined (76), IMR90 lung fibroblasts are not immortalized by telomerase. Their life span is modestly extended by telomerase in 20% oxygen and significantly extended by telomerase in cells grown in 1% oxygen (in preparation). Tissues in the body are normally exposed to oxygen concentrations of 1–6%, and perhaps lower in fetal tissues (77). Our interpretation of these results is that most human skin fibroblasts express sufficient antioxidant reserves to withstand the insults of the usual culture environment and reach telomere-based senescence. However, IMR90 fetal lung fibroblasts (and presumably other cell types as well) are less resistant to 20% oxygen and eventually sustain sufficient damage to growth arrest prior to telomere-based senescence.

This interpretation provides a solution to some puzzling observations on the extension of life span by viral oncoproteins. Human papillomavirus (HPV) E6/E7 extends the life span of IMR90 by approximately 40–50 doublings. Given that IMR90 telomeres shorten at the rate of 70 bp/division (8), this suggested that M1 was occurring when there were still sufficient telomeric repeats to permit an additional 3.5 kb of shortening prior to M2. Why would a telomere(s) be "too short" and signal a growth arrest when there were still 3.5 kb of repeats remaining? In addition, IMR90 cells have much longer telomeres (mean length of about 7.5 kb) at proliferative arrest than senescent BJ foreskin fibroblasts (mean telomere lengths of only about 5 kb). Furthermore, E6/E7 extends the life span of BJ foreskin fibroblasts by only 10–15 divisions (in preparation). We know that BJ fibroblasts senesce because of telomere-based replicative aging, since they are immortalized by telomerase, and thus their senescent telomere length of about 5 kb

and their extension of life span by E6/E7 provide benchmarks for comparison to IMR90. Collectively, these results imply that although BJ fibroblasts divide until activating M1, IMR90 fibroblasts are growth arresting owing to inadequate growth conditions (growth in 20% oxygen plus other factors) and do not reach replicative senescence. By blocking the ability of cells to checkpoint arrest, E6/E7 or SV40 T antigen forces the IMR90 cells to divide in spite of inadequate conditions until critically short telomeres cause M2.

Studies on the behavior of IMR90 fetal lung fibroblasts (together with the fetal lung fibroblast strains WI-38 and MRC5) have formed the core literature on replicative aging. Our conclusion that IMR90 cells in culture do not stop dividing owing to replicative aging forces the reinterpretation of many of these studies. For example, many investigations have shown an elevation of $p16^{INK4a}$ as IMR90 fibroblasts reach proliferative arrest. There is now abundant evidence that $p16^{INK4a}$ is a critical tumor suppressor checkpoint factor that becomes elevated under stressful conditions, when cells find themselves in an inappropriate environment, or following DNA damage (67,78). We do not find $p16^{INK4a}$ elevated in BJ fibroblasts at telomere-based M1 and suspect that it does not play a significant role in replicative aging. Our studies implicating the RB pathway in M1 were performed in IMR90 cells (5) and may simply reflect the presence of culture-induced p16/pRB checkpoint activity. There is now good evidence that a p53-mediated DNA damage signal from a too short telomere induces M1 (see below). Although Hara (6) found that antisense p53 extended the life span of TIG-1 fibroblasts, it only did so if the life span was first extended by antisense RB. This is consistent with the interpretation that the p16/pRB pathway is being induced by inadequate culture conditions in TIG-1 cells, just as in IMR90 cells, prior to the onset of replicative aging.

IX. MECHANISMS FOR THE INDUCTION OF M1

One of the important functions of telomeres is to mask the ends of linear chromosomes from being recognized as double-strand breaks needing repair. Checkpoint arrest following the induction of a DNA damage signal from a too short telomere(s) was suggested as the mechanism inducing replicative aging in the early 1990s (24). Although certainly reasonable, additional factors might be involved, since this hypothesis does not appear fully to explain the behavior of IMR90 cells. If too short telomeres were inducing growth arrest, how could the cell deal with those chromosomes during the 40–50 doublings between M1 and M2? We thus proposed that telomere position effect (TPE) might be inducing M1 (79). In yeast, the spreading of telomeric heterochromatin can silence genes placed near telomeres (80). We hypothesized that genes regulating M1 might be located in the subtelomeric DNA, and the strength of TPE might be proportional to the length of the

telomeres. Under these circumstances, the regulatory genes would be silenced in young cells with long telomeres but activated to induce M1 once telomeres became sufficiently short.

We have now demonstrated that TPE exists in human cells, and that the strength of TPE is proportional to telomere length (80a). However, we do not believe that TPE regulates M1 for several reasons. The most important evidence is the behavior of foreskin fibroblasts, which do not prematurely growth arrest but exhibit telomere-based M1, following a transient expression of hTERT. BJ fibroblasts were infected with a retrovirus expressing hTERT near the end of their life span. The cDNA insert was flanked by loxP sites, so it was possible to excise the hTERT after seven population doublings by infecting the cells with a retrovirus expressing cre-recombinase. Telomerase is preferentially recruited/active on short telomeres (81,82). After seven doublings, the shortest telomeres had increased in length by 2.5 kb, although mean telomere length had only increased by 0.6 kb. After hTERT was removed, these cells went through an additional 50 doublings before telomeric shortening caused them to senesce at M1. The mean telomere size in these cells was then only 3.5–4.0 kb, which is considerably shorter than the 5 kb normally seen in BJ fibroblasts at senescence. Because of the elongation of the shortest telomeres, the overall size distribution of the telomeres became much more homogeneous. Consequently, although the mean telomere length was smaller, the shortest telomeres in these cells were the same size at M1 as in the parental BJ fibroblasts. The 50 doubling extension of life span can be fully explained by the observed rate of telomeric shortening of 50 bp/division and the 2.5-kb elongation of the shortest telomeres. These results imply that it is the shortest telomeres that are inducing M1 (81). The justification for invoking TPE as a possible mechanism regulating M1 was to be able to induce a growth arrest when telomeres were still moderately long. Unless the critical telomere containing the relevant regulatory locus was also among the shortest telomeres, M1 should have been induced when the average telomere size reached 5 kb (Fig. 1). The fact that the shortest group of telomeres appears to induce M1 rather than average telomere length is much more consistent with a DNA-damage signal rather than TPE.

An additional reason for favoring a DNA-damage signal as the mechanism inducing M1 is the realization discussed above that IMR90 cells growth arrest prior to M1 is due to inadequate growth conditions. If IMR90 cells are not leaving the cell cycle owing to telomeres that are too short, then there is no need to explain how the cells can maintain their chromosomes during the 40–50 doubling extension of life span conferred by E6/E7.

Blackburn (83) has hypothesized that the telomerase holoenzyme "caps" the end of short human telomeres and helps hide the ends from the DNA-damage recognition machinery, thus permitting short telomeres to become shorter than is observed in the absence of telomerase. Importantly, in addition to simply adding

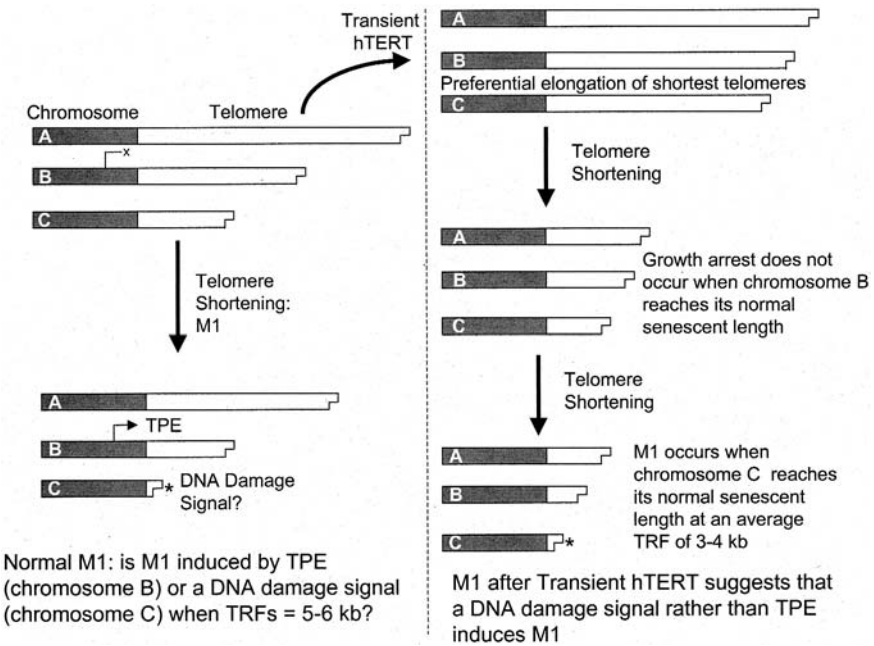

Figure 1 M1 is induced by a DNA-damage signal rather than by TPE. The growth arrest induced by short telomeres was hypothesized to be either due to telomere position effects (TPE) or a DNA-damage signal from a too–short telomere. The length of the telomere controls TPE. The relevant chromosome ("chromosome B") containing regulatory genes in the subtelomeric DNA should thus activate the growth arrest when their telomeres reach the "set point" length, regardless of the size of the rest of the telomeres. A DNA-damage signal occurs when a telomere is sufficiently short that the end can no longer be masked from the DNA-damage–sensing machinery. The DNA-damage signal from the limiting telomere ("chromosome C") should also be generated independently of the size of the rest of the telomeres. The preferential elongation of the shortest telomeres by a transiently expressed hTERT results in a change in the distribution of telomere sizes, with different results predicted for when M1 should be induced for TPE versus DNA-damage signals. The result that the average telomere length at M1following transient hTERT is less than at a normal senescence implies that M1 is induced by a DNA-damage signal.

telomeric sequences, it is proposed that the role of telomerase includes assembling a protective complex on the ends. All of the observations in vertebrates supporting this conclusion are adequately explained by the preferential activity of telomerase on short telomeres. In the experiments described above, telomerase was transiently expressed and was no longer present to provide any capping function.

The telomeres nonetheless became shorter than is normally seen in senescent cells. Although TTAGGG sequences are clearly required for the proper assembly of telomere end binding factors, and telomerase could cap denuded chromosomes by adding those sequences, we do not believe there is any evidence in vertebrates for the telomerase protein itself directly participating in capping functions that permit extreme telomeric shortening.

X. WHAT IS THE NATURE OF TELOMERIC EROSION BETWEEN M1 AND M2?

BJ fibroblasts lose 50 bp/doubling, so that 500–750 bp are lost during the 10–15 doublings extension of life span. Although the magnitude of the problem is considerably less if the extension of life span between M1 and M2 is 10–15 doublings rather than the 40–50 that was thought to be present in IMR90 cells, one still needs to explain how the cells deal with their too short telomeres: Why are massive telomeric fusions (M2) delayed until an additional 500–750 bp are lost beyond M1? We initially believed that cells might be able to cope with one or two short telomeres, and that M2 occurred only when too many telomeres became critically short. This predicted that the extension of life span would be much less in the BJ fibroblasts described above, in which the transient expression of telomerase homogenized telomere length. This should have caused many more telomeres to be closer to criticality when M1 occurred, and should have reduced the extension of life span by E6/E7. However, E6/E7 produced exactly the same 10–15 doubling extension of life span in these cells as in the parental BJ fibroblasts. Two major alternative possibilities exist to explain these results, involving t-loops and noncanonical telomeric repeats.

The 3′ overhang is proposed to invade the double-stranded regions of telomeres to produce large structures called t-loops (Fig. 2, top). The telomeric binding protein TRF2 is preferentially localized to the junction of the invading overhang and the resulting displacement loop (d-loop), and is able to stimulate t-loop formation by itself in vitro using model substrates (84). The t-loops are usually many kilobases in length, and there is likely to be a minimal telomere length below which t-loop formation becomes increasingly difficult. M1 might be induced when the shortest telomere could no longer efficiently form t-loops. It is possible that 3′ overhang end-binding factors are sufficient in most cases to prevent telomere fusion, but that t-loop formation is required to prevent activation of the DNA-damage checkpoint complex. If t-loop formation becomes compromised at telomere lengths 500–750 bp longer than end-protection, then an appropriate interval could exist between the time M1 was induced and when end-fusion/nondisjunction/apoptosis events occurred at M2.

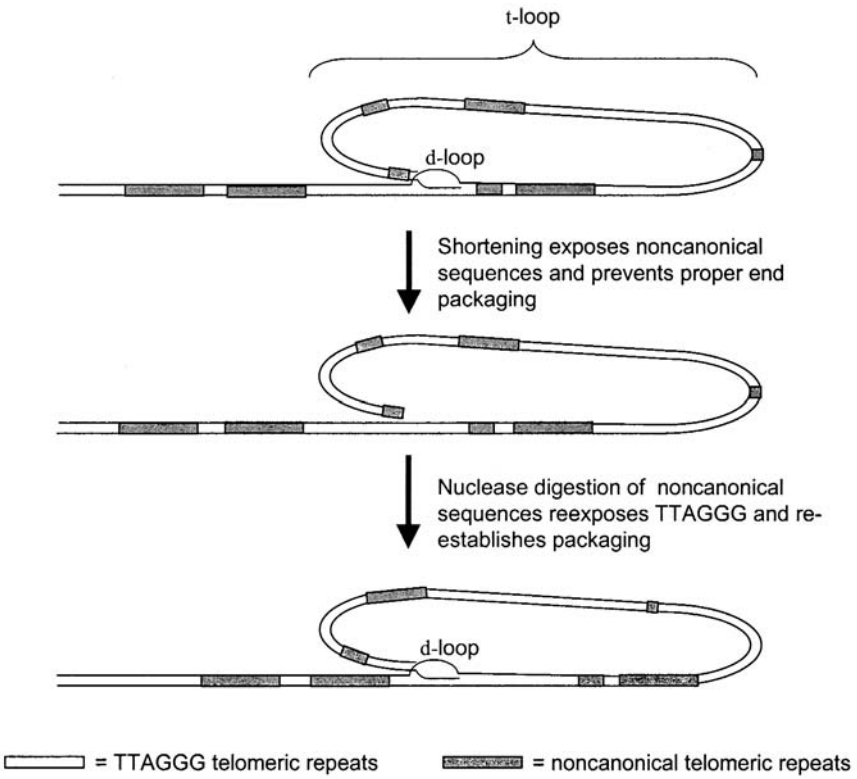

Figure 2 Telomeric structure between M1 and M2. The current model for structure of the telomere involves the telomere-binding protein TRF2 causing the 3′ overhang to invade the duplex region, forming a larger t-loop (telomeric loop) and a smaller d-loop (displacement loop) (Ref. 84). The top image illustrates the structure that might be present at a limiting chromosome as cells approached M1. The green bars represent blocks of noncanonical variants of the telomeric TTAGGG repeat. The middle panel shows that M1 might occur when the first block of these noncanonical repeats was reached, where the absence of correct binding sites for TRF2 or single-stranded end-binding factors resulted in the failure to form a t-loop and the induction of a DNA-damage checkpoint signal. If growth arrest is blocked by mutant p53 or viral oncoproteins, nuclease actions might digest the end of the telomere, removing the terminal block of noncanonical sequences and exposing functional TTAGGG repeats. These would then permit t-loop formation and further divisions (bottom panel). This would permit the additional divisions of an extended life span. M2 might occur when there were no significant TTAGGG sequences internal to the noncanonical repeats (see Fig. 3).

Some aspects of the above scenario can be combined with the observation that the base of the telomeres contains a mixture of sequences. In addition to the canonical TTAGGG, there are stretches of TGAGGG, TCAGGG, and other sequences (85,86). The detailed sequence of the subtelomeric-telomeric junction of some telomeres is becoming available, and one example is shown in Figure 3. This particular sequence stops before one reaches significant runs of TTAGGG, but it demonstrates the diverse admixture of variants that dominate the subtelomeric-telomeric junction. These sequences probably arise from mutations in the canonical sequence, followed by an expansion of the mutant hexamer due to slippage during replication or recombination. This process would be similar to that which produces the expansion of dinucleotide and trinucleotide repeats (87). Mutations that occur near the end of the telomere would be replaced as cycles of shortening and elongation by telomerase occur. However, because telomeres are maintained at large sizes in the germ line, the extent of this "breathing" will be relatively limited. Mutations in telomeric sequences near the base of the telomere would thus accumulate over time. The frequency of telomeric turnover by telomerase should increase as one gets further from the subtelomeric junction, so we anticipate that the zone of few uninterrupted TTAGGG sequences shown in Figure 3 would be followed by a zone of progressively longer stretches of TTAGGG repeats, and finally by many kilobases in which there is nothing but pure canonical telomeric repeats.

It is well established that nuclease-processing events occur at the sites of double-strand breaks. It is possible that M1 occurs when telomeric shortening exposes the first stretch of these noncanonical sequences, thus interfering with the assembly of end-binding complexes and/or the formation of t-loops (see Fig. 2). If p53 mutations or viral oncoproteins block the resulting checkpoint arrest, the cell is forced to divide. Although some end-fusion events might occur, in many cases nuclease processing might trim away the noncanonical sequences,

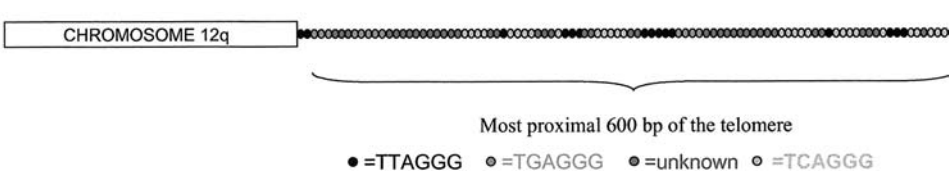

Figure 3 Noncanonical sequences at the subtelomeric-telomeric junction. The base of the telomere at chromosome 12q is shown. The first 600 nucleotides of the telomere are dominated by variants of the TTAGGG telomeric repeat, with relatively few blocks of adjacent canonical repeats. The sequence distal to this 600-bp region is not yet known. The actual sequence of tel 12q is polymorphic, and only one of many similar sequences is shown. (Data are from Ref. 86.) (See color insert.)

thus making the next block of TTAGGG sequences available. This would eliminate the clastogenic stimulus, and the cells could continue dividing until the next block of noncanonical sequences appeared. M2 would occur when there were no longer significant stretches of TTAGGG sequences remaining near the subtelomeric junction. Neither of these hypotheses has been confirmed by experimental data.

The presence of noncanonical repeats near the subtelomeric junction can alter the interpretation of telomere length measurements. For example, experiments using quantitative in situ hybridization to measure the telomeres on individual chromosomes suggest that the shortest telomere does not determine the onset of M1 (88). However, the concept that the exposure of noncanonical sequences could lead to a checkpoint arrest would change how one defined the shortest telomere. Rather than reflecting total TTAGGG, the functionally shortest telomere would be the one with the shortest length of pure TTAGGG sequences beyond the most distal noncanonical sequence. This would be the one in which telomeric shortening first exposed DNA that could not be properly packaged into an end-protective structure, and which thus first generated a DNA-damage signal. Interestingly, the chromosome that is limiting for the onset of M1 need not be the same chromosome(s) that cause the onset of M2. M2 is more likely to reflect the erosion of the last protective stretch of TTAGGG sequences, and may be directly related to the size as measured by in situ hybridization with telomeric probes.

One of the predictions of this model is that since mutations and expansion of telomeric sequences should be constantly occurring in the population, there should be individuals in which a block of noncanonical sequences is present in a moderately distal location on some telomere(s). These blocks should induce a premature telomere-based replicative arrest when these sequences become exposed even though average telomere length would still be considerably longer than normal. Because one has to have sufficient replicative capacity for growth, maintenance, and repair at least until reproduction has occurred, there will be a strong selection against these noncanonical sequences being present in very distal locations. More internal locations compatible with normal adult development could nonetheless persist, and could contribute to a variety of age-associated diseases. Many of these pathologies might have other causes, but require ongoing cell turnover in order to compensate for the proximate insult—just like muscle regeneration in Duchenne muscular dystrophy is progressively compromised by replicative aging of the myogenic satellite stem cells (89,90). Individuals with a genetic predisposition to premature onset of these diseases due to mutant telomeric sequences may be difficult to identify, since a conventional analysis would indicate a normal telomere length. Whether or not such predispositions exist, and what their prevalence in the population is, will only become evident after the contributions of replicative aging to organismal aging and disease are understood in much greater detail.

XI. CONCLUSION

The definition of the molecular mechanism underlying the ability to count cell divisions has revolutionized our understanding of the limited proliferative capacity of normal cells. In addition to opening up many new areas for investigation, it has provided a critical criterion for determining whether or not one is even studying replicative aging. We propose that the ability to immortalize cells with telomerase alone should be the current gold standard for whether or not a cultured growth arrest is a consequence of replicative aging or whether it is something else. If telomerase can be expressed, and it maintains or elongates telomeres without conferring essentially indefinite growth, then we believe the burden of proof lies with those who would call this replicative aging. They must demonstrate that there is some relationship with donor age and in vivo processes and mechanisms before it makes sense to use terms such as *aging* to describe the phenotype. The previous inability to apply this criterion has led to a very confusing literature, in which many different phenomena, with different characteristics and giving contradictory results, have been indiscriminately lumped together. The identification of telomeric shortening as the cause of limited cellular life span and telomerase expression as the mechanism conferring the indefinite proliferative capacity of most cancer cells should now provide the foundation for rapid progress in our understanding of the physiological role of replicative aging and our ability to use this information to advance human health.

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5
Telomeric Regulation in Eukaryotic Cells

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I. INTRODUCTION: TELOMERIC DNA

The telomere is a specialized structure at the extremity of linear chromosomes that is essential for the maintenance of chromosomal stability. Sixty years ago, it was determined that unlike broken DNA ends, which are sticky and undergo fusions, natural chromosome ends are stable (1,2). Most eukaryotic telomeres are composed of long arrays of short tandem repeats bound by telomere-specific and general chromosomal proteins.

Watson (3) first pointed out the difficulty in completing the replication of linear chromosome ends. Whereas the leading strand of a chromosome can be replicated to its end, the lagging strand cannot unless an RNA primer is placed at the very end of the lagging strand. Even so, this strand would be shortened by the length of the primer and would terminate in a 3′ single-stranded extension, whereas the other end of the newly replicated DNA (leading strand end) would be blunt. As Okazaki fragments in most eukaryotic cells are believed to be ~150 nucleotides (nt) in length, the finding that telomeres in somatic cells shorten by 50–200 nt at each division (4) has been taken as evidence for Watson's "end replication problem." However, telomere length can be maintained through a variety of mechanisms, including the telomere-specific reverse transcriptase telomerase (5,6) or alternative recombination-based methods (ALT) (7). Since the activity of telomerase is negligible in human somatic cells, telomeric DNA gradually shortens with continued cell cycles. As a result, somatic telomeres from

young individuals are longer than those from older individuals (8,9), and hence, telomere erosion has been proposed as the "molecular clock" that regulates cellular life span.

Both the sequence and the length of telomeric DNA are organism specific (Table 1). In mammals, telomeric DNA is composed of a tandem repeat of the double-stranded sequence ([5′-TTAGGG-3′/3′-AATCCC-5′]$_n$) oriented in this direction toward the chromosome end (10). Although the tract length varies among chromosome ends and cell types, the size range is organism specific. Telomeric tracts range from 5 to 30 kb in humans but can be up to 20–60 kb in mice (11–14). Telomeres in plants most often consist of a related repeat, TTTAGGG, and can vary from ~3 kb in *Arabidopsis* to more than 100 kb in tobacco and *Barley calus* (15). Telomeres in *Caenorhabditis elegans* have a less stringent repeated sequence ([C_{1-3}(A/T)G_{1-3}]) and are approximately 350 bp long (16).

In addition to the duplex repeat tract, a 3′ single-stranded overhang of the G-rich strand is a conserved feature of higher eukaryotic telomeres (17–19). The 3′ extensions are 75–150 nt in length for human telomeres and may result either from incomplete replication of the telomere, an exonuclease acting on the 5′ telomere ends, or from a combination of these processes. While the end replication problem would predict that one end would be blunt, current thinking is that both ends have extensions, suggesting a possible processing event. Evidence for this prediction has been observed at the telomeres of the minichromosome of *Trypanosoma brucei* in a recent electron microscopic (EM) study (20), where long

Table 1 The Sequence and Length of Telomeric DNA for a Variety of Organisms

Vertebrates		
Homo sapiens	$(TTAGGG)_n$	5–30 Kb
Mus musculus	$(TTAGGG)_n$	20–60 Kb
Xenopus laevis	$(TTAGGG)_n$	10–50 Kb
Yeasts		
Saccharomyces cerevisiae	$(TG_{1-3})_n$	350 bp
Schizosaccharomyces pombe	$(TTACAG_{1-8})_n$	200–300 bp
Protozoa		
Trypanosoma brucei	$(TTAGGG)_n$	10–20 Kb
Tetrahymena thermophila	$(TTGGGG)_n$	1–2 Kb
Oxytrichia nova	$(TTTTGGGG)_n$	20 bp
Plants		
Arabidopsis thalliana	$(TTTAGGG)_n$	2–7 Kb
Barley calus	$(TTTAGGG)_n$	>100 Kb
Nicotiana tabacum	$(TTTAGGG)_n$	>100 Kb

single-stranded extensions were directly observed on both ends of the minichromosome.

The single-stranded overhang likely plays multiple roles in telomeric maintenance and structure in addition to providing a template for de novo synthesis of telomeric repeats by telomerase. It had been suggested that chromosomal end protection may occur as a result of specialized G–G interactions within the overhang (G quartets) (20,21) or through association with single-stranded telomeric binding proteins, such as those isolated from *Oxytricha*, *Euplotes*, and human cells (23–28). Furthermore, the 3′ extension is required for the formation of telomeric loops (t-loops), a specialized telomeric structure that has been observed in humans and mice (29), *Oxytricha* (30), *T. brucei* (20), and *Pisum sativum* (our laboratory, unpublished results) chromosomes and have been proposed to disguise and protect the telomeric terminus (Fig. 1A).

II. TELOMERIC CHROMATIN

The DNA of eukaryotic interphase chromatin and condensed metaphase chromosomes is packaged into nucleosomes consisting of 145 bp of DNA wrapped 1.75 times around an octamer of the four core histones, H2A, H2B, H3, and H4. Nucleosomes are spaced by linker DNA that varies from as little as ~15 bp in length to in some cases more than 70 bp, as in the chromatin of avian erythrocytes (31; reviewed in Ref. 32). Linker DNA is complexed by histone H1 and in avian erythrocytes by H1 and H5. The sum of the invariant core and variable spacer yields a repeat unit of 160–220 bp, which is organism- and tissue-specific. The nucleosomal chain is then believed to undergo further condensation into either a smooth solenoid (33) or possibly a zigzag arrangement (34). Segments of chromatin are thought to be attached to the nuclear matrix every 100 kb (roughly) at SAR and MAR elements, and in metaphase chromosomes, these segments may be organized as loops (35).

Telomeres from various organisms differ in their chromatin structure. Yeast and tetrahymena telomeres have not been observed in nucleosomal arrays (36,37). In yeast, whereas the subtelomeric region is packaged into nucleosomal arrays, the TG_{1-3} repeat tract is not (36, 38). Instead, it is arranged into nonnucleosomal particles containing between 245 and 395 bp of DNA (telosome).

Vertebrate and plant telomeres consisting of multikilobase strings of simple repeats are assembled into nucleosomal arrays, but the length of the repeat unit is usually shorter, and thus, by inference, the length of the linker DNA must be reduced. For example, when total rat chromatin is digested by micrococcal nuclease, a repeating ladder of DNA fragments (n × 200 bp) is observed by gel electrophoresis. However, when the same gel is probed for telomeric sequences, a

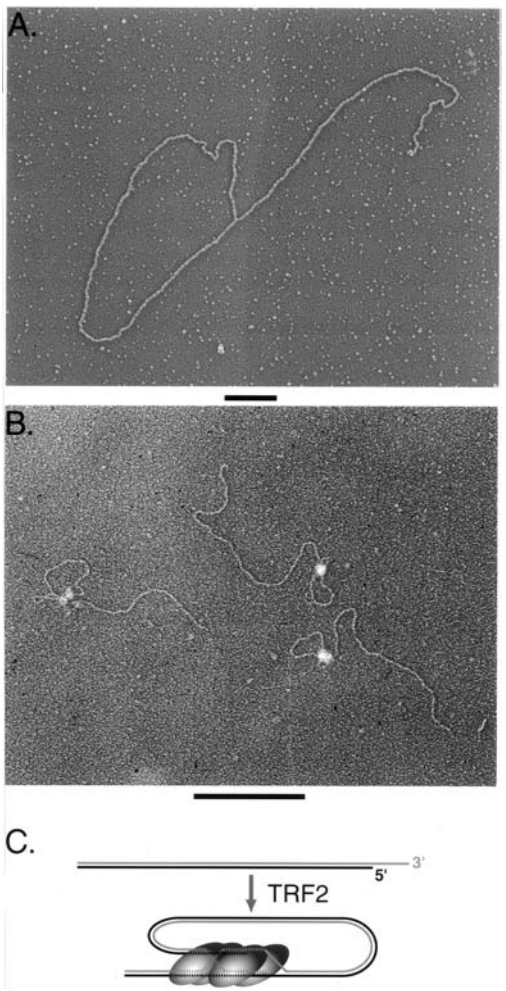

Figure 1 Telomeres are protected by specialized loops structures (t-loops). T-loops have been isolated from a variety of organisms, including human (A) and mouse cells, *Oxytricha nova*, and trypanosomes. The formation of these loops on model telomeres in vitro required the telomere-binding protein TRF2, which binds at the base of the loop (B). The loops are proposed to be formed by the insertion of the 3′ overhang into the duplex to form a d-loop (C). Bars are equivalent to 1 kb.

ladder of DNA fragments (n × 157 bp) is detected (39,40). This length (157 bp) would provide just enough DNA to form nucleosomal cores arrayed into a close-packed, possibly linear chain. Whether or not this indicates the absence of histone H1 is unclear, but in *Saccharomyces cerevisiae*, for which little evidence of H1 exists, a nucleosomal ladder with a repeat closer to 160 bp is seen. Indeed it has been suggested that H1 may be lacking in some organisms (40,41), and that the core histone protein, H4, is hypoacetylated in telomeric chromatin (14,42). Finally, in nuclease digestion experiments the telomeric chromatin is digested more rapidly than the bulk chromatin (43), but the exact cause for this hypersensitivity is not clear.

The higher order structure of telomeric chromatin may differ significantly from that of the bulk chromatin owing to the monotonous repeating nature of telomeric DNA (Table 2). Studies have shown that the free energy of nucleosome assembly for a given segment of DNA can vary significantly in a sequence-dependent manner (44). Certain sequences have been found strongly to inhibit nucleosome formation, whereas other sequences strongly encourage this formation. Such sequences tend to consist of monotonous tracts of simple repeats. Thus, DNA containing the repeating trinucleotide CTG that is expanded in myotonic dystrophy strongly favors nucleosome assembly (low free energy of association) (45), whereas DNA containing the repeating triplet CCG that expands in the fragile X syndrome strongly excludes nucleosomal assembly (high energy of association) (46). The underlying reason why repeating CTG tracts favor nucleosomal assembly has not been determined. However, experiments from this laboratory modeling different CCG-based sequences have demonstrated that the latter repeat has difficulty wrapping about a histone core owing to the unfavorable placement of major and minor grove wedges (47). Thus, what may be a minor difference in DNA flexibility becomes greatly amplified when such an element is repeated hundreds (or thousands) of times in these long repeat tracts—with the net result being

Table 2 Free Energy of Nucleosome Assembly for Various Repeated DNA Sequences

DNA sequence	ΔΔG, kcal/mol
Tetrahymena thermophila telomere $(TTGGGG)_{28}$	2
Saccharomyces cerevisiae telomere $(GGTGTGTG)_{20}$	1.65
Mammalian telomere $(TTAGGG)_{28}$	1.35
Arabidopsis thalliana telomere $(TTTAGGG)_{24}$	1.10
Mononucleosomal DNA	0
Xenopus borealis 5S rRNA gene	−1.75
Myotonic dystrophy repeat $(CTG)_{62}$	−2.80

Source: Refs. 44 and 46.

exhibited macroscopically as major differences in chromatin stability and/or structure.

These features of repeating DNA and nucleosomal assembly must play a role in chromatin assembly into long telomeric arrays. Repeating TTAGGG DNA shows a lower propensity to form nucleosomal arrays than almost any other sequence (48), and DNase I footprinting shows a loss of the normal rotational positioning of histone cores along the telomeric DNA (49). Thus, although nucleosomes clearly do form on telomeric DNA, the underlying DNA–histone core association is weaker than most genomic sequences, and as a result, the histone octamers would be expected to slide more readily along the telomeric DNA. Such sliding would be greatly facilitated by the lack of the sequence-dependent energy barriers normally present in genomic DNA. These effects are illustrated in Figure 2 in which we have shown

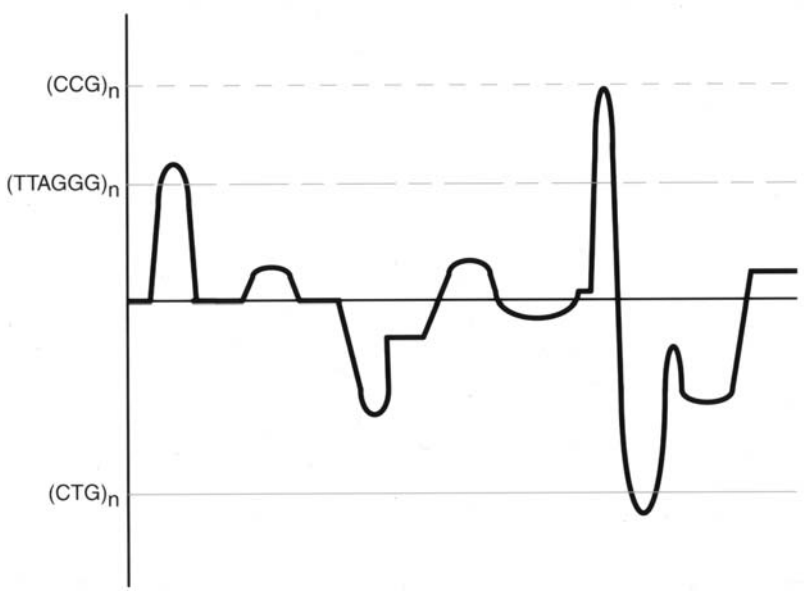

Figure 2 Hypothetical relative free energy of nucleosome assembly for DNA molecules. Assembly consists of repeating CTG, CCG repeats, for repeating telomeric DNA and for a hypothetical segment of genomic DNA (*solid black line*). The DNA–histone core association is weaker for TTAGGG and CCG repeat tracts than most genomic sequences, and as a result, the histone octamers would be expected to slide more readily along the telomeric DNA. Such sliding would be greatly facilitated by the lack of the sequence-dependent energy barriers normally present in genomic DNA. Nucleosomal sliding and hence close, dense packing may be inhibited by repeating CTG tracts. The net effect may be the formation of highly regular structures on the repeating CCG and TTAGGG tracts that may be quite different from what would be formed on bulk cellular chromatin.

the relative free energy of nucleosomal assembly for DNA molecules consisting of repeating CTG, CCG repeats, for repeating telomeric DNA, and for a hypothetical segment of genomic DNA. Nucleosomal sliding, and hence close, dense packing, may be inhibited in the case of repeating CTG and for genomic DNA (where there will be occasional low, free energy wells and tightly bound sequence-specific proteins presenting barriers to nucleosomal movement). Conversely, nucleosomes may move relatively freely on long segments of repeating CCG and telomeric DNA. The net effect therefore may be the formation of highly regular structures on the latter sequences that may be quite different from what would be formed on bulk cellular chromatin. Direct electron microscopic studies are underway in our laboratory using chromatin reconstitution and EM to examine this important structural issue.

III. CELLULAR LOCALIZATION OF TELOMERES

In 1984, chromosome ends were observed to localize to one end of the nucleus during mitotic prophase (50). Cytological methods including three-dimensional microscopy and in situ hybridization have been used to show that chromosome ends are in close proximity to the nuclear periphery (51–55). Nuclear fractionation approaches have revealed strong interactions between the telomeric DNA and the nuclear matrix (56,57), and although these interactions are maintained throughout interphase, it is not known if the telomeres are released during mitosis.

The telomeric DNA is anchored to the matrix by proteins. A study in *S. cerevisiae* proposed that yKu, a duplex DNA end-binding protein involved in double-stranded break repair, and two nuclear envelope–specific factors, M1p1p and M1p2p, are essential for this attachment (58). The relevance of this discovery to the human system is unclear, although recent data suggest hKu may play a role in telomeric structure and function (discussed below).

The protein (ATM) that is mutated in ataxia-telangiectasia (AT), a rare autosomal recessive disorder, functions in cell cycle control, telomeric maintenance, chromatin structure, and matrix association. Cells from individuals with AT have shortened telomeres (59), show aberrant telomeric clustering during mitotic metaphase (60–64), contain extrachromosomal telomeric fragments of 5–25 kb (65), and arrest in meiotic prophase (66–68). ATM, a PI3 kinase family member, shows homology to Tel1, which is involved in yeast telomeric maintenance (69). In cells from patients with AT, the telomere/matrix associations formed during meiosis are not properly released resulting in cell cycle arrest possibly due to an increased telomeric DNA/matrix association (70,71). These associations are dispersed by the introduction of a wild-type ATM gene into the AT cells, supporting a direct role of the ATM protein in the telomere/matrix structure (71).

Localization of telomeres has been closely examined in germ line cells where telomerase is active. The telomeres of these cells are approximately twice

the length of those in somatic cells (12,72). In premeiotic cells, mammalian telomeres are dispersed throughout the nucleus (56,73,74). Once cells enter the first meiotic prophase, the telomeres are attached to the nuclear periphery by a number of proteins (attachment plaques (75,76). These attachment sites are clustered to one spot on the envelope in a bouquet formation (74,77,78–80). In human sperm, these clusters are in close proximity to an increased number of nuclear pore complexes (81). Based on similar findings in yeast and plants, it is thought that this organization may enable proper chromosomal pairing (82–83).

Telomeric clustering corresponds to their localization to the nucleolus during meiosis. Specifically, during the zygotene stage, the nucleolus is located adjacent to the telomeric cluster (84). A truncated form of nucleolin has been found to bind double-stranded and single-stranded telomeric DNA in vitro (85). These findings may be functionally relevant, since in HeLa and mouse fibroblasts, telomerase RNA localizes to the nucleolus (reviewed in Ref. 86).

A variety of proteins have been shown to colocalize with telomeric DNA and the nuclear envelope. A complex of proteins containing a sperm-specific variant of histone H2B has been isolated from human sperm based on its ability to interact with telomeric DNA (87), and this variant has been observed to colocalize to the nuclear periphery. A 70-kD protein, suggested to be TRF2, has been identified in Rana oocytes as an integral component of the nuclear envelope (88). If it is proved to be TRF2, it will be one of the first telomeric DNA-binding factors shown directly to interact with the nuclear membrane.

IV. TELOMERE-ASSOCIATED PROTEINS: TELOMERIC DNA–BINDING FACTORS

Telomeric proteins can be divided into two main groups: those that bind directly to the telomeric DNA and those that do not bind telomeric DNA but rather bind indirectly through the former class. Both groups play a major role in the structure of the telomere and consequently in the regulation of telomeric function.

In human cells, there are two related duplex TTAGGG repeat binding factors, TRF1 and TRF2 (89–92) (Fig. 3). TRF1 (439 amino acids) and TRF2 (500 amino acids) are Myb-related nuclear proteins with both proteins containing a single Myb-like helix-turn-helix DNA-binding domain at their carboxy-termini. These domains are highly conserved (56% identity) (93). Myb repeats, usually found in pairs or triplets, are a common component of telomeric binding proteins (90–96). It is less common to find a single Myb domain, although other examples have been identified (97–99).

A centrally located dimerization domain is also conserved between TRF1 (Fig. 4A) and TRF2 (Fig. 4B). The TRFs form stable homodimers in solution but do not heterodimerize (91). Efficient binding requires a preformed dimer—and

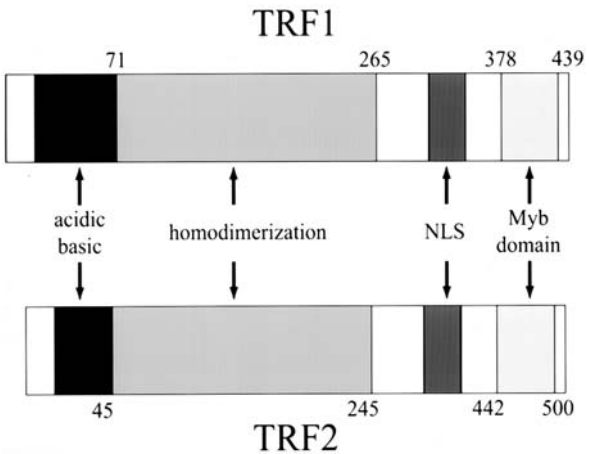

Figure 3 Schematic diagrams of the structures of hTRF1 and hTRF2.

therefore the presence of two Myb domains—to bind telomeric DNA (91, 100). Unlike the Myb and dimerization domains, the amino-terminal domains differ substantially, with TRF1 being enriched in acidic amino acids and TRF2 being enriched with basic amino acids. Owing in part to these amino-terminal domains, the predicted pI of the two TRF proteins are quite different (6.0 for TRF1 and 9.1 for TRF2). When compared with the mouse homologs, TRF1 shows a lower conservation (65% identity) than TRF2 (82% identity) (101). TRF1 and TRF2 display similar patterns of expression and localization. Both are expressed throughout the cell cycle at a fairly constant, low level and localize to the chromosome ends (55,90–92,102).

Both TRFs show a high degree of specificity for duplex TTAGGG repeats. Neither protein binds single-stranded telomeric DNA as seen by electrophoretic mobility shift assays (91,102). Each TRF1 monomer binds to the sequence YTAGGGTTR (90,103) and two such sites, each bound by the Myb domain of one of the monomers in the dimer, are required for stable binding (103). The affinity of TRF1 for telomeric DNA increases with increased tract length (102,104). TRF1 can coat extended TTAGGG tracts and pair coated tracts preferentially in a parallel orientation through protein–protein associations (104).

The relative location of the two telomeric half sites required for TRF1 binding is flexible. Bianchi et al. (103) have shown that the sites could be located up to 200 bp apart and in either orientation and still provide a stable binding site for a single TRF1 dimer. In this case, a loop in the DNA was observed with a TRF1 dimer at the base of the loop (103). The ability of TRF1 to associate two telomeric tracts and to bring together distant binding sites suggests a possible role in

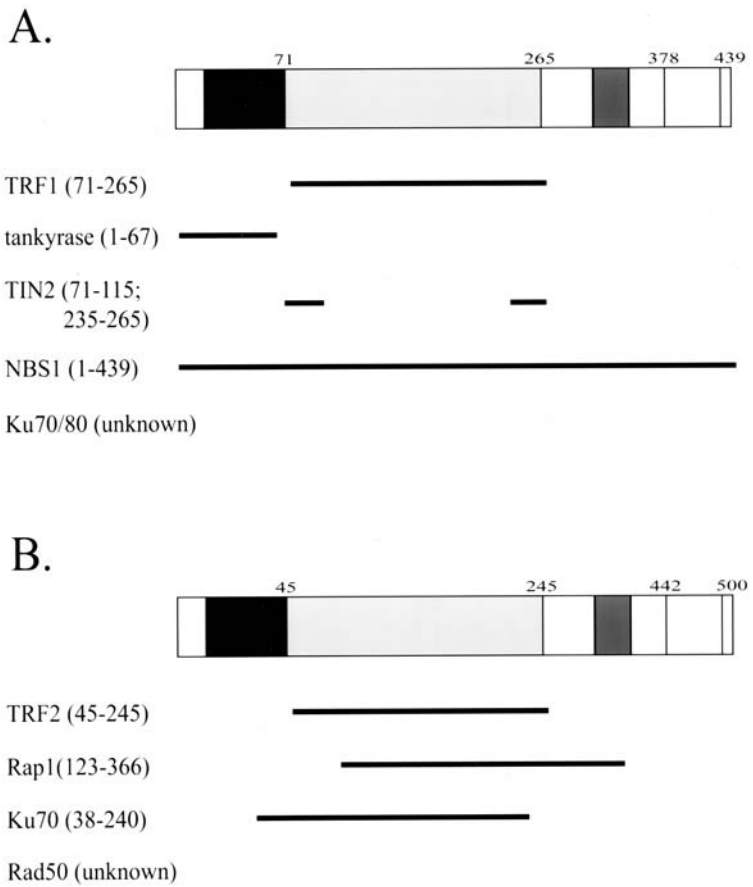

Figure 4 Schematic diagram of domains of interactions for hTRF1 and hTRF2. The solid lines indicate the portion/s of TRF1 (A) and TRF2 (B) required for interaction with the particular telomere associated factor.

telomeric organization such as bouquet formation and telomeric condensation. Owing to the high degree of identity between the DNA-binding domains of TRF1 and TRF2, it is thought that the minimum binding site of TRF2 is equivalent to that of TRF1, but this has not been proved experimentally. Whether or not TRF2 dimers are able to pair distant half sites has not been addressed. Further pairing properties of these proteins is discussed below (t-loops).

Recent evidence suggests that the DNA end–binding protein Ku can associate with mammalian telomere ends both in vivo and in vitro (105,106). Ku is a heterodimer of a 70-Kd and an 80-Kd subunit and was first identified as being in-

volved in V(D)J recombination and double-stranded break repair in mammalian systems (reviewed in refs. 107 and 108). In yeast, the absence of Ku results in a variety of telomere-related effects, including the alteration of telomeric clustering (discussed above) (109), critical telomeric shortening (110,111), disrupted telomeric silencing potentially through alterations of the telomeric chromatin (112–114), and elongation of the single-stranded overhang from 20 nt to 50–90 nt (115,116). The association of Ku with mammalian telomeres raises an interesting problem: specifically, how does the cell prevent telomere-associated Ku from functioning in a double-strand break repair pathway?

V. TELOMERE-ASSOCIATED PROTEINS: PROTEINS THAT BIND TELOMERIC DNA INDIRECTLY

In addition to the proteins that directly bind telomeric DNA, other proteins have been identified that associate with the telomere by binding the former factors, for example TRF1 (see Fig. 4A) or TRF2 (see Fig. 4B). To date, seven proteins have been shown to associate with telomeric DNA in this manner: TIN2 (also known as TINF2); tankyrase; Rap1; the Mre11 complex of proteins including Mre11, Rad50, and NBS1; and the Ku heterodimer.

A. TIN2/TINF2

A human TRF1–interacting nuclear protein, TIN2/TINF2, has been identified from a yeast two-hybrid screen as a unique TRF1-associated protein with no homology to known proteins (117) (Fig. 5A). The amino-terminus of TIN2 contains two adjacent basic domains followed by an acidic domain. These three regions may form α-helices. TIN2 and TRF1 interact via an ~80–amino acid domain within the carboxy-terminal half of TIN2 and a portion of the homodimerization domain of TRF1 (see Fig. 4A). This interaction is stable both in vivo and in vitro. TIN2 colocalizes with the telomeres as well as with TRF1 in vivo throughout the cell cycle and is expressed in a wide variety of tissue types. The colocalization of the proteins with the chromosome ends suggests that the DNA-binding activity of TRF1 is not affected by its interaction with TIN2. Since this interaction involves the dimerization domain of TRF1, it remains possible that the way in which TRF1 binds DNA at distant sites or its ability to synapse two telomeric tracts is altered by TIN2/TINF2 association.

B. Tankyrase

A second TRF1-interacting protein, tankyrase, was also has been identified through a yeast two-hybrid screen (118) (see Fig. 5B). Tankyrase, a 142-Kd

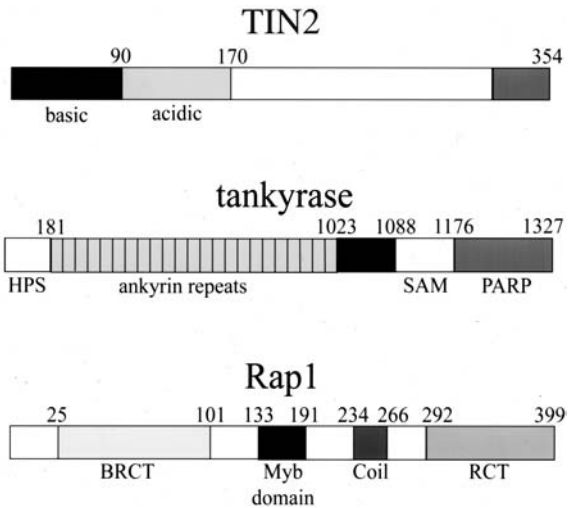

Figure 5 Schematic diagram of the structures of hTin2, tankyrase, and hRap1.

(1327–amino acid) protein, has homology to both ankyrins and poly-ADP ribose polymerase (PARP). Ankyrins are structural proteins involved in associating the cytoskeleton to integral membrane proteins via a repeated 33–amino acid motif (ankyrin repeat) (119). Twenty-four tandem ankyrin repeats are centrally located within tankyrase, but no other homology exists between tankyrase and other ankyrins. TRF1 and tankyrase associate via the amino-terminal acidic domain of TRF1 and the ankyrin repeats of tankyrase (see Fig. 4A). Owing to the involvement of the acidic domain of TRF1 in this association, TRF2 is not expected to form a complex with tankyrase. Consistent with this, yeast two-hybrid screens have failed to detect a TRF2/tankyrase interaction (120).

In addition to the homology with ankyrins, tankyrase contains a carboxy-terminal domain with homology to the catalytic domain of PARP (118). PARP catalyzes the addition of chains of ADP-ribose onto protein targets in response to DNA damage using nicotinamide adenine dinucleotide (NAD^+) as a substrate (reviewed in Refs. 121 and 122). The degree of homology is low (~30% identity), but all of the amino acids required to bind the NAD^+ substrate and to catalyze the addition of ADP-ribose polymers are conserved. Tankyrase is capable of self-ribosylation, as well as ribosylation of TRF1, but does not modify TRF2 (118). The modification of TRF1 results in an inhibition of TRF1 binding to telomeric DNA in vitro.

Tankyrase localization and expression differ from that of TRF1. The protein is expressed consistently throughout the cell cycle in a wide variety of cell lines (118,123). A portion of the cellular tankyrase always colocalizes with TRF1 and

therefore with telomeres (123). The remaining tankyrase associates with the nuclear pores and centromeres in a cell cycle–dependent manner. Throughout interphase, tankyrase resides at the nuclear pores and at the cytoplasmic side of the nuclear envelope at the Golgi apparatus (123,124).

When cells enter mitosis, tankyrase relocates to the centrosomes (123). Nuclear pore localization may reflect a structural function or may simply provide an available pool of tankyrase. Centrosomal localization raises the interesting possibility of involvement of tankyrase in meiotic telomere-induced bouquet formation. This possibility is strengthened by the finding of high levels of tankyrase expression in rat testis and therefore in meiotic prophase I (S. Smith, P. Morris, T. deLange, unpublished results).

C. hRAP1

hRap1 (see Fig. 5C) interacts with telomeric DNA through TRF2 (125). hRap1 is an ortholog of yRap1, the most abundant double-stranded telomere-binding protein in yeast (126–129). The orthologs share three domains in common: a 95–amino acid BRCT domain at the amino-terminus (130,131), a centrally located Myb domain (132), and a Rap1-specific protein–protein interacting domain at the carboxy-terminus. The main difference lies in the central Myb domains: whereas hRap1 has only one, yRap1 has two adjacent domains, which bind DNA directly. Although hRap1 appears to be capable of forming dimers through interactions between the carboxyl-terminal regions of the monomers, hRap1 does not bind telomeric DNA directly (125). hRap1 interacts with TRF2 through its carboxy-terminal domain (see Fig. 4B) (amino acids 123–366) (125), and hence would not be expected to interact with TRF1, which contains a very different C-terminal sequence. The stoichiometry of the two proteins bound to DNA has not been established. Examination of the intracellular localization of hRap1 reveals a punctate pattern that corresponds to the chromosome ends throughout the cell cycle in all cell types examined (125). Since DNA-associated TRF2 is required to observe this pattern, hRap1 is not thought to disrupt TRF2 binding to the TTAGGG repeat tract.

D. Mre11 Complex

The Mre11 complex is composed of three components, Mre11, Rad50, and NBS1, and plays a central role in the processing of double-stranded breaks (reviewed in Refs. 133 and 134). In vitro, Mre11 functions as a single-stranded (ss) and double-stranded (ds) nuclease with 3′–5′ directionality that can also cleave hairpin structures (135–139). Rad50 binds ds DNA, and, through hydrolysis of ATP, functions as a helicase to unwind DNA (140–143). NBS1 increases the nuclease activity of Mre11 and the helicase activity of Rad50 (142). The yeast Mre11 com-

plex is composed of Mre11, Rad50, XRS2 and is similar to the human Mre11 complex. The yeast complex is essential for telomeric maintenance, with a mutation in any of the components resulting in telomeric shortening (112,113,144).

A number of proteins, including TRF1 and TRF2, interact with the Mre11 complex, with interactions between the Mre11 complex and TRF2 being detected by nanoelectrospray polypeptide sequencing and coimmunoprecipitation (see Fig. 4B) (145). Based on the coimmunoprecipitation data, only a small fraction of the cellular Mre11 is associated with TRF2. Similarly, only a small fraction of TRF2 interacts with the Mre11 complex. This is reflected in the observation that only a small amount of the total cellular Mre11 and Rad50 colocalize with TRF1, and therefore the telomeres in vivo. Neither Mre11 nor Rad50 bind TRF1 directly (146).

NBS1 localizes to the telomere in a cell cycle–specific manner (145). During interphase, NBS1 is localized to the nucleoli, and only during DNA replication is it seen at the telomere. In meiotic chromosomes, NBS1 colocalizes with Mre11 and the telomere (147). The telomeric localization of NBS1 appears to be due to its association with TRF1 (146). This association is promoted by interaction between the carboxy-terminus domain of NBS1 and full-length TRF1 (see Fig. 4A). When associated with NBS1, TRF1 is still capable of binding telomeric DNA. Although the purpose of this complex is unclear, association with NBS1 does not eliminate the TTAGGG-binding properties of TRF1.

Several roles have been suggested for the Mre11 complex in telomeric maintenance. One popular idea is that the complex is required to generate the 3′ TTAGGG overhang at one or both chromosome ends. The finding that Mre11 is associated with TRF2, which is required for end protection (discussed below), lends some support to this possibility. The Mre11 complex might also play a role in regulating cellular life span (the "aging clock") by facilitating the regulated loss of telomeric repeats.

E. Ku70/80

Ku associates with mammalian telomeres indirectly through interactions with both TRF proteins (148,149). Surface plasmon resonance has revealed a stable, high-affinity interaction between TRF1 and the Ku heterodimer (149). TRF1 and Ku can be coimmunoprecipitated from cell lysates, supporting the presence of TRF1/Ku complexes in vivo. The interaction with Ku does not alter the DNA-binding activity of TRF1, since the proteins colocalize to telomeres of human cells (100,149). This indirect association of Ku with the telomere suggests a function for Ku distinct from its role in nonhomologous end joining, although the specific function is unclear. Both Ku and TRF1 have been shown to associate with the nuclear matrix, and thus Ku may function to anchor and localize the telomere to the matrix.

A yeast two-hybrid screen has been used to demonstrate that Ku70 associates specifically with TRF2 (148) via the central region of Ku70, including a Leu-Ser repeat and the central region of TRF2, including a large portion of the dimerization domain (amino acids 38–240) (see Fig. 4A). The Ku70-interacting domain of TRF2 overlaps substantially with the Rap1-interacting domain, raising the question of their competition for TRF2 association. The TRF2-interacting domain of Ku70 does not overlap with the known Ku80-associating region (150–152). The three proteins, Ku70, Ku80, and TRF2, are capable of forming a trimeric complex in vitro (148). It appears, therefore, that Ku80 and TRF2 do not compete for Ku70 binding. In vivo, however, the trimeric complex has not been isolated. Rather Ku70/Ku80 and Ku70/TRF2 complexes are observed. It may be that TRF2/Ku70 is no longer capable of associating with Ku80 once the complex is bound to the DNA, or that an additional factor may exist that binds to Ku70, thereby preventing Ku80 association. It has been shown that the level of matrix-associated Ku70 is higher than that of Ku80 (153), and therefore a trimeric complex may not be common owing to the low availability of Ku80 at the telomere.

VI. TELOMERIC TRACT LENGTH REGULATION IN HIGHER EUKARYOTES

Regulation of telomere length has been proposed to provide the means by which cells count the number of cell divisions from the time of their exit from germ line status. Some mechanism for cell division counting is demanded by the work of Hayflick and colleagues (154), who demonstrated that cultured human cells have a limited capacity to divide in culture. They further showed that cells taken from a young individual have the capacity to divide a greater number of times in culture than cells taken from an older person (155). When cultured human fibroblasts stop dividing, they enter a state termed *senescence* (Fig. 6). Senescence can be divided into two stages: mortality stage 1 (M1) and mortality stage 2 (M2) (156). M1 occurs when normal replication ends. If the genes involved in maintaining this checkpoint are mutated or blocked by introduction of viral oncogenes, the cells will continue to divide and proceed to crisis (M2). In culture, M2 is characterized by large-scale apoptosis. The observation that M1 and M2 can be prevented by introduction of telomerase (157–161) provides further evidence for a direct involvement of telomere length regulation in senescence and aging.

Such experiments led to the hypothesis of a cellular aging clock based on telomere length. This model was first proposed by Olovnikov (162). Whatever combination of factors (see above) leads to progressive telomeric shortening, it is presumed that below some critically short telomere tract length the telomere is no longer able to protect the chromosome end. At this point, events similar to what is observed when TRF2 function is ablated ensue, including chromosome fusions,

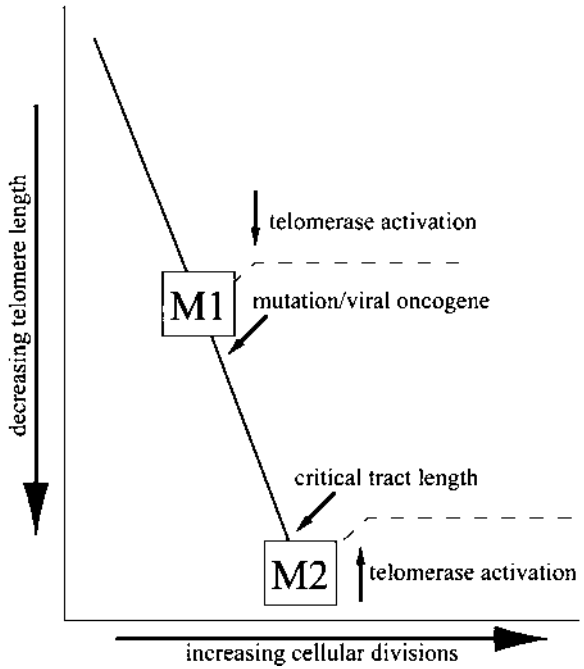

Figure 6 Hayflick's theory of the limited capability of cellular divisions. M1 occurs when normal replication ends. If the genes involved in maintaining this checkpoint are mutated or blocked by introduction of viral oncogenes, the cells will continue to divide and proceed to crisis (M2), which is characterized by large-scale apoptosis in the culture. M1 and M2 can be prevented by introduction of telomerase.

induction of p53, and apoptosis. Whether this is triggered by the passage of one chromosome in the cell past this "gate" or by an average shortening past this value is not known.

The existence of a telomere length "measuring stick" raises a fundamental structural problem. In human cells, entry into a nondividing state appears to occur when telomeres decrease in length to ~3 kb. This length of DNA could still accommodate a large number of proteins. Further, it is unclear why the length of the preceding telomeric DNA, be it 3 or 10 kb, would influence telomeric protection, were protection of the single-stranded overhang the critical factor. The answer may lie in part in the ability of long repeat tracts to sequester large numbers of the telomere-binding proteins, in particular TRF1 and TRF2, and further, as discussed in the following section, in the ability of telomeric tracts to assemble into a complex folded structure within the context of highly regular nucleosomal chains. In

this section, we review what is known about the effect on telomere length of altering the copy number or function of the major telomere-binding proteins in higher eukaryotic cells. It is not clear that an age/length–counting mechanism similar to that in human cells exists in yeast. Hence these last two sections will focus only on higher eukaryotic telomeres.

Studies in which the amounts of TRF1 or TRF2 are artificially increased in cells, or alternatively, in which their activities are greatly reduced, have been carried out by de Lange and her colleagues using two approaches. In one approach, viral vectors have been used to introduce additional copies of wild-type TRF1 or TRF2 into cultured cells. In a dominant negative approach, the fact that both proteins form highly stable homodimers was used to oblate TRF1 or TRF2 activity in cells. First, an inactive mutant of either protein was generated that lacked the Myb domain, and hence would not bind DNA. Introduction of this mutant in excess over wild-type protein is assumed to have driven most of the endogenous protein into mixed dimers with the inactive species. The homodimer, having only a single Myb domain, would thus be inert (100).

In these experiments, overexpression of TRF1 was found to cause progressive telomeric erosion at a very slow rate (3–11 bp per population doubling [PD]), whereas expression of a dominant negative TRF1 resulted in elongation of the tract at a faster rate (~35 bp per PD) to a new stable length (100). Telomerase activity was not affected in either case. These results suggest that TRF1 plays a role in telomere length regulation through some negative feedback mechanism.

The regulation of telomere length was suggested to occur in cis, with TRF1 affecting telomere length at each chromosome end (163,164). According to this model, long telomeric tracts recruit more molecules of TRF1 that will inhibit further extension by telomerase. As the telomere shortens, less TRF1 would be associated, and at some critical length telomerase would gain access to the telomere end and extend the telomeric tract, or in somatic cells, the cells would senesce. The number of TRF1 molecules bound to the tract could potentially serve as a mechanism of maintaining tract length and of recruiting telomerase. This type of mechanism is seen in yeast, where yRap1 may serve as the tract length–counting factor (165). It also remains possible that TRF1 affects telomere length by controlling the rate of telomeric erosion. The physical mechanism by which a higher density or greater number of TRF1 molecules along a telomeric tract could function to sequester the telomere end from telomerase is not described in these models.

TRF2 can also negatively regulate telomeric tract length (166). Overexpression of TRF2 results in telomeric erosion at a relatively fast rate (47–87 bp/PD). TRF2 accumulates on the telomere in a length-dependent manner, suggesting a role in the negative feedback loop of telomere length regulation.

TRF2 has a direct role in protecting chromosome ends that may strongly influence the regulation by controlling access to the telomere end. In mammalian systems, a telomeric looped structure (t-loop), formed between the duplex tract

and the single-stranded overhang, serves to protect the telomere end (discussed below) (29). In studies using a dominant negative allele of TRF2, TRF2 has been shown to be involved in telomere end protection (167). When expressed in cells, end-to-end chromosome fusions have been observed. At the fusion site, telomeric repeats have been observed, suggesting that the loss of end protection, and therefore chromosomal stability, is not due simply to loss of the telomeric DNA. Instead, the chromosomes lost the G-rich 3′ overhang. Finally, while some cells entered senescence, others underwent p53/ATM–dependent apoptosis (168). Together, these results support a role for TRF2 in the formation of a specialized structure at the telomere terminus (discussed below). In addition, they raise the possibility that apoptosis or senescence can be signaled by a lack of TRF2 binding to the telomere that might occur owing to shortened telomeres.

The ability of TRF1 negatively to regulate telomere length is mediated through interaction with TIN2 and potentially with tankyrase (117,169). Overexpression of wild type in TIN2 does not alter the length of the telomeric tract substantially, although a slight decrease is observed. Expression of mutants of TIN2, which retain the TRF1-interacting domain but lack either the C-terminal or N-terminal domain, result in an increase in tract length from 3 kb to more than 15 kb even though telomerase levels are not affected. These results lead to the conclusion that TIN2 modulates telomere length through an indirect inhibition of telomerase. Since TIN2 does not displace TRF1 upon association, TRF1 alone may not control telomere length. Instead, TRF1 may function to recruit TIN2, which is then able to modulate tract length.

VII. TELOMERE END PROTECTION AND HIGHER ORDER ARCHITECTURE

Several fundamental questions regarding telomeric function and telomeric DNA–binding proteins have been addressed in the studies reviewed above. The central questions of how the cell protects the telomere end and how the cell is able to measure the length of the telomeric tract remain fundamentally unresolved. In this section, we will describe recent work on end-binding proteins as well as the discovery and potential biological significance of t-loops.

Any current model of telomeric protection must take into account the work of de Lange and colleagues (167), who showed that abrogation of TRF2 function results in a series of changes typical of what would be expected were the telomere end no longer protected. Based on these results, one could argue that TRF2 functions to bind and protect the G-strand overhang. However, unpublished data from this and the de Lange laboratory have shown that TRF2 does not bind single-stranded telomeric DNA. Thus, an alternate explanation is required.

Elegant studies conducted by several groups (24,170) have described the mechanism by which the ends of the short multicopy DNA fragments in the

macronuclei of *Oxytricha nova* are protected. A protein has been isolated that has led to a model of telomere end protection based on a single protein species that would bind and sequester the single-strand overhang. Until recently, few examples of single-stranded telomere overhang binding proteins outside of *Oxytricha* were known. One such example was found in *Candida parapsilosis* (171), where a single-stranded binding protein was shown to bind a single-stranded overhang of linear mitochondrial DNA. Recently, a single-stranded telomere-binding protein termed *Pot1* (protection of telomeres 1) was isolated and characterized in fission yeast and human cells (28). Pot1 has high affinity for single-stranded DNA with the sequence motif of *Schizosaccharomyces pombe* telomeric repeat or the G-strand overhang in mammalian telomeres. It has been proposed that Pot1 might serve to protect the telomere end from degradation and regulate the ability of telomerase to elongate the telomere. Whether this protein interacts with the other known telomere-binding proteins is presently unclear, and how elimination of TRF2 function may alter Pot1 activity has not been suggested. Another mechanism of end protection, including the formation of G-quartets through a fold-back structure of the G-rich strand, has been proposed (20,21). No evidence for G-quartet formation has been provided, and, as above, no relation to TRF2 function has been suggested.

The two conserved features of nearly all eukaryotic telomeres are a long array of repeating six to eight nucleotide units and a single-stranded overhang on one strand. One facet of this structure that has not been discussed in detail is the highly recombinogenic nature of such a DNA template. Studies of homologous recombination catalyzed by RecA protein have shown that a single-stranded DNA terminating in a 3′ hydroxyl is highly recombinogenic when presented with a homologous duplex DNA, and that such a single-stranded tail can find its homologous duplex partner rapidly even in the presence of a 100,000-fold excess of nonhomologous DNA (172). Given this, DNA in the form of a telomere would be expected rapidly to undergo self-recombination in the presence of proteins having activities like those of RecA and Rad51. During this event, the 3′ single-stranded tail would invade the internal duplex segment, which is fully homologous to the tail throughout its length. Consideration of these facts led us to propose that mammalian telomeres might be arranged as giant loops in which the single-stranded overhang would invade the duplex segment somewhere along its length. The biological consequences of this novel structure would be that it could provide a means of hiding the telomere terminus within the internal repeat segments. Such structural folding also provides additional interesting biological properties. In collaboration between our group and the de Lange laboratory, two means of testing this hypothesis have been developed.

In one approach to test this model, a slipped structure polymerase chain reaction (PCR) method (173) was used to generate a model template containing several kilobases of duplex TTAGGG repeats on the end of a plasmid DNA. The 5′ ends were resected to create a DNA with the essential features of a mammalian telomere: long duplex repeats and a G-strand 3′ overhang. The effects of the dom-

inant negative TRF2 allele in cells (discussed above) suggest that TRF2 might be involved in the formation of a looped structure that protects the extreme telomere end. Incubation of the model telomeric DNA with purified TRF2 protein led to the observation of DNA molecules arranged into loops, with TRF2 protein at the loop junction (29) (see Fig. 1B). These looped structures were present at high frequency, but only in the presence of TRF2; neither TRF1 nor tankyrase showed any looping activity. The DNA loops were stabilized by covalent cross linking at A/T steps in the DNA using psoralen and ultraviolet (UV) light, providing evidence that the G-rich overhang had undergone strand invasion into the preceding duplex DNA to form a d-loop structure (Fig. 1C). In these studies, TRF2 localized exclusively to the base of the loop (t-loop junction). It remains unclear how TRF2 may function to form these loops; however, detailed analysis has clarified some of the DNA features required for TRF2-mediated t-loop formation. Specifically, a 3′ G-strand overhang and the natural junction between the single-strand and double-strand repeats are required for loop formation (173).

Direct isolation of telomeric looped molecules from human and mouse cells was also undertaken. Beginning with 3×10^8 HeLa cells, nuclei were treated with psoralen and UV to stabilize the t-loop structures. The t-loops were then purified by digesting the genomic DNA with four base restriction enzymes and size fractionating the large telomeric fragments away from the small digested genomic fragments using gel filtration. Examination by electron microscopy revealed DNA molecules greater than 10 kb in length, and in several preparations, 30% of these DNA molecules were present as looped structures (see Fig. 1A). The loops were frequently 15–20 kb in size when the DNA was isolated from mouse cells or from a HeLa strain with long telomeres. Key findings in this study (29) were the following: (a) the t-loop molecules were present in human cultured and primary cells and mouse liver cells in abundance; (b) the looped molecules were formed from telomeric DNA, as shown by their binding TRF1 protein; (c) a single-stranded segment, consistent with a d-loop at the loop junction, could be observed; (d) the size of the t-loop molecules was in good agreement with the known size of the telomeric DNA isolated from the same cells, as determined by Southern blotting; and (e) to observe the t-loops, the telomeric DNA had to be cross linked in the form of chromatin in situ.

Recently, t-loops have been found to be present not only at human and mouse telomeres, but also at the termini of the micronuclear chromosomes of *Oxytricha nova* (30) and at the telomeres of *Trypanosoma brucei* minichromosomes (20). Further, telomeres in *Saccharomyces cerevisiae* appear to form folded-back structures through protein–protein interactions (174–177). Thus, telomeric looping may be a common theme in telomeric architecture.

The discovery that telomeres are arranged into large duplex loops offers insights into telomeric function. For example, the ability of the cell to distinguish between telomere ends and double-strand breaks has never been explained. Ending chromosomal DNA in loops would provide a simple means of disguising them from the double-strand break recognition and repair factors. Loss of TRF2 had

been shown to cause loss of the 3′ overhang and apoptosis (167,168). The t-loop model suggests that loss of the overhang may be due to the inability to protect the telomere terminus in a t-loop structure without being TRF2 present.

VIII. REPLICATION INITIATION FROM A CLEAVED T-LOOP JUNCTION: PLACING AN ORIGIN AT THE END

If an origin of replication in the middle of a chromosome fails to fire, or if replication from that origin stalls, bidirectional forks moving in from the left and right should complete the synthesis of both strands. On the other hand, if an origin close to a telomere fails to fire or stalls, then the chromosome may not replicate to its very end and will be lost at metaphase. Thus, mechanisms for ensuring that chromosomes replicate to their full ends may exist. The t-loop formation provides a possible means for initiating replication within the telomere (Fig. 7). Once the G-strand overhang has strand invaded to form a d-loop, this structure could be cleaved by Holliday junction-resolving enzymes. This would result in the loss of the TTAGGG overhang from the end of the telomere and would leave

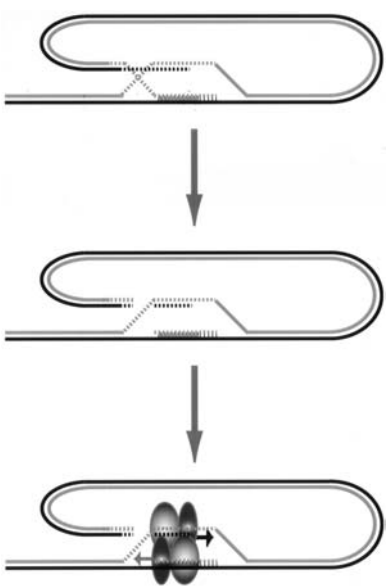

Figure 7 Initiation of replication from a t-loop. A t-loop structure may serve as a site for initiation of replication following cleavage by Holliday junction-resolving enzymes. These enzymes may cleave the d-loop resulting in the loss of the TTAGGG overhang from the end of the telomere. Replication primers would be left annealed to the C-rich and G-rich strands and may allow replication of the telomeric DNA.

behind a replication primer annealed to the C-rich strand. Recent data from this laboratory suggest that t-loops formed in vitro by TRF2 may involve not only the G-strand overhang, but also a portion of the C-rich strand as well (173). Hence, cleavage would leave primers on both strands. Bidirectional replication also could result from priming on the G-rich strand once synthesis on the other strand had begun. Such a cleavage event also could provide an alternative mechanism for progressive telomeric shortening by increments of ~150 nt per replication cycle. Although this model has not been suggested for telomeres, it has a general parallel in the late phase of bacteriophage lambda and T4 replication (178–180).

IX. T-LOOP–DEPENDENT PATHWAY OF TELOMERIC ELONGATION

Alternative pathways not involving telomerase exist for telomeric maintenance and may utilize enzymes involved in other pathways of DNA metabolism (7). Once a t-loop junction has formed, simple polymerase/helicase action could result in telomeric elongation (Fig. 8). Here the 3′ end is extended by DNA polymerase with

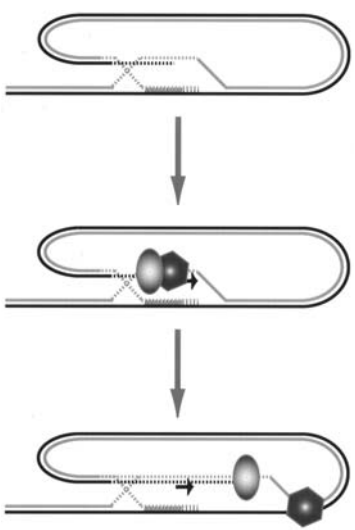

Figure 8 T-loop–dependent telomeric elongation. The extension of the telomere would not require cleavage of the G-strand overhang. Instead, DNA polymerase (*circle*) may use the inserted 3′ end to elongate the telomeric tract. A helicase (*hexagon*) would be required to unwind the duplex ahead of the DNA polymerase. This reaction would be expected to increase the length of the telomeric tract by the size of the t-loop.

a helicase unwinding the duplex ahead of the polymerase. These reactions would be similar to those above except that the G-strand overhang would not be cleaved off. This is a self-templating reaction that could occur with any repeated DNA having a 3′ ssDNA extension. If evidence for such reactions can be obtained, it would provide an important mechanistic insight into how alternative pathways of telomeric maintenance may occur in vivo and what proteins could be involved.

X. COMPACTION OF THE T-LOOP INTO A REGULAR PARTICLE AS A MEANS OF MEASURING TELOMERE LENGTH

A central unanswered question remains: How does the cell determine that its telomeric DNA has shortened to 2–3 kb, a point at which it activates senescence pathways? Structurally, this can be restated by asking how the cell can measure the difference between a segment of telomeric DNA that is, for example, 3 kb from one that is 6 kb? Such a mechanism may reflect t-loop formation and the unusual structure of telomeric chromatin. The latter is based on the assumption that telomeric DNA is condensed into a nucleosomal array that is highly regular and possibly more compact than normal genomic sequences owing to the properties of repeating TTAGGG DNA as discussed in the first section of this chapter.

Previous work on SV40 minichromosomes may provide a useful analogy for a chromatin structure–based counting mechanism. In the minichromosome, the 5-kb viral DNA is assembled into a chain of 21 nucleosomes by cellular histones (181). Further compaction by histone H1 or other binding proteins forms a compact chromatin particle about which the viral shell forms. In the SV40 minichromosome, the viral origin of replication remains nucleosome free but is bound by T antigen. We suggest that the origin lies sequestered at the center of the viral core particle surrounded and protected by nucleosomes until the appropriate time for the core particle to open and allow entry of replication/transcription factors. For the telomere analogy, it becomes important to know how small the nucleosomal circle could be and still sequester the origin within a compact particle. Simple ball and string modeling suggests that 12–15 nucleosomes would be required to form a shell around one segment of the circular DNA. Translated into base pairs, this suggests a length of at least two and no more than 3 kb of DNA.

Application of the SV40 model to telomeres provides a possible mechanism for measuring a critical telomere length. In this model, the G-strand overhang would be protected from nucleases and double-stranded break recognition factors by the formation of a t-loop with the DNA end hidden within the t-loop junction, and further by the assembly of the telomeric DNA into nucleosomes and compaction by TRF1 to generate a dense particle with the t-loop junction sequestered

at its center. We presume that TRF2 would remain bound to the d-loop region, possibly along with Pot1 and Ku.

If such a compact telomeric particle is formed, once the telomeric DNA shortens to a critical length of 2–3 kb, then this length may be too small to provide a sufficient number of nucleosomes adequately to surround the d-loop. Exposed it would then be recognized by a variety of proteins, which could signal an exposed telomere end to the cell. These might include p53, which binds avidly to Holliday junctions (182), the Mre11 complex, and other nucleases. Presumably, during S phase, the t-loop structure must transiently open to allow the replication proteins to proceed through. In addition, there must be some mechanism for reforming the loop and compact particle. The features of such a model that distinguish between senescence and apoptosis are not clear. Entry into senescence is a normal event, resulting from progressive telomeric shortening. This may be triggered by the first few telomeres that reach the critically short length and begin the signaling process. If the cells are forced to divide further by, for example, the expression of SV40 T antigen, then the presence of additional short telomeres may override the senescence signal and induce apoptosis.

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6
Role of Ku at the Telomere

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I. INTRODUCTION

Ku, originally identified as the autoantigen associated with systemic lupus erythematosus (1), is an abundant nuclear protein with fascinating multifunctionality. Ku exists primarily in a heterodimer form composed of ~70 and ~86 kD subunits (referred to as Ku70 and Ku80), which can also form into a larger functional complex, termed the *DNA-dependent protein kinase* (DNA-PK) complex. In addition to Ku, the DNA-PK complex contains the 470-kD DNA-dependent protein kinase catalytic subunit (DNA-PKcs) (2,3).

Surprisingly, Ku—along with several other proteins that play critical roles in double-stranded break repair through the nonhomologous end-joining (NHEJ) pathway or during site-specific recombination of V(D)J gene segments—recently has been shown to play additional roles in capping telomeres or preventing chromosome end fusions (4–10). Therefore, Ku can function in seemingly opposite ways—either joining DNA ends or preventing telomeric fusions—depending on its nuclear context or microenvironment. Ku also has been reported to play critical roles during transcriptional regulation and in modulation of chromatin structure (11–17).

This chapter will focus primarily on the role of Ku at the telomere. Current understanding of the role Ku plays at the telomere has come mainly from yeast and mammalian studies. Ku has been shown to function in telomeric capping (or in preventing telomere end fusions), telomeric DNA length control, and telomeric silencing. Ku is known to interact with certain telomeric proteins in yeast and mammals (see details below), although the exact role that Ku plays at the telomere and how it localizes to the telomere are not presently known. Importantly, despite the

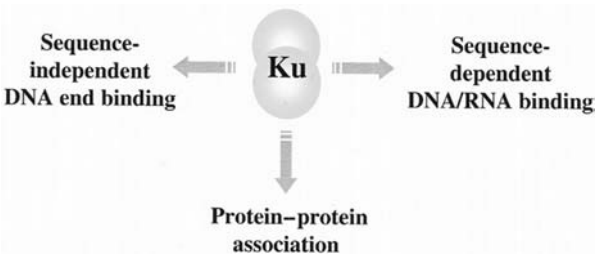

Figure 1 Ku has been observed to form three different types of intermolecular contacts:s (a) sequence-independent DNA end binding, (b) sequence-dependent DNA/RNA binding, and (c) protein–protein associations. These interactions are likely important for its functional role in the cell.

well-known binding affinity of Ku for DNA termini, there is currently no evidence that Ku binds directly to the ends of telomeric DNA in a sequence-independent manner. Furthermore, mammalian Ku does not bind directly to internal regions of telomeric DNA in a sequence-dependent manner (8). It is likely that the telomere is a dynamic structure with various telomeric components forming many different intermolecular interactions, depending on the cell cycle, age of the organism, and other biological conditions. This chapter will explore possible roles for Ku in telomeric maintenance and several possible intermolecular contacts that may facilitate its function.

Before focusing on the telomere, a brief description of Ku protein's intermolecular contacts during other important cellular processes is provided. These background sections are not intended to provide the reader with a complete review of Ku, but are meant to inform the reader of the capabilities and potentials of Ku and provide an understanding of how Ku might function at the telomere. For a more comprehensive summary of Ku, the reader is referred to several excellent reviews (3,18).

Remarkably, three basic types of intermolecular interactions have been assigned to the Ku heterodimer: (a) sequence-independent DNA end binding, (b) sequence-dependent DNA/RNA binding to internal regions of nucleic acids, and (c) protein–protein interactions (Fig. 1). Understanding the basic molecular associations that Ku forms at the telomere is required for a continued understanding of the basic actions of Ku at the telomere.

II. KU BINDS DNA ENDS IN A SEQUENCE-INDEPENDENT MANNER

Analyses of x-ray–sensitive mutant rodent cell lines or of mice deficient for Ku reveal its requirement for joining DNA double-strand breaks via the NHEJ path-

way and during V(D)J recombination (15,19–23). NHEJ functions to repair DNA double-strand breaks created throughout the genome by exogenous and endogenous DNA-damaging agents. NHEJ does not require sequence homologies between the two recombining DNA ends. V(D)J recombination is the recombinational joining of gene segments to form functional immunoglobulin (Ig) and T-cell receptor (TCR) genes during the development of lymphoid cells. V(D)J recombination involves cleavage of double-stranded DNA between coding sequences and neighboring recombination signal sequences. Recombination of these DNA segments after modification of their ends permits the assembly of an immense variety of immunoglobulins and TCR genes. Therefore, the NHEJ and V(D)J recombination processes involve the joining of double-stranded DNA ends composed of immense DNA sequence variation (2,16,24).

The Ku heterodimer exhibits intriguing in vitro sequence-independent or nonspecific, double-stranded DNA end-binding properties that most likely play an important role in the recognition and joining of cleaved nonhomologous DNA ends in NHEJ and V(D)J recombination. The dissociation constant for the end-binding activity of Ku is reported to be between $1.5–4.0 \times 10^{-10}$/M (25,26). The Ku heterodimer not only binds directly to double-stranded DNA ends, but also binds with high affinity to single-stranded nicks and gaps in a sequence-independent manner (25,27,28). Additionally, when bound at a DNA end, Ku is capable of translocating to internal DNA positions in an energy-independent manner (27,29,30). Biochemical studies of the DNA-PKcs and Ku components of the DSB repair pathway have revealed that the Ku heterodimer is required for the stable interaction of the DNA-PK catalytic subunit (DNA-PKcs) with DNA ends and for kinase activation (31,32). The Ku heterodimer is believed to target DNA-PKcs to DNA double-stranded breaks in vivo, and the resulting activation of serine/threonine kinase activity of DNA-PKcs likely initiates critical aspects of the DSB joining process in mammalian cells (2,23). However, essentially nothing is known about the specific function of Ku within the cell. Understanding the role the Ku protein plays in vivo during the joining of double-stranded DNA ends is an active and important area of current research.

III. KU BINDS TO DNA AND RNA IN A SEQUENCE-SPECIFIC MANNER

In addition to DNA sequence-independent end binding, many groups have reported the binding of Ku to internal regions of DNA in a sequence-dependent manner (18). Several reports show a DNA sequence–specific binding activity of Ku to upstream promoter elements during the regulation of gene expression. For example, it has been reported that Ku functions to repress glucocorticoid-induced MMTV transcription by binding to negative regulatory element 1 (NRE1) (11,33). The latter study was the first to use covalently closed microcircles in elec-

trophoretic mobility shift assays (EMSAs) to test Ku for sequence-specific binding in vitro. This methodology was a major advance in the study of Ku protein's sequence-dependent DNA binding, allowing Ku protein's sequence-independent end-binding activity to be experimentally separated from its sequence-specific binding. Ku binds with nearly the same affinity to the internal NRE1 binding site ($K_d = 0.84 \times 10^{-9}$/M) as it does to DNA ends via its sequence-independent DNA end-binding activity (11). Additionally, it has been reported that Ku70 binds to the enhancer region of the T-cell receptor beta-chain gene (34) and is a negative regulator of heat shock protein 70 (hsp70) gene expression (35,36). Since Ku possesses a high-affinity, nonspecific DNA end-binding activity, it is essential that special experimental care be taken to determine whether any putative interaction with a specific nucleic acid sequence or structure is truly direct or is a DNA mediated or bridge associated.

Yet another functional role for Ku involving its sequence-specific DNA-binding activity was identified by the discovery that Ku binds with high affinity to matrix attachment regions (MARs) contained within DNA microcircles (13). MARs are DNA sequences located at the base of chromatin loops, which are thought to attach to the nuclear matrix (37). Base unpairing regions are found within MARs and are thought to contribute to the formation of the chromatin loop domain structures (37). Base unpairing region affinity chromatography has been used to copurify poly(ADP-ribose) polymerase, Ku, and DNA-PKcs from a nuclear cell extract (13). Therefore, Ku may play a role in the formation of loop domain structures of chromatin, thereby participating in the modulation of chromatin structure.

Similar to the ability of Ku to bind specifically and nonspecifically to DNA, it also has been reported to bind directly to certain RNA sequences (18). One reported example of this is the specific association of Ku to the human immunodeficiency virus (HIV) RNA transactivation response element (38). Additionally, using SELEX (systematic evolution of ligands by exponential enrichment) technology, an in vitro study has been initiated to identify specific RNA sites that bind Ku with high affinity (18). Three classes of Ku-RNA–binding sites have been found, with most sites binding Ku with affinities less than or equal to 2×10^{-9} M. Recently, Ku was reported to have a possible genetic link with the yeast telomerase RNA (TLC) (39) (discussed in more detail below), but whether Ku physically binds directly to the yeast telomerase RNA has yet to be determined (39). Finally, although Ku can clearly bind with high affinity to either DNA or RNA in a site-specific manner, the biological significance of these interactions requires further study.

IV. KU FORMS PROTEIN–PROTEIN INTERACTIONS

Ku has been reported to form many different protein–protein interactions. As discussed above, Ku forms a complex with the DNA-dependent protein kinase cat-

alytic subunit (2). Additionally, Ku has been reported to interact with the Werner syndrome protein (40,41), the heterochromatin protein 1 alpha (42), the XRCC4/Ligase IV complex (43), terminal deoxynucleotidyl transferase (44), the proto-oncogene p95vav (45), poly(ADP-ribose) polymerase (13), and several other proteins (2,46,47). In addition, the only reported intermolecular Ku protein interactions with the telomere occur through the protein–protein complexes of the telomere protein, Sir4, in yeast and the telomeric DNA-binding protein, TRF1, in mammals (8,48) (see below for more details). It is important to reiterate that owing to the high-affinity, nonspecific end-binding activity of Ku, differentiating between DNA-mediated interactions and true protein–protein interactions requires special experimental care.

V. TELOMERES AND TELOMERASE

Telomeres, composed of repetitive DNA sequences bound by telomeric protein complexes, protect the chromosome termini from fusion events and promote chromosomal end replication (49). Telomeric DNA is synthesized by telomerase, a specialized ribonucleoprotein reverse transcriptase. Known essential core components of telomerase include the telomerase RNA (containing a short template sequence that is copied into telomeric DNA) and a protein reverse transcriptase subunit (TERT) (50–52). A minimal telomeric DNA length, and in some situations an active telomerase, are required for chromosomal stability and cellular viability (53,54). Telomeric maintenance requires a homeostatic balance between addition of telomeric sequences by telomerase, repression of telomerase, and persistent capping activity by telomeric proteins and the formation of structures such as the t-loop (48,49,55). Failure to maintain telomere length or function can lead to a form of replicative senescence in ciliates, yeast, and mammalian cells (50,56–58). Most differentiated human somatic cells lack telomerase activity, and activation of telomerase is characteristic of most established human cell lines and tumors (59). Correspondingly, experimental activation of telomerase allows certain virus-transformed human cell lines to bypass normal cellular replicative senescence and crises and continue proliferation (53,58).

The well-known capacity of Ku for DNA binding, either sequence independent or dependent, suggests that Ku might bind directly to telomeric DNA (see Figs. 2C and 3C). It was initially hypothesized that Ku may bind telomeric DNA ends sequence independently, or bind to internal regions of telomeric repeats through its reported sequence-dependent mode, or both. At present, however, there is no clear evidence that Ku binds directly to telomeric DNA, and Ku does not specifically recognize internal regions of vertebrate telomeric DNA—$(T_2AG_3)_n$ repeats—within covalently closed microcircular DNA (8). Indeed, it is important to consider that the function of Ku at the telomere is likely very dis-

tinct—possibly in direct opposition—from its action in joining DNA ends, since the critical functional role of telomeres is to cap or prevent telomere end fusions (8,10,60,61). Some fascinating insights into the role of Ku at the telomere have recently been discovered, which will be discussed in the remaining sections of this chapter.

VI. KU AT THE YEAST TELOMERE

Several lines of evidence have converged over the last several years strongly supporting the conclusion that the Ku heterodimer plays a critical role in yeast telomeric maintenance. Initially, two groups working with *Saccharomyces cerevisiae* found that nulls for either yKu70 or yKu80 had abnormally short telomeres (4–6,62). To identify components that interact with Ku, a two-hybrid screen for factors that interact with yKu70 was used, and the SIR4 protein was identified (63). The SIR4 protein is known to be important for telomeric silencing, a chromosomal position effect in which genes adjacent to yeast telomeres are repressed similar to the repression associated with heterochromatin in higher eukaryotic cells (64). In agreement with the involvement of Ku in *S. cerevisiae* telomeric silencing, yKu70 nulls are defective in telomeric silencing (6,65,66). In contrast, recent studies have revealed that Ku plays an important role in telomeric maintenance in *Schizosaccharomyces pombe* (67,68), yet is not required for telomeric silencing (69). Direct evidence that Ku is physically localized to telomeres was initially provided by in vivo cross-linking experiments using chromatin immunoprecipitation (CHIP) (70). Studies during the last 10 years have suggested that Rap1 and SIR4 interact directly at the telomere in yeast (48,71). Rap1 is a multifunctional protein, which in addition to binding yeast upstream-activating sequences, binds yeast telomeric DNA directly in a sequence-depedent manner and plays an important role in telomeric silencing (72). Therefore, it has been hypothesized that a Ku/SIR4 complex could localize to the telomere by an interaction via Rap1. However, Ku localizes to telomeres in a SIR4-independent manner, indicating that SIR4 is not a direct protein bridge for Ku localization to the telomere (Fig. 2A) (73). In addition, there is no evidence that Ku and Rap1 interact directly. CHIP experiments have demonstrated that Ku is in close proximity to the telomere, but whether Ku binds directly to yeast telomeric DNA cannot be determined by this method. The possibility clearly exists that yKu localizes to the telomere via a telomere protein component (Fig. 2B).

Cellular studies using *S. cerevisiae* have provided evidence that Ku localizes to telomeric foci near the nuclear periphery, and that Ku80 colocalizes with the telomere-binding protein Rap1 at these telomeric foci (73,74). Similar experiments in *S. pombe* and mammals have not been possible owing to the large abundance and uniform distribution of Ku throughout their nucleus (69) (our unpub-

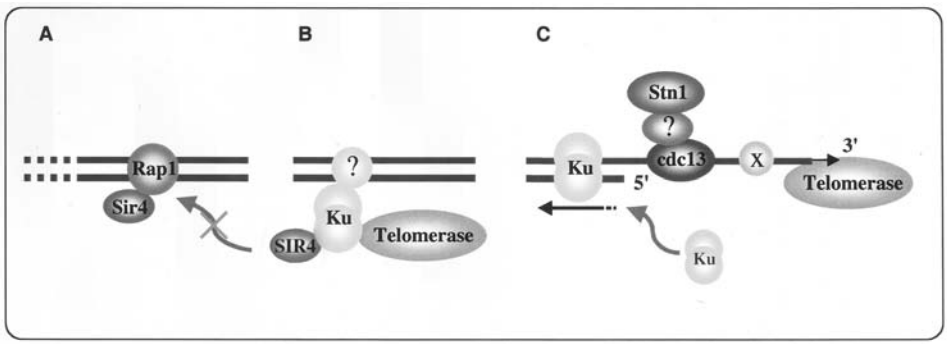

Figure 2 Model of Ku at the yeast telomere. This schematic does not attempt to show all known telomeric components at the yeast telomere but rather centers on proteins related to the Ku protein's function at the telomere. Telomeric DNA is shown as lines. (A) Rap1 and Sir4 may form a complex at the telomere. (B) Model suggesting that yKu might localize to the telomere via an interaction with an unidentified protein (denoted by a question mark) that binds directly to telomeric DNA. (Ku has been reported to interact with Sir4 (Refs. 48 and 71). Ku localization to the telomere is not bridged directly through Sir4 (Ref. 71)). (C) Ku may localize to the telomere by binding directly to the double-stranded DNA end, although direct telomeric DNA end binding of Ku might not be possible owing to capping of the telomere end by telomerase, Cdc13, Stn, telomerase, and other putative telomeric end-binding proteins (Refs. 54 and 92–94).

lished results). Deletions of either Ku70 or Ku80 in *S. cerevisiae* have been shown to alter the positioning of telomeric DNA in the yeast nucleus, and Ku, Rap1, and the SIR proteins (SIR2, 3, and 4) are released from the telomere in response to DNA damage (73,74). It has been suggested that *S. cerevisiae* telomeric DNA serves as a reservoir for Ku and other proteins that respond to physiological signals such as DNA damage (73). Additionally, it has been reported that the nuclear pore protein Mlp1 physically tethers Ku70 to the nuclear periphery, thus forming a link between the telomere and nuclear envelope in *S. cerevisiae* (75).

The *S. cerevisiae* telomerase RNA gene (TLC1) was originally isolated using a screen for genes that, when expressed in high amounts, would suppress telomeric silencing (39). TLC1 involvement in telomeric silencing was generally unexpected, and until recently the nature of TLC1 RNAs role in telomeric silencing remained unclear. Some recent clarification of this role has come in a report that demonstrated that overexpression of a 48nt stem-loop structure found within the TLC1 RNA (~1.3 kb) is responsible for this suppression of telomeric silencing, and additionally, causes telomeric shortening (76). Furthermore, the phenotypes associated with TLC RNA overexpression have been found to be similar to the phenotypic characteristics associated with yKu70 and yKu80 deficiencies.

Overexpression of the 48nt stem-loop TLC RNA in cells deleted for Ku70 or Ku80 has no additional impact on telomere length, suggesting that they share the same functional pathway. This is contrasted by additional telomeric shortening resulting from the deletion of several other telomeric components—TEL1, TEL2, RAD50p, or Sir4—in combination with overexpression of the 48nt stem-loop TLC RNA. However, it is important to recognize that a direct molecular interaction between the full-length Ku protein and TLC has yet to be demonstrated (76). Therefore, based on this rather tenuous genetic analysis, the investigators concluded that Ku might be linked to the yeast telomerase RNA (76).

VII. KU AT THE MAMMALIAN TELOMERE

The discovery that Ku plays a critical role at the yeast telomere, along with the fact that Ku and certain other components of the telomere are highly conserved from yeast to mammals, has inspired studies into whether Ku plays a similar role at the mammalian telomere. Using CHIP, Ku80 has been found to localize to human and hamster telomeres in the same manner as has been observed in yeast (77). It has also been determined that the DNA-dependent protein kinase catalytic component (DNA-PKcs) is not required in vivo for the association of Ku80 with telomeric repeats (77). Additional CHIP in vivo cross-linking studies have confirmed that Ku80 and Ku70 are localized to the mammalian telomere (60) (Fig. 3). As with the yeast studies, these CHIP findings indicate a direct physical link between Ku and the telomere in mammalian cells but do not determine whether Ku binds directly to telomeric DNA. In regard to Ku binding directly to telomeric DNA, it has been reported that purified Ku protein is capable of binding to mammalian telomeric DNA ends in vitro (78). However, as previously stated, it is well known that the Ku protein binds nonspecifically to DNA double-stranded ends, so the biological significance of this result is unclear. Nonetheless, it is clear from these studies that some portion of Ku is in close proximity to the telomere.

Additionally, it has been demonstrated that Ku plays a biological role at the mammalian telomere by the accumulation of telomeric fusions in mouse cell lines deficient for Ku (10). However, high background levels of telomeric fusions are often observed in transformed mouse cell lines derived from wild-type primary cells (our unpublished results). The telomeric fusions observed in these transformed cell lines may result from spontaneous genomic alterations associated with the transformation process. Thus, in this case, genomic alterations associated with transformed cells may cause the telomeric fusions and be unrelated to a Ku deficiency. However, a corroboration of this finding recently came from several groups reporting the accumulation of telomeric fusions in various primary mouse cells deficient for either Ku70 or Ku80 deficiencies (8,60,61), confirming that Ku is critical for telomere capping.

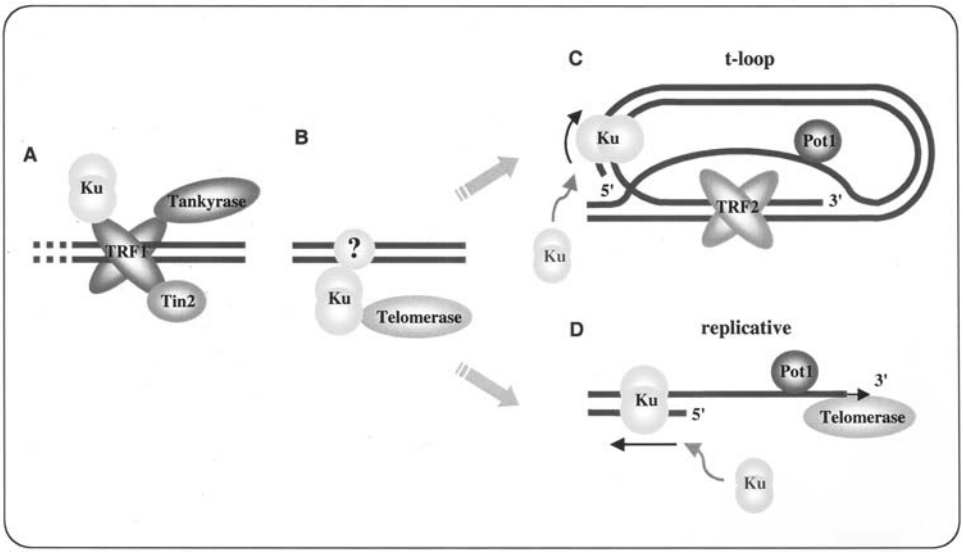

Figure 3 Model of Ku at the mammalian telomere. As with the yeast telomeric model in Figure 2, this schematic focuses on telomeric components that may interact with Ku at the telomere and does not attempt to show all known mammalian telomeric components. Lines represent telomeric DNA. (A) TRF1 interaction with several telomere proteins: Ku, Tin2, and Tankyrase (Refs. 8,95,96). (B) Ku might localize to the telomere via an unidentified interaction with a telomeric component (putative unidentified protein denoted by question mark) and may form a complex with telomerase. (C) T-loop and replicative conformations of the mammalian telomere (Ref. 55). Ku could load onto telomeric DNA directly via its nonspecific end-binding activity at a double-stranded DNA junction either in the t-loop or in the replicative telomere form. TRF2 is shown bound to the t-loop junction. Pot1 is shown bound to the G-rich single-stranded telomeric DNA (Ref. 91). Telomerase must bind to the 3′ telomeric DNA during the telomeric DNA replication.

The mammalian telomere-binding protein TRF1 localizes at telomeres by binding specifically to telomeric DNA and plays a role in telomere length regulation (79,80). Recently, it was reported that Ku forms a high-affinity protein/protein interaction with TRF1 (8). The Ku and TRF1 complex is a specific high-affinity interaction (K_d of 0.4×10^{-9}M), as determined in vitro by Biacore analysis, coimmunoprecipitation, and Far Western analysis. Coimmunoprecipitation experiments have determined that the Ku/TRF1 complex exists in human cells. An EMSA, using microcircular DNA substrates containing internal telomeric repeats, has shown that Ku does not bind mammalian telomeric repeats site specifically but rather localizes to telomeric repeats via its high-affinity interaction with TRF1 (8). Thus, Ku protein function at the telomere is different from its function

when joining nonhomologous DNA double-stranded breaks. This suggests that once Ku forms a complex with TRF1, Ku-specific DNA repair domains required to tether broken DNA ends and recruit other DNA repair proteins may be obscured to prevent DNA end-joining activity at the telomere (29).

TRF2, a telomeric repeat binding protein that localizes to t-loop junctions (see Fig. 3C) (55), can interact with an undetermined affinity to a truncated version of Ku70 (consisting of amino acids 200–385) (81). However, since full-length Ku70 and TRF2 fail to form a complex (81), the biological significance of this finding is unclear. Regardless, if not DNA mediated, a Ku70/TRF2 complex might form transiently at the telomere depending on the conformation or exposure of a specific domain within Ku70.

Although Ku has been localized to the mammalian telomere and plays a critical role in capping, it was still uncertain whether Ku also had a role in telomere length maintenance. Two independent groups using quantitative fluorescence in situ hybridization (qFISH) have published different results on this topic. Initially, one group reported that telomeric DNA remains at wild-type lengths in Ku80-deficient mouse embryonic fibroblasts (MEFs) (61). Then, it was reported recently that telomere length is reduced significantly, about 40% from wild-type telomere length, in Ku70- and Ku80-deficient cells taken from a variety of different cell types at various developmental stages (60). It is likely that these conflicting data are the result of technical differences in performing qFISH. Clarification of whether Ku functions in telomere length maintenance in mammals is essential to understand the basic role of Ku at the telomere.

In contrast to the action of yeast Ku in response to DNA-damaging agents (73), human Ku does not dissociate from the telomere upon exposure to ionizing radiation and the radiomimetic drug phleomycin (60). Since Ku is distributed throughout the mammalian nucleus, apparently its recruitment from the telomere to sites of DNA repair is not necessary (73).

VIII. TELOMERIC SHORTENING AND CELLULAR SENESCENCE

Before a final discussion concerning the possible role of Ku in cellular senescence and aging, we will briefly review the role of the telomere in these processes. Chromosomal stability and cellular viability require a minimal telomeric DNA length. Failure to maintain telomere length leads to replicative senescence in yeast and mammalian cells (49). Telomeres in somatic cells shorten by 50–200 bp per cell division in the absence of telomerase activity, and telomere length has been correlated with the proliferative capacity of normal cultured human cells (82). In contrast to most somatic cells, telomeres are maintained by an active telomerase in germ line and most immortalized cells (83). Accordingly, activation of telomerase is a characteristic of most established cell lines and tumors (84,85). One model

that accounts for the involvement of telomeres in the proliferative capacity of normal human cells proposes that telomeric shortening during successive cell divisions serves as a counting mechanism that prevents unlimited proliferation of human somatic tissues (59). Programmed telomeric shortening in normal human cells has been viewed as a tumor-suppressor mechanism, limiting the growth potential of certain cells that may acquire genetic defects (86). Intriguingly, activation of telomerase, via the ectopic expression of telomerase component TERT, allows certain human primary and virus-transformed cell lines to bypass senescence and crises and continue proliferation (58,87). However, although telomeres seem to play a critical role in the replicative capacity of certain human cells cultured in the laboratory, whether or not telomeric maintenance is a critical factor in human aging has yet to be established and is an area of active debate.

IX. PREMATURE REPLICATIVE SENESCENCE IN MICE DEFICIENT FOR KU

Embryonic fibroblasts taken either from Ku70 or Ku80 knockout mice reveal a pleiotropic phenotype, which includes premature cellular senescence. Telomeric maintenance appears to be a critical event that controls the ability of cells to continue proliferating in cell culture (58,87). Therefore, factors that control telomere maintenance are potential candidates for components that block proliferation and lead to cellular senescence. Because of the link between replicative capacity and telomere length, the reduction in the replicative capacity of Ku-deficient MEFs may simply be due to loss of telomere length regulation (14, 88). Furthermore, the accumulation of DNA damage that occurs during aging has been suggested by many as a possible cause of aging and cellular senescence (89). In fact, during aging and cellular senescence, mutations accumulate in genomic DNA. Therefore, loss of DNA repair capacity might increase the accumulation of DNA damage and play an important role in cellular senescence and aging. In this regard, Ku70 and Ku80 show significant decreases in steady-state levels during replicative senescence of human fibroblasts (90). Determining the actual mechanistic operation of Ku during the process of cellular and organismal aging will be important to further our understanding of the aging process.

X. CONCLUSION

The telomere is likely a dynamic structure, changing conformations depending on the stage of development or cell cycle, with proper maintenance of telomeric capping being required at all times to prevent telomeric fusions. Therefore, it seems paradoxical that the DNA repair enzymes involved in NHEJ are localized to the telomere. It is conceivable that DNA repair proteins were recruited to the telom-

ere at some time during or after the evolution of linear genomes for a divergent telomere-specific function.

At the mammalian telomere, the 3′ telomeric DNA single-stranded overhang can be buried in the t-loop form with telomeric proteins bound to stabilize and conceal the t-loop junction (see Fig. 3C) (55). However, during telomeric DNA replication, the 3′ end must be single stranded to allow telomerase access to anneal to the telomerase RNA template for telomeric DNA replication (see Figs. 2C and 3D). Although no evidence currently exists, Ku could load onto telomeric DNA using its nonspecific end-binding activity at double-stranded DNA junctions (see Figs. 2C and 3C). Moreover, even as the 3′ telomeric DNA overhang is unwound, it is likely to be associated with specific telomere-associated proteins and telomerase (see Figs. 2C and 3D) (91). In yeast, the telomere is apparently not exposed as naked double-stranded DNA but has been shown to be associated with specific telomeric single- and double-stranded DNA-binding proteins and telomerase (see Fig. 2C) (92). Given that the telomeric DNA end is capped or physically coated with telomere-associated proteins, it is possible that Ku is not physically capable of loading directly onto the telomeric DNA termini. Since Ku and TRF1 form a high-affinity complex in the human cell, and essentially all TRF1 localizes to the telomere, these results give a possible mechanism for Ku localization to the capped telomere via complex formation with TRF1 (see Fig. 3A). Yeast Ku protein also might localize to the telomere via a protein–protein interaction (see Fig. 2B).

Ku protein's functional mode likely depends on its nuclear microenvironment. The nuclear microenvironment depends on potential protein, DNA, and even RNA contacts associated with Ku. In summary, as Ku has many different functional capabilities and interacting partners, it is important clearly to assess findings in relation to Ku protein's role at the telomere and not be biased by preconceived ideas from its possible roles in other cellular processes.

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7

Chromosomal Instability in Normative Aging

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Every man desires to live long, but no man would be old.
Jonathan Swift

I. INTRODUCTION

Mankind has always been fascinated, but also frightened, by the phenomena of aging and mortality. Especially during the last 50 years, many scientific theories have emerged trying to explain senescence; for example, the free radical theory (1), the somatic mutation theory (2), the theory of an "error catastrophe" originating from inaccurate protein synthesis (3), the neuroendocrine theory (4), and the theory of programmed aging by "gerontogenes" (5,6).

In the era of the human genome project, the involvement of genes in aging is widely accepted. DNA contains all basic information that ensures normal cellular function. Since DNA is organized in chromosomes, chromosomal structure and stability play an important role in the regulation of DNA-encoded production of all cellular proteins. Aging is believed to result from an organism's inability to maintain its cellular integrity. Therefore, chromosomal instability may be the key initiator of altered cell function and aging.

This chapter presents a discussion of numerical and structural chromosomal aberrations associated with normative aging and their underlying mechanisms, as well as the influence of genetic and environmental factors on chromosomal instability.

II. NUMERICAL CHROMOSOMAL ABERRATIONS

Jacobs and coworkers first demonstrated the increasing proportion of aneuploid cells in aging humans (7). Based on the analysis of metaphase cells in mitotically stimulated cultures of T lymphocytes from 97 probands aged 1–82 years, they found significant increases in hypoploid cells (chromosome number < 46) and in hyperploid cells (chromosome number > 46) as a function of aging. The increase in hypoploid cells significantly exceeded the increase in hyperploid cells. In probands aged 5–14 years, 3.0 and 1.1% of all somatic cells were found to be hypoploid and hyperploid, respectively. These percentages increased to 9.5 and 3.3% of hypoploid and hyperploid cells in probands aged 65 years and older. Since this first study population consisted of a mixture of volunteers, hospitalized patients, and parents of children with chromosomal disorders, the study was widely criticized, because its results were derived from a nonrandom population. However, various other studies later confirmed the age-related increase of chromosomal aberrations as well as the excess of hypoploid cells versus hyperploid cells (8–16). The ratio of hypoploid to hyperploid cells is approximately 10:1 (17).

In patients with congenital aneuploidies involving 47 chromosomes (Klinefelter and Down syndromes), the same increase of hypoploid and hyperploid cells with advancing age can be demonstrated (7).

A. Numerical Chromosomal Aberrations Involving the Sex Chromosomes

With the advent of banding techniques (18,19), it became possible to establish the identity of the chromosomes involved in chromosomal loss or gain in aneuploid cells. It was found that the sex chromosomes are most likely to be lost or gained in hypoploid and hyperploid cells.

Most examinations concentrated on hypoploid cells, because their number greatly exceeds that of hyperploid cells (17). In hypoploid cells, it can be demonstrated that the X chromosome is preferentially lost in female cells, whereas in male hypoploid cells the Y chromosome is often lacking (8,10,15,20–22) (Fig. 1).

In addition to cultured lymphocytes, sex chromosome loss also has been described in bone marrow cells (23,24) and in corneal fibroblasts (25).

1. Sex Chromosome Loss in Different Age Groups

Sex chromosome loss increases with age in men and women. It has been suggested that endocrine factors, especially the concentrations of gonadotropins, may play a role in the age-related increase in sex chromosome aneuploidy (26). Unfortunately, very few studies are of the size necessary for regression analysis of sex chromosome aneuploidy in different age groups. One of these studies was performed by Guttenbach and coworkers (10). They analyzed a large population of 90 women and 138 men and performed in situ hybridization with sex chromosome specific probes in 228,000 lymphocytes (Table 1).

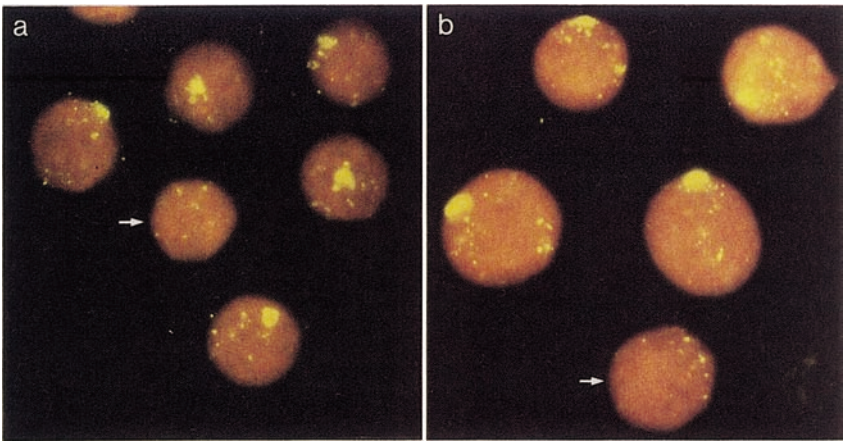

Figure 1 (a, b) Interphase nuclei of a male proband hybridized in situ with biotinylated Y chromosome–specific pHY2.1 DNA. Signals were detected by avidin–FITC. Nuclei lacking a hybridization signal are indicated by arrows in the two samples. (See color insert.)

Table 1 Incidence of Sex Chromosome Loss in Different Age Groups

Age group (years)	No. of probands		Average age (years)		Nuclei (%)	
	Male	Female	Male	Female	Lacking a Y-specific signal in males	Exhibiting a sole X-specific signal in females
0–5	10	5	1.5	2.6	0.03	1.58
6–10	7	6	7.7	8.8	0.05	2.82
11–15	8	5	12.8	13.0	0.05	2.50
16–20	5	4	19.6	19.0	0.24	2.30
21–25	13	8	24.3	22.5	0.23	2.23
26–30	6	5	28.0	27.8	0.32	2.00
31–35	8	5	32.8	32.6	0.38	3.32
36–40	10	6	37.6	37.2	0.63	2.95
41–45	7	5	42.4	42.8	0.54	2.52
46–50	10	4	48.7	47.5	0.60	3.13
51–55	12	4	52.7	54.0	0.70	3.20
56–60	7	5	57.9	57.2	0.87	4.35
61–65	9	5	62.0	62.8	0.89	3.68
66–70	8	5	67.6	67.5	0.95	4.94
71–75	8	7	72.6	72.0	1.04	4.17
76–80	5	4	77.8	78.3	1.34	4.58
81–85	4	6	82.3	84.0	1.30	4.73
>85	1	1	93.0	91.0	1.00	5.10

Source: Ref. 10.

Female probands were divided into three age groups according to the reproductive function (younger than 12.8 years, 12.8–51.2 years, and older than 51.2 years) and regression analysis was performed in each group. No significant age correlation of X chromosome loss was found prior to menarche and menopause. Significant age correlation could be shown only in postmenopausal women. The postmenopausal endocrine milieu, combining a rise in gonadotropin levels with a decrease in sex hormones, might be the origin of the increased loss of the X chromosome after menopause.

Unlike X chromosome loss in women, Y chromosome loss in men is already significantly increased after the onset of puberty, although at an overall lower incidence. In prepubescent boys aged 0–15 years, the incidence of Y chromosome loss is very low, but after age 15, there is an even stronger correlation of Y chromosome loss with age than can be observed for X chromosome loss in postmenopausal women (10).

B. Numerical Chromosomal Aberrations Involving the Autosomes

In contrast to the loss of sex chromosomes, loss of autosomal chromosomes is not correlated with age. Cells aneuploid for different autosomes have been found with similar frequencies in young and old probands (14,15,27).

Chromosomal size seems to influence the incidence of the involvement of specific chromosomes in aneuploidy. Most investigators have found that small chromosomes are lost much more frequently than large chromosomes (14, 15,27–31). Chromosome 21 is the most frequently lost autosome. It is still unclear whether preparation methods contribute to the preferential loss of small chromosomes (14,27). Only one study failed to confirm the correlation of autosomal loss and chromosomal length. According to Guttenbach and coworkers (10), autosomes 1 and 17, representing a large and a small chromosome, are lost at approximately the same prevalence (1%).

C. Mechanisms of Loss of Specific Chromosomes with Aging

1. Selection

There are two alternate explanations for the loss of specific chromosomes:

1. Primary loss of chromosomes is random, but cells monosomic for chromosomes that are less essential for survival have a selection advantage and therefore will be more frequently found in cultures.
2. Primary loss of chromosomes is specific and occurs at different rates for different chromosomes; there is no influence of selection.

In all probability, both theories are valid, and in fact their combination may explain the predominant loss of specific chromosomes. In studies pursuing this question, attention has once again focused on the sex chromosomes, since these are most frequently lost as a function of aging.

The Y chromosome contains genes important for spermatogenesis and male sexual development (32,33), but it cannot harbor important "survival information", because women are perfectly able to survive without a Y chromosome. The mitotic loss of the Y chromosome in men leads to 45,X cells. Nonmosaic monosomies are associated with an extremely high prenatal mortality. Only a small percentage of fetuses with monosomy X (Turner syndrome) is able to live. 45,X cells appear to have a lower selection disadvantage and are therefore frequently found in men and women.

The phenomenon of X inactivation in women as a mechanism for equalizing the amount of active X information in women and men was first proposed by Lyon in 1961 (34). Although a number of X chromosome–specific features have been discovered, such as DNA hypermethylation (35), underacetylation of histones (36,37), and the required presence and transcription of the XIST gene (38–40), the exact mechanism of X inactivation remains a mystery (41). Inactivation of X seems to occur randomly: Approximately half of all female cells contain the paternally inherited active X, whereas the remainder harbor the maternally inherited active X chromosome. By this mechanism, all females are mosaics for the X chromosome, but every cell contains 45 active chromosomes: 44 autosomes and 1 X chromosome. Consequently, it has frequently been hypothesized that X chromosome loss in female hypoploid cells would preferentially affect the inactive X chromosome, thus leaving 45 active chromosomes. A low selection disadvantage would explain a high percentage of hypoploid 45,Xa (Xa = active X chromosome) cells in females.

Several studies, all using peripheral blood lymphocyte culture, have since shown a preferential involvement of the inactive X chromosome in X chromosome loss (42,43). Only one study found that not only the inactive, but also the active X chromosome is commonly lost in human lymphocytes of karyotypically and mentally normal women, and that this effect increases dramatically with age (44). This study differs from other studies in its use of cytochalasin B, a substance that blocks cytokinesis at anaphase. Thus, X chromosome loss is observed only immediately following the anaphase when primary production of aneuploidy occurs. At this time, selection advantages or disadvantages have not yet come into force. In contrast, experimental data obtained from studies not using cytochalasin B only refer to the final product of primary production of aneuploidy and selection.

Apparently, the X chromosome is involved in primary aneuploidy production more frequently than other chromosomes. It is possible that the active and inactive X chromosomes are primarily lost at similar frequencies. Their differences

concerning functional aspects only start to play a role in subsequent selection: Cells containing an active X chromosome may then have a selection advantage. Studies showing the preferential loss of the inactive X chromosome did not use cytochalasin B, and therefore, analyzed cultures at a later stage, when selection in favor of cells with an active X chromosome had come into force in addition to primary production of aneuploidy.

A comparison of these studies—each with different use of cytochalasin B—is highly interesting, because it seems to justify the combination of the two theories mentioned above: primary chromosomal loss may occur specifically according to chromosomal size and structure, and subsequently cells with functional advantages may undergo additional positive selection.

These two effects are probably modified with aging, thus explaining the increased frequency of aneuploid cells as a function of aging. Although it has not been demonstrated yet, it is likely that an increased production of aneuploidy, as well as a decreased selection against aneuploid cells, may occur with aging.

2. Exclusion of Chromosomes into Micronuclei

A micronucleus is a separate nucleus in addition to the main nucleus of a cell. Micronuclei are produced during the telophase of mitosis at the time of nuclear membrane formation. They often contain chromosomal fragments or entire chromosomes (45).

Several studies have shown an increase in micronuclear formation with age (9,46–50). The analysis of umbilical blood samples from newborns indicates a very low frequency of micronuclei after birth (approximately 0.1%) (11). Several studies have shown a four- to five-fold increase of micronuclei in lymphocytic cultures during the course of life. The frequency of micronuclei in probands older than age 70 years is approximately 0.5% (9,46). Some investigators have reported a significant difference between the number of micronuclei in men and women with a man-to-woman ratio of approximately 1:1.5 (11,13,48). Apparently, nearly all micronuclei observed in adults are the result of their accumulation since birth. This accumulation occurs at a higher level and is more accelerated in women than it is in men.

Since hypoploid cells are much more frequent than hyperploid cells and their number increases with age, a relation between the loss of chromosomes and the formation of micronuclei has been hypothesized: the loss of chromosomes could be explained by chromosomal exclusion into micronuclei. The chromosomal content of micronuclei has been analyzed by a variety of different techniques, such as the use of antikinetochore antibodies (51–56), chromosome identification by staining techniques (57), and in situ hybridization with chromosome-specific probes (58–62). Rapid and reliable identification of particular chromosomes in micronuclei and the distinction between micronuclei containing chromosomal fragments and those including whole chromosomes is made possible by in situ hy-

bridization with probes scattered around a specific chromosome (9,60,62). By applying this method, it can be shown that predominantly entire sex chromosomes, rather than chromosomal fragments, are excluded into micronuclei. Additionally, a significant increase has been observed in the number of sex chromosomes containing micronuclei with age (9,11,13). These findings explain the excess of hypoploid over hyperploid cells and the predominance of sex chromosome aneuploidy over autosomal aneuploidy.

The range of X chromosome exclusion into micronuclei has been reported to be between 20 and 70% (9,11,44,63). In two studies, the proportion of X chromosome–bearing micronuclei in females was shown to be age related, rising from 8 to 15% in young adults (younger than 30 years) to 20 to 40% in probands older than 55 years (9,44). The fact that the results obtained for X chromosome exclusion into micronuclei are rather inhomogeneous might be explained by different experimental conditions; for example, the use of cytochalasin B (see Sec. II.D).

The exclusion of the Y chromosome into micronuclei occurs at a lower incidence (approximately 10–20%) (9,13) but also seems to be age related (10).

3. Chromosomal Lagging and Nondisjunction

The mechanism responsible for eliminating entire chromosomes rather than chromosomal fragments from cells and their inclusion into micronuclei has not yet been defined, but it is likely to involve chromosomal lagging at anaphase.

Ford and coworkers (58) have suggested that chromosomes displaced at metaphase will subsequently be more likely to fail to attach to the spindle during mitosis. These chromosomes are predisposed to "lag" at the metaphase plate, whereas the other chromatids are already moving to the spindle poles. Lagging chromosomes are then prone to exclusion into micronuclei during nuclear membrane formation. In cells where only one chromatid is lagging, the other chromatid seems to have been able to perform a normal kinetochore–spindle attachment that allows the chromatid to segregate into a daughter cell.

Chromosomal lagging might be a plausible alternative to the mechanism of aneuploidy formation by nondisjunction. For a long time nondisjunction was believed to be the only origin of constitutional aneuploidy by meiotic errors and mosaicism originating from mitotic errors. Zijno and colleagues (63) were the first to suggest that both mechanisms contribute to malsegregation of the X chromosome. To distinguish between chromosomal lagging and nondisjunction, X centromeric probes were used. These probes produce two signals in each nucleus and four signals in binucleated cells, which occur shortly after anaphase before cytoplasm division.

Cells with a signal distribution pattern deviating from the expected 2:2 segregation of X chromosomes into the two daughter nuclei have been investigated in detail. Single and double nondisjunction lead to a 3:1 and 4:0 distribution of X centromeric spots, respectively. In comparison, chromatid or chromosomal loss

resulting from chromosomal lagging at anaphase with subsequent exclusion of the lagging chromosome into micronuclei would result in several possible distribution patterns:

1. A 2:1:1 mn (mn = micronucleus) distribution in case of chromatid exclusion into a single micronucleus; the two daughter nuclei contain two and one X centromeric signals, respectively
2. A 1:1:2 mn distribution in case of the exclusion of a whole chromosome into a single micronucleus
3. A 1:1:1 mn:1 mn distribution when both chromatids are included in two separate additional micronuclei

Other distribution patterns are imaginable, but have been shown to occur at a very low incidence (63).

By performing this analysis, it can be shown that X chromosome and chromatid exclusion into micronuclei occur at a slightly higher frequency than nondisjunction (0.75 vs 0.58%). Nondisjunction can be demonstrated to involve only one X chromosome (3:1 distribution) in nearly all cases; a 4:0 distribution resulting from double nondisjunction is very rare. In cells with chromosomal loss and exclusion of chromosomal material into micronuclei, the exclusion of whole X chromosomes has been observed more frequently than the loss of single chromatids (0.40 vs 0.27%). The total rates of malsegregation of one or both X chromosomes are estimated as 1.15 and 0.17%, respectively. X chromosome malsegregation can be shown with greater frequency in primarily aneuploid cells than it can in primarily euploid cells (63).

From these results, it can be deduced that, in all probability, chromosomal lagging and nondisjunction contribute to malsegregation of the X chromosome. However, the correct interpretation of these data remains difficult: it is unclear whether the two postulated mechanisms of chromosomal malsegregation are strictly distinguishable by this method.

4. Premature Division of the Centromere

As discussed in the preceding section, chromosomal lagging may lead to chromosomal exclusion into micronuclei, but what is the underlying mechanism of nondisjunction? *Nondisjunction* is a term describing the failure of equal chromatid separation at mitosis and the division of meiosis, resulting in unequal chromatid or chromosomal distribution to daughter cells. The centromere is responsible for reversible binding between the chromatid and the microtubule protein of the spindle; therefore, it is an essential structure during mitosis. Centromeres of different chromosomes have been observed to divide at different times. Human chromosomes 2, 17, and 18 divide early, whereas chromosomes 1, 16, and chromosomes of the D and G group as well as the Y chromosome are the last to divide. The X

chromosome is among the first half of chromosomes to divide (64,65). Chromosomal length and centromeric position do not seem to have any influence on the sequence of centromeric division (64). In some female metaphase cells, one of the X chromosomes displays a much earlier division of the centromere than observed in the other "normal" X chromosome. Those X chromosomes with early dividing centromeres appear as two entirely parallel chromatids that can easily be mistaken as acentric fragments. The phenomenon of prematurely dividing centromeres in relation to the centromeres of other chromosomes has been described as premature centromeric division (PCD), and most frequently affects one of the X chromosomes in females (PCD,X) (66–68) (Fig. 2).

A close relation among the number of mitoses showing PCD,X and the number of 45,X cells has been noted (69). This correlation was less pronounced for the cells with a 47,XXX karyotype. Centromeric defects leading to nondisjunction of the X chromosome have been observed with increasing frequency in women of advancing age. In population studies, the frequency of PCD,X was four times greater in women aged older than 60 years than it was in women aged younger than 40 years (8,66,67).

Figure 2 Metaphase of a female proband after C-banding showing premature chromosomal division (PCD) in one X chromosome. Note the separated centromeric region in this X chromosome. (Modified from Ref. 69.)

The same age correlation was found in men, albeit at only one-ninth of the incidence observed in women (66,67). This significantly lower frequency of PCD,X in male cells compared with female cells suggests the preferential involvement of the inactivated X chromosome in PCD,X in women, whereas the activated X chromosome in men and women may possess centromeres dividing within the sequence observed in normal cells. So far, few attempts have been made to determine whether PCD,X predominantly involves the inactivated X chromosome; existing studies favor this hypothesis (8,67,70).

Fitzgerald and coworkers (67) were the first to observe PCD,X. They describe a patient with aneuploidy of 13% of all cells, and in the euploid and aneuploid cells, many metaphase cells displayed PCD,X. Interestingly, premature division of the X chromosome occurred in 70% of aneuploid cells but in only 3% of euploid cells. These observations provide strong evidence that PCD,X is the underlying mechanism of nondisjunction that causes aneuploidy of the X chromosome.

D. Effects of Experimental Conditions on Numerical Chromosomal Aberrations

So far, most analyses have been performed on peripheral lymphocytes. These lymphocytes are treated with a variety of chemical substances, such as mitogens (e.g., phytohemagglutinin), to stimulate cell proliferation in vitro. It therefore has been speculated that in vitro conditions do not precisely reflect in vivo conditions. Chromosomal aberrations observed in cultured lymphocytes may, at least partly, be due to culture conditions (71).

Cell division begins 24–48 hrs after mitogen stimulation. Cell cultures are usually harvested after 72 hrs when lymphocytes have replicated two to five times (72). Assuming that chromosomal aberrations are influenced by culture conditions, their incidence would be expected to increase as a function of in vitro culture time. However, early studies produced contradicting results: Some investigators found an increased incidence of aneuploidy after 72 hrs in contrast to 48-hr culture time (17,73), whereas others did not confirm this time-dependent difference (74). More recent studies have demonstrated that autosomal, but not gonosomal, loss increases from 48- to 72-hr cultures (10,15). Gonosomal loss in 48- and 72-hr cultures was not significantly elevated when compared with directly isolated lymphocytes (10). Furthermore, structural aberrations were found with similar frequencies in 48- and 72-hr cultured cells (75).

To evaluate the contribution of cell culture to the incidence and content of micronuclei, Surrallés and coworkers (71) compared uncultured with cultured human lymphocytes. Cytochalasin B was used to differentiate among micronuclei produced in vivo and in vitro. This substance inhibits cytokinesis but not

karyokinesis, and therefore only cells that have divided in vitro appear as binucleated (46). A significantly increased frequency of micronuclei was observed in cultured cells with or without cytochalasin B treatment as compared with uncultured cells. Moreover, the difference between the two types of culture was significant. Cells from cytochalasin B–treated cultures formed less micronuclei than cells in cultures without cytochalasin B (71). The frequency of micronuclei containing acentric fragments also was strongly increased by cell culture—to a 2.9-fold rate without cytochalasin B, and to a 2.3-fold rate with cytochalasin B.

Interestingly, the frequency of micronuclei including an X chromosome was significantly higher in cultures treated with cytochalasin B than in those cultures without cytochalasin treatment (42 vs 24%). This led to the conclusion that cells treated with cytochalasin B develop less micronuclei compared with those not treated with this substance, but micronuclei are more frequently X chromosome positive in cytochalasin B–treated cell cultures.

In summary, these results show that the formation of micronuclei and the preferential exclusion of X chromosomes into these micronuclei are not in vitro artefacts but occur in vivo. Different experimental conditions influence the frequency of micronuclei in a cell culture and the percentage of micronuclei containing specific chromosomes.

III. STRUCTURAL CHROMOSOMAL ABERRATIONS

A. Translocations, Chromosomal Breaks, and Fragile Sites

Chromosomal translocations are structural aberrations characterized by relocations of chromosomal segments within one or among different chromosomes. As chromosomal translocations are relatively stable structural changes (76–78), they would be expected to accumulate with age. Several investigators have found that the increase in translocation frequencies in a nonsmoking normal population follows a curvilinear relationship with age (79,80). Although ionizing radiation is known to induce chromosomal translocations, it cannot alone account for the strong age-related increase of chromosomal translocations (80). It has been argued that age may play a role in an individual's response to radiation exposure, as the capacity of DNA repair mechanisms may decline with age (81,82). However, it has been demonstrated that the susceptibility to translocation formation induced by acute (83) and chronic (80) ionizing radiation does not change with age. Apparently, other factors are involved in this process.

Marlhens and coworkers (84) have analyzed the incidence of structural chromosomal alterations in young (<30 years) and old (>70 years) probands. Their study shows that chromatid and chromosomal breaks are most likely to in-

crease as a function of age, with an estimated factor of three to four from 30–75 years. There are several "hot spots" for chromosomal breakage at well-known fragile sites, such as 3p14.3 and Xq27 [fra(X)]. These sites were affected in up to 3% of cells from older individuals. The observation of isolated cells exposing fragile sites therefore must not be considered to be a pathological finding but a normal age-related characteristic.

Terminal deletions occur at a lower incidence, with a maximum of 2 per 100 cells. Their age-related increase is not significant. Most frequently, chromosome 9 and the X chromosome are affected, but these sites—particularly prone to deletions—do not correspond to the most common fragile sites. As for the analysis of chromosomal translocations (80), the results obtained for chromosomal breakage are fairly homogeneous within different age groups (84). Interindividual variation is also negligible for probands from different ethnic backgrounds (80).

Prieur and colleagues (75) have found that specific translocations involving chromosomes 7 and 14 represent an exception to the frequently observed age-related increase of structural aberrations (Table 2). The frequency of rearrangements among specific bands of chromosomes 7 and 14 is higher in newborns (0.43%) than in adults (0.24%). This excess in newborns is most probably related to rearrangements within the immunoglobulin gene family (85). These rearrangements are mainly generated before birth and in early childhood during differentiation of the immune system. Once this differentiation is complete, rearrangements of immunoglobulin gene family members occur at a decreasing frequency. The average incidence of these specific translocations is low (0.3%) compared with the mean frequency of rearrangements involving other bands (2.5%). Rearrangements of other chromosomal bands follow the "normal" age-related pattern and are more common in adults (3.8%) than in newborns (1.3%). However, this example illustrates the importance of discriminating among different types of structural rearrangements, especially when comparing specific structural aberrations occurring at low incidences.

Table 2 Frequencies of Structural Rearrangements Involving Either Specific Bands or Other Bands in Newborns and Adults

	Structural rearrangements (%) involving	
	Specific bands: 7p14, 7q35, 14q11.2, 14q12, and 14qter	Other bands
Newborns	0.43	1.3
Adults	0.24	3.8
Average incidence	0.30	2.5

B. Heterochromatin Structure

Aging also correlates with changes in the structure of heterochromatin. Throughout the interphase, heterochromatin possesses a dense and compact structure associated with transcriptional inactivity. A multitude of functions have been ascribed to heterochromatin. It is thought to act mainly as a modifier of gene action, excluding specific regions from transcription, and stabilizing the chromosome, thereby protecting it from mutation (86). DNA contained in heterochromatic regions replicates late in the cell cycle. Each round of the cell cycle harbors a risk of new failures. Thus, with increasing rounds of the cell cycle, the risk increases with age that heterochromatic regions will be inaccurately reassembled in dense domains after replication. This damage may result in the loss of repressive chromatin domains by alterations of the acetylation status in heterochromatin. The core histone H4, which is normally underacetylated in heterochromatin, has been found to be predominantly acetylated in damaged DNA (87). In vitro studies examining the chromatin structure reveal an increase of single-stranded DNA in aging cells. This was indicated by a reduction of the melting temperature of these cells, reflecting a decreased supercoiling potential of the DNA and a loss of transition IV (88).

Several genes that are activated depending on the heterochromatic structure have been detected. One of these genes, Sdi1, is involved in the cell cycle progression from the G1 to the S phase and functions as an inhibitor of a specific kinase complex (89–91). Based on these results, the phenotype of senescence is thought to be dominant, because changes in the heterochromatic organization of only one chromosome can be sufficient to activate specific genes, such as Sdi1 (92).

IV. INFLUENCE OF DIET ON CHROMOSOMAL INSTABILITY

Chromosomal instability—reflected by an age-related increase in balanced translocations, chromatid and chromosome breaks, and aneuploidy associated with chromosome loss and the formation of micronuclei—has been considered a possible cause of accelerated aging and carcinogenesis (93–95). There is a strong interest in determining whether dietary factors influence an individual's rate of chromosomal breakage. By understanding the contribution of life style and diet on chromosomal instability, it may subsequently be possible to delay aging and reduce susceptibility to cancer by modification of dietary factors.

The formation of lymphocytic micronuclei has been analyzed in vegetarians and nonvegetarians, and differences in vitamin B_{12}, vitamin C, and folic acid in-

take between the two groups were noted. Vegetarians, in general, were found to have significantly higher plasma levels of vitamin C and folic acid and significantly lower levels of vitamin B_{12} compared with nonvegetarians (96,97). For vitamin B_{12} and micronucleus formation, a significant negative correlation in both women and men was detected. Additionally, in young females (<30 years), a low folic acid blood level was associated with a high rate of micronuclei. These data suggest that vitamin B_{12} and folic acid have an impact on the formation of micronuclei (97,98). However, micronuclear frequencies are similar in vegetarians and nonvegetarians. This is due to an enhanced intake of folic acid in vegetarians, whereas nonvegetarians are known to have higher blood levels of vitamin B_{12}. Thus, the effects of both substances are roughly equalized in vegetarians and nonvegetarians.

It has been shown that deficient levels of vitamin B_{12} and folic acid are often accompanied by high levels of homocysteine (99). The micronuclear formation rate was found to be significantly increased not only in probands with low levels of vitamin B_{12} and folic acid in combination with high levels of homocysteine, but also in individuals with normal levels of vitamin B_{12} and folic acid and merely increased plasma homocysteine (97). Dietary changes did not lead to a significant reduction of micronuclear formation.

Moderate wine drinking also has been examined to assess whether it can protect against DNA damage and micronuclear formation. A protective effect was observed for red and white wine, although statistical significance was achieved only for red wine. This effect is most probably due to flavonoids contained in high concentration, especially in red wine (97,100). Moreover, the consumption of black tea also significantly reduces micronuclear formation (101).

In summary, chromosomal damage seems to be influenced by diverse dietary factors, but results are not yet conclusive enough to allow general dietary advice. Results seem to point to the formation of micronuclei as an expression of chromosomal damage. It would be interesting to examine other cytogenetic changes, such as translocation formation or the loss of specific chromosomes, under the influence of altered dietary factors.

V. EFFECT OF GENOTYPE ON CHROMOSOMAL INSTABILITY

To analyze the impact of the genotype on chromosomal instability and breakage, it is useful to study monozygotic twins and compare the results with those obtained for dizygotic twins or brothers and sisters as well as for unrelated probands. If genotype has a major influence on chromosomal instability, one would expect high concordance rates in monozygotic twins, whereas interindividual differences of chromosomal breakage rates would be more widely spread in less-related (dizy-

gotic twins or brothers and sisters) or nonrelated subjects. However, in a study performed by Jarvik and Kato (102), monozygotic twins did not show a higher concordance rate than less-related or unrelated subjects.

In the search for factors causing or influencing premature division of the X chromosomal centromere (PCD,X), a genetic influence also has been considered. However, incidences of PCD,X within a family appear to be inhomogeneous (67,70).

Although these preliminary results make the case for genetic involvement in the process of aging seem doubtful, there are other observations supporting a genotypic influence on aging. A dominant phenotype of senescence has been proposed, because heterochromatic structure has been shown to play a major role in the regulation of gene expression (92,103). Genes located in dense heterochromatic domains are activated depending on the conformation of these heterochromatic regions. Decondensation of DNA due to failures occurring in only one chromosome may allow transcription of otherwise repressed genes. Activation of specific genes involved in the cell cycle, such as Sdi1 (89–91), may lead to accelerated aging.

Another argument in favor of a genetic influence on aging is the occurrence of premature aging in the so-called chromosomal instability syndromes. These represent a group of inherited diseases (e.g., xeroderma pigmentosa, ataxia-telangiectasia, and Bloom syndrome). Based on a higher rate of chromosomal instability than that found in the normal population, patients affected by these diseases exhibit signs resembling aging and an increased susceptibility to cancer (104–107).

VI. CONCLUSION

Numerical and structural chromosomal aberrations increase significantly with age. In older probands, up to 10% of somatic cells are affected by chromosomal aberrations. Sex chromosomes especially are frequently involved in chromosomal loss or gain in aneuploid cells. In hypoploid cells, which greatly exceed the number of hyperploid cells, the X chromosome is mainly lost in females, whereas the Y chromosome is often lacking in male hypoploid cells. A significant age-related X chromosome loss has been shown only in postmenopausal women. In males, Y chromosome loss already increases significantly after puberty, although at an overall lower incidence. In contrast, the loss of autosomes, predominantly involving chromosome 21, is not correlated with age.

Several mechanisms for the loss of specific chromosomes with aging have been proposed. Primary chromosomal loss may occur specifically in relation to chromosome size and structure, and subsequently cells with functional advantages

may undergo additional positive selection. These two mechanisms may explain the predominance of specific aneuploidies (e.g., 45,X) in men and women. The loss of the Y chromosome in men and the inactive X chromosome in women seems to be associated with a relatively low selection disadvantage. Primary production of aneuploidy and selection may be altered with aging, thus leading to a higher frequency of chromosomal aberrations in older probands. The exclusion of specific chromosomes into separate small nuclei (micronuclei), which are produced during nuclear membrane formation, constitutes another mechanism of chromosomal loss. Entire sex chromosomes are more likely to be excluded into micronuclei than chromosomal fragments. Chromosomal lagging and nondisjunction lead to a predisposition toward chromosomal exclusion into micronuclei. Nondisjunction may be related to premature centromeric division (PCD). The phenomenon of prematurely dividing centromeres of specific chromosomes in relation to the centromeres of other chromosomes has especially been described for the X chromosome (PCD,X), and its incidence is closely related to the number of 45,X cells in females.

Structural chromosomal aberrations, especially chromatid and chromosomal breaks, are also found with an increasing frequency in aging humans. Several hot spots for chromosomal breakage at well-known fragile sites have been described. The finding of isolated cells exposing fragile sites therefore should not be considered to be a pathological finding but a normal age-related characteristic. Translocations involving specific bands of chromosomes 7 and 14 are the only exception to the overall age-related increase of structural chromosomal aberrations. These specific translocations are related to rearrangements within the immunoglobulin gene family. Their incidence declines after differentiation of the immune system in early childhood.

Age-related changes in the heterochromatic structure may result in the loss of repressive chromatin domains, which in turn may lead to activation of "senescence" genes (e.g., Sdi1).

Dietary factors seem to have only a minor influence on chromosomal instability. High levels of vitamin B_{12} and folic acid and moderate wine drinking may protect against DNA damage and the formation of micronuclei.

The impact of genotype on chromosomal instability is still unknown. Chromosomal breakage and PCD,X were found to underly the same differences among related and unrelated subjects. On the other hand, the feature of presumptive premature aging in chromosomal instability syndromes is a strong argument in favor of a genetic influence and of chromosomal instability on aging.

Finally, the importance of large longitudinal cytogenetic studies must be emphasized. The detection of factors that influence chromosomal instability is of crucial importance. It could subsequently be possible to decrease the risk of malignancy and develop new strategies for therapeutic intervention in disorders associated with a shortened life span.

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8

Chromatin, Aging, and Cellular Senescence

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I. INTRODUCTION

A common theme in this volume is the association of chromosomal instability with aging. The notion of chromosomal instability, of course, can be considered at multiple levels, with breaks, heteroploidy, and other cytogenetic abnormalities being only its most readily visualized aspects. Cumulative defects are known to accumulate at the submicroscopic level, including, for example, point mutations, small deletions, and shortening of telomeres. Each of these types of age-related instabilities produces heritable alterations in DNA sequence and associated gene function. Evolutionarily conserved mechanisms exist, however, to generate heritable changes in the cell state without altering the primary DNA sequence—such mechanisms provide the basis for epigenetic inheritance. The term *epigenetic* encompasses both covalent modifications to DNA (e.g., methylation of cytosine residues in mammals) and self-templating higher order chromatin structures. The latter have been studied intensively in a number of systems (Table 1), but only recently have received more than passing attention within the aging field. This chapter will provide a brief overview of the current understanding of chromatin-based structures, emphasizing how instability in such chromosomal elements might contribute to senescence at cellular and organismal levels.

Table 1 Selected Examples of Epigenetically Inherited Silencing[a]

In mammals	In yeast
X chromosome inactivation	Mating-type silencing
Imprinting	Silencing at telomeres, rDNA repeats
In Drosophila	
Position effect variegation	
Polycomb-mediated silencing	

[a] Several particularly well-studied experimental systems are listed and discussed in the text: X chromosome inactivation (Refs. 1, 2); imprinting (Ref. 3); position-effect variegation (PEV) (Refs. 4,5); Polycomb group-mediated silencing (Ref. 6); mating-type silencing (Refs. 7–9); and silencing at telomeres and rDNA repeats (Refs. s10,11).

II. CHROMATIN VERSUS OTHER DETERMINANTS OF AGING

At the outset, it is important to place links between senescence and chromatin within the broader context of research on aging. Current studies on cell senescence and life span are focused increasingly at the molecular level using experimental model systems amenable to genetic manipulation. In budding yeast, several gene loci have been identified that can extend the proliferative life span of mother cell lineages. Examples include *LAG1*, *RAS1*, and *RAS2*, products of which have been implicated in metabolic regulation and genetic stability. Of particular interest here are additional examples, which include *SIR4*, *SIR2*, and *RPD3,* loci known to modulate silencing and higher order chromatin structures (12,13).

In *Caenorhabditis elegans*, a number of genes have been discovered that, when mutated, extend organismal life span dramatically (14). The mechanisms by which these genes act are not fully understood, but they have been implicated in dauer and other signaling pathways, as well as in metabolic regulation. The insulin/IGF1 receptor homolog DAF-2 appears to transduce signals that regulate entry into the dauer pathway in a DAF-16–dependent manner (15). Along different lines, the *CLK-1* gene product is a predominantly mitochondrial protein that may be involved in coordinating mitochondrial and nuclear function (16). In *Drosophila melanogaster*, a mutation in the *methuselah* gene product prolongs life span and confers resistance to a variety of stresses (17); *methuselah* is most similar to GTP-binding protein–coupled 7-transmembrane domain receptors.

In the mouse, several gene knockout strains have been serendipitously discovered to influence life span. For example, ablation of the *ku86* locus yields mice that manifest a number of pathologies suggestive of premature senescence (18). Conversely, life span extension is observed in association with *Pit1* and *Prop1* mutations in the Ames and Snell dwarf mouse models, respectively. Homozygous defects at *Pit1* or *Prop1* loci compromise anterior pituitary development, leading to reductions in growth hormone, prolactin, and thyroid-stimulating hormone, as

well as to indirect deficiencies in insulinlike growth factor-1 (IGF-1) and thyroid hormones (19).

The identification of specific gene loci that modulate the life span generates interesting avenues for study; nevertheless, one must of necessity consider such findings in light of more classic evolutionary approaches to aging and cell senescence. The latter areas have been reviewed, among others, by Finch (20,21), Martin (13), Campisi (22–24), Kirkwood (25), Smith and Pereira-Smith (26), and Jazwinski (27,28). Drawing from this literature, several broad issues deserve emphasis: First, there is enormous variation in the natural history, or temporal organization, with which senescent changes develop during the life spans of different organisms (21). This underlines the notion that multiple molecular mechanisms may contribute to aging or age-related pathology, and that these may vary in relative importance depending on taxonomic group or species. Second, the details with which senescence evolves in a given organism are characterized by plasticity. Thus, developmental cues and environmental conditions experienced by the developing organism can, in some cases dramatically affect the life span or the pattern of age-related pathology.

An intriguing example in this regard is the maternal-effect phenotype of mutations in the *C. elegans CLK-1* gene (16). In *Drosophila*, an unexpected environmental input during development has been observed for flies selected for late-life fitness (13). In this case, the extended longevity characteristic of such flies is not evident unless the larvae are reared at a high density. Numerous examples of alternative life spans encoded by a single genome are cited by Finch (21), but remain at the phenomenological level. Of these, the most striking involves social insects such as honeybees, where alternative developmental pathways, queen versus worker, are associated with 10- to 100-fold differences in life span (21). Third, despite variation and plasticity, senescence can be described as having public versus private components (13). In this context, mechanisms involved in resistance to oxidative and other stresses may apply across multiple taxonomic groups. By contrast, age-related, tissue-specific changes in gene expression (sometimes termed *dysdifferentiation*) tend to be species or even strain specific. Finally, organismal senescence (at least the gradual form manifested by iteroparous mammals) is unlikely to be genetically programmed in a strict sense. In fact, there is broad agreement among investigators involved in aging research that senescent changes that emerge late in the life span of the organism either are not subject to strong evolutionary selection or they reflect negative pleiotropy.

Taken together, the results of genetic manipulation in model systems and evolutionary approaches have been reconciled into what might be termed *a consensus view*. This view suggests that oxidative damage and other cumulative stresses interact with signaling pathways in a manner that leads to genomic (i.e., chromosomal) instability. Such instability in turn has the potential to trigger abnormal gene expression as well as terminal effector programs. Apoptosis is one such accepted program, which strikingly involves regulation of mitochondrial function as an integral part of the effector mechanism. Recent evidence suggests

that cellular senescence may function, at least in part, as a second effector program. Consistent with this, cell senescence or senescence-like phenotypes can be induced through oxidative stress, a variety of DNA-damaging agents, activated oncogene expression, or agents known to disrupt higher order chromatin structure (29–34). Here as well, the suggestion has been made that mitochondrial nuclear interactions play an important role (28). As for the genome, it presumptively influences the progression and pattern of senescence at the organismal level in several ways. It sets the metabolic rate as well as levels of oxidative defense and DNA repair pathways. It encodes determinants of signaling pathways and a variety of gene products that protect the integrity of the genome or that trigger either checkpoint(s), apoptosis, or senescence in response to severe lesions (24,33). In addition, its structure may determine what aspect of chromosomal stability is likely to be critical for a given organism or tissue. Thus, ribosomal DNA repeat arrays may

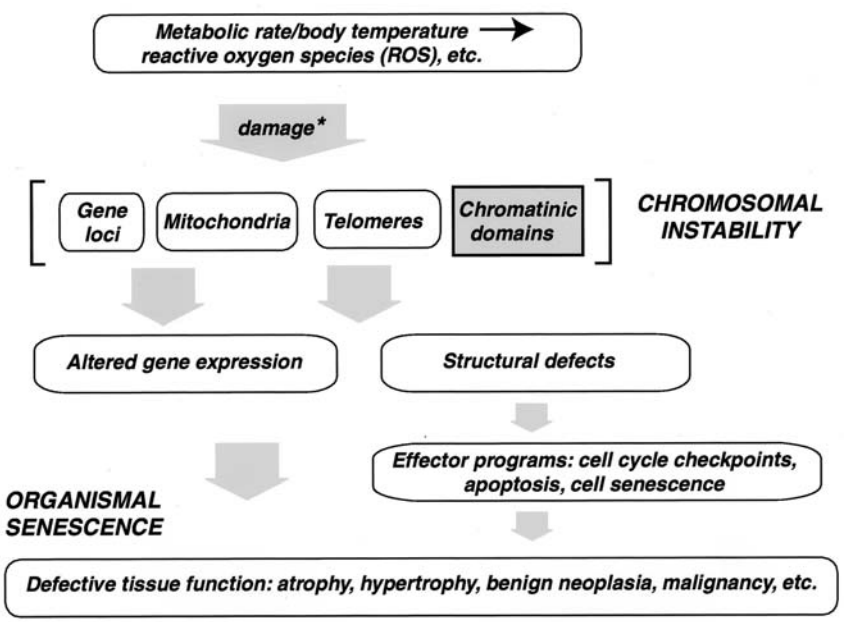

Figure 1 Chromatin viewed in the context of age-related chromosomal instability. In addition to genetic loci (single copy genes, mitochondria, and telomeres), epigenetically heritable chromatin structures are considered as targets for metabolic or oxidative insults. The uppermost arrow subsumes multiple types of "damage,"* with the asterisk indicating that such damage may be primary, but may also be secondary owing to errors in DNA replication and repair. Chromatin domains are thought to be locally disassembled and reassembled in association with replication/repair; accordingly, their instability may not require errors at the DNA sequence level (see Fig. 2).

be particularly subject to damage in budding yeast (10), whereas mitochondrial DNA may be prone to instability in certain other fungi (13), and telomeres may be limiting in some human tissues that are subject to chronic damage and replication-dependent healing (35).

In general, the current consensus is well supported, so potential links between chromatin and senescence should be considered within this framework. Along these lines, it seems most straightforward simply to extend the notion of genome instability to include a higher order chromatinic organization (Fig. 1) (see also Refs. 31 and 36–38). As emphasized above, heritable, genome-associated chromatin structures play essential roles during development. One implication, therefore, of their inclusion under the general rubric of genome instability is that senescence may reflect the enormous complexity of developmental pathways. Although daunting, this could help to explain some otherwise puzzling observations (e.g., the plasticity and variability of aging in different organisms, segmental and tissue-specific aspects of senescence).

III. EPIGENETIC MEMORY: DISTINCT REQUIREMENTS FOR INITIATION AND MAINTENANCE OF HERITABLE CHROMATINIC DOMAINS

A longstanding issue in aging research concerns the immortality of germ line and primitive stem cell lineages. Immortality implies that such cells escape cumulative chromosomal instability—in obvious contrast to most somatic cells. A variety of explanations have been offered to account for this difference. Most often, it is argued that germ cells are far better protected from the cumulative oxidative and other metabolic insults than are somatic cells. This is in keeping with the theory of antagonistic pleiotropy and is thought to reflect allocation of limited metabolic resources. A second approach to the problem is taken by those interested in mitochondrial chromosomes. Here it is suggested that the germ line has a mechanism to sense and select for mitochondrial genome integrity. If so, the age-related accumulation of mitochondrial DNA defects in somatic cells does not apply to germ line cells. The same hypotheses apply, at least in principle, to the protection of higher order chromatinic domains, but with an additional aspect—epigenetic memory. The paradigm for epigenetic memory derives from work on budding yeast. Rine and coworkers were the first to demonstrate rigorously that the molecular requirements for establishing inherited chromatinic structures can be distinct from those essential for their maintenance (7,39). The importance of this work merits a brief digression.

Four members of the silencing information regulator (SIR) family are required to establish heritable repression at the silent mating cassettes. SIR1 plays an essential role in recruiting the other members of the apparatus. This is accomplished via its interaction with ORC1, a component of the origin recognition com-

plex (40). Of the remaining SIR proteins, SIR2 is known to be a NAD-dependent deacetylase (as discussed in detail elsewhere in this volume), whereas SIR3 and SIR4 appear to be structural components of the silencing apparatus. Silencing is established in association by passage of cells through S phase (40,41), although not necessarily by transit of a DNA replication fork through the region to be repressed (8,9). The critical observation in this system is that SIR1, the recruiting element, is no longer required for mitotic inheritance once a self-templating "protein shell" is formed that mediates silencing. In a manner yet to be fully delineated, the shell can perpetuate itself through multiple subsequent rounds of disassembly and reassembly that occur during S phase and mitosis. Heritable memory appears to be acquired by the chromatin structure itself, encoded by an appropriate assembly of modified histones and SIR2–4, together with, most likely, a limited number of additional proteins.

Two further points merit emphasis with respect to epigenetic memory. First, such memory mechanisms appear to have been adopted and widely used through evolution. In addition to budding yeast, well-studied examples exist in the fission yeast *Schizosaccharomyces pombe* and in *D. melanogaster*. These two systems reveal the high degree to which the molecular mechanisms for silencing are conserved. Second, in higher organisms, the information required to establish heritable chromatinic domains can be restricted to discrete developmental windows. The paradigm for this latter concept derives largely from studies on Polycomb-mediated silencing in *D. melanogaster* (42).

Again, a brief summary is in order. The Polycomb group (PcG) gene family and its role in epigenetic silencing first came to light in studies on homeobox protein function. Positional information for antennae, wing, and other structures is specified by transient gradients of transcription factors, typically belonging to the gap and pair-rule families. These gradients establish appropriate expression patterns for homeobox proteins, which in turn control developmental pathways. The initial gap and pair-rule transcription factor gradients disappear within a few hours after the onset of embryogenesis, raising an important problem: How is the positional information preserved? The answer to this question has been revealed by studies on mutant organisms in which memory for the initial transcription factor gradients is defective. The affected gene loci encode a family of interacting chromatinic proteins that, like SIR proteins in yeast, mediate epigenetic inheritance. Members of the Polycomb group and their function are very well conserved from *Drosophila* to mammals. Moreover, their inappropriate expression in mammals has been linked to defects in growth regulation and tumorigenesis. Taking the notions of developmental windows and epigenetic memory together, it is tempting to suggest a remarkably simple—although certainly only partial—explanation for the immortality of the germ line versus the mortality of somatic lineages. In essence, reproduction of the organism, sexually or asexually, may provide a natural "reset mechanism" for higher order chromatin structures, erasing

the cumulative disorder in these that contributes to senescence. According to this view, the germ line, and presumably some primitive stem cell lineages, maintain the requisite information for this reset process, whereas—for developmental reasons—epigenetic memory is used in most or all somatic cell lineages.

IV. AGE-RELATED SILENCING DEFECTS IN MAMMALS

The preceding provides a brief conceptual framework regarding chromatin and senescence. Needless to say, however, such frameworks are of limited interest in biology without experimental support, so the next section turns to a historical perspective.

Evidence for age-related chromatinic remodeling in mice dates back to a seminal study published by Cattanach more than 25 years ago (43). Cattanach, who wished to establish a mouse model of position-effect variegation, chose to study coat color genes inserted into the X chromosome. Through a series of genetic maneuvers, he obtained female mice with one X chromosome carrying an insertion of the wild-type tyrosinase gene, and a second X chromosome defective for silencing due to Searle's X autosome translocation. Both copies of the tyrosinase gene on chromosome 7 were inactivated by an albino mutation. Incomplete silencing of the X-linked autosomal chromosome 7 insert in such mice yielded animals with variegated coats. More importantly, Cattanach observed that coat color changed in these mice in an age-related manner—by the age of 12 months, dramatic darkening of initially white and brown regions was evident. The simplest interpretation of these results is that X chromosome–dependent silencing over the inserted tyrosinase gene is unstable with age, reactivating expression of that and neighboring genes within the autosomal region.

Complementing the above findings are those of Wareham et al. (44). Here again, the stability of X chromosome silencing was examined in female mice heterozygous for Searle's translocation. This time, the ornithine carbamoyltransferase *(OCT)* gene, a native X-linked locus, was examined, avoiding the potential criticism that age-related defects in repression might only be observable in rearranged chromosomes. A histochemical assay allowed detection of reactivated OCT expression in single cells and clusters of liver cells, which likewise exhibited strong age dependence. Remarkably, comparison of young mice (8–10 weeks) and older mice (14–17 months) revealed a 50-fold increase in OCT-positive clones. Subsequent to these two key reports, several groups searched for additional examples of age-related reactivation in X-linked gene expression. Overall, their results were consistent with the notion that instability in silencing patterns is localized, occurring at only a relatively small number of such loci.

The Cattanach and Wareham reports are emphasized here for two reasons. First, they have received relatively little attention in the aging field. This may reflect the fact that epigenetic memory was not discovered until after the Wareham

study. Further, evidence has since accumulated that establishment of X chromosome silencing is restricted to the embryonic blastocyst; that is, a discrete developmental window in the mouse (1). Such information bears strongly on the potential importance of the earlier work. Second, the Cattanach and Wareham studies raise a number of interesting questions. Do age-related defects in silencing reproducibly arise in regions other than on the X chromosome? If so, what is the frequency and how do mitotically active versus postmitotic tissues compare? Can evidence for instability in epigenetic inheritance be detected in humans as well as mice? Does the converse of reactivation occur; that is, do heritably active states of gene expression exist, and are there regions in the genome subject to ectopic silencing in an age-dependent manner?

V. HERITABLE ACTIVE STATES AND AGE-RELATED DE NOVO SILENCING

Of these questions, the last is most readily addressed. Although epigenetic memory is for the most part studied in the context of gene silencing, strong evidence exists that memory can exist as well for active states. A pertinent example of the latter is provided by work on PcG-mediated silencing in *Drosophila*. Paro and coworkers mapped functional domains within Fab-7, a cis regulatory element that controls homeotic gene expression. They demonstrated that this element can be set to a silent state maintained by PcG gene products, but *also* can be converted to a mitotically active heritable state by transcriptional regulators present only during early embryogenesis. Members of the trithorax group (trxG) family have been implicated in maintaining the active state, which is marked by histone H4 hyperacetylation (see below). Heritable active states are associated with heterochromatin as well as with PcG-associated chromatinic structures. The *Drosophila* gene *light* and several other genes require a heterochromatinic environment for transcriptional activity. Such genes behave in a manner opposite to conventional genes in that their expression is attenuated by transposition into a euchromatic chromosome region. In contrast to conventional transcription units, these genes function less well as heterochromatinic proteins become limiting within the cell. Dosage compensation on the *Drosophila* X chromosome is a related system of interest. Here, an epigenetically determined active state specifies a two-fold increase in gene expression specific to the male X chromosome. This is mediated by the MSL complex, an entity that contains both MOF family acetyltransferases and roX RNAs (45,46).

Imprinting provides yet another example of gene regulation in which alternative heritable states of gene expression are believed to exist. Imprinted genes exhibit parent-specific gene expression; that is, they are active if maternally inherited or vice versa. One allele is set to a silent state, typically marked by DNA

methylation within a CpG-rich island associated with the promoter, whereas the other allele is expressed. Recent evidence points to an intimate relationship between DNA methylation and chromatin-based silencing, but little is known concerning chromatin structure and its determinants at imprinted loci. Accordingly, studies on imprinting have remained (somewhat artificially) distinct from those on epigenetic inheritance in model organisms. Still, work by Issa et al. on the human IGF2 promoter provides a notable instance of age-related instability in an epigenetically heritable state (47). In young individuals, promoter methylation is selective for the maternal allele, with only the paternal IGF2 gene copy being expressed. During aging, a switch is frequently observed to biallelic promoter methylation, presumably resulting in de novo silencing. Broadening the discussion briefly to include DNA methylation permits mention of age-related inactivation of rRNA gene clusters in CBA/Ca mice (48) and of age-associated CpG island methylation within the promoters of several human genes (49). At such loci, de novo methylation is highly variable when multiple individuals are compared, although the statistical trends are unambiguous. Importantly, it remains to be determined whether such changes are initiated by errors in DNA methylase activity or reflect remodeling of higher order chromatin structures followed by increased methylation—or perhaps both.

VI. MOLECULAR MECHANISMS INVOLVED IN CHROMATIN-DEPENDENT INHERITANCE

The fields of gene regulation and developmental biology have already revealed enormous complexity in chromatin-mediated epigenetic processes. A number of excellent reviews are available (4–6,50,51), and it is far beyond the scope of this chapter to summarize work in this area. Nevertheless, a much-oversimplified schematic of silencing may be illustrative (Fig. 2). As outlined, three steps are thought to be involved in *establishment*: First, one or more "address-specifying" DNA binding factors mark, for example, a promoter element, CpG-island, or enhancer, to be set to a silent state. Second, chromatin-modifying enzymes are recruited. Depending on the locus involved, these may include histone deacetylases of the RPD3 family, deacetylases belonging to the SIR2 family, or histone methylases such as Su(var)3–9. Appropriate modification of histone tails promotes interactions among nucleosomes, likely permitting the formation of a more highly ordered 30-nm fiber. According to the histone code hypothesis (50), modifications of histones also create a surface for the third step—secondary recruitment of silencing proteins. A recently described example of secondary recruitment involves HP1 heterochromatinic structural proteins. These bind through chromo domains to methyl-lysine residues at position 9 on the amino-terminal tail of histone H3 (52). Once established, silencing domains persist via *maintenance* mechanisms

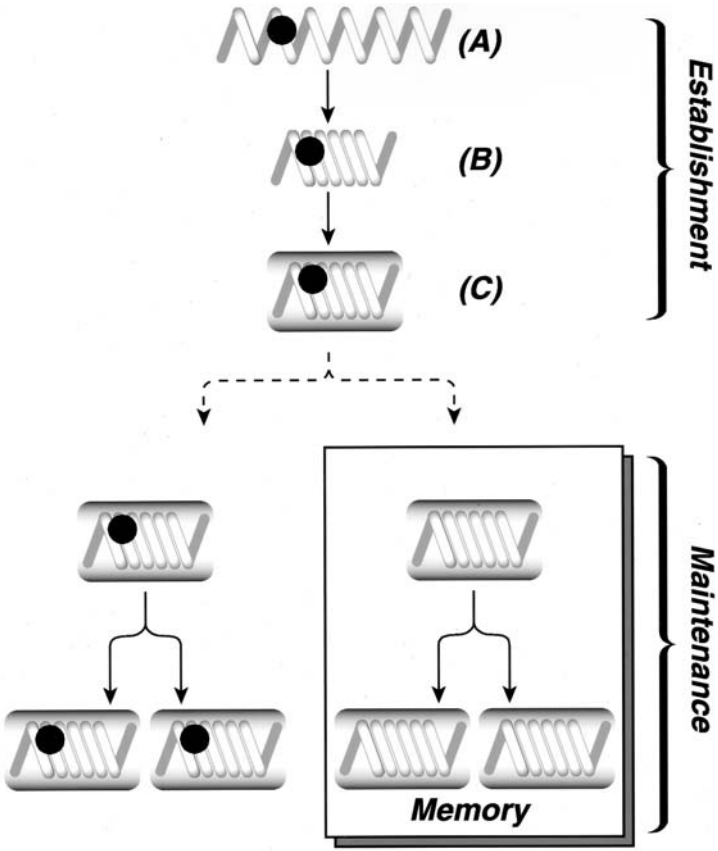

Figure 2 Requirements for establishment and maintenance of heritable chromatin structures. The black circle denotes a sequence-specific DNA-binding factor that acts to specify the initial address for establishment. Steps (A) through (C) are described in the text. Subsequent to establishment, the address-specifying DNA-binding factor may dissociate from the higher order domain; whether or not this requires DNA replication is unknown. Maintenance implies one or more duplications in association with DNA replication and mitosis. For such duplications to occur in the absence of address-specifying factor(s), the structure is presumed to be self-templating and to persist by virtue of epigenetic memory.

either in the presence or absence of address-specifying DNA-binding factor(s). The preceding schema applies to heterochromatin-like structures characterized in organisms ranging from yeast (in particular the fission yeast *Schizosaccharomyces pombe*) to mammals.

Yet it is clear that there are numerous unique as well as common aspects to the various repressive chromatinic subtypes. In general, histones H3 and H4

in heterochromatin are underacetylated, presumably reflecting recruitment of RPD3 and SIR2 family deacetylases as well as exclusion of histone acetyltransferases such as PCAF, GCN5, and CBP/p300. At some loci, however, putative acetyltransferases belonging to the SAS family contribute to or are required for silencing (53,54). Inactivation of X chromosome expression involves histone underacetylation and DNA methylation, but differs from silencing at centromeres, and other heterochromatin-associated regions in that HP1 family members are not preferentially recruited. Rather Xist RNA molecules (and presumably associated proteins) contribute to the establishment and maintenance of the silenced state on the X chromosome (2). Centromeric silencing in yeast involves the incorporation of variant H3-like histones (55), and it has been proposed that X chromosome silencing involves the atypical histone variant macroH2A (56). Most distinct is Polycomb group–mediated silencing, which differs genetically and biochemically from that associated with heterochromatin. In *Drosophila*, most mutations that suppress or enhance PcG silencing have no effect on position-effect variegation mediated by heterochromatin. One exception to this rule is the gene locus *Enhancer of zeste*, which has been implicated in the assembly of repressive domains in both systems (57). It will be interesting to determine whether this locus encodes a protein directly or indirectly involved in chromatinic modification.

VII. CHROMATINIC STRUCTURES: MANIPULATION IN MODEL SYSTEMS

In so far as instability in higher order chromatin structures accompanies aging, what mechanisms might be involved? The answer(s) to this question remains to be determined, but may depend in part on whether mitotic or postmitotic compartments are being considered. For tissues characterized by continuous or intermittent cell proliferation, errors in chromatinic reassembly after DNA replication and/or mitosis are an obvious possibility. A prediction that follows, in principle, is that proliferation of somatic cells under conditions favoring instability in higher order chromatinic domains could accelerate senescence, whereas factors that stabilize such structures should extend life span. As mentioned at the outset, the budding yeast model of replicative senescence appears to conform to this prediction. Mutations at the SIR2, SIR4, or RPD3 loci shorten or prolong the replicative life span in a manner that is consistent with their influence on gene silencing. Further, overexpression of a yeast SIR2 homolog in the nematode *C. elegans* extends the life span. In the yeast model, increased longevity and enhanced silencing require the NAD-dependent deacetylase associated with SIR2. Presumably this enzymatic activity is also required for life span extension in *C. elegans*. Additionally, one report has appeared concerning histone deacetylation and senescence in the

plant model system *Arabidopsis thaliana*. Antisense-mediated downregulation of the *Arabidopsis* RPD3 histone deacetylase homolog HD1 is associated with pleiotropic effects on development and ectopic expression of silenced genes and early senescence (58). Manipulation of the life span in mammalian organisms is of course a far more complex task than it is in yeast or other simple model organisms. Transgenic mice may provide a means to test the role of epigenetically inherited chromatinic structures in organismal senescence, although to obtain unambiguous evidence, conditional expression systems as well as reliable biomarkers for aging might prove to be necessary. A further consideration is the redundancy typically observed in mammalian gene regulatory circuits. At present, the number of RPD3-like histone deacetylases (HDACs) in humans stands at nine (59), whereas seven SIR2-like proteins have been described (60).

In the meantime, limited information can be obtained from studies on replicative senescence as measured in tissue culture. Senescence in this system can be seen, as noted, as a stereotypic effector program akin to apoptosis and activated by a variety of environmental insults. That such insults can target chromatin-based structures and thereby induce premature senescence is suggested by experiments with inhibitors of RPD3-like histone deacetylases. Sodium butyrate and trichostatin A, two such inhibitors, elicit cell cycle–dependent commitment to growth arrest and associated phenotypic changes in human embryonic lung fibroblasts and mouse skin fibroblasts (31). The specificity of these pharmacological agents should be verifiable by further experiments in which dominant negative mutants of RPD3 family deacetylases are overexpressed. Specifically, mutant HDACs of this category should mimic the actions of butyrate and trichostatin A—with the proviso that the latter simultaneously inhibit multiple different deacetylase family members. Indeed, overexpression of mutant, but not wild-type, forms of HDAC1, HDAC2, or HDAC3 can cause rapid growth arrest and induce markers of senescence. Not surprisingly, the relative importance of each enzyme in this context appears to depend on the cell type under study (our unpublished data).

VIII. POTENTIAL CONSEQUENCES OF CHROMATIN INSTABILITY

Broadly speaking, there are two ways in which instability in higher order chromatin domains might contribute to age-related deficits. The first is by altering patterns of gene expression, and thereby the differentiation state, within a cell. It has been proposed that abnormal gene expression in even a small fraction of cells in tissue can lead to field effects that compromise the overall integrity of the tissue (23,24). Such changes, which could affect adult stem cells as well as more differentiated lineages, would most likely fall within the category of private or dysdif-

ferentiation mechanisms of aging. A second consequence of instability in chromatin domains—or structures based on those domains—could be an activation of stress-induced checkpoint or terminal effector programs. Tentative evidence for links between chromatin instability and stress-activated terminal effector programs derives from studies in budding yeast.

Guarente and coworkers (37) have reported a *sir4-42* mutant strain that produces a C-terminally truncated SIR4 protein. This longevity-extending mutation exhibits elevated resistance to starvation in the cold as well as to heat stress. Conversely, loss of the *Sif2* gene product, an SIR4-interacting protein that antagonizes telomeric silencing and localization of SIR4 to telomeres, dramatically reduces resistance to heat and starvation (61). In mammalian cells, senescence and apoptosis effector programs appear to be modulated by bmi-1, a member of the PcG family of chromatin regulators. The *bmi-1* gene was initially identified owing to its association with B-cell lymphomas in mice (62). Overexpression of bmi-1 promotes cell proliferation and immortalization and also antagonizes apoptosis. In contrast, its underexpression is associated with premature cell senescence (63). The mechanism by which *bmi-1* modulates these pathways is at least in part through interference with upregulation of ink4a-ARF in response to c-myc activation (64).

IX. MITOTIC VERSUS POSTMITOTIC COMPARTMENTS

Cycles of chromatin disassembly and reassembly accompany each round of DNA synthesis, so it is easy to imagine how ectopic silencing and other errors in heritable chromatin patterns could arise. Less obvious is how such errors could occur in tissues where most cells are nonreplicating. Nevertheless, one special case does illustrate that epigenetic silencing can be unstable in a postmitotic state.

Paro and coworkers (65) constructed a *Drosophila* strain carrying a Pc-G response element–linked reporter gene. In temperature shift experiments, they reported loss of silencing over time during adulthood (i.e., an age-related loss) at this recombinant locus. How might relaxation or initiation of heritable silencing be established in the absence of DNA replication? A speculative but potentially important possibility is that chromatin domains could be remodeled by repeated cycles of DNA damage and repair. Studies on budding yeast provide a precedent for this idea. In that organism (as in mammalian cells), double-stranded DNA breaks lead to activation of one or more nonhomologous end-joining (NHEJ) pathways. Ku proteins are thought to bind to, and facilitate, end joining, at least in part through mobilization from telomeric binding sites. Notably, SIR proteins are recruited to double-stranded DNA breaks in much the same manner (11,66). The product of the *SIR3* gene, in particular, has been shown by chromatinic immunoprecipitation experiments to be present at broken DNA ends.

How often errors in chromatinic restoration occur after NHEJ remains to be seen. In one experimental system—the yeast *ADE2* gene with an adjacent silencer—further repression is readily observed after DNA damage (11). This would suggest that persistent local inactivation of gene expression is rather frequent. A corollary suggestion then is that genetic defects in DNA repair pathways might impact chromosomal stability indirectly as well as directly. Although hypothetical, this suggestion raises the possibility that specific DNA repair defects could differentially affect the rates of DNA mutation and chromatinic remodeling. In turn, effects on cancer and aging could be partly dissociated.

X. REMAINING ISSUES AND CONCLUSION

Numerous questions remain concerning silencing mechanisms, some of which can be posed with specific reference to senescence. For example, do stress and developmental pathways overlap in a manner that links them to the silencing apparatus? If this is the case, activation of stress pathways might have broader consequences than heretofore considered. Heterochromatin-based structures appear to be very temperature sensitive in *Drosophila*. Is the same true for heterochromatin-mediated silencing in vertebrates? What, if anything, might this have to do with the temperature dependence of the life span in poikilothermic species, or even the slight hypothermia associated with dietary restriction? Ecdysone, a steroid hormone that regulates differentiation in *Drosophila*, influences HP1-dependent heterochromatin during embryogenesis and the late stages of the third larval instar (67). How might hormonal and other environmental factors affect the formation of inherited chromatinic structures during mammalian development? Could this impact on subsequent risk patterns for cancer (e.g., adenocarcinoma in daughters of women treated with diethylstilbestrol during pregnancy [68]) or other age-related diseases? Variegation is a characteristic aspect of heritable silencing—and indeed, in at least one experimental model, the *Avy/a* mouse, variable epigenetic regulation of a retroviruslike element inserted near the *agouti* gene influences longevity (69). Could variegated silencing in human siblings, or even in identical twins, contribute to variation in life span? If so, this might lead to an overestimate of the importance of environmental factors in aging.

In sum, there are numerous facets to the study of senescence, including metabolic control and signaling pathways, oxidative stress and mitochondrial regulation, and DNA repair and chromosomal instability. Of these facets, the general notion of chromosomal instability is in some ways the most intriguing. Here, a case has been made that such instability can be extended to include higher order chromatin structures. Much work remains to be done to develop a more complete picture as to which types of heritable chromatinic domains are subject to age-related damage in, for example, various organisms, and tissues. Although no sim-

ple picture may emerge, one potential reward is linked to a special aspect of these structures; namely, their natural linkage to developmental programs that depend on epigenetic memory. This may provide a means to tie such programs to the plasticity of aging, as well as to understand how developmental inputs can be "imprinted" in a manner that influences patterns of age-related disease.

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9
Werner Syndrome

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I. INTRODUCTION

Until recently, Werner syndrome (WS) had been considered to be a model of accelerated aging. The Werner gene (*WRN*) was identified in 1996 (1) and was subsequently shown to act as a DNA helicase and as an exonuclease (2,3). Since then, cancer researchers and those who study DNA metabolism have collaborated to further characterize WS and the function of the *WRN* gene. The more we understand about the *WRN* gene, the more we realize that WS is not merely accelerated aging. WS certainly does not represent premature aging, in the sense that the characteristic aging phenotypes seen in WS are considerably different from those observed in normal older adults. WS is now being more correctly recognized as a condition in which the lack of *WRN* protein (WRNp) results in an overall decline in the normal physiological functions of various organs, including those most frequently used to estimate chronological age (e.g., skin and hair).

Since the identification of the *WRN* gene, various in vitro biochemical studies of WRNp have answered many of our initial questions regarding the helicase and exonuclease functions of this enzyme. Considerably more time will be required to answer the more difficult questions concerning the in vivo functions of *WRN* at the cellular and organismal levels. The lack of WS mouse models that mimic the human disorder currently limits our ability to carry out such studies. In this chapter, the known in vitro functions of WRNp and the cellular characteristics of WS cells will be summarized. From this, the putative in vivo functions of *WRN* will be extrapolated.

II. CLINICAL FEATURES

The clinical phenotypes of WS can best be summarized as onset of an aged appearance and age-related common disorders (4–7). Unlike people with Hutchinson-Gilford progeria (discussed in Chapter 12), patients with WS usually develop normally until they reach the second decade of life. Some patients may present with flat feet. Generally, the first sign is a lack of the pubertal growth spurt during the teen years. Patients frequently recall that they were of average height when they entered grade school, but were the shortest in their class by the time they graduated from high school. In their 20s and 30s, patients begin to manifest skin atrophy, loss of hair, and graying hair. Subcutaneous fat tends to deposit on the trunk, and combined with osteoporosis of the limbs, patients exhibit a stocky appearance. The other most common age-related disorders seen in patients with WS are bilateral cataracts and type 2 diabetes mellitus. Penetrances are 98% for cataracts and 90% for diabetes mellitus (6). These numbers obviously depend on the age of the patient when clinical reports are made and how rigorously patients are examined. Demographically, WS cases are most frequently reported in Japan. This can be attributed partly to an awareness of the syndrome among Japanese physicians and the higher consanguinity among the Japanese population as compared to the U.S. population. The chronological order of the onset of these complications is similar in Caucasian and Japanese patients with WS (4,7). The International Registry of Werner Syndrome (Seattle, Wash.) has documented more than 100 cases of WS. New cases, with a diagnosis confirmed by genetic analysis are mostly in patients over age 30 years. This is probably due to the lack of prominent symptoms before age 20 years.

Two common causes of death among patients with WS are malignancy and myocardial infarction (4,8). Although these are also two common causes of death in the general population, unique characteristics are observed in patients with WS. An extensive review of malignancies has been carried out in WS cases in Japan, where the prevalence of *WRN* mutations is relatively high (9). Strikingly, the ratio of cancers of epithelial origin and sarcomas of mesenchymal origins is 1:1 in patients with WS, whereas this ratio is approximately 10:1 in general population. A possible explanation for the high representation of mesenchymal tumors in patients with WS may be related to the mechanism by which telomere length is maintained (10) (discussed in Sec. III.F). Review of the pathological studies of these malignancies revealed unusual primary sites for cancers in patients with WS. For example, melanomas in patients with WS are of the acral lentiginous type in mucosa, and are unrelated to sun exposure (9). The primary sites of osteosarcomas in patients with WS are more likely to be in the lower extremities, whereas these are more common in the upper extremities in the general population (11). This variation may be related to the threshold of WRNp required to maintain DNA stability in each cell type within a given organ. Among patients with WS, the spe-

cific cell type in which cancer develops may differ depending on the type of mutation in the *WRN* gene. Papillary carcinoma has been associated with an N-terminal mutation, whereas follicular carcinoma is more frequently observed with a C-terminal mutation (12). This finding clearly contradicts our original assumption that all identified mutations within *WRN* result in truncation of the nuclear localization signal of WRNp, and thereby act as null mutations (see Sec. II.B). Further studies may reveal an additional correlation between specific genotypes and phenotypes.

Several other important differences have been noted between patients with WS and normal older adults. Atherosclerosis exhibits unique characteristics in patients with WS. Atherosclerotic lesions are more extensive in arterioles in WS patients. Calcification of cardiac valves is also sometimes observed in WS patients, possibly reflecting excessive cell death due to the constant pressure of blood flow. Skin ulcers around the ankles and elbows that are more severe than those typically seen in the progression of diabetes mellitus are not uncommon in WS. Dementia of the Alzheimer type is relatively rare (13) despite the fact that WRNp is expressed in the brain (1). Whereas in the general population osteoporosis has a more pronounced effect on vertebrae, long bones (particularly those of the lower limbs) tend to be more affected by osteoporosis in patients with WS (14).

Werner syndrome was characterized and first described by Dr. Otto Werner at the Kiel University in his doctoral thesis at the turn of 20th century (15). Two symptoms that drew his attention were bilateral cataracts and scleoderma-like skin. The latter turned out to provide insight into the interaction between WRNp and the Ku complex (see Sec. III.E.), the autoantigen that triggers scleroderma, an autoimmune disorder.

III. *WRN* GENE PRODUCT

A. Functional Domains of *WRN* Gene Product

The *WRN* gene comprises 35 exons on the short arm of chromosome 8 and encodes protein of 1,432 amino acids. It was identified in 1996 using a conventional positional cloning approach (1) and the methods that were available at that time (16). Simple alignment in database searches and more complicated structural studies showed four defined regions of WRNp. These include exonuclease domains I, II, and III in the N-terminal region (17,18); RecQ-type helicase domains I, Ia, II, III, IV, V, and VI in the central region (19); a RecQ-conserved motif immediately following the helicase motifs (20); and a helicase ribonuclease D C-terminal (HRDC)–conserved motif in the C-terminal regions (20).

Biochemical studies confirmed the helicase and exonuclease activity of WRNp in vitro (2,3,21). The function of the RecQ-conserved motif has not been

defined. The HRDC motif is thought to form a scaffold that interacts with substrate DNA with relatively low affinity, based on the three-dimensional structure, which resembles the auxiliary DNA-binding domains of bacterial DNA helicases (22). Cell biological studies identified two other functional domains. One of these functions as a transcriptional activator within a highly acidic region between the exonuclease and helicase domains (23,24); the other functions as a nuclear localization signal in the extreme C-terminus of WRNp (25,26). The relative locations of these structural domains are shown in Figure 1.

B. *WRN* Mutations

To date, at least 35 different *WRN* mutations have been reported from all over the world (1,8,27–30) (shown in Fig. 2). The known mutations correspond to either stop codons, insertions, or deletions that result in a frame shift, or splicing donor or acceptor site mutations, which cause an exon to be skipped, resulting in a frame shift. All of these mutations result in truncation of the nuclear localization signal. Unlike the *BLM* gene (31), no missense mutation has been identified in WS. As we expand WS screening, this type of mutation may be identified in future WS cases.

In addition to the loss of the nuclear localization signal in *WRN* mutations, the mutant mRNAs and the resulting mutant proteins exhibit shorter half-lives

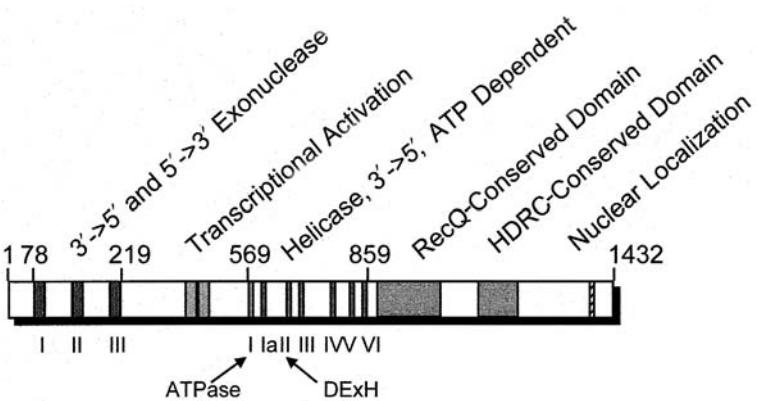

Figure 1 Functional domains of the *WRN* protein. Arabic numbers indicate amino acids of the *WRN* protein. The designated functions of the exonuclease domains (I-III), the acidic repeat transcriptional activator regions, helicase domains (I-VI), and nuclear localization signal have been demonstrated in various biological assays. The roles of the RecQ C-terminal–conserved region and the helicase RNaseD C-terminal (HDRC)–conserved regions are based on the structural studies.

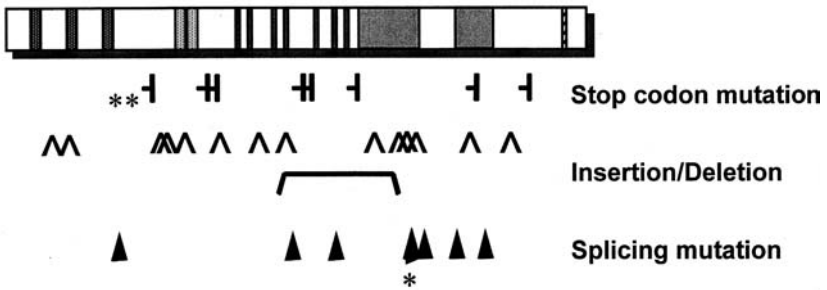

Figure 2 *WRN* mutations in patients with WS. The rectangular boxes indicate the functional domains of the *WRN* protein (see Fig. 1). Known *WRN* mutations are grouped based upon the type of mutation, shown above and underneath the *WRN* protein diagram along with Registry codes. Parentheses indicate the heterozygous mutations. R367Stop (**) is the most common mutation seen in Caucasian patients with WS (approximately 25%) and the second most common mutation in Japanese patients with WS (approximately 18%) (83). Deletion of exon 26 (*), which is due to a splice junction mutation, is the most common mutation in Japanese patients with WS (approximately 52%) (Rsef. 83).

than do the wild-type mRNA and WRNp (32,33). Mutant products truncated at N-terminal to the helicase regions were more labile than a mutant protein truncated C-terminal to the helicase region. The biological significance of low levels of mutant WRNp in the cytosol is unknown. Depending on the specific mutation, certain WRNp mutations can retain enzyme activity or other functions. These proteins can modify DNA during the mitotic phase or modify RNA during all phases of the cell cycles. It is possible that various mutant forms of WRNp, which may be retained in the cytosol, could contribute to the slight differences in phenotype observed with various *WRN* mutations. For example, thyroid cancer associated with the two major *WRN* mutations differs (34). Papillary carcinoma is associated with the N-terminal mutation, whereas follicular carcinoma is seen more often with the C-terminal mutation.

C. *WRN* Helicase Activity—Effect of Single-Stranded Binding Proteins and Substrate Specificity

The helicase activity of WRNp is a RecQ-type helicase, named after the prototypic *Escherichia coli* RecQ. A number of laboratories have demonstrated that WRNp exhibits ATP-dependent $3' \rightarrow 5'$ helicase activity in an oligo-displacement assay (2,21,35–37,39). One study showed that this activity was present in an immunoprecipitated sample of WRNp (40). WRNp appears to first bind to the single-stranded portion of the longer strand of the DNA duplex and proceeds in a

3′→5′ direction with respect to the longer strand (39). This 3′→5′ movement corresponds to the direction of proofreading, as opposed to the direction of DNA synthesis, which proceeds from 5′→3′. Thus, *WRN* has been proposed to play a role in some aspects of DNA strand repair, although the specific mechanisms involved are not known. WS cells are hypersensitive to 4-nitroquinoline-1-oxide (4NQO) (38), although WRNp does not have a higher binding affinity for DNA that has been damaged by 4NQO or by ultraviolet (UV) light (39). The *WRN* helicase can also function to unwind DNA–RNA duplexes (21).

The helicase activity of WRNp can be enhanced by the presence of various single-stranded binding proteins, such as *E. coli* SSB, the T4 gene 32 product or, more efficiently, human replication protein A (hRPA) (2,21,35,37,39,41). These single-stranded binding proteins are believed to facilitate *WRN* helicase activity by stabilizing single-stranded structures and by preventing their reannealing. Several investigators have demonstrated that WRNp and hRPA are colocalized in *Xenopus* replication initiation complexes and in HeLa cells arrested in S-phase with hydroxyurea (42–44). Moreover, recombinant WRNp and purified hRPA have been shown to coimmunoprecipitate (37). hRPA might play an additional role, besides stabilizing the single-stranded DNA structure, in the *WRN*-catalyzed unwinding reaction that requires direct interaction with WRNp.

Several unusual substrates have been tested as potential physiological targets for *WRN* helicase activity. *WRN* was able to efficiently unwind a G4-quartet made by two hairpin loops (G′2 biomolecular tetraplex) of d(CGG)n (45). In this study, the G4 structure was generated under a high salt concentration in vitro. Though its presence has not been demonstrated in vivo, a G4-quartet can potentially be formed from two GC-rich regions of unwound single-stranded DNA (or RNA) during replication, repair, recombination or transcription. Another interesting structure is a recombination intermediate or α structure. WRNp was able to promote branch migration of a Holliday junction (43). In fact, the *WRN* helicase appeared to dissociate the α structure better than a simple DNA duplex, as assessed by the length of the migration. This structure can be formed during recombination and replication, such as break-induced DNA replication or repair of the stalled replication. The complex secondary structures described above were also substrates for the *BLM* helicase in vitro. Thus, in vivo, WRNp may participate in the dissociation of these structures as well.

D. Characteristics and Significance of *WRN* Exonuclease Activity

The 3′→5′ exonuclease activity of *WRN* has been demonstrated by at least four independent laboratories (3,41,46–50). One group measured 5′→3′ exonuclease activity (36), although no laboratory has demonstrated both 3′→5′ and 5′→3′ exonuclease activities in WRNp.

Although exonuclease activity in the 3′→5′ direction does not require ATP, enzyme activity is enhanced in the presence of ATP and is specific for the 3′ recessed end of the duplex (3,46,47,49). The *WRN* exonuclease uses the 3′ end of the blunt end as well as the 3′ overhang end in presence of Ku70/80, the regulatory subunit of DNA protein kinase (DNA-PK) (48,50). Similar to its *WRN* helicase activity, the exonuclease activity of *WRN* also digests RNA-DNA heteroduplexes (49). The N-terminal region of the mouse WRNp also shows 3′→5′ exonuclease activity (49).

Double-stranded DNA substrates with multiple base pair mismatches within the duplex (termed "bubbles") are more susceptible to digestion by human or mouse *WRN* exonuclease than those without mismatches, but the presence of more than two mismatches at the end of the substrate make them relatively more resistant to digestion (47,49). Structures resembling Holliday junctions are also known to be more susceptible to *WRN* exonuclease digestion than are simple DNA duplexes (47).

Relative substrate specificity suggests that, like the *WRN* helicase, *WRN* exonuclease activity may be involved in repairing DNA damage. The exonuclease and helicase activities of *WRN* are physically and functionally separable (3). How exonuclease and helicase functions are specifically coordinated during the DNA repair process remains unknown.

E. Regulation of Transcriptional Activation by *WRN*

Helicases play a critical role in transcription. Transcriptional activation by the *WRN* protein was originally suggested by findings in the yeast one-hybrid system (23). Subsequently, transcription was measured using nuclear extracts on a reporter plasmid containing the RNA pol II specific adenoviral major late promoter. Transcription catalyzed by nuclear extracts from WS cell lines showed a 50–60% reduction compared with wild-type nuclear extracts (24). This defect in transcription was reversed by the addition of recombinant wild-type *WRN* protein but not by the addition of a mutant (K577M) *WRN* protein defective in helicase activity. In a yeast hybrid-protein reporter system, an acidic 27–amino acid repeated sequence of WRNp functioned as a strong transcriptional activation domain alone or in the context of the full-length WRNp. Therefore, the repeated sequence and the ATPase/helicase activity of WRNp may be involved in the stimulation of transcription in vitro (24).

IV. WRN PROTEIN COMPLEXES AND CELLULAR FUNCTION OF *WRN*

Identification of proteins that interact with *WRN* has helped to shed light on the in vivo functions of WRNp. Some laboratories have characterized the association of

WRN with specific candidate interacting proteins, whereas others have screened cDNA libraries by the yeast two-hybrid system, isolated the *WRN* complex from the cell extracts, or captured the interacting proteins by binding to a WRNp affinity column. Interestingly, each method identified different sets of *WRN*-interacting proteins. Thus, *WRN* may associate with different protein complexes depending on the status of DNA metabolism during the time that the *WRN* protein is recruited.

Cell biological studies also suggest that *WRN* has multiple functions. Two characteristics of WS cells that had been well known even before the *WRN* gene was identified are that these cells exhibit a shortened replicative life span and genomic instability. More recently, studies of drug sensitivities and telomeric metabolism led to a better understanding of the specific functions of WRNp (discussed in more detail in Sec. III.E). The identification of proteins interacting with WRNp along with results of cellular and subnuclear studies suggest that *WRN* may be involved in a wide variety of DNA metabolic processes.

A. DNA Replication

FFA-1 is a *Xenopus* ortholog of *WRN* and was originally identified in the replication initiation complex of *Xenopus* oocytes as being required for the formation of replication foci during DNA replication (42). FFA-1 and the p70 subunit of RPA also have been coimmunoprecipitated from lysates of *Xenopus* oocytes (44). The immunodepletion of FFA-1 from total interphase egg extracts has no significant effect on DNA replication. However, RPA stimulates the DNA helicase activity of FFA-1, and this stimulation can be prevented in a dominant negative manner by the addition of glutathione S-transferase FFA-1 fusion proteins. Therefore, it remains to be determined how essential FFA-1 is for DNA replication, and to what extent other helicases can compensate for their absence (44). WRNp interacts with several components of the DNA replication complex, including proliferating cell nuclear antigen (PCNA) and topoisomerase I (51). The N-terminal region within the exonuclease domain of the WRNp, particularly the region including amino acids 168–246, strongly interacts with the C-terminal portion of PCNA (51), as indicated in Figure 3. The N-terminal region of recombinant WRNp appears to form mostly trimers, as does PCNA, suggesting that the interaction between *WRN* and PCNA involves quaternary structure (49).

B. DNA Polymerase δ

DNA polymerase δ (polδ) participates in DNA replication and repair of DNA damage. This enzyme is also found in the telomeric complex. Evidence of physical interaction between *WRN* and polδ initially came from a yeast two-hybrid screen, in which the C-terminal region of WRNp was used as the "bait" to capture

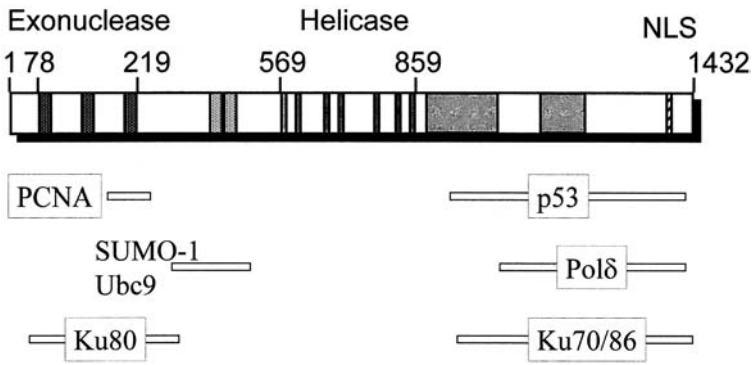

Figure 3 Proteins that interact with the *WRN* protein. Proteins known to interact with WRNp at specific sites are shown schematically. The approximate regions of protein–protein interaction are indicated.

the p50 subunit of polδ, as shown in Figure 3 (52). Immunoprecipitation with an anti-p50 antibody resulted in a complex including p120, the catalytic subunit of polδ. In yeast, physical and functional interaction between *WRN* and polδ requires the third subunit pol32p (53).

Functional interaction of WRNp and polδ appears to be unilateral. In a simple primer extension assay, the addition of WRNp enhanced polδ activity, whereas the addition of polδ did not stimulate exonuclease or helicase activities (53). In contrast, WRNp did not stimulate either polα- or polϵ-mediated DNA synthesis (53, 54). The presence of WRNp also enabled polδ to transverse hairpin and G′2 bimolecular tetraplex structures to complete DNA synthesis (54). One of the functions of WRNp may be to recruit polδ to regions of complex secondary DNA structure to alleviate stalled DNA synthesis (52,54).

C. Homologous Recombination

One of the important biological functions of *E. coli* RecQ is to suppress homologous recombination by disrupting its intermediate structure (55). The yeast homolog of RecQ, Sgs1, is involved in homologous recombination and in illegitimate recombination or nonhomologous end joining (NHEJ). The *WRN* and *BLM* helicases are able to complement increased homologous recombination and illegitimate recombination of an Sgs1 deletion mutant (56). However, the cytogenetic characterization of cells from Bloom syndrome patients show increased exchanges of sister chromatids, whereas cells from patients with WS exhibit variegated translocation mosaicism (57). These findings suggest that the *BLM* helicase may play a more prominent role than WRNp in the suppression of homologous recombination in vivo.

D. Repair of Breaks in Double-Stranded DNA

DNA-PK is a protein complex including a protein kinase catalytic subunit, DNA-PKcs, and a regulatory subunit, Ku70/80 (58). This protein complex is involved in the initial stages of nonhomologous end joining (NHEJ). WRNp has been shown directly to interact with DNA-PKcs and Ku80 (48,50,58). Association of *WRN* with the Ku complex enhances its exonuclease activity but has no effect on its helicase activity (50). Upon binding to Ku, *WRN* is able to degrade DNA from the 5′ recessed end (5′→3′ exonuclease activity) and from the blunt end. This may explain why both 3′→5′ (3,46,60) and 5′→3′ (36) exonuclease activities were observed in recombinant WRNp. The presence of exonuclease activity has been speculated to be a necessary step for DNA-PK–mediated NHEJ prior to the polymerization and ligation step mediated by XRCC 4 and ligase IV (58). In addition, the *WRN* helicase may unwind broken ends of double-stranded DNA in the search for microhomology.

E. Telomeric Maintenance

In a high-quality immunofluorescence study, Shiratori et al. (61) identified nuclear dots suggesting that WRNp is localized on telomeres. Johnson et al. (10) demonstrated WRNp colocalizes with various telomeric components, including TRF1 and TRF2 in six ALT cell lines. ALT cells lack telomerase activity, and the telomeres in these cells are presumably maintained by recombination. Recombinant WRNp did not unwind a G′2 tetraplex containing a telomeric repeat sequence (45). However, WRNp did appear to unwind up to 23 kb of a polymerase chain reaction (PCR)–generated telomeric repeat sequence to single-stranded DNA, and this process was stabilized by hRPA (62). These findings collectively suggest that WRNp plays an important role in telomeric maintenance.

During the serial passage of primary WS fibroblasts, telomeres became shortened more quickly, and telomeres in cells that have stopped dividing were longer than those in control fibroblasts (63). Telomeres of WS lymphoblastoid cell lines (LCLs) were unstable, and telomere length varied more widely in LCLs from patients with WS as compared with those from normal subjects (64). The telomere length at which LCLs from patients with WS go into crisis also varied widely. It has been suggested that one way in which WRNp may be involved in the ALT pathway is by regulating the number of extrachromosomal telomeric repeats (65). This may explain why the catalytic subunit of human telomerase (hTERT) is able to extend the replicative life span of WS cells indefinitely (66–68). hTERT may be able to circumvent the early halt of cell division in WS cells because the WRNp complex functions in pathways that are distinct from hTERT-dependent telomeric maintenance (65). These findings provide significant insight into the possible mechanism of mesenchymal tumorigenesis in patients with WS, as ALT cell lines are frequently mesenchymal in origin (10).

However, other important factors also may be involved in regulation of telomeric function. Hisama et al. (71) have shown that either *WRN* or hTERT could complement 4NQO sensitivity in simian virus 40 (SV40) transformed WS fibroblasts. Introduction of hTERT appears to reprogram gene expression (68). WRNp may play an additional role in telomeric maintenance aside from repairing damaged telomeres and immortalizing cells that lack telomerase activity.

F. p53

As shown in Figure 3, the C-terminal region of WRNp interacts with the p53 tumor suppressor, as these proteins can be coimmunoprecipitated (69,70). In the absence of *WRN*, p53-mediated apoptosis is attenuated (69). Overexpression of *WRN* enhances p53-dependent transcriptional activation of p21Waf1 (70) and potentiates p53-mediated apoptosis (70). Synergistic actions of p53 and *WRN* have been observed in mouse models of WS (72,73) (as discussed in Sec. IV.D).

G. SUMO-1

A yeast two-hybrid screen using mouse *WRN* as the bait identified Ubc9 and SUMO-1 as *WRN*-interacting proteins (74). Ubc9 (ubiquitin-conjugating 9) protein is involved in the covalent attachment of SUMO-1 to its target proteins, and SUMO-1 is a ubiquitinlike protein that modifies other cellular proteins, enhancing their stability or modulating their subcellular compartmentation. The N-terminal domain (amino acids 272–514) of *WRN* interacts with Ubc9 and SUMO-1.

V. MOUSE MODELS OF WERNER SYNDROME

There are currently three mouse models of the Werner syndrome: *WRN* helicase domain deletion mice (*WRN*Δhel/Δhel) (75), *WRN* truncation mutant mice (presumably null, *WRN*−/−) (72), and transgenic mice expressing dominant negative *WRN* (K577M-*WRN*) (76). These various genetic mutations are depicted schematically in Figure 4.

WRN-deficient mice carry a *WRN* gene with an in-frame deletion of the helicase domains III and IV, as described by Lebel and Leder (75). These mice express a mutant *WRN*, *WRN*Δhel/Δhel, which retains the exonuclease domains and nuclear localization signal. Mouse embryonic stem cells showed hypersensitivity to campthothecin and mitomycin C, and the growth rate of embryonic fibroblasts was progressively reduced as the cells were passaged. The mice exhibited a normal phenotype at least until the age of 12 months (73).

Another mouse line expresses a mutant *WRN* protein that is truncated in the middle of helicase region, resulting in mice that are functionally null (*WRN*−/−)

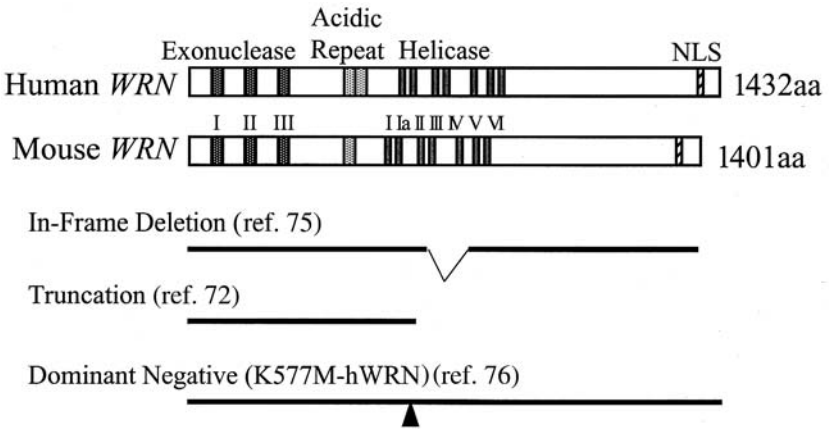

Figure 4 Mouse models of Werner syndrome. Structures of human and mouse *WRN* proteins and the alterations in the *WRN* gene product in the three mouse models of WS are shown.

(72). This mutation resembles many of the mutations in patients with WS. Embryonic fibroblasts showed accelerated replicative senescence, and this was enhanced in cells from mice on a BLM+/− background. They did not show hypersensitivity to campthothecin or 4NQO. Histopathological studies failed to show any unusual lesions in these mice up to the age of 17 months (72).

Mice with either a deletion or a truncation in the *WRN* helicase domain showed gross abnormalities when they were crossed to mice on a p53−/− background. *WRN*+/− mice showed increased rates of mortality, as assessed by median survival and maximum survival of the cohort of 48 *WRN*−/−; p53−/− and 59 *WRN*+/−; and p53−/− mice (72). Median life span of *WRN*−/−; p53−/− mice was approximately 20% shorter than that of *WRN*+/−; p53−/− mice. Interestingly, *WRN*Δhel/Δhel; p53−/− mice exhibited increased numbers of tumors and a larger variety of tumors types at an earlier age, as compared with *WRN*+/+; p53−/− mice (73). Both studies suggest that *WRN* and p53 play a synergistic role in the maintenance of genomic stability.

The dominant negative *WRN* transgenic mouse line, K577M-*WRN*, was developed in my laboratory (76). These mice expressed a full-length mutant (K577M) human *WRN* and endogenous mouse *WRN*. The K577M mutation abolishes the helicase and ATPase activity of *WRN* in vitro but not the exonuclease activity (2,3). Tail fibroblasts from K577M-*WRN* mice showed hypersensitivity to 4NQO and a reduced replicative life span, as determined by clone size distribution (76). These mice did not show any histopathologic abnormalities at least up to the age of 18 months.

To date, there is no animal model of WS that accurately mimics the human disease. This is also true for some other mouse models of other RecQ helicase–deficient disorders. This lack of success in reproducing human disease states in rodents may be due to the difference between humans and mice in maintaining genomic instability. Nevertheless, these mouse models have greatly facilitated the characterization of the functional interaction of WRNp with other proteins and of the pathogenesis of WS phenotypes.

VI. *WRN* POLYMORPHISM AND THE ROLE OF *WRN* IN "NORMAL" AGING

A systematic search of the *WRN* polymorphism across a variety of ethnic groups identified 58 single nucleotide polymorphisms (SNPs) in 35 exons and flanking introns. Of these, 15 are localized within the coding region, and 11 of these result in alterations in amino acid sequence (77,78). A limited association study of the two known *WRN* SNPs with relatively high heterogeneity suggests that *WRN* might be involved in determining longevity, possibly by modulating the risk of a variety of common age-related disorders, including atherosclerosis. Statistically significant associations with longevity and atherosclerosis were observed for the 1074Leu/Phe mutation (79). In addition, the 1367Cys/Arg mutation was associated with myocardial infarction (80), atherosclerosis (79,81) and long-term hemodialysis (82). These two polymorphic sites are in linkage. The functional manifestations of these two SNPs, with regard to *WRN* function, are unknown. However, it is of interest to note that 1074Leu/Phe resides within the RecQ consensus domain and 1367Cys/Arg is four amino acids away from the nuclear localization signal. Interestingly, in 1367Cys/Arg, the beneficial allele, Arg, is a minor allele. This might be explained by the presence of linkage disequilibrium between 1074 Leu/Phe and 1367Cys/Arg in some populations (78,79). The relative contribution of *WRN* polymorphisms to the risk of age-related disorders or longevity may not be nearly as high as other major known genetic risk factors; for example, polymorphisms in ApoE (77). However, these studies are beginning to provide hints that *WRN* may indeed be involved in the "normal" aging process in general population.

VII. CONCLUSION

Following the initial biochemical characterization of the helicase and exonuclease activities of WRNp, the identification of *WRN*-interacting proteins has led to a variety of studies exploring various ways in which WRNp may be involved in DNA metabolism. These include DNA repair at the site of stalled replication and nonhomologous end joining. *WRN* also may have a special role in transcription by

RNA polymerases I and II, as well as in homologous recombination. Cell biological studies raise some interesting questions regarding the role of *WRN* in telomeric maintenance. If *WRN* is capable of all these functions, how do cells regulate the functions of *WRN* at any given time? How is the switching of these roles mediated? How are the relationships with other RecQ helicases regulated? Since symptoms of WS are relatively mild (not lethal), *WRN* may have evolved for the "fine tuning" of these various DNA metabolisms. If this hypothesis is true, then what are the driving forces for the origin of *WRN*? At the level of the whole organism, how is *WRN* involved in the progression of "normal" aging phenotypes? It will be exciting to learn the answers to these and other important questions during the coming decades.

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10

Bloom Syndrome: Genetic, Cellular, and Molecular Features as Compared to Werner Syndrome

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I. INTRODUCTION

Genetically determined chromosomal instability was first discovered in Bloom syndrome (BS), a clinical entity that is now known to be the result of mutations in the *recQ* gene *BLM*. The RecQ proteins are a large family of evolutionarily conserved DNA helicases, which are enzymes that unwind double-stranded DNA (dsDNA), and they function in ways not completely understood in the maintenance of genomic stability (1). In all biological systems studied so far, mutations in the *recQ* helicase genes result in various genomic instabilities that are detectable at both the level of the chromosomes and the genomic DNA. In humans, there are five *recQ* genes, three of which are associated with human diseases, namely BS, Werner syndrome (WS), and Rothmund–Thomson syndrome (RTS). A comparison of the clinical and genetic features of these three syndromes is presented in Table 1. WS results from mutations in the *recQ* helicase *WRN*, and it is most remarkable clinically for the development prematurely of features of aging (see Chapter 9). Consequently, WS has served as a model for understanding the aging process.

BS is a rare autosomal recessive disorder first recognized as a clinical entity by the New York dermatologist David Bloom, who brought together three children affected by growth deficiency with normal proportions and a sun-sensitive, telangiectatic facial erythema that resembled lupus erythematosus (2). Since this initial description of BS, a number of other features of the entity have become apparent, including a characteristic facies and voice, hypopigmented and hyerpig-

Table 1 Comparison of the Clinical Features of the Syndromes in Which RecQ Helicases Are Known to Be Defective

Syndrome	Major clinical features	Cancer diathesis	Cytogenetic findings
Bloom syndrome	Small size, sun-sensitive facial erythema, other skin lesions, immunodeficiency, hypogonadism	All types, including non-Hodgkin's lymphomas, leukemias, and carcinomas	Isochromatid breaks and gaps Telomeric associations Translocations Quadriradials Sister chromatid exchanges
Werner syndrome	Small size in the adult, alopecia, cataracts, atherosclerosis, osteoporosis, hypogonadism	Soft-tissue sarcomas, osteosarcomas, melanoma, meningioma, hematological disorders	"Variegated chromosomal mosaicism" for inversions, translocations, and chromosomal losses
Rothmund–Thomson syndrome	Small size, poikiloderma of the face and extremities, skeletal abnormalities, alopecia, juvenile cataracts, hypogonadism	Osteosarcomas and skin cancer	Translocations

mented skin lesions that can appear virtually anywhere on the body, a moderate immunodeficiency that very likely explains the susceptibility of persons with BS to otitis media and pneumonia, a notably sparse distribution of subcutaneous fat, infertility in males and subfertility in females, and two major complications—predispositions to the development of diabetes and of cancer (3). The small size of persons with BS, which is probably the most obvious and least understood feature of the syndrome, is manifest at birth with birth weights and heights under the third percentile; it is detectable by ultrasound analysis in utero and it is present throughout life. The major cause of death is from the development of cancers, which on average occur earlier in life in the person with BS compared to the average ages of diagnosis of sporadic cancers. The types of cancer include leukemias, lymphomas, and, as the group of persons with BS has grown older, carcinoma of most of the sites and types that develop in the general population. The development of metachronous primary cancers is common.

Particularly relevant to the theme of this book, BS is completely unremarkable with respect to the premature development of the characteristics of aging. Because both WS and BS are associated with mutations in homologous *recQ* heli-

case genes and with chromosomal and genomic instability, in this review chapter we will compare and contrast the genetic, cellular, and molecular similarities and differences of BS and WS in order to shed light on functional distinctions that might explain how mutations of these two RecQ helicases can lead to such differing clinical outcomes.

II. GENOMIC INSTABILITY

When cells from persons with BS are stimulated to proliferate in culture, a large number of different chromosomal aberrations are detectable. These aberrations include the following: abnormal nuclei and micronuclei in interphase cells; in metaphase preparations, excessive numbers of chromosome breaks, gaps, telomeric associations, and translocations between nonhomologous chromosomes; anaphase bridges; and excessive numbers of a symmetrical four-armed figure referred to as a quadriradial (Qr), which is very likely the cytogenetic manifestation of crossing over between homologous chromosomes (4,5). In bromodeoxyuridine (BrdU)–treated cells, the frequency of sister chromatid exchanges (SCEs) in BS cells is 10-fold higher compared to the frequency in normal cells (6) (Fig. 1). The elevated SCEs in BS has been observed in all cell types that can be stimulated to

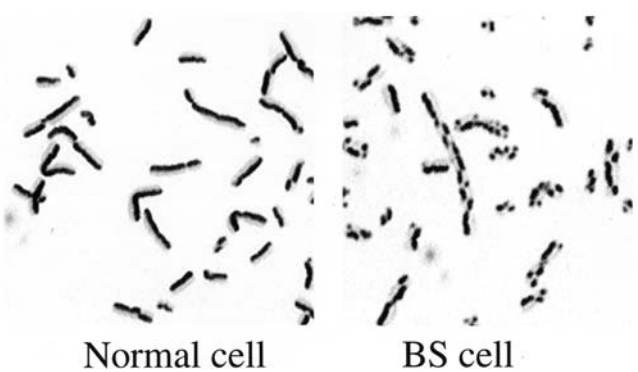

Figure 1 Sister chromatid exchanges in normal and BS cells. The cells were cultured in medium containing BrdU to make possible the differential staining (light or dark) of sister chromatids; alternating regions of light and dark staining signify exchanges between sister chromatids (SCEs). Shown here are partial metaphases from each of two SV40-transformed cell lines, one established from a nonmutant person and the other from a person with BS. The number of SCEs in cells from normal persons averages <10/metaphase, whereas BS lymphocytes show (as here) 60–90/metaphase or more; a greatly elevated SCE frequency is diagnostic of BS.

divide in culture, and it is diagnostic of the syndrome. The elevated frequencies of Qrs and SCEs demonstrate that exchange between homologous DNA sequences occurs excessively frequently in BS cells.

Underlying the chromosomal abnormalities are presumed DNA lesions that lead to both the excessive chromosomal breakage and exchange. Although the primary DNA lesions have not been identified, they inevitably result in the accumulation of mutations in BS cells. The frequency of mutations in lymphocytes from persons with BS has been measured at the glycophorin A (*GPA*), hypoxanthine guanosine phosphoribosyl transferase (*HPRT*), *HLA*, and T-cell receptor (*TCR*) genes and the numbers of mutated cells that accumulate is as much as 50 times the numbers in cells from normal persons (7–10). Moreover, an accumulation of DNA abnormalities has been documented in cultured BS cells (11,12); one of the most striking genetic abnormalities is increased loss of heterozygosity (10,13), occurring as the result of the excessive exchange between homologous chromosomes. As expected, the mutation rate of BS cells examined is approximately 10 times higher than normal (14,15).

Persons with WS also accumulate mutations at the *GPA* and *HPRT* loci (16–18), but to a lesser extent than in BS. It is important to note, however, that excessive chromatid exchange and chromosomal breakage are not abnormalities featured by WS cells. Instead, WS cells exhibit an unusual cytogenetic aberration that has been referred to as "variegated translocation mosaicism" (19–21). Detected in cultured WS cells are different clones of cells that feature a different characteristic chromosomal gain, loss, or translocation. However, although fewer mutations accumulate in the WS cells examined compared to BS cells, the mutation rate estimated in WS is similar to the rate estimated in BS (16).

BS cells exhibit increased sensitivities to various DNA-damaging agents (22–25), including ultraviolet light, N-ethyl-N-nitrosourea, and ethyl methane sulfonate, whereas WS cells display hypersensitivities to the nitrogen mustard 4-nitroquinilone-1-oxide, camptothecin, and interstrand cross-linking agents (26–28). Thus, major differences between BS and WS emerge both in the type of chromosomal instability that can be detected and in the drug sensitivities that can be elicited. These differences may reflect differences in the abnormalities occurring on the DNA that accumulate and subsequently generate the genetic mutations and chromosomal abnormalities. The fundamental differences in the underlying genomic instabilities in BS and WS could be explained by differences in the substrates upon which and the biochemical pathways in which the two proteins operate.

III. GENETICS OF BLOOM SYNDROME

Characterization of the genetics of BS as well as its natural history has been facilitated by the accession in and regular follow-up of persons with the syndrome

through the Bloom's Syndrome Registry (29). This registry is a cohort of nearly all persons in the world diagnosed with BS from its identification as a clinical entity as reported in 1954 until 1991 when the Registry was closed arbitrarily to new accessions. The Registry contains 168 persons representing all but one of the major continental population groups, the exception being Aboriginal Australians/Oceanic peoples. By segregation analysis of the families ascertained in the Registry (30), BS was demonstrated to be an autosomal recessive genetic disorder. Although underdiagnosis certainly occurs for persons with BS, it is equally certain that the frequency of BS worldwide is extremely low.

A. Homozygosity Mapping

Limited somatic cell hybridization experiments suggested that a single disease gene was mutated in BS (31). Efforts to map the BS gene, *BLM*, to a specific region of the chromosomes culminated in the early 1990s with the localization of *BLM* to chromosome 15 by SCE analysis of a panel of BS somatic cell clones into which different human chromosomes had been introduced by microcell-mediated chromosome transfer (32) and with regional localization of *BLM* to chromosome band 15q26.1 by a linkage method referred to as "homozygosity mapping" (33). Homozygosity mapping entailed the genotyping of polymorphic DNA markers around the genome in multiple persons with BS whose parents are cousins. In these persons, the mutated *BLM* gene is inherited identical by descent from a recent common ancestor. By the identification of linked loci at which more than the expected numbers of affected persons were homozygous, *BLM* was genetically linked to the proto-oncogene *FES* and polymorphic markers near to *FES*. The results from homozygosity mapping confirmed that mutation of the *BLM* locus on chromosome 15 was responsible for most, if not all, cases of BS.

B. Linkage Disequilibrium

Although BS is rare worldwide, it is relatively more frequent in the Ashkenazi Jewish population. The cause of this relative increase in the incidence of BS in this population was postulated to be "founder effect" (34). By founder effect, a relative increase in the descendents of one person who happened to carry a particular mutated *BLM* gene was generated by genetic drift, a stochastic process that occurs when the numbers of persons in a founding population are small. This hypothesis was supported by the observation of linkage disequilibrium at *BLM*; that is, the nonrandom association of particular alleles of marker loci linked to *BLM* in Ashkenazi Jews with BS (35). Proof of this hypothesis followed the isolation of the *BLM* gene and the identification of the particular mutated allele *BLMc.2207–2212delATCTGAinsTAGATTC* (36), which we refer to as blm^{Ash}. Such a mutation could not have occurred more than once in human genetic history. It was identified in 58 of 60

Ashkenazi Jewish BS chromosomes examined (37). The linkage disequilibrium in the Ashkenazi Jewish BS chromosomes confirmed the localization provided by homozygosity mapping, and both methods specifically pointed to a region tightly linked to *FES* as containing *BLM*.

C. High-SCE/Low-SCE Mosaicism

As mentioned above, the high-SCE phenotype of BS cells is present in all cell types that can be stimulated to divide in culture in all persons with BS. However, in approximately 30% of persons with clinical BS there is present a subpopulation of cells that have a low-SCE phenotype (38). Because chromosomal breakage and quadriradials are returned to their normal frequencies in the low-SCE cells examined, these cells are phenotypically normal by other cytogenetic criteria as well. So far, low-SCE cells in BS have only been detected in B- and T-cell lymphocyte populations, but their frequency in those cell populations ranges in different individuals from below 1% to as high as 75%. Because somatic cell hybrids of high-SCE BS cells and low-SCE BS cells have a low-SCE phenotype just as hybrids of high-SCE BS and normal cells do (31), the low-SCE cells contain a stable genetic factor that can be supplied in trans, and that factor functions to correct the defective *BLM* gene product.

The first clue to the genetic mechanisms by which this phenotypic reversion occurs came from analysis of the BS population itself (39). The high-SCE/low-SCE mosaicism only rarely occurs in persons with BS who are homozygous at *BLM* due to identity by descent; conversely, the mosaicism is detected predominantly in the outbred persons with BS for whom compound heterozygosity is possible. These data implied that the genetic reversion event occurs at *BLM* itself. Based on these observations, the following model was developed (Fig. 2). The type of somatic mutational event in which compound heterozygosity would be a strict requirement for correction to normal of the *BLM* gene is a crossing-over event between the two sites of *BLM* mutations on the homologous chromosomes 15 (40). If the crossing-over event within *BLM* occurred during or after replication of the homologous chromosomes 15, two types of segregation of these chromosomes are possible: The chromatid with the corrected *BLM* gene could segregate with the reciprocally recombinant chromatid, in which case heterozygous markers both proximal and distal to *BLM* would remain heterozygous. Conversely, the chromatid with the corrected *BLM* gene could segregate with the nonrecombinant chromatid, in which case heterozygous markers distal to *BLM* would become homozygous, whereas proximal markers would remain heterozygous. This hypothesis was tested by the analysis of polymorphic DNA markers proximal and distal to *BLM* in DNAs isolated from high-SCE cells and from low-SCE lymphoblastoid cells from 11 persons with BS (40). Reduction to homozygosity

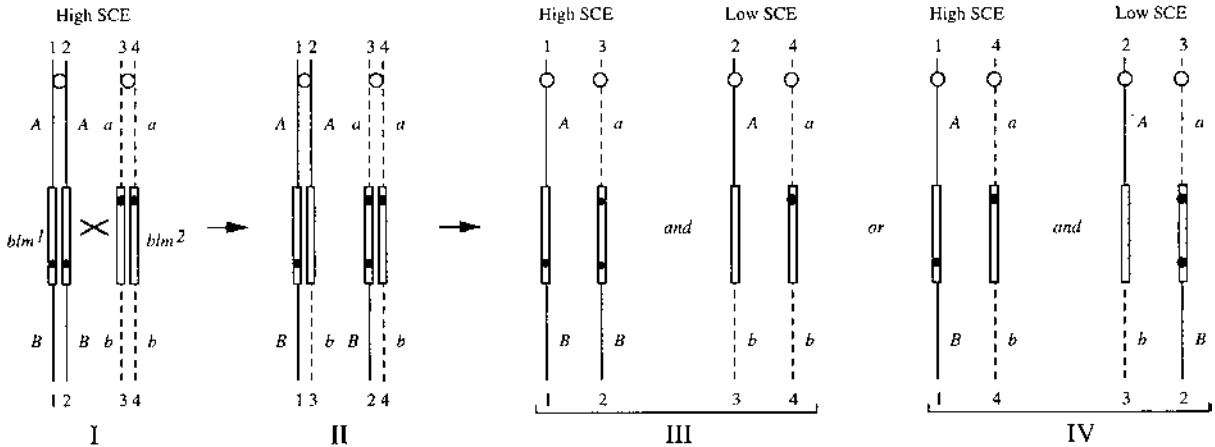

Figure 2 Model to generate a wild-type *BLM* locus via somatic intragenic recombination: (I) The two pairs of sister chromatids of the homologous chromosome Nos. 15 in a G2 somatic cell of a BS genetic compound (blm^1/blm^2) are numbered 1–1 to 4–4. Each of the two mutations in *BLM* (the open rectangle), represented by black dots, one inherited from each parent, is at a different site in the gene. Flanking markers proximal to and distal to the mutated loci are heterozygous *A*/*a* and *B*/*b*. (II) After homologous interchange between chromatids 2–2 and 3–3 at a point between the sites of mutation within *BLM* (the X in I), a wild-type gene is reconstituted on chromatid 2–3 that corrects to normal the high-SCE phenotype of BS cells. Simultaneously, the distal marker *b* becomes associated with the wild-type gene on chromatid 2–3. (III and IV) By segregational events at mitosis, two pairs of daughter cells are possible. If chromatids 2–3 and 4–4 cosegregate to the same daughter cell, the distal marker becomes homozygous *b*/*b* (the diagram on the right side of III). On the other hand, if chromatids 2–3 and 3–2 cosegregate, the distal marker remains heterozygous *b*/*B* (the diagram on the right side of IV). The proximal marker remains heterozygous *A*/*a* in both cases. In the sister cells, segregation of chromatids 1–1 and 3–2 (the diagram on the left side of III) or of chromatids 1–1 and 4–4 (the diagram on the left side of IV) do not give rise to a low-SCE phenotype. (*Note*: Cells of heterozygous carriers of a mutation at *BLM*, that is, *blm*/BLM parents of persons with BS, display a low-SCE rate.) (From Ref. 40.)

was detected in 5 of the 11 low-SCE cell lines examined at markers distal to *BLM*, whereas constitutional heterozygosity was maintained at markers proximal to *BLM* in all the low-SCE cell lines. These results demonstrated that a recombination event within *BLM* had occurred in a progenitor lymphocyte whose progeny thereafter transmitted a functionally normal *BLM* gene. The recombination event permitted the precise localization of *BLM* to a small region bounded on the proximal side by the most distal marker that remained heterozygous and on the distal side by the most proximal marker that exhibited reduction to homozygosity. This precise localization led directly to the positional cloning of *BLM* (36).

D. Mutational Analysis of *BLM*

With the cloning of *BLM*, the laboratory embarked on a large-scale mutational analysis of the gene in persons with BS. The analysis has disclosed 64 unique disease-causing mutations in *BLM* identified in 124 of the 133 persons examined. Of these 64 mutations characterized, 54 resulted in truncation of the BLM protein (20 nonsense mutations; 21 frameshift mutations caused by small insertions or deletions; 9 splice site mutations, some of which were shown to cause exon skipping; and 4 deletions consisting of 1 or more exons) and 10 were missense mutations at conserved residues the alteration of which putatively destroys the helicase activity of BLM. Both mutated alleles were detected in 116 persons, only one mutated allele in 8 persons, and no *BLM* mutation in 9. These data argue that most, if not all, persons with BS contain a mutation in *BLM*, but some mutations were not detected. By the methods we have used to detect mutations, large deletions could be difficult to detect, and the promoter region and most intronic sequences have not been screened for mutations. In addition to the disease-causing mutations, 17 mutations were identified that very likely have no effect on BLM function.

Forty-six of the 64 mutations were identified in a single person with BS and the remaining 18 were identified in multiple persons (they were recurrent). The most striking example of a recurrent mutation is blm^{Ash}, which was found on 70 of the 240 *BLM* chromosomes characterized. Of the 75 persons with BS who are homozygous for mutations at *BLM*, 26 carry blm^{Ash}. For the remaining 49 homozygotes, identity by descent was clearly the reason for homozygosity in 24, because their parents were cousins. It is probably the cause in most of the remaining 25 as well, because most of the mutations in these persons were detected in other unrelated persons with BS. Theoretically, a mutational hot spot could explain recurrent mutation. Arguing against this possibility is the observation that by the analysis of the parents of persons with BS no new mutations have been identified.

The genetics of BS and WS are remarkably similar. Both are rare, single-gene disorders that follow an autosomal recessive mode of inheritance. In each disorder, the gene was localized by homozygosity mapping (33,41,42), because

there were many persons with the syndrome born of consanguineous marriages. Linkage disequilibrium between the disease gene and tightly linked polymorphic DNA markers has been described in certain human populations—it was found in the Ashkenazi Jews in BS (35) and in Japanese persons in WS (43). Founder mutations have been identified in both these and other populations in both *BLM* and *WRN* (3,44). Positional cloning strategies were used successfully to isolate these genes (36,45), and the mutational analysis, besides providing proof that the correct gene had been identified, has revealed the molecular genetics of the two syndromes and aspects of the genetics of the human populations in which the mutated genes are segregating. A striking difference in the mutational spectra of the two genes is the absence of missense mutations in *WRN* in persons with WS, suggesting that these sorts of alleles may have clinical manifestations different from those of WS. In addition, somatic mosaicism is an important feature of BS. Mosaicism had not been described in WS; however, a powerful and convenient test, such as the SCE assay, is not available for monitoring the function of the WRN helicase in a single cell. It is possible that such mosaicism has gone undetected.

IV. PRIMARY STRUCTURE OF BLM

Analysis of the protein databases for sequence homologies using the protein sequence encoded by *BLM* reveals that it has a central region (residues 650–1000) containing seven motifs that are characteristic of DNA helicases (36). DNA helicases are a large group of nuclear proteins that catalyze the unwinding of the DNA duplex, a process that is required for all manipulations of DNA, including DNA replication, repair, recombination, and RNA transcription. The region of BLM that contains these seven motifs exhibits 40–45% identity to homologous regions of proteins in the RecQ subfamily of DNA helicases. The first RecQ helicase was identified in *Escherichia coli* by the *recQ* mutation, which confers resistance to thymineless death (46). Besides the *E. coli* protein, RecQ helicases have been identified in many other organisms, including *SGS1* in *Saccharomyces cerevisiae* (47), *rqh1*$^{+}$ in S*chizosaccharomyces pombe* (48), and multiple RecQ helicases in *Caenorabditis elegans*, *Drosophila melanogaster* (49), *Arabidopsis thaliana* (50), *Xenopus laevis* (51,52), chickens (53), mice (54–59), and humans. In humans, five RecQ helicases have been found: *RECQL1* (60,61); *BLM* (36); *WRN* (45); *RECQL4*, which is also referred to as *RTS* (62,63), and *RECQL5* (64). A comparison of the helicase regions of BLM with the helicase regions of the other known human RecQ helicases is shown in Figure 3.

C-terminal to the helicase domain in BLM, there is an approximately 200–amino acid region (residues 1000–1200), referred to as the C-terminal extended homology region, that is 20% identical to similar regions in several other RecQ helicases (48). Because mutations that abolished the helicase activity of

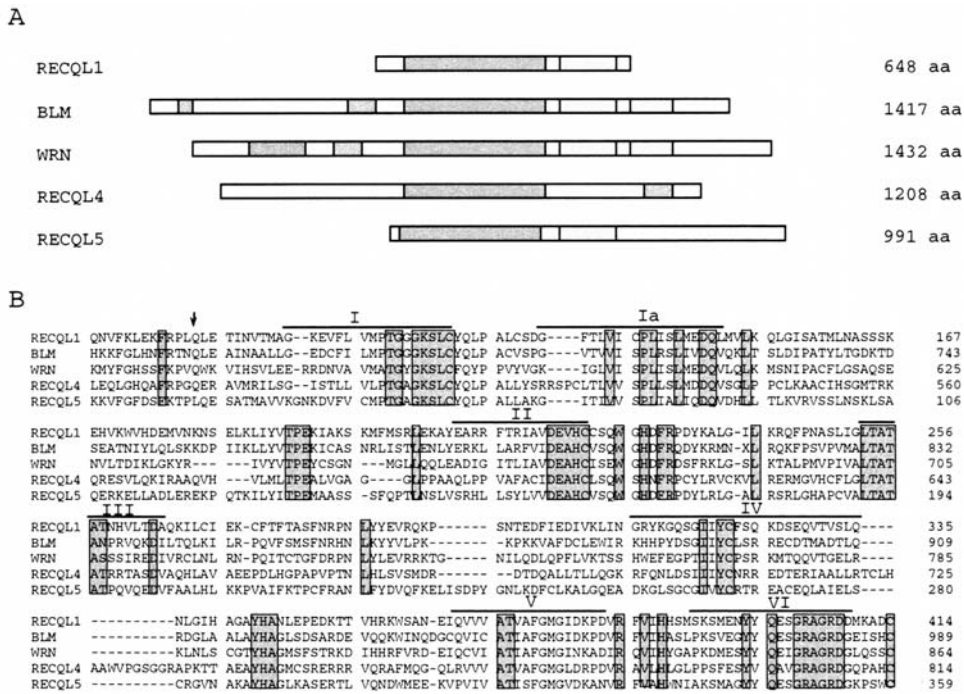

Figure 3 Comparison of the five human RecQ helicases. (A) Schematic representation of the RecQ helicase proteins, indicating the known regions of shared similarity. The proteins are aligned with reference to the helicase domains. Shown are the C-terminal extended homology and helicase RNAseD C-terminal regions, the stretches of acidic amino acids, and the WRN exonuclease region. The protein names are at left and the length of the protein in amino acids is indicated at right. (B) Alignment of the amino acid sequences of the helicase regions. Sequence alignment was performed by the Clustal program. The seven motifs that are characteristic of the RecQ helicases are indicated by the overlined sequences marked by the roman numerals. Identities shared by all five proteins are shaded. Numbers at right indicate the amino acid positions in each protein and the protein names are at left. The arrow points to the conserved glutamine that was mutated to an arginine in a person with BS.

BLM have been found in this region (65,66), it is important for the helicase activity of BLM. Another region of protein homology, referred to as the helicase and RNAseD C-terminal region (HRDC; residues 1212–1299), has been identified C-terminal to the extended homology region that is postulated to participate in DNA binding (67). A bipartite nuclear localization signal (NLS; residues 1334–1349) is present at the C-terminal end of BLM (68). BLM also contains an N-terminal

region (residues 1–649) that contains stretches of acidic residues and overall is negatively charged. Large negatively charged N-terminal regions are also present in Sgs1, rqh1^{+}, and WRN but not in RECQL1 and RECQL5. RECQL4 has a negatively charged region C-terminal to its helicase region.

WRN and proteins of the RecQ family that are WRN-like are unique in comparison to the other RecQ helicases characterized, because they possess a 3′ to 5′ exonuclease activity in the N-terminal part of the protein. An exonuclease activity so far has not been associated with BLM. This functional difference between the BLM and WRN helicases could help explain some of the observed cytogenetic and cellular differences between BS and WS cells. The existence of two enzymatic activities in WRN also could explain why no inactivating missense alleles have been identified in the WRN helicase region. Such alleles would probably leave the exonuclease activity intact. The known disease-causing alleles of WRN are nulls and as such ablate both activities at once.

V. ENZYMATIC ACTIVITY OF BLM

DNA helicases bind to single-stranded DNA (ssDNA) and translocate along the substrate either in a 5′ to 3′ or in a 3′ to 5′ direction using nucleoside triphosphate (NTP) hydrolysis for energy. Therefore, DNA helicases are defined by two biochemical activities: (a) DNA-dependent or DNA-stimulated ATPase activity and (b) NTP- or deoxyribonucleoside triphosphate (dNTP)–dependent strand-displacement activity, invariably with Mg^{2+} as a cofactor. BLM has been expressed in and purified from yeast and has been shown to exhibit both of these activities (65,71). In the strand-displacement assay, a standard substrate (Fig. 4) is prepared that contains a radiolabeled oligonucleotide annealed to M13 ssDNA or another oligonucleotide. BLM unwinds this type of substrate when a 3′ single-stranded tail is available, indicating that BLM moves along ssDNA in a 3′ to 5′ direction, as do other RecQ helicases.

Compared to the standard substrate, purified recombinant BLM protein is more active in unwinding several unusual DNA substrates in vitro, including G4 DNA (72), triplex DNA (73), Holliday junctions (74), and D-loops (75). G4 DNA is a structure of four parallel DNA strands that are stabilized by Hoogsteen bonds between guanines. Triplex DNA can be generated when a third strand composed of either pyrimidines or purines lies in the major groove of duplex sequences. A Holliday junction is a four-way junction of two DNA duplexes that is formed during homologous recombination. A D-loop is a recombination intermediate that forms when the end of a ssDNA molecule invades and anneals with the homologous sequences of another DNA duplex. Other RecQ helicases exhibit similar capabilities, and the WRN helicase in particular can unwind G4 DNA (76), triplex DNAs (73), and Holliday junctions (77). (D-loops have yet to be tested.) The sub-

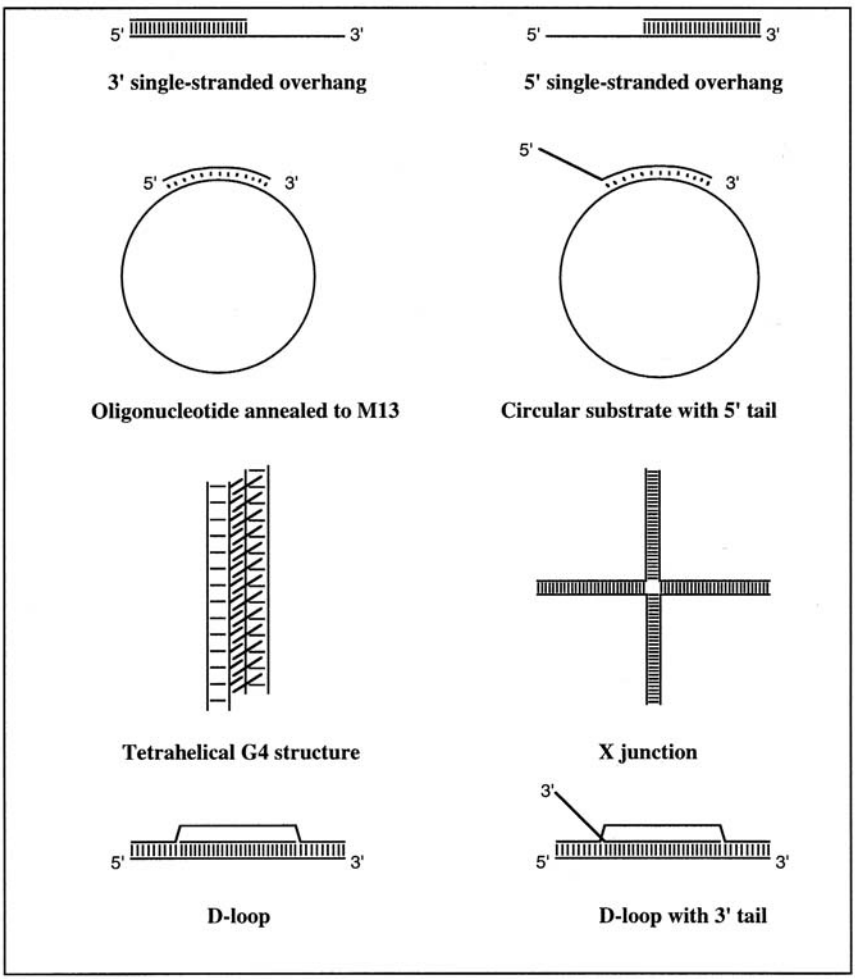

Figure 4 Selected substrates used for helicase assays. Conventional helicase substrates include the linear duplexes with 3′ or 5′ single-stranded overhangs and the circular substrates with or without nonhomologous tails. More unusual substrates that BLM binds and unwinds preferentially are the tetrahelical G4 DNA, the X junction, which resembles a Holliday junction, and D-loops, which resemble the joint molecules made by Rad51. Typically, one of the oligonucleotides is radiolabeled either with T4 polynucleotide kinase or with the Klenow fragment, and a annealing reaction is performed. Before incubating the substrate with enzyme, the substrate is purified. The products of the reaction are detected by polyacrylamide gel electrophoresis.

strate specificities of the BLM and WRN helicases presently cannot be used to explain the differences between BS and WS cells.

Of the 11 missense mutations found in BS-affected persons, all occur either in the helicase domain or in a 50–amino acid stretch of the RecQ C-terminal extended homology region. Experimentally, two mutations have been studied in some detail (65). One mutation changes the glutamine at residue 672 to an arginine (Q672R); this glutamine lies 10 amino acid residues N-terminal of helicase motif I, and it is conserved in many RecQ helicases. The other mutation replaces a conserved cystine at residue 1055 with a serine (C1055S). Two mutant BLM proteins each containing the Q672R and C1055S substitutions were expressed in and purified from yeast, and their biochemical activities were measured. The BLM Q672R protein had 40% of the normal DNA-dependent ATPase activity and lacked detectable DNA strand-displacement activity, and the BLM C1055S lacked both of these activities. By extension, we expect the other missense alleles in the helicase and C-terminal extended homology regions to either reduce or abolish the helicase activity of BLM.

Measurement of the SCE frequencies in BS cells in which different experimentally mutated BLM proteins have been stably expressed is the most powerful method of determining the function of a mutant BLM in vivo. Particularly useful for this purpose are the simian virus (SV40)–transformed BS fibroblasts, GM08505, which can proliferate in culture indefinitely, transfect easily, and exhibit the characteristic high-SCE phenotype. These cells harbor the blm^{Ash} null allele at *BLM*, and by Western analysis BLM protein is undetectable. Stable expression of a normal BLM protein in GM08505 cells resulted in a correction of the high-SCE phenotype nearly to normal (78). In contrast, expression of either of the two missense mutant BLM proteins (Q672R and C1055S) failed to correct the high-SCE phenotype (65). Since the mutant proteins are defective helicases, their inability to rescue the high-SCE phenotype of BS cells indicated that the helicase activity is required for the cellular function of normal BLM.

VI. CELL BIOLOGY OF BLM

Immunofluorescence using BLM-specific antibodies reveals two striking distributions of BLM protein in the nucleus (78) (Fig. 5): (a) diffuse, finely granular microspeckles that can vary in local concentration from region to region within the nucleus and (b) bright, discrete foci that range in size from 0.2 to 1.0 μm and in number from 3–20. These discrete foci have been identified as the promyelocytic leukemia (PML) nuclear bodies (PML-NBs; also known as ND10s or PODs) (79). The PML-NBs were identified initially with human antibodies to a major component of the bodies known as Sp100 (80). These antibodies develop sporadically in persons with hepatic inflammatory disease. Later, antibodies to the PML protein identified this

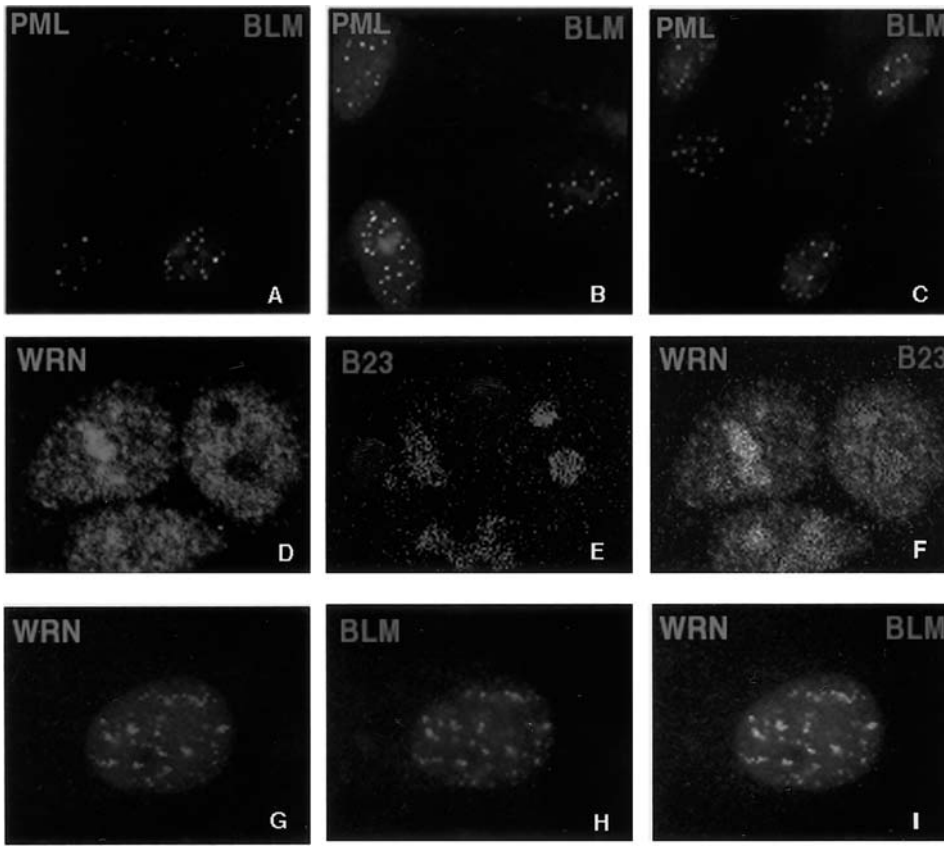

Figure 5 (A–C) Spatial and temporal colocalization of BLM and PML in PML nuclear bodies. Normal diploid fibroblasts blocked first in G0 by serum deprivation and then blocked again at the G1/S boundary by culture in medium containing serum (10%) plus hydroxyurea. Cells were sequentially incubated in anti-BLM antibody followed by Texas Red–conjugated secondary antibody; then in anti-PML antibody followed by fluorescein-conjugated secondary antibody. All cells in these fields contain PML nuclear bodies. (A) The three nuclei presumed to be in G1 contain no detectable BLM, and so the PML bodies are green. An increase in the amount of nuclear BLM is demonstrated by the fourth cell in which BLM colocalizes with the PML bodies (yellow dots); in this cell an increase in diffuse nuclear granularity and the beginnings of the formation of larger patches of BLM (red) are seen. (B) and (C) are representative of cells incubated 10 h and 12 h in fresh medium following release from the HU block. All cells also contain abundant BLM, with the exception of two in (C). BLM when present may be in dots, in large patches that vary in size and intensity, or as a diffuse granularity. When BLM is detectable in the cell, it is present in PML nuclear bodies. Digital imaging microscopy at 630×. (A–C from Ref. 88.) (D–I) Comparison of the nuclear distributions of BLM and WRN. (D–F) Localization of WRN

protein as another major component of the bodies (81). Translocations between the *PML* gene and the retinoic acid receptor gene, *RARα*, are observed in ~99% of cases of acute promyelocytic leukemia (APL). The oncogenic fusion protein PML-RARα disrupts the organization of the PML-NBs probably by binding normal PML protein in the PML-RARα/RAR complex and sequestering it presumably at sites where the RAR complex binds. The oncogenic fusion protein PML-RARα suppresses transcription at these sites by blocking response of the RAR complex to retinoic acid. The disruption of the PML-NBs in APL cells results in a redistribution of proteins such as BLM to a more diffuse and microspeckled pattern (79).

PML-NBs have been found to contain many other proteins, including, for example, the RAD50/MRE11/NBS1 complex, Daxx, hypophosphorylated Rb, and p53, implicating the PML-NBs in transcription, apoptosis, DNA-damage response, and maintenance of genomic stability (82). Certain DNA viruses, including herpes simplex virus, ctyomegalovirus, and adenovirus 5, encode proteins that disrupt the PML-NBs, and other viruses, including Epstein–Barr virus and SV40, segregate key viral proteins to the PML-NBs (83). The viral genomes themselves associate physically with the bodies early in the infection cycle where they are transcribed and viral replication is initiated.

Another indication of the important organizational role of the PML protein in the PML-NBs has been revealed by analysis of mouse *PML*−/− knockout cells (82). For those protein components of the PML-NBs examined, homozygous deletion of *PML* results in failure of these proteins to concentrate in brightly staining foci characteristic of PML-NBs (79,84). These data suggest that PML is one of the key structural proteins that holds the PML-NBs together. *PML*−/− animals are viable; obviously, formation of PML-NBs is not essential for cell viability. Nevertheless, the *PML*−/− cells have defects in transcription, apoptosis, and responses to DNA damage, emphasizing the broad role the PML-NBs play as organizing centers for multiple nuclear processes (82).

The SCE frequency in *PML*−/− cells is two times higher than that in normal cells (79), suggesting that BLM might carry out some part of its function in the maintenance of genomic stability under the auspices of the PML-NBs. The PML-NBs may regulate the availability of BLM in the nucleus generally or they may provide access to specific substrates.

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in nucleolus and nucleoplasm in HeLa cells. Cells were stained by incubation with anti-WRN followed by a fluorescein conjugated secondary antibody (green) and with B23, an antibody to a nucleolar protein, followed by a Texas Red–conjugated secondary antibody (red). (D) shows the staining with anti-WRN, (E) the staining with B23, and (F) is the merger of (D) and (E). (G–I) Colocalization of BLM and WRN in SV40-transformed normal fibroblast that have activated the ALT pathway and contain AA-PML bodies. (G) shows staining with anti-BLM (Texas Red), (H) the staining with anti-WRN (fluorescein), and (I) is the merger of panels (G) and (H). (See color insert.)

The amount and distribution of BLM in the nucleus is cell cycle regulated (85–88). The level of BLM protein is the lowest in early G1 phase and reaches the maximum by late S/G2 phase. Throughout the cell cycle, BLM is consistently found in the PML-NBs except in metaphase, where the protein becomes diffusely distributed in the cell despite the persistence of the PML-NBs. BLM is also hyperphosphorylated at metaphase (89), which may explain in part the dissociation of BLM from the PML-NBs.

In a two-hybrid screen for potential BLM-binding proteins, we have found three factors that are involved in the same small molecule–modification pathway—UBC9, SUMO-1, and SUMO-2 (79). SUMO-1 and SUMO-2 are small proteins 20% identical to ubiquitin (90). Like ubiquitin, they can modify target proteins via an isopeptide bond between a carbonyl group on a C-terminal glycine on the SUMO protein and an available amine group on one or more lysines on the target protein. The modification process requires E1 proteins that process the C-terminal ends of SUMOs and load them onto the E2 protein, the SUMO-conjugating enzyme UBC9. E3 proteins have been identified (138); the SUMO substrates can also function as their own E3s in vitro (91).

SUMO-1 has been shown to modify numerous nuclear proteins (90). SUMO-1 modification of PML is essential for the formation of PML-NBs, because expression in *PML*−/− cells of a PML protein that cannot be modified by SUMO-1 fails to cause reformation of the PML-NBs, whereas expression of normal PML does (92). Consequently, the yeast two-hybrid data suggest that BLM might be modified by SUMO. Modification of BLM can be demonstrated in vitro with purified components, and interaction of BLM with UBC9 has been detected in vivo (unpublished observations with M. Matunis, Johns Hopkins University). Modification of BLM in vivo has been difficult to detect; however, the region of BLM that is modified by SUMO proteins in vitro correlates with the region that is necessary and sufficient for the localization of BLM to the PML-NBs (residues 133–458). SUMO modification of BLM may play a role in the localization of BLM to the PML-NBs.

The nuclear distribution of WRN, however, is different from that of BLM. In normal, untreated human cells, in addition to finely granular microspeckles evenly distributed in the nucleoplasm, WRN is concentrated in the nucleolus in some cells (93, 94). It has been suggested that the intertrafficking of WRN between the nucleoplasm and the nucleolus may depend on the transcriptional state of the cell (95). For example, in serum-starved cells, WRN is absent from the nucleolus. In mouse cells, WRN is not found in the nucleolus (93). Interestingly, the mouse WRN can be modified by SUMO-1 (96). Therefore, as with human BLM, SUMO modification of WRN may play a role in guiding the nuclear distribution of mouse WRN. Those aspects of the nuclear distributions of BLM and WRN that are distinct from each other could point to differences in their cellular functions. For example, WRN could play a role in telomeric maintenance, the failure of which might explain the premature replicative senescence of WS cells (97).

Colocalization between WRN and a subset of the BLM foci has been observed in ALT (alternative lengthening of telomeres) cells (Fig. 5). ALT is a telomerase-independent mechanism of maintaining telomeres that is used by some transformed cell lines (e.g., some SV40-transformed cells and some tumor cell lines). In ALT cells, PML-NBs are often associated with telomeric elements, such as telomeric repeat DNA and telomeric binding proteins, forming a structure referred to as ALT-associated PML-NBs (AA-PML) (98). WRN is present in these AA-PMLs, providing evidence that WRN may be involved in alternative maintenance of telomeres (139). BLM remains present in the AA-PMLs, but it is unlikely BLM and WRN interact in ALT cells.

In addition to the colocalization of BLM foci and the PML-NBs, other focal concentrations of BLM are observed, transiently or under certain conditions, in nucleoli, at telomeres, and at or near some replication foci. Further investigations will be required to determine the significance of these observations. Because many fine granular microspeckles in the nucleus are detected with antibodies to both BLM and WRN, at the level of confocal microscopy, it has not been possible to determine whether the sites of action of the two helicases are sometimes coincident.

VII. BLM COMPLEX

A major effort has been underway in many laboratories to determine what proteins interact with BLM. Such proteins, whether they bind BLM physically or functionally interact with BLM as part of a biochemical pathway, provide some of the most powerful clues to the substrates on which BLM acts and to BLM's cellular function. In turn, from the study of the BS cellular phenotype, and the phenotypes of microbes with mutations in RecQ helicases, we have guessed at the cellular function of BLM, and these suppositions have led to testing specific candidate proteins for interaction with BLM. As the list of BLM-interacting proteins grows, we are beginning to put together "BLM complex(es)," which from its composition must have a key role in preserving the integrity of the genetic material.

The first feature of the BLM complex derives from BLM itself. Electron microscopy and gel filtration analyses of BLM expressed in and purified from yeast suggested that BLM forms tetrameric and hexameric ring structures in vitro (99). Similarly, the N-terminal 431 amino acids of BLM when expressed in and purified from *Escherichia coli* forms hexamers and dodecamers, showing that the N-terminal region probably mediates the oligomerization of full-length BLM (100). In contrast, analyses by gel filtration of purified WRN proteins suggest that WRN forms trimers in vitro and that the N-terminal part of WRN also mediates its oligomerization (101). Since RecQ proteins are known that can function without the long N-terminal regions, the oligomerization of BLM and WRN may not be critical for their helicase activities. Oligomerization could modulate enzymatic activity by cooperativity, or it could deliver multiple independently active units to cellular substrates.

Purified BLM has been shown to interact in vitro with the 70-kD subunit of the heterotrimeric human single-stranded DNA–binding protein (ssDNA) replication protein A (RPA). RPA is involved in many, if not all, DNA metabolic processes that require the exposure of ssDNA and therefore its protection and stabilization. Physical interaction between BLM and RPA is detectable by far-Western analysis and addition of RPA to helicase assays increases the extent of unwinding by BLM (102). For example, purified BLM protein is inefficient at unwinding substrates containing more than 100 bp of duplex DNA. With the addition of RPA, BLM becomes efficient at unwinding substrates up to 300 bp. Physical interaction between RPA and WRN and stimulatory effects of RPA on the extent of unwinding by WRN in helicase assays have also been observed (103), suggesting that the interaction with RPA is not specific to either the BLM or WRN helicases and probably does not explain the specificities of BLM and WRN functions.

When a protein complex moves through DNA, topoisomerase activity is needed to relax superhelical strain created by the opening of the DNA (104). Topoisomerase III (TopoIII) is a type IA topoisomerase that acts by breaking and rejoining DNA via an enzyme–DNA intermediate in which a protein tyrosine forms an ester bond with a 5′-phosphoryl group in DNA. In *Saccharomyces cerevisiae*, *top3* mutant cells grow slowly and have a greatly elevated mitotic recombination frequency (105). The *top3* mutant phenotypes can be suppressed genetically by deletion of the *S. cerevisiae* RecQ gene *SGS1* (106), and Sgs1 physically interacts with Top3 via its N-terminal region (107–109). The *sgs1* mutant cells also exhibit increased mitotic recombination frequencies as well as increased chromosomal missegregation (106,110). By coimmunoprecipitation and far-Western analyses, BLM has been found to interact with topoisomerase IIIα (TopoIIIα) (111,112), which is one of the two known TopoIII proteins in mammalian cells. Like Sgs1, BLM physically binds to TopoIIIα via sequences within the N-terminal 133 amino acids (111,113). Expression of a BLM mutant lacking the TopoIIIα interaction domain in GM08505 cells results in intermediate levels of suppression of the high-SCE BS phenotype as compared to suppression by normal BLM (113). Because some SCE suppression was observed, the data suggest that the two proteins can function independently in the absence of the interaction domain and that the interaction increases the efficiency of SCE suppression. In yeast, complementation of *sgs1* mutant phenotypes can be achieved with a fusion protein of TopoIII and Sgs1 deleted for the N-terminal interaction domain (114). This experiment suggests that the interaction domain does not have a function distinct from interaction with TopoIII.

TopoIIIα is colocalized with BLM in the PML-NBs, but in BS cells that contain null mutations of *BLM*, TopoIIIα fails to localize there (111,112). Expression in BS cells of full-length BLM but not the BLM mutant lacking the TopoIIIα interaction domain restores TopoIIIα to the PML-NBs (113). Therefore, Topo IIIα is recruited to the PML-NBs by direct physical interaction with BLM (see model in Fig. 6).

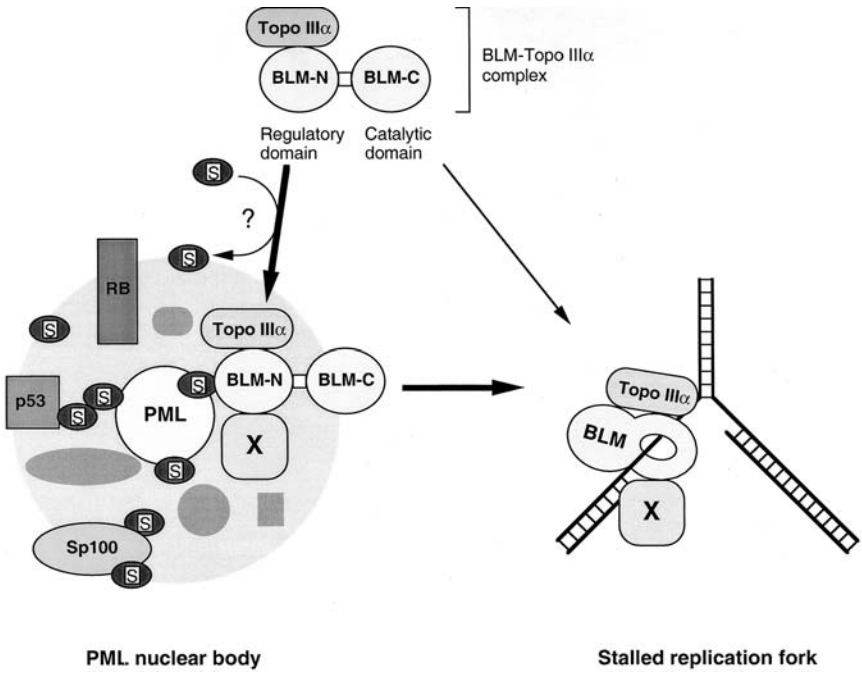

Figure 6 Model for the role of the BLM/TopoIIIα complex and the PML-NBs in the maintenance of genomic stability. BLM consists of two major regions. The N-terminal region (BLM-N) contains domains for the binding of TopoIIIα, localization to the PML nuclear bodies, and homo-oligomerization of BLM. The C-terminal region (BLM-C) contains domains for DNA binding and the catalytic functions of BLM, including the DNA-dependent ATPase and the ATP-dependent DNA helicase activities. Interaction between BLM and TopoIIIα can occur in the nucleoplasm, because BLM fragments that contain the TopoIIIα interaction domain but not elements required for PML-NB localization can interact with TopoIIIα. BLM recruits TopoIIIα to the PML-NBs by an unknown mechanism that may involve the small ubiquitinlike modifier (SUMO-1, the protein indicated by the letter S), as evidenced by yeast two-hybrid data. BLM does not appear to interact directly with the PML protein, but it may do so through a bridging partner, such as SUMO-1 that itself modifies PML. Once the BLM/TopoIIIα complex is localized to the PML-NBs, hypothetically it may bind other proteins (e.g., in the figure, unknown protein X). The complete BLM complex is then released from the PML-NBs as the result of an unknown signal and is targeted to specific sites of action to regulate the formation or maturation of substrates; here imagined to be a stalled replication fork. BLM can find and act on these substrates in the absence of TopoIIIα binding or localization to the PML-NBs; however, in the absence of these functions, BLM activity is compromised, as evidenced by the failure of mutant BLMs to suppress to normal the high-SCEs of BS cells.

BLM also interacts with Rad51, the eukaryotic homolog of the *E. coli* RecA protein (115,116). Rad51 mediates the formation of joint molecules, which resemble the D-loops that form during an early step in homologous recombination. In approximately 15% of untreated normal cells proliferating by mitosis, Rad51 is detected in some PML-NBs with BLM. The frequency of cells containing Rad51-positive PML-NBs and the percentage in each cell of PML-NBs that contain Rad51 increase after treatments with gamma rays or aphidicolin. Physical interaction between Rad51 and BLM is detectable in vitro by far-Western analysis, but interaction can be detected in vivo by coimmunoprecipitation only in cells that have accumulated at S or G2 phase of the cell cycle using a treatment such as aphidicolin (116). In the far-Western analysis, part of the interaction with Rad51 is mediated by the N-terminal 212 amino acids of BLM—the same region that mediates interaction with TopoIIIα. This observation raises the question whether TopoIIIα and Rad51 bind to BLM at the same time or whether they compete for the same binding site(s). Physical interaction between BLM and Rad51 nicely links the biochemical evidence that BLM unwinds D-loops and Holliday junctions and the hyperrecombination evidenced in BS cells, and it suggests a possible antagonistic role of BLM on Rad51 during recombination. Appearance of Rad51 in a subset of PML-NBs and an increase in this localization after treatments with gamma rays and aphidicolin support the interpretation that these bodies play a role in the response to DNA damage.

BLM has been recently found to interact with MLH1, a protein involved in mismatch repair (117,140). The interaction was initially detected in a yeast two-hybrid screen with a C-terminal fragment of BLM, and it was subsequently confirmed by immunoprecipitation and far-Western analyses. Mismatch repair, however, is normal in BS cells, suggesting that BLM is not involved in mismatch repair. MLH1 may have some other function(s) in association with BLM that is linked to meiotic and mitotic recombination. For example, MLH1 may help through its own interactions to provide a proofreading capacity that distinguishes those substrates that BLM will unwind, preventing the recombination machinery from processing them, and those substrates that BLM leaves alone, allowing the recombination machinery to process them.

BLM and WRN both have functional interactions with p53 (122–124), implicating these RecQ helicases in the checkpoint response to DNA damage. However, there is no evidence that WRN interacts with TopoIIIα, Rad51, or MLH1. Instead, WRN interacts with proteins with which BLM does not appear to interact, for example, the Ku70/80 complex (121,122), which functions in DNA double-stranded break repair, and polymerase δ (123,124), the replicative DNA polymerase. How each of the helicases interacts functionally with these different proteins remain to be elucidated; however, the different protein–protein interactions provide clues to identification of the in vivo substrates favored by the two helicases. Such information will undoubtedly lead to an understanding of the

molecular events triggered by the absence of either the BLM or WRN helicase and help explain the different cellular outcomes of mutations in each. Because the Ku70/80 complex functions in the nonhomologous end-joining pathway of repair of double-stranded DNA (dsDNA) breaks, it seems likely that WRN functions in some aspect of this pathway. In the next section, we discuss the possible in vivo substrates on which BLM acts and biochemical pathway(s) in which it functions.

VIII. POSSIBLE MOLECULAR MECHANISMS OF BLM FUNCTION

The challenge for understanding the function of BLM and other RecQ helicases generally has been to deduce correctly the molecular mechanism in which they participate from the consequences of their failure to act. Because hyperrecombination is such a characteristic feature of BS cells, it has been tempting to postulate that BLM acts in an antirecombinogenic capacity. The hyperrecombination of BS cells could result because BLM specifically regulates recombination events in a cell, suppressing illegitimate recombination events or influencing their initiation or resolution. A nonexclusive, alternative view is that in the absence of BLM, a DNA structure that is normally processed by BLM inappropriately persists and accumulates, and this structure stimulates recombination directly or secondary to the action of some other enzymatic activity in the cell. For example, the unrepaired dsDNA breaks that are detected in BS cells at metaphase may arise directly from a process that is defective because BLM is absent and secondarily they could be the stimulus for recombination.

Recombination is used in cells proliferating by mitosis to repair a dsDNA break when a homologous chromosome or the sister chromatid is available. In mammalian cells, recombination between homologous chromosomes is infrequent, but sister chromatid exchanges can be detected at nearly every metaphase in chromosomes appropriately labeled with BrdU. It is now commonly believed that the dsDNA breaks that induce these sister chromatid exchanges are generated during replication. One mechanism (Fig. 7A) that would generate a dsDNA break is the situation in which the replication fork encounters a ssDNA break on either the leading or the lagging strand. The replication fork falls apart when the replicative helicase unwinds the DNA at the site of the ssDNA break and the two DNA strands dissociate. A dsDNA break may also occur at sites at which the replication fork is stalled. Once a dsDNA break has been generated, it can be a substrate for the recombination machinery. This machinery would restart the replication fork by establishing a joint molecule with the 3′ end from the broken chromosome invading the unbroken chromosome. Under these circumstances, a Holliday junction can be formed and, depending on how it is resolved, the recombination event can lead to a sister chromatid exchange. A postreplication dsDNA break, such as

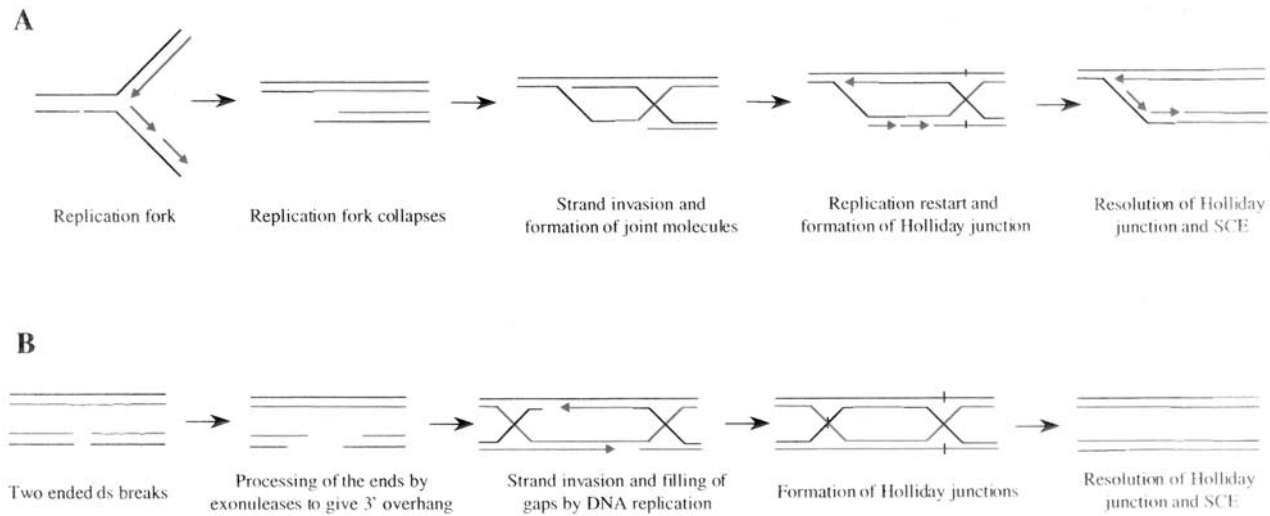

Figure 7 Model for the generation of an SCE by a homologous recombination event. (A) A single-stranded DNA break leads to replication fork collapse and the generation of a broken chromatid with a single DNA end. For DNA replication to restart, a homologous recombination event is initiated by processing and unwinding the DNA at the end of the broken chromatid and by invasion of a strand with a 3′ end into the unbroken chromatid. The strand invasion can generate a Holliday junction. DNA replication is reinitiated from the end of the DNA strand that invaded and the fork is restarted. Resolution of the Holliday junction with breaks as indicated by the small vertical lines would lead to a SCE. (B) A dsDNA break occurring during late S or G2 phases on one of two chromatids generates two broken DNA ends that can be repaired by a homologous recombination event using the unbroken sister chromatid as template. Again, a homologous recombination event is initiated by processing and unwinding the DNA at the broken ends, strand invasions, and the formation of two Holliday junctions. Replication occurs across the DNA between the annealed ends and resolution of the Holliday junctions as indicated would lead to an SCE. Newly synthesized DNA strands are depicted by lighter lines.

might be induced upon gamma irradiation of G2 cells, also can be repaired by a homologous recombination event, using the adjacent sister chromatid as template, possibly leading to an SCE (Fig. 7B).

Whenever DNA is damaged, for example, by ultraviolet radiation or chemicals that produce DNA adducts, the DNA repair machinery cleaves out the damaged DNA. The last step of the major repair pathways, such as nucleotide excision repair, mismatch repair, and base excision repair, is to seal the nicked DNA by the action of a ligase (125). In the base excision repair pathway, the ligase III–associated protein XRCC1 coordinates the final step between the outgoing polymerase and the incoming ligase. Mutation of the *XRCC1* gene in Chinese hamster ovary cells was until recently the only other mammalian mutation known besides *BLM* in which a high-SCE phenotype is featured (126). Theoretically, in the absence of XRCC1, ssDNA breaks arising from failure to complete base excision repair lead to single dsDNA breaks during replication and the stimulation of repair by recombination. Consistent with this view, many agents that damage DNA induce SCEs, and the damaged DNA must pass through S phase in order for SCE formation to occur (127). Following the argument made above, we postulate that in BS cells there is an increase in persistent dsDNA breaks arising during replication when such breaks would stimulate recombination. This postulate is consistent with the cytological evidence of chromatid breaks and with the observation in BS cells of retardation of the rate of replication fork progression (128) and the accumulation of abnormal replication intermediates (129).

Elucidation of the mechanism that generates the postulated dsDNA breaks in BS cells requires identification of the in vivo substrates of BLM. Biochemical studies have implicated the recombination intermediates D-loops and Holliday junctions, supporting hypotheses such as the reverse branch-migration model (Fig. 8A). In this model, BLM acts at replication forks that have stalled. When a fork is stalled, the nascent, newly replicated DNA strands may anneal to form a Holliday junction. The position of the strand crossing over then can move in a direction opposite to that of the replication fork. This structure has been called the "chicken foot" and, although its existence is hypothetical, an enzyme in *E. coli* RecG is known that can produce such a structure. The proposed function of BLM in the model is to reverse chicken foot formation and reestablish the replication fork.

One proposed function of chicken foot formation is to provide an opportunity to repair the DNA. If a replication fork encounters damaged DNA, such as an abasic site, the fork will stall at that site. Reversing the fork direction to reanneal the old DNA strands would allow repair to be completed at the site of damage. If the replication fork is allowed to idle at the site of damage, the DNA becomes prone to breakage, and, as described above, a dsDNA break generated at that site could be repaired by a homologous recombination event. Consistent with these ideas, agents that stall the replication fork, such as aphidicolin, also induce SCEs (130). Therefore, even if BLM does not specifically reverse the formation of the

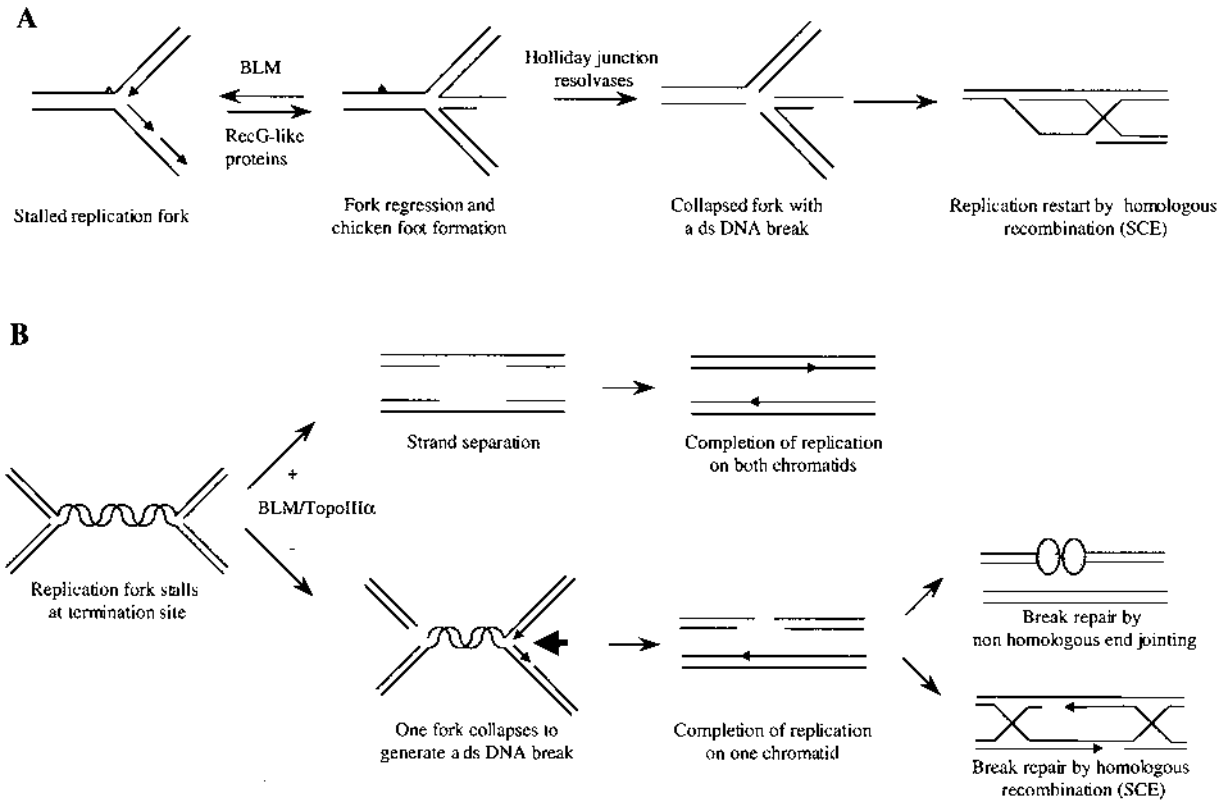
A
BLM
RecG-like proteins
Holliday junction resolvases
Stalled replication fork
Fork regression and chicken foot formation
Collapsed fork with a ds DNA break
Replication restart by homologous recombination (SCE)
B
Strand separation
Completion of replication on both chromatids
+
BLM/TopoIIIα
-
Replication fork stalls at termination site
One fork collapses to generate a ds DNA break
Completion of replication on one chromatid
Break repair by non homologous end jointing
Break repair by homologous recombination (SCE)

Figure 8 Two possible cellular functions of BLM, the absence of which may lead to SCEs. (A) The replication fork can stall when it meets certain forms of DNA damage. At a stalled replication fork, newly replicated DNA can anneal to each other and may propel fork regression, forming the so-called chicken foot structure. Such a structure could be catalyzed by RecG-like proteins. The chicken foot structure at the regressed replication fork is structurally similar to a Holliday junction and could be resolved by Holliday junction resolvases, resulting in a dsDNA break. Repair of the dsDNA break by homologous recombination can lead to an SCE as described in Figure 7A. In this pathway, BLM may function to prevent replication fork regression and Holliday junction resolution by stabilizing the stalled replication fork while the damaged DNA is being repaired. This model is supported by the observation that the BLM protein preferentially catalyzes branch migration of the Holliday junction structure. How the direction of branch migration would be controlled remains to be elucidated. (B) When two replication forks meet from opposite directions, they stall prior to the completion of DNA replication, possibly due to the topological tension on the DNA between the two forks. BLM and TopoIIIα may function together at the site of termination to process the separation of parent strands. This model specifically utilizes both activities of the BLM/TopoIIIα complex. As the BLM unwinds the duplex DNA, it provides the ssDNA substrates which TopoIIIα relaxes and decatenates. After separation of the chromatids, replication is completed across the two single-stranded gapped molecules. In the absence of BLM/TopoIIIα function, the stalled replication fork is hypothetically prone to breakage. Breakage allows one fork to finish replication but a dsDNA break occurs on other chromatid. Repair of the two-ended dsDNA break can be performed by the nonhomologous end joining pathway or the homologous recombination pathway as described in Figure 7B. If a homologous recombination event occurs, it can lead to an SCE.

chicken foot, a defect in BS cells that increases replication fork stalling or decreases the preservation of a stalled replication fork is consistent with the high-SCE phenotype of these cells.

Replication forks may slow or stop not only upon encounter of DNA damage but also in normal, nonpathological situations. Pausing occurs as two replication forks approach one another from opposite directions at the site of termination of replication (see Fig. 8B). The pausing probably occurs because torsional strain created by the two approaching forks in the last approximately 200 nucleotides is not efficiently removed by topoisomerases. Theoretically, there are two mechanisms that can be used to resolve the DNA at termination sites (131). In one mechanism, one of the replication forks completes DNA synthesis leaving the two sister chromatids catenated. The sister chromatids can then be decatenated by a type II topoisomerase. In the other mechanism, the two strands of DNA that remain to be replicated are separated by combined DNA helicase and topoisomerase activities. In mammals, such combined activities are available in the BLM/TopoIIIα complex.

TopoIIIα and other TopoIIIs prefer ssDNA to dsDNA, and they can cleave a ssDNA molecule and rejoin the ssDNA ends (132–134). These enzymes are in fact poor topoisomerases, but they are impressive as decatenases with the capacity to disentangle intertwined single DNA strands (135). As a helicase, BLM generates ssDNA that could be made available to the associated TopoIIIα. These combined activities could act to separate the two strands of the DNA duplex at termination sites, allowing replication to be completed (see Fig. 8B). In the absence of efficient separation of the strands, the DNA may break at some of the forks. The break relieves the torsional strain and allows completion of replication on one strand. Completion of replication creates two broken chromatids; these broken ends could be repaired either by a nonhomologous end-joining event or by a homologous recombination event that can potentially lead to an SCE. Because the ragged ends of the broken chromosomes would have to be processed for nonhomologous end joining to be performed, this repair pathway potentially results in deletions. DNA deletions are common in both BS and WS cells (11,136). If the BLM/TopoIIIα complex performs the strand-separation function at sites of replication termination, then in BS cells replication termination would often be associated with DNA breaks that are repaired by homologous recombination, nonhomologous end joining, or both. A prediction of this model is that induction of the nonhomologous end-joining pathway, for example, by overexpression of proteins in the pathway, should suppress the high-SCEs in *BLM*−/− cells. Indeed, such suppression has been observed in *Drosophila melanogaster BLM* mutants (137).

Biochemical studies of the WRN helicase have disclosed that the substrate specificity of the helicase in vitro is similar if not the same as BLM's. The molecular biology of WRN and WS cells however differs from that of BLM and BS cells

in four major ways: (a) WRN interacts with proteins of the nonhomologous end-joining pathway Ku70/Ku80; (b) WRN protein possesses an exonuclease activity that potentially processes substrates for this pathway; (c) the cytogenetics of WS cells, which is described as "variegated translocation mosaicism," is consistent with an inappropriately regulated end-joining activity; and (d) WRN is present throughout the cell cycle and it localizes to the nucleolus and to microspeckles in the nucleoplasm and not to the PML-NBs except under rare conditions. We propose that WRN functions in a class of nonhomologous end-joining events, processing the DNA ends of substrates with structures that require the helicase/exonuclease activities of WRN. BLM, on the other hand, is probably called into play during S phase to act at particular replication forks; namely, those that have stalled or paused. These forks are processed or stabilized by the BLM/TopoIIIα complex, thereby preventing the formation of recombinogenic dsDNA breaks.

How these molecular differences translate at the organismal level into a premature aging syndrome in the case of WS and a syndrome of small size in the case of BS will require further elucidation of the molecular biology of WRN and BLM. As our understanding of the function of each of these two helicases increases, we are characterizing at the molecular level the chromosomal instability present in WS and BS cells. The differing abnormal effects of these instabilities on cell proliferation and cell death in the different organs and during development and the life of the individual, and the systemic sequelae of these abnormalities, will eventually lead to a complete understanding of both of these syndromes. It will also enhance our understanding of the basic biology of DNA recombination, replication, and repair.

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11

Molecular Biology of Rothmund–Thomson Syndrome

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I. INTRODUCTION

One hundred and thirty-five years ago, August Rothmund, a German ophthalmologist, saw several children with clinical phenotypes of what was later referred to as Rothmund–Thomson syndrome and reported the cases to the domestic ophthalmology journal (1). His report starts with,

> In April 1866, a five-year old boy was brought into my clinic, who suffered from cataract in one eye. According to his parents this had been noticed four days earlier without any foregoing accident. At the same time, a strange marbled skin was noticed. Two weeks later, two other children with the same cataracts living in the same area and with the same degeneration of the skin were brought to me. This made me quite alert, first because the parents told me that there were even more such children with the same skin and eye problems in the same area and second because all my colleagues assured me they had never to seen anything similar. Therefore I decided to look at this problem directly.

Rothmund visited the small valley where all these children came from.

> The valley is small, surrounded by high mountains open only towards the north and it has three villages, Ritzeln, Kirshek and Mittelberg, which are in the suburbs of present Munich. It has an altitude of 3816 ft, and about 1500 people live there. In each of the villages there are at least one family with several children who always show cataracts and exanthema at the same time.

In his report, Rothmund describes his visit, together with the observation of the cases found in his clinic, and he concludes that the phenotypes are the results of some kind of hereditary disease. Rothmund's paper might have markedly influenced Otto Werner, another German ophthalmologist, who lived near Hamburg and reported on his young patients who had cataracts and a hard skin phenotype in 1905, which was later referred to as Werner syndrome (WS).

In 1936, Sidney Thomson, a British dermatologist, reported additional bony abnormalities, such as frontal bossing, and hypoplastic thumbs in some of his patients with phenotypic features similar to those described by Rothmund (2). As these two reports shared several features in common, such disease phenotypes were assumed to be an identical disease. The disease has been referred to as Rothmund–Thomson syndrome (RTS), although this naming has been disputed because of the wide variability in the phenotypes (3–5). RTS and WS have often been cited as "premature-aging diseases with a high predisposition of cancer," because individuals affected with either disorder share disease phenotypes or symptoms associated with elderly people. Patients with RTS in general show skin abnormalities such as poikiloderma, skin sensitivity to light in infancy, and osteosarcoma (6). Several excellent articles have reviewed the clinical features of RTS (7–9).

The gene responsible for these complex clinical phenotypes was not known until Kitao and coworkers (10) found that the RECQL4 gene (*RECQL4*) encoding a DNA helicase belonging to the RecQ helicase family was mutated in the cells from a subset of an RTS patient population. Studies to clarify the biological and biochemical natures of the function of RECQL4 helicase are underway. In this chapter, we review the clinical features of RTS and describe what is known of *RECQL4* and its gene product, its mutation in patients with RTS, and some of the molecular and biological aspects of RTS helicase.

II. CLINICAL FEATURES

A. Cutaneous Signs

Rothmund–Thomson syndrome is classified as a hereditary dermatosis. Most patients with RTS develop erythematous patches or red edematous plaques in infancy, sometimes accompanied by blistering. These symptoms are initially on the cheeks and spread to the forehead and ears and then to the neck. The characteristic appearance of poikiloderma shows dry atrophic skin with areas of hyperpigmentation and hypopigmentation in a marblized pattern (7). The poikiloderma is most marked in, but not limited to, sun-exposed areas, and skin changes commonly occur on the buttocks. Acute photosensitivity eruptions are uncommon. Brown skin pigmentation without atrophy is also seen. Skin atrophy does not involve subcutaneous fat. RTS is often associated with telangiectasia similar to the Bloom syndrome (BS) (11) (see Chapter 10). Other variable features include short stature,

small saddle nose, graying of the hair, alopecia, sparse eyebrows and eyelashes, dystrophic teeth and nails, and early onset of cataracts, as initially reported by Rothmund. These are typical findings associated with natural aging; thus, RTS is classified as a premature aging syndrome along with the Werner syndrome (10,12).

B. Malignancy

Patients with RTS are susceptible especially to osteosarcoma and squamous cell carcinoma (7). Eight cases of skin malignancies have been reported in patients with RTS: four squamous cell carcinoma (SCC) (13–16), two Bowen disease (17,18), one basal cell carcinoma (19), and one spindle cell carcinoma (20). No melanomas have been reported. In 1975, Werder and colleagues (20) reported a parathyroid adenoma in a patient who also developed a spindle cell carcinoma. Since Tokunaga and colleagues (21) reported the first case of osteosarcoma in an RTS patient in 1976, more than 20 cases of osteosarcoma have been reported. Patients with RTS show some bony abnormalities, most commonly metaphyseal chondrodysplasia and abnormal trabeculation. Pujol and coworkers (23) suggested that the diagnosis of RTS should be considered in all patients with osteosarcoma, particularly if the bone changes are associated with skin changes (22). Lindor and coworkers (6) reported a brother and sister with typical clinical features of RTS. At age 31 years, the brother was diagnosed with osteosarcoma of the distal right tibia, and the sister was diagnosed at age 15 years with multicentric osteosarcoma of the right tibia, left fibula, and right humerus. They received some courses of chemotherapy, and the sister had limb salvage surgery. After their diagnosis, both showed no evidence of osteosarcoma recurrence. Survival of patients with RTS with osteosarcoma is 14 years, well within the usual age of onset of this malignancy (7). However, these features might identify cancer-prone patients, because bony malignancies often arise in previously dysplastic or dysostotic long bones, similar to other diseases such as hereditary bone dysplasia and erythroid macrocytosis (23,24).

C. Short Stature

Patients with RTS are born after a normal gestation. However, most affected infants have low birth weight and develop short stature or dwarfism (7). The majority of patients have normal intelligence, but exceptions exist. Some patients have hypogonadism or delayed sexual development. Several patients also have shown low levels of serum estrogen, testosterone, or 17-keto steroids. Accordingly, infertility has been described as inevitable in patients with RTS, although this conclusion has been contradicted by reports of pregnancy (25,26). Studies of patients with short stature showed that most patients have normal levels of growth hormone release (21,27–29). Figure 1 shows a typical RTS patient. Table 1 compares

Figure 1 Portrait of a patient with Rothmund–Thomson syndrome at age 21 years. The patient (boy) was born with a normal weight of 3.5 kg, but he suffered from postnatal growth restriction beginning in infancy. He had marked photosensitivity, developing red scaly skin in sun-exposed areas. At the age of 21 years, he was diagnosed as having osteosarcoma in the distal radius. He had chemotherapy-related scalp alopecia and nearly complete absence of eyebrows and lashes. He had diffuse erythema on his face with faint marbleized pigmentation on one cheek, but he had no telangiectasias. Lindor and coworkers (Ref. 11) give more precise descriptions. This patient has compound heterozygous *RECQL4* gene mutations of mut-5 and mut-6.

the clinical phenotypes of patients with RTS with several other disease symptoms that patients with WS and BS often share with patients with RTS.

III. *RECQL4*, A GENE CAUSING RTS

A. Cloning of *RECQL4*, a Member of the RecQ Helicase Gene Family

In 1998, Kitao and coworkers (30) reported the cloning of the then new genes *RECQL4* and *RECQL5*, at 8q24.3 and 17q25.2–25.3, respectively, that have homology with the RecQ gene of *Escherichia coli*. Cells of *E. coli* that have a mutation on the RecQ gene (*RecQ*) show phenotypes of chromosomal instability, be-

cause the DNA helicase coded for by *RecQ* directly participates in suppressing hyperrecombination (31). *RECQL4* and *RECQL5* also share homologies with human *WRN* and *BLM*—genes responsible for WS and BS, respectively. WS and BS are autosomal recessive genetic diseases, and the mutations of *WRN* and *BLM* result in the onset of diseases (32–34) (see Chapters 9 and 10). Seki and coworkers (35) have cloned *RECQL*, the other human gene homologous to *E. coli RecQ*, which also encodes the DNA helicase. This *RECQL* has not yet been shown to be relevant to any known human disease. Collectively, the five genes, *RECQL*, *BLM*, l*WRN, RECQL4*, and *RECQL5,* form the human RecQ gene family, and *RECQL4* and *RECQL5* were named following the historical order of the gene cloning (30). Further efforts to seek additional RecQ-related genes in human cells have been unsuccessful (A.S., unpublished results). *RecQ*-related genes exist in other organisms: *SGS1* in the yeast *Saccharomyces cerevisiae*, and *rqh1*$^{+}$ in the *Schizosac-*

Table 1 Clinical Phenotypes of Werner, Bloom, and Rothmund-Thomson Syndromes: WS, Werner Syndrome; BS, Bloom Syndrome; RTS, Rothmund–Thomson Syndrome

Symptoms	WS	BS	RTS
Short stature	+	+	+
Early onset cataracts	+	+	+
Narrow nose	+	+	+/−
High pitched voice	+	+	?
Maturity onset-type diabetes	44%	8%	−
Radial ray anomalies	−	−	+
Premature atherosclerosis	+	?	?
Hypogonadism	+	+	+
Valvular sclerosis	+	−	−
Osteoporosis	+	?	?
Dystrophic teeth	−	+	+
Dystrophic nails	+	−	+
Intellect usually normal	+	+	+
Immunoglobulins low	−	+	?
Early graying of hair	+	−	+
Sparse hair, brows lashes	+	−	+
Photosensitivity	−	+	+
Telangiectasia	−	+	+
Poikiloderma	−	−	+
Areas of hyperpigmentation	+	+	+
Scleroderma-like skin	+	−	−
Neoplasia predisposition	+	+	+
Subq calcifications	+	−	−
Acral skin ulceration	+	−	−

charomyces pombe. Both have been extensively studied and participate in suppressing hyperrecombination (36–39). Multiple species of RecQ-type genes are in higher eukaryotes, exemplified by three genes in *Drosophila melanogaster* (an insect) and four genes in *Caenorhabditis elegans* (a nematode) (40,41), whereas single-celled organisms (such as *E. coli* and yeast) contain single species of the RecQ-type DNA helicase gene (30). All five human *RecQ*-type genes code for DNA helicases, enzymes that unwind DNA. Figure 2 shows representative members of the RecQ-type helicase family. BLM, WRN, and RTS DNA helicases have large molecular weights of 130K–160 kD. One *RECQL5* gene product, the RECQL5β helicase, is 110 kD (42), and the RECQL helicase has a small molecular weight (75 kD). Of these, BLM and WRN helicases with a large molecular weight are correlated with human genetic diseases (32,33). Thus, it was natural to suspect that a mutation of *RECQL4* and its defective helicase could be related to the RTS, based on its similarities to the BLM and WRN helicases (i.e., molecular biological and genetic features). Additionally, some clinical phenotypes of patients with RTS overlap with those of WS and BS (see Table 1). In collaboration with Lindor at the Mayo Clinic and Miller in the National Cancer Institute in the United States, we identified mutations in *RECQL4* from several patients with RTS (10, 11). These data are summarized in Figures 3 and 4.

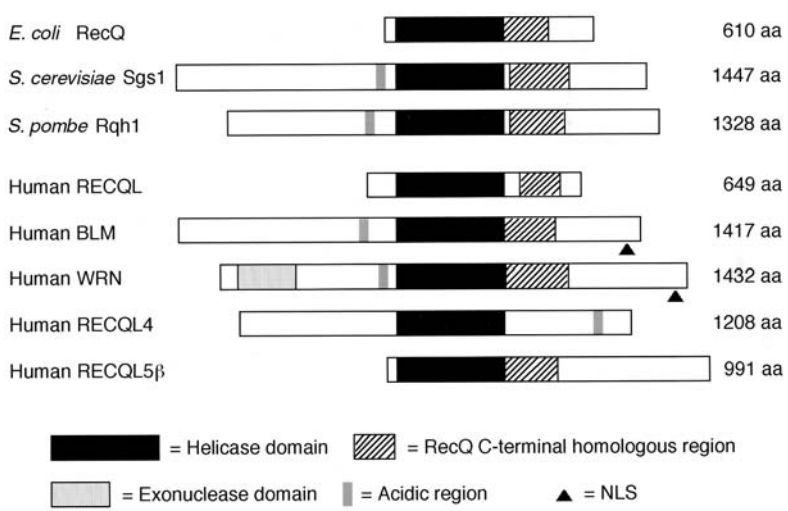
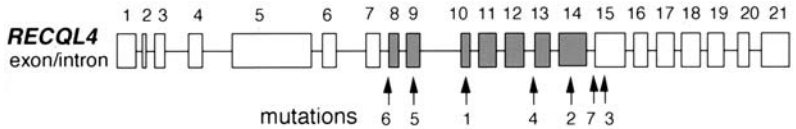


Figure 2 Schematic representation of the RecQ family proteins. The names of the gene products and the organisms are shown on the left. The size of each protein is given on the right. RECQL4 helicase is quite different from the others, as it has no exonuclease domain, HRDC, or NLS. The amino acid polarity of RECQL4 is also opposite to that of WRN and BLM helicases. These differences may distinguish the specific biological functions of RECQL4 helicase.



Mutation type	Mutation	Exon	Consequence
mut-1	1650 del 7	10	frameshift
mut-2	C 2269 T	14	nonsense
mut-3	2492 del 2	15	frameshift
mut-4	3' splice site, ag→at	13	frameshift
mut-5	1573 del 1	9	frameshift
mut-6	3' splice site, ag→aa	8	altered splicing
mut-7	3' splice site, ag→ac	15	altered splicing

Figure 3 *RECQL4* gene mutations in patients with RTS. Seven types of mutations are in patients with RTS. An arrow shows each mutation. The *RECQL4* gene consists of 21 exons indicated as boxes, and the intervening lines show the intron regions. The shaded boxes indicate the exons coding for a helicase domain. The seven mutation types and their consequences are listed at the bottom, and the nucleotide numbers are based on the genomic sequences of the *RECQL4* published by Kitao and coworkers (Ref. 75). All mutations are presumed to cause either inactivation of the helicase or incomplete molecules.

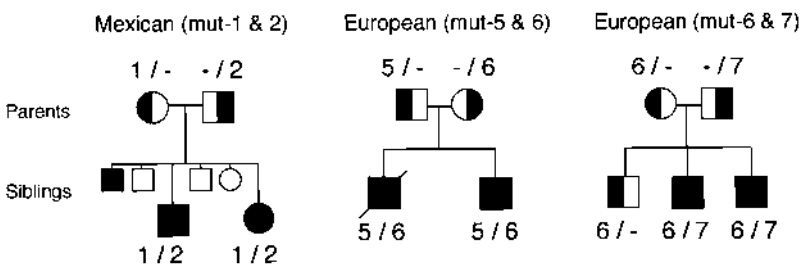


Figure 4 Three RTS patient pedigrees carrying hereditary heterozygous *RECQL4* gene mutations. The patients with RTS have inherited two mutated alleles from their parents, and the affected brothers or sister have the same mutations. The Mexican family with mut-1 and mut-2 was reported by Lindor and coworkers (Ref. 6) and by Kitao and coworkers (Ref. 10). The European family with mut-5 and mut-6 also was reported by Lindor and coworkers (Ref. 11), and the other European family has not been reported.

B. *RECQL4* Gene Mutation in Patients with RTS

Thus far, we have found seven different types of gene mutations in *RECQL4* from patients with RTS of various ethnic backgrounds (see Fig. 3). The mutations are distributed throughout *RECQL4* and are predicted to result in incomplete protein products either by truncation owing to early termination in protein synthesis or by deletion of part of the protein owing to exon skipping. All patients whose *RECQL4* mutations are recognized are compound heterozygous and have no background of consanguinity (mut-1,2; mut-3,4; mut-5,6; mut-6,7; and mut-3,6). In three patient pedigrees, we confirmed that each mutated allele was inherited from each parent (see Fig. 4). These findings suggest that different mutations have arisen over time in the human population, and are transmitted as Mendelian autosomal recessive disorders in which the carrier parents are apparently healthy and asymptomatic. We analyzed 20 patients (including brothers and sisters) who were clinically diagnosed as having RTS and identified the *RECQL4* mutations in seven patients, all carrying the compound heterozygous mutations. These results indicate that mutation of *RECQL4* may explain the pathogenesis of a subset of patients with RTS. Further studies are needed to determine if the gene expression of *RECQL4* is reduced in the patients with RTS in whom we failed to find a distinct mutation in the coding region of *RECQL4*. Alternatively, patients clinically suspected to have RTS may have a defect in another gene(s) that causes similar clinical phenotypes as RTS. In any case, *RECQL4* provides an excellent tool for diagnosing RTS, as well as for clarifying the molecular mechanism underlying RTS. In a later section, we will discuss the diagnosis of RTS using an immunologic method that assesses the presence (or absence) of the complete molecule of the helicase protein produced from *RECQL4*.

IV. CELL BIOLOGY OF RTS CELLS

A. Chromosomal Instability of RTS Cells

Chromosomal analysis shows most patients with RTS have a normal karyotype (7,43). However, some cytogenetic studies have shown structural abnormalities—especially trisomy 8 or isochromosome 8 mosaicism in lymphocytes or fibroblasts in patient cells (6,44–47). Ying and coworkers (44) first described trisomy 8 mosaicism in 2.5% of peripheral lymphocytes accompanying RTS, which were all derived by centrosomal misdivision or breakage. This low-frequency mosaicism was later reported in four other studies. Lindor et al. (6) performed extensive cytogenetic and molecular genetic studies on the cells from two siblings with RTS and their phenotypically normal parents. They also investigated the osteogenic sarcomas obtained from the patients. Their findings suggest that acquired somatic mosaicism may be characteristic of classic RTS resulting from chromosomal abnor-

malities. In the study, high frequencies of trisomization of chromosome 8 were found in vitro in the phytohemaglutinin (PHA)–stimulated blood cells of patients as well as in vivo in the uncultured cells from blood or buccal smears of patients, whereas this did not occur with cells from their obligate heterozygotic parents for RTS which showed normal karyotypes or were within the normal ranges upon various cytogenetic analyses. In the PHA-stimulated blood cells from patients, the spontaneous breakage of chromosomes did not increase over controls even after the treatments with bleomycin and mitomycin-C, and the sister chromatid exchange was within normal laboratory limits. Chromosomal analysis of the tissue cultured from the osteogenic sarcoma from a patient revealed grossly hyperdiploid metaphases with multiple chromosomes and many different structural abnormalities, whereas no evidence of microsatellite instability was found at any locus, suggesting that DNA mismatch repair genes were functioning normally. From these results they concluded that RTS does not show the same type of chromosomal instability found in ataxia-telangiectasia (bleomycin sensitive), Fanconi anemia (mitomycin-C sensitive), or Bloom syndrome (increased sister chromosome exchange). Miozzo and coworkers (47), looking for a common genetic background, performed cytogenetic analysis of tumors in patients with RTS and suggested that chromosomal instabilities including trisomy 8 explain the predisposition to tumorigenesis. Miozzo and coworkers also pointed out an increased risk of mesenchymal tumors in patients with RTS. Whether trisomy or mosaicism involving chromosome 8 influences cell proliferation and tumorigenesis of RTS patient cells remains to be studied.

B. Sensitivity to Ultraviolet Light and DNA-Damaging Agents

There is a suggestion of increased risk of skin cancers in RTS patients and recurrent reports of osteosarcoma. The most common cutaneous malignancies associated with RTS include squamous cell carcinoma, basal cell carcinoma, spindle cell carcinoma, and Bowen disease. Because skin eruptions occur mainly in sun-exposed areas and a high risk exists of squamous cell carcinoma in the skin of patients with RTS, several cases have been investigated to determine the sensitivity to specified or unspecified wavelengths. However, the results of phototesting with RTS cells have been inconsistent. Studies have shown normal sensitivity to ultraviolet B (UV-B), UV-A, or unspecified wavelengths (19,48–52). Other studies have shown a high sensitivity by phototesting: one study used an unspecified wavelength (53) and other studies used UV-A (18) or UV-B (54). Because these studies were done prior to identification of the causative gene, it is unknown if these patients had RECQL4 mutations or not. Thus, clinical histories showing the degree of sun sensitivity may not be consistent with results from cultured cells in laboratory phototesting. This discrepancy may be due to

differences in genetic background of cells or in the gene(s) responsible for RTS phenotypes.

Studies have shown normal DNA repair after UV irradiation of cultured fibroblasts or in biopsied skin cells (5,18,43,48,52,55–57). Other studies, however, have shown the decreased unscheduled DNA synthesis (UDS), which is derived from nucleotide excision repair after UV-C irradiation (58,59). DNA repair after gamma ray irradiation has been reported to be either abnormal (60,61,12) or normal (62). Studies have been made of chromosomal instabilities in RTS cells. In one of these studies, mitomycin-C–treated blood cultures from one patient with RTS showed increased chromosomal breakage compared with control cultures (63). Another study showed that cultured fibroblasts have a consistent clonal rearrangement of chromosomes, suggesting chromosomal instability in RTS cells (45). These discrepancies in the sensitivities to UV or gamma ray irradiations, and the genomic instabilities in the presence of DNA-damaging agents, may reflect the heterogeneity of the genetic background of each patient, such as having a functional or defective *RECQL4*. For example, patient cells that show only a slight decrease in UDS after UV-C irradiation actually have an intact RecQ4 helicase protein (Kitao et al., unpublished results) (57). Further study of the relationship between impaired DNA repair and the defective RTS gene is required to substantiate a high occurrence of tumors in patients with RTS, as well as continued investigation of the biochemical causes(s) of the onset of other RTS symptoms.

C. Dynamics of the RECQL4 Helicase in Cells

The amount of RECQL4 helicase in the cultured skin fibroblasts from normal individuals is low, but it is detectable by Western blot analysis using specific antibodies (64). The amount of RECQL4 helicase protein in each cell is higher in growing cells than in resting cells, suggesting that the expression of *RECQL4* is probably dependent on cell growth. Consistent with this, the copy number of RECQL4 helicase decreases as the cells senesce by continued culturing: nonsenescent TIG-3 cells at population doublings (PDL) 39 contain at least a five-fold greater amount of RECQL4 helicase in each cell than do TIG-3 senesced cells at PDL 82 (65). By contrast, rapidly growing TIG-3 cells immortalized by transformation with simian virus (SV40) large T antigen increase the copy number of RECQL4 enormously (more than 10-fold). This finding implies that a large copy number of RECQL4 helicase is beneficial for rapid cell growth. High copy numbers of helicase in rapidly growing TIG-3 human fibroblast cells have been observed with other human RecQ helicases, including WRN, BLM, and RecQL5, but not with the RecQL helicase (64). Other quantitative studies of the copy number of human RecQ helicases have been made with B lymphoblastoid cells trans-

formed by Epstein–Barr virus (EBV) infection. The transformed B cells grow rapidly, but they are not necessarily immortalized (66). Immortalized cells expressing telomerase activity appear occasionally from mortal, but transformed, cell populations with a maximum PDL of approximately 100. Here, the normal resting B cells contain about 120 copies of RECQL4 helicase in each cell, but EBV-transformed lymphoblastoid cell lines (LCLs) acquire much greater (1100) copies in each cell, and immortal LCLs derived from mortal LCLs have more than 1000 copies in each cell (64). Similar large increases in the copy number occur with WRN and BLM helicases when the resting B cells are transformed by EBV infection or by transfection with SV40 large T antigen. In addition to viral proteins, the tumor-promoting drug (and the B-cell mitogen) phorbol-12-myristate-13 acetate (PMA) upregulates the expression of RECQL4, WRN, and BLM helicases in B cells in a very short time, indicating that these helicases are probably required to replicate cells, and that the regulations of the gene expression are mediated mainly by transcription (64).

D. Immunological Diagnosis of RTS, WS, and BS

An immunological procedure to diagnose RTS, WS, and BS (67) was developed based on the observations that (a) normal RECQL4, WRN, and BLM helicases are highly upregulated in EBV-transformed LCLs and (b) incomplete helicase molecules produced by mutated genes are scarcely detected in patient LCLs. This method has been proven to be practical for WS, BS, and a subset of patients with RTS. It avoids difficulties in finding new mutations when analyzing large genomic DNAs by nucleotide sequencing and has been used several times for diagnosis in our laboratory. In brief, B-cell fractions in blood specimens are infected with EBV and are cultured for 3–4 weeks to obtain sufficient numbers of transformed cells for protein analysis. The protein levels of the individual helicases are subsequently measured using Western blot analysis using monoclonal antibodies specific to RECQL4, WRN, or BLM helicases and are compared with those of normal individuals. The reason underlying the specific reduction of defective protein is not well understood, but the fact that mRNAs containing gene mutation(s) that are preferentially degraded by the nonsense-mediated RNA sequestering mechanism (68–70) may partly explain it. Another possible explanation is that incomplete protein products are conformationally unstable and are more susceptible to proteolytic degradation than the complete molecule.

In the light of the facts that most truncated helicases produced from mutated *WRN* and *BLM* lack a nuclear localization signal in the C-terminal region and are thus unable to migrate to the nucleus and remain in the cytoplasm (71–73), it is conceivable that the turnover of protein in the cytoplasm may be faster than in the nucleus.

V. BIOCHEMICAL FEATURES OF THE RECQ4 HELICASE

A. Structural Similarities to Other RecQ Helicase Family Members

The RECQL4 helicase has a helicase domain that contains seven consensus motifs in the middle of the molecule (see Fig. 2). The amino acid sequence of the helicase domain is 40.8% homologous to that of the *E. coli* RecQ helicase, the prototype of this family, within the same range as the other four family members, RECQL, WRN, BLM, and RECQ5 helicases. Of the five human RecQ helicases, defective WRN, BLM, and RECQL4 helicases are related to diseases that show phenotypes of genomic instability and a high risk of cancer. Because RecQ helicase–deficient mutants of *E. coli* and yeast show genomic instability and a high level of recombination, it is conceivable that the function(s) of the RecQ helicase family is conserved across the species and participates in maintaining genomic integrity. In fact, the yeast Sgs1 helicase suppresses illegitimate recombination, and the human BLM, WRN, or RECQL4 helicase can substitute this function in the *sgs1*-defective yeast mutant (74) (Kitao et al., unpublished results).

The WRN helicase contains an exonuclease domain at the N-terminal region of the molecule, and both WRN and BLM helicases have distinct nuclear localization signals (NLS) consisting of basic amino acid arrays at the C-terminal region (71–73). However, the RECQL4 helicase does not contain an exonuclease domain, nor does it contain a clear NLS, although the RTS helicase is transported to the nucleus and is distributed in the nucleolus and the nucleoplasm (75) (see Sec. V.D). These findings suggest that each helicase in the RecQ family has evolved from a progenitor helicase and contains diverged structural features even though its enzymatic action that hydrolyzes ATP and unwinds DNA remains the same. The WRN and BLM helicases are intact in RTS patients with defective *RECQL4*-defective patients (and conversely WRN patients have intact RECQL4 helicase (Kitao et al., unpublished results), suggesting that these helicases have specific roles and are unable to compensate for one another's functions. In remains to be clarified in what kind of DNA structures these helicases unwind, or with what kind of nuclear factors they cooperate (or both), and in what kinds of cellular events they function.

The RecQL4 helicase has high amino acid homologies to the homologue helicase in *Drosophila* (41) and in the mouse (76) (Kitao et al., unpublished results): They have homologies of 53.9% and 78.2% in the helicase domain, respectively. Particularly, the mouse RecQL4 helicase is very similar to that of humans, showing an overall homology as high as 60.7%, whereas the homologies of the *Drosophila* homolog are restricted to the short N-terminal end (34.0%), the helicase domain (53.9%), and the C-terminal region (32.1%). These data imply that two domains, the helicase and the C-terminal domains, are conserved among species and may be important for the RecQ4 protein's enzymatic activity.

B. Promoter and Tissue-Specific Expression of *RECQL4*

The upstream region of the open reading frame of *RECQL4* contains 1 Sp1 and several AP2 transcription regulatory element sites and no TATA-box near the capping site (Fig. 5), suggesting that the expression of *RECQL4* is probably regulated by a housekeeping-type promoter similar to the expression of *WRN*. Previously, Yamabe and coworkers (77) extensively investigated the expression of *WRN* and showed that *WRN* is mainly regulated by Sp1, modulated by tumor-suppressor proteins p53 and Rb, downregulated by p53, and slightly upregulated by Rb. A similar (but not an identical) transcriptional control is predicted to occur with *RECQL4* gene expression. In general, the transcription of *RECQL4*, *WRN*, and *BLM,* is upregulated in rapidly growing cells, such as SV40-transformed fibroblasts and EBV-infected lymphoblastoid cells, but no or only a very low level of transcription is observed for all these genes in senescent or resting cells (64).

The tumor-promoting PMA rapidly stimulates the synthesis of RECQL4, WRN, and BLM helicases in resting peripheral B cells; apparently mediated by simultaneous activation of transcription and translation (64). This stimulatory effect of PMA is different in the three helicase genes. PMA stimulates RECQL4 and WRN synthesis in a much shorter period (within approximately 5 hr) than with BLM (within approximately 40 hr). These findings prompt us to speculate that the transcription of *RECQL4*, as a housekeeping gene, is regulated by a cell cycle similar to *WRN*.

To determine if each helicase can be expressed in specified tissues or organs, we analyzed the expression of all five RecQ helicase genes in various human organs and tissues by Northern blotting (Fig. 6). The data show that *RECQL4*, *WRN*, and *BLM* are expressed in a tissue specific–fashion, and support the idea that a close correlation exists between the site of gene expression and the site (tissue or organ) of disease phenotypes. For example, *WRN* is expressed highly in pancreas, testis, and ovary, and WS patients often show clinical symptoms of diabetes and hypogonadism. Also, *BLM* is highly expressed in the thymus

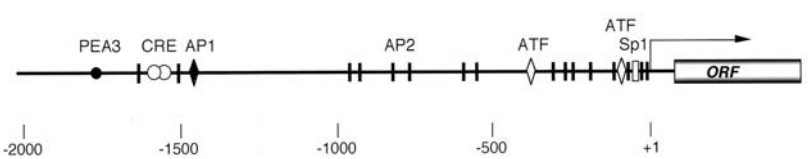


Figure 5 Potential promoter region of *RECQL4* adjacent to the transcription initiation sites. The first intron and the upstream region of *RECQL4* contained a high content of GC residues and no TATA box, characteristics of the promoter of a housekeeping gene. The high expression of *RECQL4* in the testis and thymus may be explained by the enriched Sp1 transcription factor in the testis and by Sp1 and CREB in thymocytes.

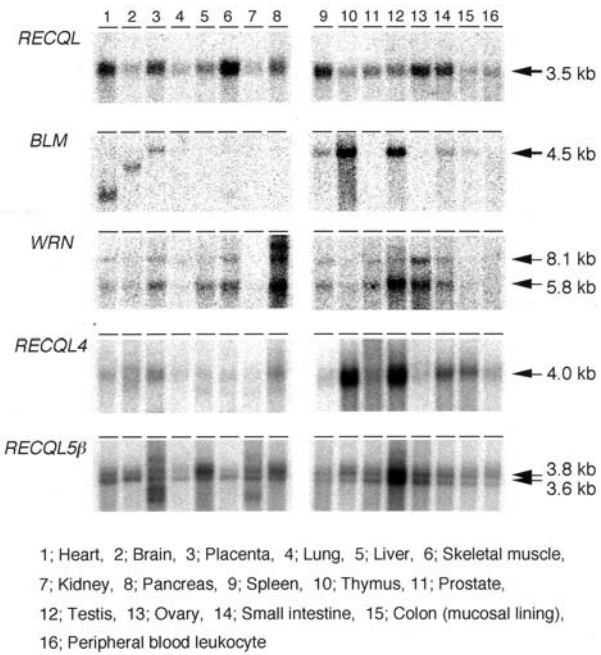


Figure 6 Expression profiles of five human RecQ family helicases in human organs. Multiple-tissue Northern blot (CLONTECH) was prepared with 2 μg of poly(A) RNA in each lane. The *RECQL* gene was ubiquitously expressed in almost all tissues tested. The other four helicase genes were expressed organ specifically to some extent. The overall expression profile of the *RECQL4* gene resembles that of the *BLM* gene.

and testis, and BS patients show immunodeficiency and male infertility. *RECQL4* is specifically expressed in the thymus and testis, and patients with RTS show hypogonadism but not immunodeficiency (30). This is because the cells expressing RECQL4 are of a different type from that of the BLM helicase, and they do not participate in lymphocytic maturation. We speculate that close correlations exist between the gene expression profile in tissue and the defective disease phenotypes, and that these helicases function in specific tissues, thus dividing the work of maintenance of the genome.

C. Biochemical Properties of RECQL4 Helicase

Based on the amino acid sequence, RECQL4 helicase is predicted to have ATPase and DNA helicase enzymatic activities. We produced a recombinant human RECQL4 helicase containing N-terminal hexa-histidine in insect cells, purified

extensively by Ni-chelate column chromatography, and analyzed their enzyme activities. Recombinant RECQL4 helicase showed a DNA-dependent ATPase activity that hydrolyzes ^{32}P-labeled ATP added as a substrate (Fig. 7). The activity depends on the presence of helicase motif I, because the recombinant K508A protein carrying a single mutation at a conserved catalytic residue in this motif does not show ATPase activity (Fig. 7B, right panel). However, the DNA-dependent helicase activity is weaker compared with that of the WRN helicase from the ATPase activity when a duplex oligonucleotide was used as a substrate (Kitao et al., unpublished data).

D. Subcellular Localization of RECQL4 Helicase

A mouse monoclonal antibody was made against the C-terminal region of the human RECQL4 helicase, and was used in immunocytochemical studies to examine the subcellular localization of RECQL4 helicase in human cells. Using indirect fluorescein immunostaining, RECQL4 helicases were detected in the nucleus,

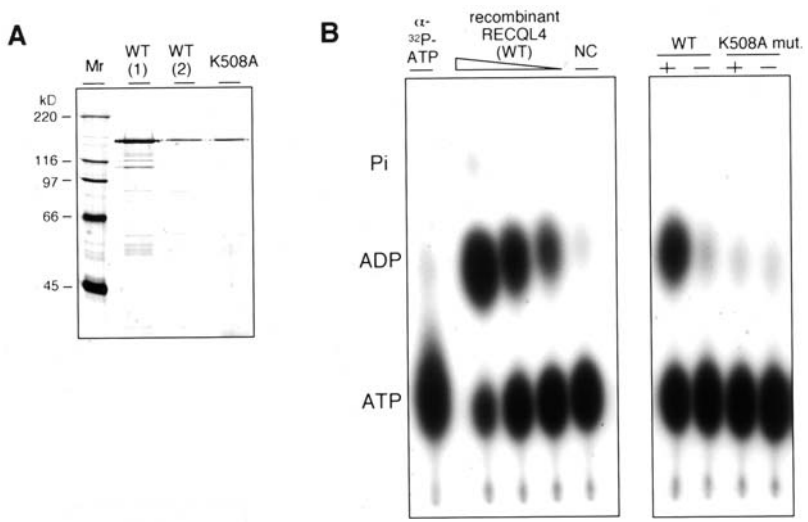


Figure 7 ATPase activity in the recombinant RECQL4 protein made in insect cells. (A) The histidine-tagged recombinant RECQL4 protein was expressed and purified from Sf9 cells using a baculovirus system. Wild-type protein (WT), fraction 1 and 2, and the dominant negative mutant type protein, K508A, were loaded onto SDS-PAGE and were stained with silver. (B) WT(1) protein shows dose-dependent ATPase activity (left panel). WT(2) and K508A proteins showing ATPase activity was DNA-dependent (+ or − lanes), and the activity was eliminated by an amino acid substitution in the catalytic site of the K508A molecule (right lane).

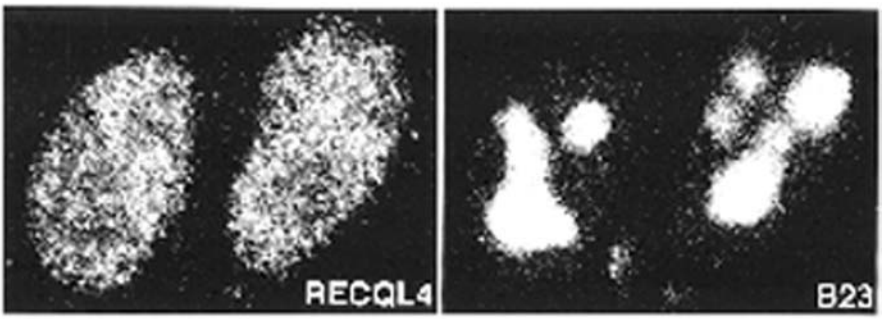


Figure 8 Subcellular localization of endogenous RECQL4 protein. Human embryonic kidney cells, 293E, were fixed by 3.7% formaldehyde and were immunostained using indirect fluorescence immunostaining. The left panel shows RECQL4 protein, and the right panel shows nucleophosmin/B23 protein. RECQL4 protein is mainly in the nucleoplasm but also exists in the nucleolus.

mainly in the nucleoplasm as small dots, and in the nucleolus as a diffused form (Fig. 8). Previous studies have shown that the WRN helicase exists both in the nucleolus and in the nucleoplasm of cells, although the distribution profile changes depending on the condition of the cells (78–80). By contrast, the BLM helicase localizes in large nuclear bodies called PML (promyelocytic leukemia) bodies (81,82). The different subcellular localizations with WRN, BLM, and RECQL4 helicases indicate that these helicases participate in different aspects of DNA metabolism.

E. RECQL4 Gene Targeting in Mice

Finally, we mention here briefly the outcome of our efforts to produce *RECQL4* gene knockout (KO) mice. To study the protein function in vivo, we replaced the intact mouse *RECQL4* with a mutant gene, or we depleted the *RECQL4* to produce null mutant mice. Heterozygous mice, in which one of two allelic genes was disrupted, were produced, and they were found to be not obviously changed from the wild-type mice. However, the disruption of two alleles resulted in early embryonic lethality. The mouse embryos died early in development, around the time of implantation into the lining of the uterus. These findings with mice are surprising and in contrast to what we know about human patients with RTS containing the *RECQL4* mutation; specifically, that newborns show no typical phenotypes of RTS. The results also contrast with the *WRN* KO mice that were born and and do not demonstrate the typical phenotypes associated with human WS patients, although the cells from *WRN* KO mice were sensitive in vitro to DNA-damaging compounds such as 4NQO and camptothecin (83). These findings indicate that a

species difference clearly exists in the biological function of RecQ helicases between mouse and humans, and suggest that the mouse RECQL4 helicase may have an essential role in the development of the mouse embryo.

VI. CONCLUSION

Despite the fact that the clinical report of patients with RTS by Rothmund was published much earlier than reports on WS and other genetic diseases including BS, the research to clarify the pathology of RTS in molecular terms has made slow progress. This situation will certainly improve because of the finding that *RECQL4* coding for RecQ-type helicase is responsible for RTS, at least for a subset of patients with RTS. Our efforts to find the distinct mutation(s) in *RECQL4* in patients by direct DNA sequencing show a success of approximately 35%. This relatively low figure, compared with our other experiences in the diagnosis of WS that showed a success of over 85%, may result partly from wide variations in the clinical phenotypes of suspected patients with RTS, ascertained in several clinics, and these factors may interfere with accurate diagnosis. This hypothesis implies that RTS could be subdivided into a few more specific syndromes. Another more plausible explanation for the low success of *RECQL4* mutation detection is that other new gene(s) may exist whose mutations cause RTS in the remaining subset of patients with RTS. In this context, Cockayne syndrome is caused by mutations in one of two separate genes, CSA/ERCC8 (chromosome 5) and CSB/ERCC6 (chromosome 10q11). These gene products work cooperatively in the nucleotide excision repair of damaged DNA, in which the CSB protein functions as a DNA helicase, and the CSA protein binds to the CSB protein (84). Further studies are required to determine if the RECQL4 helicase, as with the Cockayne CSB protein, needs to bind to a partner nuclear protein to carry out its biological role, which we believe, is to participate in the maintenance of the genomic structure.

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12

Hutchinson–Gilford Progeria Syndrome

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I. INTRODUCTION

Progeria, the Hutchinson–Gilford progeria syndrome, is a rare disease of childhood with striking features resembling premature aging (1–3). The syndrome is most likely caused by a sporadic dominant new mutation in a gene that has yet to be identified. Progeria is unlikely to represent true aging, because it is presumed to be caused by a single gene defect, whereas aging is the result of a polygenetic or multifactorial process. However, similar to Werner syndrome, important clues to the nature of the aging process may result from understanding the nature of the basic defect in progeria.

Aging appears to have a strong genetic component. This is reflected in the wide variation (approximately 50,000-fold) seen in the maximal life spans of various animal species (4). Even among mammals, an approximately 100-fold range in maximal life span is observed. As examples, the smokey shrew appears to have a life span of only about 1 year (5), whereas the oldest documented human died at the age of 122 (6). Analyses of the degree of genetic complexity underlying longevity have suggested it may be encoded by a limited number of genes, perhaps 20–50, which have a major gene effect on aging (7–9). Therefore, a useful approach to understanding the genetic basis of aging may be to study appropriate genetic mutants that appear to affect that process. Although no mutations are known to extend the maximal human life span, there are a number that shorten the life span. Several genetic diseases have mutations that appear to accelerate many features of the aging process. Because they do not appear to accelerate all segments of the aging process, they have been described as "segmental progeroid" syndromes by Martin (7). Such diseases can serve as useful models for the study

of aging. Insight into the nature of the basic mutations in these syndromes may identify the genes that play a major role in aging.

Two genetic diseases that show the most striking clinical features suggestive of accelerated aging are the Hutchinson–Gilford progeria syndrome (progeria) and the Werner syndrome (WS, also called *progeria of the adult*). The basic mutation underlying progeria is not known, whereas the gene mutation for WS is now known (see Chapter 9). Findings in several laboratories, including our own, have indicated that patients with these two diseases may excrete an excessive amount of the glycosaminoglycan, hyaluronic acid, or hyaluronan (HA) (2,10–14). Cultured cells from patients with these diseases show an excessive amount of HA accumulation (2,15). Experimentally, excess HA also has been shown to inhibit vascular development and may act as an antiangiogenesis factor (16,17). These findings raise the possibility that one or several major genes affecting aging may relate to HA metabolism. Understanding the basis of these genetic abnormalities may help to elucidate further the molecular basis of normal aging. In this chapter, we review clinical aspects, genetic features, and laboratory investigations of progeria.

II. CLINICAL FEATURES

Progeria, illustrated in Figure 1, is a rare genetic disease with striking clinical features that resemble premature aging (1–3). It has a reported birth incidence of about 1 in 8 million (1). Patients with this condition generally appear normal at birth, but by about 1 year of age, severe growth retardation is usually seen. Balding occurs, and loss of eyebrows and eyelashes is common in the first few years of life. Widespread loss of subcutaneous tissue occurs. As a result, the veins over the scalp become prominent. The skin appears old, and pigmented age spots appear. The patients are very short and thin. They average about 40 inches in height, but they usually weigh no more than 25 or 30 pounds even as teenagers. The weight-to-height ratio is thus very low. The voice is thin and high-pitched. Sexual maturation usually does not occur. They have a characteristic facial appearance with prominent eyes, a beaked nose, a "plucked-bird" appearance, and facial disproportion resulting from a small jaw and large cranium. The large balding head and small face give them an extremely aged appearance. The bones show distinctive changes, with frequent resorption of the clavicles and replacement by fibrous tissue. Resorption of the terminal finger bones (acro-osteolysis), stiffening of finger joints, elbow and knee joint enlargement, coxa valga, and a resulting "horse-riding" stance are all seen. Aseptic necrosis of the head of the femur and hip dislocation is common (18–19).

Individuals with progeria have a normal to above-average intelligence. The median age of death is 12 years. Over 80% of deaths are due to heart attacks or

Figure 1 A 10-year-old girl with Hutchinson–Gilford progeria. She had mild strokes and bilateral hip dislocations. She died at age 13 years of atherosclerotic heart disease.

congestive heart failure. Widespread atherosclerosis, with interstitial fibrosis of the heart, is usually seen at postmortem examination (20). Occasionally, marked enlargement of the thymus gland is noted. However, some features often associated with normal aging such as tumors, cataracts, diabetes, and hyperlipidemia, although occasionally reported (21–23), are not usually present.

Consideration of the mode of inheritance in progeria is important for genetic counseling and may help to understand the nature of the underlying mutation. Recessive diseases often appear to be caused by enzymatic deficiencies that lead to metabolic abnormalities. Dominant diseases often involve structural proteins. However, they may be due to partial deficiencies of rate-limiting enzymes (i.e., porphyria) or cell surface receptors (i.e., familial hypercholesterolemia) where half the normal level of the gene product can lead to a disease.

Several genetic considerations suggest progeria is most likely a sporadic dominant mutation. First, high rates of consanguinity (i.e., first-cousin marriages) are expected in rare recessive diseases. High consanguinity is not seen in progeria. Debusk (1) has noted that consanguinity was present in only 3 of 19 families in which it was specifically discussed. Some of these cases had come from areas

of the world with high background population levels of consanguinity, and the diagnosis in some may be in doubt. In addition, it was not reported to be present in 41 other families in the literature. A family history of consanguinity was not present in any of some 50 progeria cases that we have seen since then. Thus, we estimate the frequency of babies with progeria born to consanguineous marriages to be less than 3%. For rare recessive diseases, an estimate of the expected frequency of consanguinity can be derived using the Dahlberg formula (24). Assuming a birth incidence of progeria to be 1 per 8 million, and a background population consanguinity frequency of 1%, this leads to an estimate of expected consanguinity of 64% in families in which progeria occurs. Thus, the 3.6% observed consanguinity frequency in progeria is much lower than the high level that would be expected for such a rare recessive disease.

Although the reported incidence of progeria in the United States is about 1 in 8 million births (1), the true population incidence may be somewhat higher, as not all cases are reported. Based on our experience, we estimate that about 50% of all cases in the United States are reported, which leads to an estimate of incidence of 1 in 4 million. This would still lead to a much higher expected consanguinity frequency (45%) than the low frequency that is seen in families in which progeria occurs. This lack of consanguinity suggests progeria is unlikely to be a rare recessive condition.

Second, a paternal age effect is seen in progeria that is also observed in some other genetic conditions caused by sporadic dominant mutations. Jones and coworkers (25) have reported that among 18 progeria cases, the fathers were older than expected by an average of 2.56 years when controlled for maternal age, a difference that was highly significant ($P = .005$). In addition to progeria, they reported a paternal age effect in seven other disorders involving new mutations for which autosomal dominant inheritance had been clearly established (basal cell nevus syndrome, Waardenburg syndrome, Crouzon syndrome, cleidocranial dysostosis, Oculo-dental-digital syndrome, Treacher–Collins syndrome, and multiple exostoses), and in four disorders (achondroplasia, Apert syndrome, fibrodysplasia ossicans progressiva, and Marfan syndrome) in which older paternal age in the setting of a new mutation previously has been shown.

We observed a paternal age effect in 24 cases of progeria that we examined where parental ages were available (2). The fathers were older than the mothers by an average of 4.5 years, which is higher than the expected control value of 2.8 years (25). The paternal age effect observed in these 24 cases confirms the previously reported paternal age effect in 18 earlier cases (25) and also suggests dominant inheritance. The paternal age effect appears to be due to the fact that about 20% of fathers are about 20 years older than the mothers, as illustrated in Figure 2. A similar secondary paternal age peak has been reported in new cases of neurofibromatosis, another dominant disease (26).

Third, for a recessive condition, the proportion of affected siblings is ex-

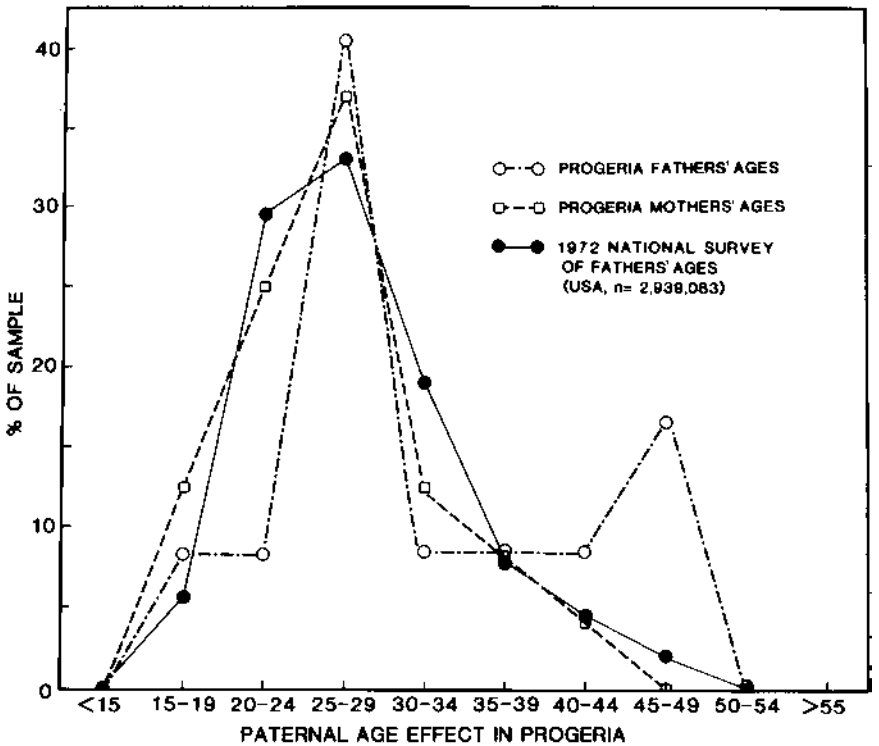


Figure 2 Paternal age effect noted in progeria. Ages of fathers and mothers of 24 cases of progeria are compared with control fathers' ages. Approximately 20% of the fathers were about 20 years older than the mothers.

pected to be 25%. In progeria, it is clearly much less than 25%. Almost all cases are sporadic, and there is no evidence of increased miscarriage rates to suggest selection against the homozygote in utero. A case of identical twins with progeria with 14 normal siblings has been reported (27). Here, three or four affected siblings would be expected if it was a recessive disease. It is recognized that for new dominant mutations, occasionally the mutation can occur in a germ line leading to somatic mutations. Several cases could then occur within one family. Probable cases of familial progeria have been reported in only a few instances among more than 100 families (28–33). We believe that these reports of progeria cases most likely represent misidentified cases of other progeroid syndromes, such as mandibuloacral dysplasia. Among the 50 cases we examined, no family had more than one affected child except for one set of identified twins. There were 80 unaffected siblings. One would expect there to be 20 of the 80 siblings affected (25%) if a recessive mode of inheritance were to apply to these progeria families.

In general, the lack of consanguinity, the paternal age effect, and the lack of affected siblings, argues that progeria is not a rare recessive disorder but most probably is a sporadic dominant mutation. Because of a lack of consanguinity, a lack of affected siblings, and a paternal age effect, we suggested progeria should be classified as a sporadic autosomal dominant mutation (34). The possibility of genetic heterogeneity in progeria, in which some cases have a similar clinical presentation but with a recessive mode of inheritance, seems possible but unlikely owing to the rarity of the condition. The majority of cases appear to represent isolated sporadic dominant mutations, although a few may be the result of a germ line mutation. For genetic counseling of families with a child with progeria, the recurrence risk can be stated to be very low, but may be on the order of 1 in 500 with each pregnancy to allow for the possibility of somatic mosaicism such as has been occasionally seen in other new dominant mutations.

III. DIAGNOSIS

Diagnosis of progeria is made by recognition of the clinical features, as there is no definitive or specific laboratory test. The syndrome in the first year or two of life is frequently misdiagnosed or confused with several other growth retardation syndromes. These include neonatal progeroid syndrome, acrogeria, mandibuloacral dysplasia (MAD), Cockayne syndrome, Hallermann–Streif syndrome, geroderma osteodysplastica, congenital generalized lipodystrophy, Petty–Laxova–Weidemann progeroid syndrome, and Ehlers-Danlos syndrome progeroid form.

The earliest signs that are suggestive of a diagnosis include failure to thrive, alopecia, and subcutaneous skin changes suggesting scleroderma. Variable degrees of insulin resistance and inconsistent abnormalities of serum cholesterol and other lipids are found, but there are no demonstrable abnormalities of thyroid, parathyroid, pituitary, or adrenal function. We studied five cases of progeria and found 24-hr growth hormone levels to be normal, but reduced levels of insulinlike growth factor I and markedly increased basal metabolic rates were found, suggesting a profile of bioinactive growth hormone (35).

IV. BASIC RESEARCH

Laboratory investigations of progeria have involved a search for a genetic marker to help define the underlying defect. The cultured life span of progeroid fibroblasts was initially reported to be greatly reduced (36). Subsequent studies have shown that although difficulties may sometimes occur in the initial establishment of a culture, once established, a normal or only a modest reduction in life span is seen (37–38). We examined the in vitro life spans of 11 progeroid cell cultures, 4

WS cultures, 4 parents of progeroid subjects, and 3 control cultures. The WS cell lines showed extremely rapid senescence, with a range of 9–15 maximal population doubling levels. The progeroid cell lines had a range from about 20–60 population doubling levels (2). This was reduced by about one-third compared with the parent lines and the normal controls. The WS line population doubling levels were greatly reduced. Thus, a markedly reduced in vitro life span of progeroid cells, such as is seen in WS, was not present. The modest and variable reduction in life span in culture is unlikely to represent a useful marker for the disease.

Goldstein and Moerman (38) have reported finding an increased fraction of abnormally thermolabile enzymes, including glucose-6-phosphate dehydrogenase (G6PD), 6-phosphogluconate dehydrogenase (6PGD), and hypoxanthine phosphoribosyltransferase (HPRT) in progeroid fibroblasts. Based in part on the Orgel error-catastrophe hypothesis of aging (39), it was suggested that diseases resembling premature aging may be the result of widespread errors in protein synthesis (40). Abnormally high thermolabile enzyme levels in circulating erythrocytes from one patient with progeria with intermediate levels in the parents also have been reported (41). It has been suggested that this would support autosomal recessive inheritance. Our studies of 3 patients with progeria and their families did not confirm these elevations, as no increased erythrocyte thermolabile enzyme elevations were seen (42). In our opinion, this lack of confirmation indicates that a defect in protein synthetic fidelity is unlikely to be the basic defect in progeria and does not support the suggestion of autosomal recessive inheritance. Subsequent work by Wojityk and Goldstein (43) on cell-free protein synthesis using progeroid fibroblast extracts also found no decreased translation ability, which also argues against a generalized defect in progeroid protein synthesis.

Abnormal immune function has been postulated as a defect in progeria. Walford has suggested that progeria could reflect an abnormality of immune function because of the similarity to experimental graft-versus-host reaction and to runting disease (44). In support of this concept, Singal and Goldstein (45) reported that HLA expression on two cultured progeroid fibroblast strains was absent. In studies of 10 progeroid fibroblast strains, we were unable to confirm this reported abnormality. We found no evidence for either qualitative or quantitative abnormalities in HLA expression, and no association with HLA type was detected (46). Thymic hormone levels have been reported as normal for the range of patient ages tested (47). No immune abnormalities have been established thus far for progeria.

An abnormality in x-ray DNA-repair capacity in progeroid fibroblasts has been suggested by Epstein and colleagues (48,49), who detected decreased single-strand rejoining of gamma-irradiated DNA using alkaline sucrose gradients. The presence of altered DNA-repair capability was found in another study. Using a somewhat modified method for the assay of single-stranded rejoining, no differences between one progeroid strain and two atypical progeroid strains were seen

as compared with normal strains (50). Brown and coworkers (51,52) have shown that cocultivation of two progeroid cell strains with normal strains or with each other reverses the single-stranded DNA-rejoining defect and have suggested that complementation groups for DNA repair might exist in progeria. Weichselbaum and colleagues (53) assayed the x-ray sensitivity of various types of human fibroblasts by measuring their ability to form colonies following irradiation. They found two progeroid strains with increased sensitivity and three strains with normal sensitivity. These studies suggest some increase in radiosensitivity but leave open the possibility that damage to cellular components other than DNA might be responsible. Rainbow and Howes (54), using a sensitive host-cell–reactivation (HCR) assay of x-irradiated adenovirus, have reported that two progeroid strains show a deficiency of DNA-repair capacity. Brown and coworkers (55) studied HCR in two other strains and found one strain showed decreased HCR, whereas another showed normal HCR under a variety of cell growth conditions. These results suggest that heterogenicity of DNA-repair capacity exists among progeroid fibroblasts, but defective DNA-repair capacity does not appear to be a consistent marker for progeria. Wang and coworkers (56) showed that prior irradiation of progeroid cells with gamma rays caused a marked reduction in ultraviolet-induced unscheduled DNA synthesis compared with controls. The basis for this difference is unclear, because progeroid cells are not more sensitive to gamma ray irradiation. Following cloning of the Werner gene, it was examined for similar mutations in a series of progeroid cell lines, and no abnormalities were found (57).

Various studies have suggested other types of basic abnormalities in progeria. Elevated levels of the blood coagulant tissue factor have been reported in progeria and WS fibroblast cells (58). This could reflect variations in culture conditions or growth state of cells unrelated to genotype, such as has been reported for other cell types (59). A normal insulin-binding receptor response, but decreased binding of insulin to nonspecific receptors in progeroid cells, has been reported (60). The significance of nonspecific receptor binding is unclear. An increased level of elastin mRNA and increased in vitro levels of elastin have been reported for cultured progeroid fibroblasts (61). The reasons for this increase are unclear, but may be due to loss of normal regulatory mechanisms in vitro or increased sensitivity to regulation in vitro by serum growth factors or some unusual cellular selection in vitro. No evidence of increased elastin production in patients has yet been seen. Elevated levels of a glycoprotein (gp200) in cultured progeroid fibroblast but not in lymphoblasts have been reported (62). It was suggested this abnormality could reflect a perturbation in glycosylation, which was cell-type specific.

Gene expression profiling has been employed in two different studies of progeroid cells (63,64). Approximately 1% of genes showed at least a twofold elevation or reduction in gene expression levels, although the genes identified were different in the two studies. The reproducibility and interpretation of these types of potentially very powerful approaches have yet to be determined.

V. HYALURONIC ACID URINARY LEVELS IN PROGERIA

A potential marker for both progeria and WS appears to be urinary HA excretion. HA excretion has been found to be elevated in these two syndromes and has not been reported to be elevated for any other genetic disease. HA levels in controls are normally considered to represent less than 1% of total glycosaminoglycans (GAGs). Elevated HA levels have been reported to vary from 2 to 22% of total GAGs in a series of Japanese WS subjects (65). Urinary HA, as a percentage of total GAGs present, also was reported to be elevated to 4.4% in one Japanese patient with progeria compared with controls of 0.2 and 0.3% (10).

To test the generality of this observation, we determined the total urinary excretion of GAGs and HA in three patients with progeria, one patient with an atypical progeroid syndrome, one patient with WS and a control subject, using standard methods for GAG analysis using cetyl pyridinium chloride (CPC) precipitation, pronase digestion, tri chloro acetic acid (TCA) treatment, ethanol precipitation, and uronic acid determination before and after hyaluronidase digestion (13). Normal levels of total urinary GAGs were observed in all affected individuals and the control. HA analyses for the patients and for the control showed that the patient with WS and three patients with progeria had increased levels of urinary HA that ranged from a high of 16% to a low of about 5%. The normal individual and the atypical progeroid subject showed no significant elevation of HA excretion. Although it generally has been accepted that urinary HA levels are normally less than about 1% of GAGs, there was no systematic study available.

To determine HA levels in progeria and normal subjects as a function of age, we developed an high-performance liquid chromatography (HPLC) method to assay HA and the GAGs (66). Using this method, we studied 30 normal individuals to determine HA excretion as a function of age (14). Our studies verified that HA content in young children and adolescents is low, but with age, an elevation to 5–6% is found. The 10 patients with progeria studied ranged in age from 3 to 14 years. On average, there was approximately a 20-fold elevation in HA levels, as illustrated in Figure 3. These results suggest that elevated excretion of urinary HA may be a normal characteristic or biomarker of aging that occurs at an accelerated rate in progeria and patients with WS.

To determine if the elevated HA excretion seen in progeria also was reflected in cell culture, we analyzed steady-state HA and GAG levels in normal, progeroid, and WS fibroblasts (2). HA and GAG levels in 3-day cultures of progeroid, WS, and control cells were assayed by measuring total glucosamine and sulfate incorporation into cells and media. HA levels were elevated in progeria and WS compared with normal cultures at all cell densities measured. A pronounced difference in total GAG levels also was observed when normal fibroblasts were compared with WS and progeroid fibroblasts as a function of cell density. A similar difference in GAG and HA excretion into the media was seen

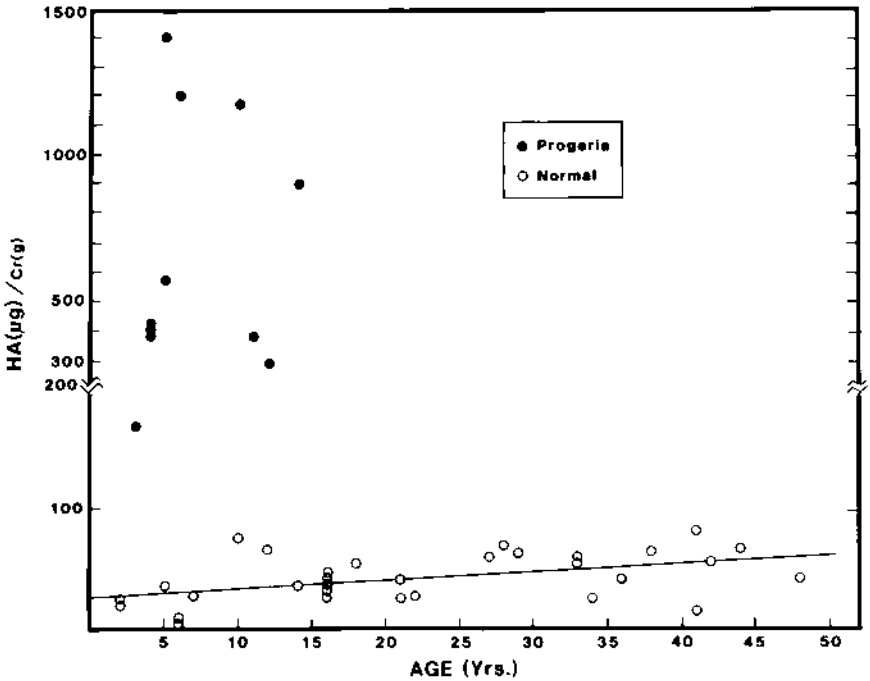


Figure 3 Urinary hyaluronic acid levels in 10 patients with progeria compared with a series of 30 controls. An approximate 20-fold elevation was found in progeria.

in comparing WS and progeria to normals as a function of cell density. Non–HA-containing GAGs, as assayed by sulfur incorporation, also were found to decrease as a function of cell density and were found to be present in excess in WS and progeroid fibroblasts.

HA and GAG production during embryogenesis is believed to play a very important role in morphogenesis. HA production in particular is associated with the formation of the primary mesenchyme and the first cell-free spaces in the rat embryo (67). In chick embryos, a striking correlation between hyaluronate synthesis and cell movement and proliferation is observed, as well as between HA degradation and differentiation. For example, in the chick embryo cornea, a close correlation exists between the presence of HA and the period during which corneal mesenchyme migration and proliferation in the hydrated primary stroma occur. Both corneal mesenchyme migration and HA production occur during days 3–9 of development. The removal of HA by hyaluronidase begins

on day 10 and corresponds with the cessation of migration and proliferation of the mesenchymal cells as well as their differentiation into corneal keratocytes (68,69).

HA appears to act as an antiangiogenesis factor. During development, tissue regions that are high in HA concentration are invariably avascular zones. HA-containing implants were shown to cause avascularity when implanted into normal vascular wing mesoderm (16). HA thus appears to be crucial in the morphogenesis of blood vessels in the embryo and may be expected to play an equally important role as an antiangiogenesis factor during maturation and aging. West and colleagues (70) have reported that partial degradation products of HA (oligosaccharides between 4 and 25 residues in length) have the opposite effect. They stimulated angiogenesis on the chick chonoallantoic membrane when these partial degradation products were applied. Slevin and coworkers (71) have shown that angiogenic oligosaccharides of HA induce a protein tyrosine kinase activity in endothelial cells that results in cellular proliferation.

Our results in progeria suggest that abnormalities of excess HA excretion or abnormal degradation may provide a marker. Mutations of HA metabolism may underlie these diseases, perhaps owing to their pervasive effects on angiogenesis. This might explain the profound failure to thrive seen in progeria patients. Elucidation of the nature of a genetic mutation that may lead to an increase in HA production or accumulation may help to understand the cause of progeria. We believe that insight into the nature of the mutation that underlies progeria, this most remarkable experiment of nature, may help in understanding a gene or genes with a major effect on aging.

VI. CHROMOSOMAL ANALYSIS IN PROGERIA

Of more than 20 cases of progeria of which we are aware that have been analyzed for chromosomal abnormalities (1–3), none has been found to be abnormal with one exception. This was an identical twin with progeria (3). He died of heart disease at the age of 8 years and 1 month following the death of his twin brother. Both twins had a severely affected phenotype, with hypoplastic leg and arm development, although both were of normal intelligence. A postmortem skin biopsy was obtained and cultured. Cytogenetic analysis of cultured fibroblasts revealed an inverted insertion of chromosome 1 [46 XY, inv ins (1:1) (q32;q44q23)] in 21 of 30 cells examined (W.T. Brown and E.C. Jenkins unpublished results). Neither the first twin nor the parents were available for cytogenetic studies. Although mosaic in nature, this unique chromosomal abnormality does raise the possibility that a site on the q arm of chromosome 1 could be the map location for the elusive progeria gene.

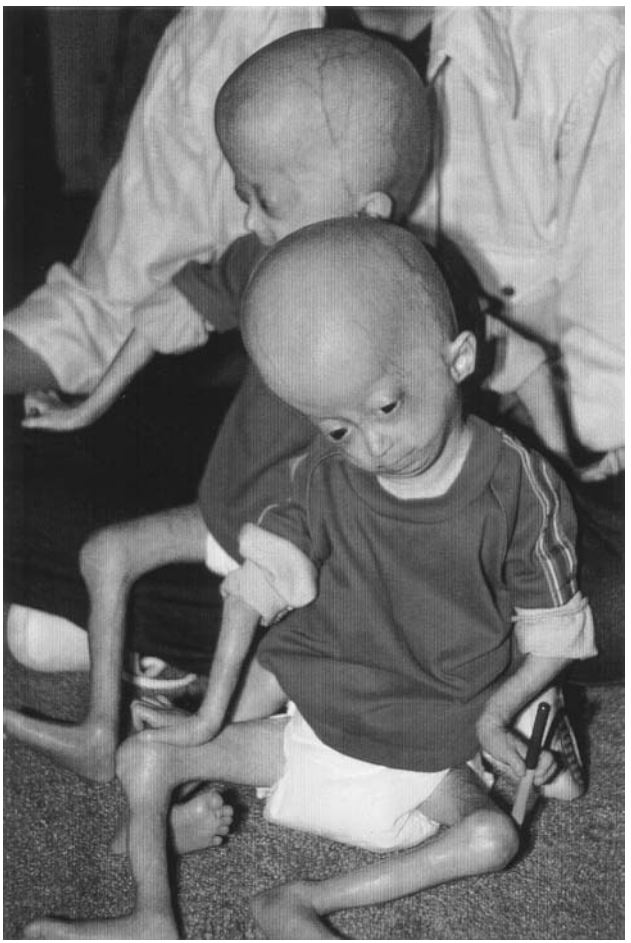
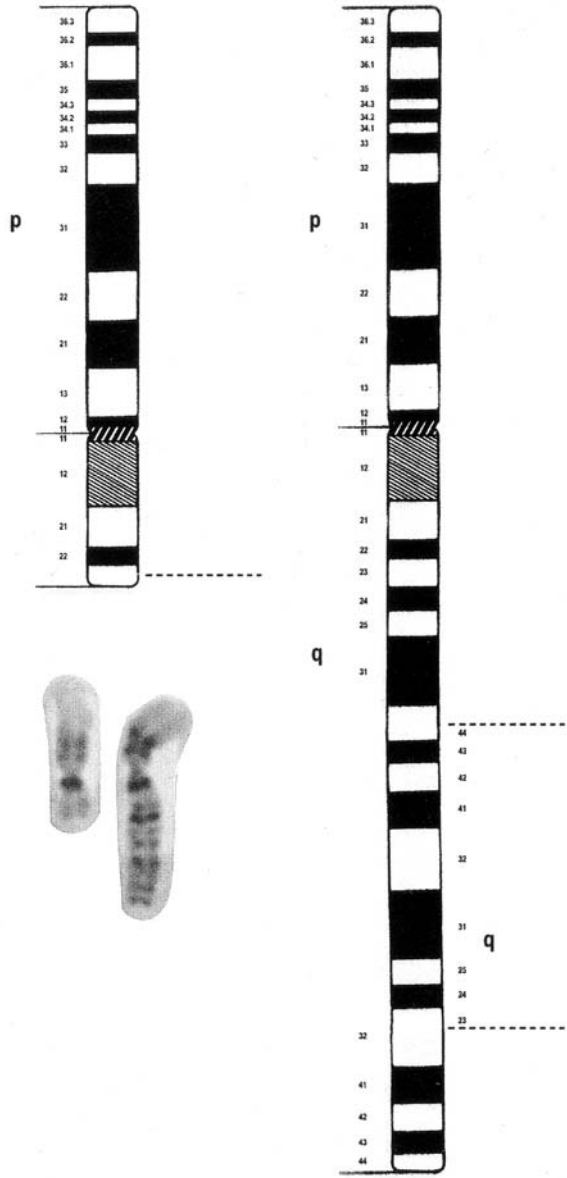


Figure 4 A chromosome 1 abnormality detected in 70% of skin fibroblast cells from a progeroid identical twin. A photo of the twins is on the left, and the location of the inverted insertion is on the right.



Inv Ins(1;1) (q32;q44q23)

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13
Ataxia-Telangiectasia

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I. INTRODUCTION

The ability to maintain genomic integrity in the face of DNA damage is critical for survival. Biological organisms are not merely passive targets of DNA-damaging agents but actively respond to DNA damage in a variety of ways; for example, the SOS system in *Escherichia coli*. (1). The means by which cells protect the genome against DNA damage are complex and involve DNA repair, genetic recombination, alterations in the cell cycle, and programmed cell death. This chapter reviews recent developments in our understanding of the *ATM* gene and ataxia-telangiectasia (AT), a human disease in which *ATM* mutations cause these homeostatic processes to break down.

Ataxia-telangiectasia is a member of a group of inherited diseases that includes Werner syndrome, Bloom syndrome, Fanconi anemia, Nijmegen breakage syndrome, Li–Fraumeni syndrome, and ataxia-telangiectasia–like disorder (ATLD). These chromosome instability syndromes share in common a marked degree of genetic instability, symptoms of premature aging, and high risk of cancer. As described in the other chapters of this book, these rare diseases are easily distinguished from one another clinically. However, recent genetic, molecular, and biochemical studies provide evidence that the genes involved in the chromosome instability syndromes interact with each other in DNA-damage–response networks that coordinately act to preserve the integrity of the genome.

II. ATAXIA-TELANGIECTASIA

Ataxia-telangiectasis occurs in individuals who completely lack functional ataxia-telangiectasia mutated (ATM) protein. Originally known as Louis–Bar syndrome (2), this autosomal recessive disorder occurs worldwide, with an prevalence of 1:40,000–1:100,000 in outbred populations (reviewed in Ref. 3). The pleiotropic clinical phenotype of individuals with AT is summarized below.

A. Degenerative Neurologic Disease

AT patients rarely have functional abnormalities as infants. However, the vast majority develop gait abnormalities in their second year of life. All patients lose cerebellar function over time, resulting in progressive ataxia, ocular apraxia, dysarthric speech, drooling, and choreoathetoid movements (4,5). Most index cases are initially thought to have a form of cerebral palsy (6). The classic AT patient is wheelchair-bound by the end of the first decade of life, and older AT patients may develop intellectual arrest. Dysphagia and silent aspiration become major problems when the neurodegeneration reaches an advanced stage (7). Functional neurological abnormalities in AT patients are accompanied by a continual loss of neurons from both the central and peripheral nervous system (reviewed in Ref. 4). The cerebellum is particularly affected owing to the cumulative effects of ongoing Purkinje cell death (Fig. 1).

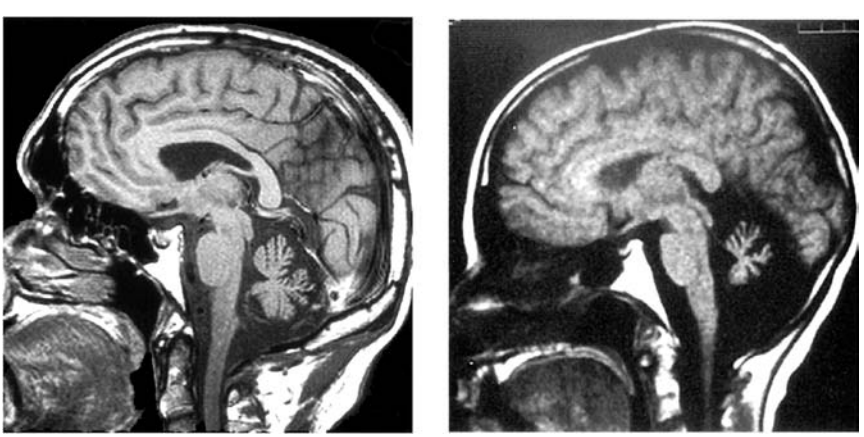

Figure 1 Ongoing Purkinje cell death leads to loss of cerebellar volume in AT. Left panel: CT image from a normal child. Right panel: CT image from an AT homozygote.

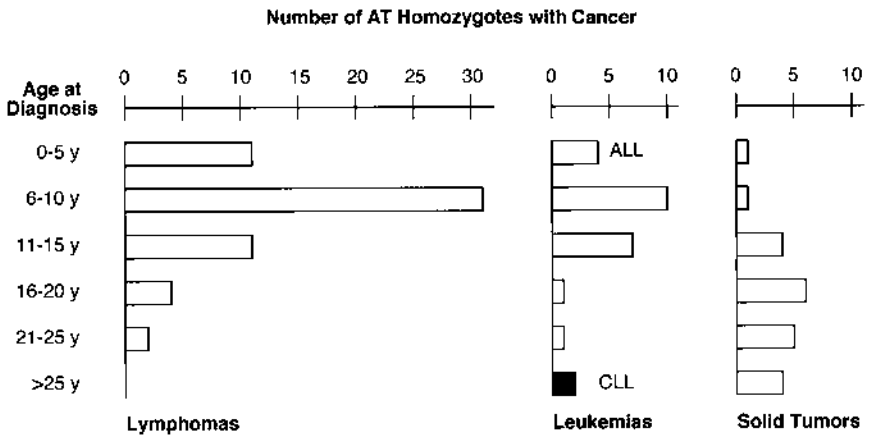

Figure 2 Malignancies in AT homozygotes as a function of age of onset (based on Immunodeficiency Cancer Registry data from Ref. 17).

B. Immunodeficiency

Most, but not all, AT homozygotes express clinically significant, but nonprogressive, humeral and cellular immune defects. These can include one or more of the following: thymic hypoplasia, low numbers of circulating T cells, functional impairment of T-cell–mediated immunity, abnormally high levels of IgM, oligoclonal expansions, and/or selective deficiencies of IgA, IgE, IgG_2, and IgG4 (8–10). Opportunistic infections are rare; however, otitis media and sinus infections are frequent. The risk of lower respiratory infections (pneumonia and bronchitis) increases with age, and the combination of immunodeficiency and progressive loss of cerebellar function makes aspiration pneumonia the leading cause of death in AT patients, whose median life expectancy was estimated in a recent survey to be ~30 years (11).

C. Malignancy

Cancer is a frequent complication of AT, with the lifetime risk estimated to be 30–40% (12). The most common malignancies are tumors of the immune system that occur in the first 15 years of life (Fig. 2). More than 40% of all tumors in AT patients are non-Hodgkin's lymphomas, another ~20% are acute lymphocytic leukemias, and ~5% are Hodgkin's lymphomas (4,13–15). Older AT patients are at risk to develop solid tumors and chronic T-cell leukemias (4,14,16). A wide range of solid tumors have been reported (4,13,17), the most frequent being gastric carcinoma, breast carcinoma, medulloblastoma, basal cell carcinoma, ovarian dysgerminoma, hepatoma, and uterine leiomyoma (in approximate order of frequency).

D. Impaired Fertility

Many female AT patients have congenital hypoplasia of the ovaries and menarche may be delayed or absent (4,18). Male patients have been shown to have histological abnormalities of their testes, and incomplete spermatogenesis has been reported (19,20), although hypogonadism is less frequent and milder than in affected females.

E. Premature Aging

In addition to an increased susceptibility to malignancy and occasional insulin-resistant diabetes (21), AT homozygotes also show multiple dermatological changes associated with premature aging. Telangiectasias usually appear on the sclera, face, and antecubital/popliteal fossae by 6–7 years of age (6). In the second and third decades of life, AT homozygotes develop additional cutaneous changes, including graying of the hair, senile keratoses, skin atrophy, and areas of hyperpigmentation and hypopigmentation (4).

Atherosclerotic heart disease and stroke are not a major problem for AT homozygotes, perhaps because of their early death. However, a recent epidemiological study found that AT carriers died an average of 7.8 years earlier than noncarriers in the same families, a difference that was found to be highly significant ($P<.001$). Although cancer caused most of the excessive deaths in this study, a 2.6 relative risk of ischemic heart disease caused the remainder (22).

F. Other Clinical Abnormalities

Serum alpha-fetoprotein (AFP) levels are elevated in >90% of older children with AT (18). AT patients usually exhibit mild postnatal growth retardation, and nutrition is a problem in those older individuals with dysphagia and advanced cerebellar degeneration. AT homozygotes tend to have sad, hypotonic facies, but congenital malformations are not a feature of the syndrome.

G. Genetic Instability

Genetic instability is a hallmark of the AT phenotype. Spontaneous in vivo chromosomal aberrations occur frequently in both lymphoid and nonlymphoid cells from AT patients (reviewed in Refs. 3, 23, and 24). The range of spontaneous karyotypic aberrations includes chromosomal breaks, acentric fragments, dicentric chromosomes, structural rearrangements, and aneuploidy. Although the breakpoints in nonlymphoid cells appear to be distributed randomly, AT lymphocytes also have 30- to 50-fold increases in the frequency of rearrangements involving four specific sites: 7p14, 7q35, 14q11.2 and 14q32 (25). These rearrangements interrupt T-cell receptor (TCR) and Ig heavy chain genes, genes that

sustain obligatory double-strand breaks (DSBs) and subsequent recombination during the maturation of the immune system (24). Genetic instability has been documented at individual loci (26–30), and spontaneous intrachromosomal recombination is elevated 30- to 200-fold in AT cells grown in culture (31,32).

H. Abnormal Responses to DNA Damage

The first clue that defects in the *ATM* gene disrupt normal cellular responses to DNA damage were provided by case reports of AT patients who died from severe reactions to radiation therapy directed at their lymphoid tumors (33–37). Although not unusually susceptible to the induction of DNA breaks by ionizing radiation (38,39), cultured cells from AT patients are typically three to five times more sensitive than control cells to the cytotoxic effects of ionizing radiation as well as other agents that induce DSBs (40–44). AT radiosensitivity is also manifest by an increase in number of chromosomal aberrations induced by ionizing radiation or radiomimetic agents (reviewed in Ref. 23).

I. DNA Repair Abnormalities in AT Homozygotes

Cells from AT homozygotes can be exquisitely sensitive to the cytotoxic effects of ionizing radiation, but they differ from most other radiosensitive mammalian cells in that their ability to repair DNA damage appears to be largely intact. Base excision repair is apparently normal (45), and multiple biochemical studies have failed to detect gross abnormalities in the kinetics of single-strand and double-strand break repair in AT cells (e.g., see Refs. 46, and 47). Other reports have found no evidence that AT cells are functionally defective in DNA repair (48,49). On the other hand, AT homozygotes appear to have subtle defects in their ability to process DNA breaks, as the accuracy of strand rejoining is reduced in AT cells (50–52). In addition, several studies have demonstrated increases in the fraction of DNA breaks left unrepaired in irradiated AT cells (38,53). This may indicate an inability to repair a small but critical fraction of DSBs (38,54–56) such as those that give rise to chromosomal breaks or that arise at replication forks (57). Alternatively, these persistent breaks may be early signs of incipient apoptosis (58).

J. Absence of Cell Cycle Checkpoints

In normal mammalian cells, multiple cell cycle checkpoints are triggered following exposure to ionizing radiation (reviewed in Refs. 59, and 60). These checkpoints also may restrain the cell cycle temporarily in response to the generation of strand breaks, shortened telomeres, stalled replication forks, and other sites of DNA damage that occurs spontaneously during the course of normal DNA metabolism (e.g., site-specific gene rearrangements, genetic recombination, and repair of replication errors). AT homozygotes lack the p53-mediated G1/S dam-

age-sensitive checkpoint (61), and AT cells do not have the S phase checkpoint, resulting in the phenomenon of radioresistant DNA synthesis (62). The G2/M and mitotic spindle checkpoints also appear to be defective in AT cells (63–66). However, the late-onset G2/M checkpoint that results in prolonged G2 arrest after x-irradiation is intact in AT cells (67).

K. Defective Radiation Responses

Following exposure to ionizing radiation, intracellular concentrations of p53, p21, GADD45, c-jun, and ku70 normally increase and nuclear factor-κB (NF-κB), inhibitor κB kinase (IκB), and the p56lyn kinase are activated. In addition, a growing number of proteins have been shown to be phosphorylated following x-irradiation or other treatments that induce DSBs (S Table 1)]. These responses are all impaired in AT cells grown in culture, suggesting that they are ATM-dependent events (68–82).

L. Apoptosis, AT, and *ATM* Function

Several years ago, we noted that the sensitivity of AT cells to the killing effects of ionizing radiation was not due to a lack of cell cycle checkpoints or to a defect in repair of the bulk of DSBs. As an alternative, we proposed that ATM normally acts to protect cells from DNA damage–induced apoptosis, and that DSB-induced apoptosis was the primary cause of radiation sensitivity and gonadal abnormalities in AT (83,84). This was based, in part, on our finding that cultured AT fibroblasts and lymphoblasts are unusually susceptible to the induction of late-onset (>48 hr postirradiation) apoptosis by low doses of x-ray and streptonigrin (83,85). Our observations have since been confirmed by others (58,66,86–91). Indirect support for underlying hypothesis also has been provided by observations that x-irradiation–induced caspase 3 and 9 activities are particularly high in AT fibroblasts, ectopic expression of ATM exerts an inhibitory effect on c-myc–mediated apoptosis, and ATM is a target for proteolytic cleavage during apoptosis (92–95).

Subsequent work with Epstein–Barr virus (EBV)–transformed human lymphoblasts and $Atm^{-/-}$ mice has made it clear, however, that the original hypothesis cannot fully explain the complex relationship between ATM, apoptosis, and DNA damage. As predicted, $Atm^{-/-}$ mice are easily killed by ionizing radiation, and $Atm^{-/-}$ spermatocytes and oocytes undergo spontaneous apoptosis during meiosis I, resulting in sterility (96–98). However, although epithelial cells from the gut and the parotid glands from $Atm^{-/-}$ mice are exquisitely sensitive to radiation-induced apoptosis, lack of functional ATM protein does not appear to radiosensitize most tissues, including the brain (99). Surprisingly, inactivation of the *ATM* gene can actually protect mouse thymocytes and embryonic neurons from radiation-induced apoptosis and save HeLa cells from apoptosis induced by defective telomeres (100–103). In addition, several laboratories have shown that

Table 1 Known Substrates for DSB-Induced, ATM-Dependent Phosphorylation

Protein	Kinase	Serine position	Reference
P53	ATM, PLK3	9, 15 (ATM)	73–75, 298, 299
	CHK2	20 (Chk2 & PLK3), 46	300, 301
BRCA1	ATM, ATR	1387, 1423, 1457, 1524	182, 302, 303
MDM2	ATM	393	304, 305
CHK2	ATM	68	306–308
NBS1	ATM	343 and others?	303, 309, 310
CtIP	ATM	664, 745	311, 312
43-BP1	ATM	111	313
hRAD17	ATM, ATR	635, 645	170, 171
H2AX	ATM, DNA-PK		179
Hrad9	ATM	272	172
Pin2/TRF1	ATM	219	314
SMC1	ATM	957, 966	165, 315
PHAS-1	ATM		140
RPA (32k)	ATM		140
BLM			316
FANCD2	ATM	222	317
c-abl			318
BP53			319
Rad51	c-abl		318
TopBP1			181
ERK6/SAPK3			320
c-jun			321
E2F1			322
Cdc25	CHK2		323
Plk3			298

EBV-transformed wild-type human lymphoblasts undergo an early wave of ATM-dependent radiation-induced apoptosis (104,105). Thus, although sensitivity to the killing effects of ionizing radiation has long been considered a cardinal feature of AT, the *Atm*$^{-/-}$ mouse studies indicate that, for most tissues, loss of ATM function does not result in increased radiation sensitivity and, for some cell types, ATM may actively promote radiation-induced apoptotic cell death.

M. Cellular Senescence and Telomere Abnormalities

AT fibroblasts grown in culture show accelerated telomere loss and premature senescence (106). Telomere fusions also are found in AT cells (107–109), perhaps as the result of shortened or structurally abnormal telomeres (110–112). Abrogation of ATM function by dominant negative ATM fragments resulted in telomere shortening and an increase in telomere associations (110). Another unusual find-

ing in AT cells is that they possess extrachromosomal telomere DNA (113). Taken together, these observations suggest a defect in telomere maintenance in AT cells. The hypothesis that *ATM* is involved in telomere maintenance is supported by the observations that *Tel1,* the closest yeast *ATM* homologue, is required for normal telomere maintenance in *S. cerevisiae* (114) and can suppress telomere shortening in AT cells (115).

III. *ATM* GENE

An intensive search for the genes responsible for AT culminated in 1995 with the positional cloning of the *ATM* gene by Shiloh and colleagues (116). The *ATM* gene is conserved in vertebrates and codes for a 12 kb transcript that is abundantly expressed in multiple tissues *in vivo.* The carboxy terminus of the ATM protein shares homology with several other checkpoint/damage response proteins, including *Schizosaccharomyces pombe* Rad3p, *Saccharomyces cerevisiae* Mec1p, *Saccharomyces cerevisiae* Tel1p, *Drosophila* MEI-41, *Arabidopsis* AtATM, *Aspergillus* uvsB, human DNA-PKcs and human ATR (Fig. 3). The *ATM* gene also

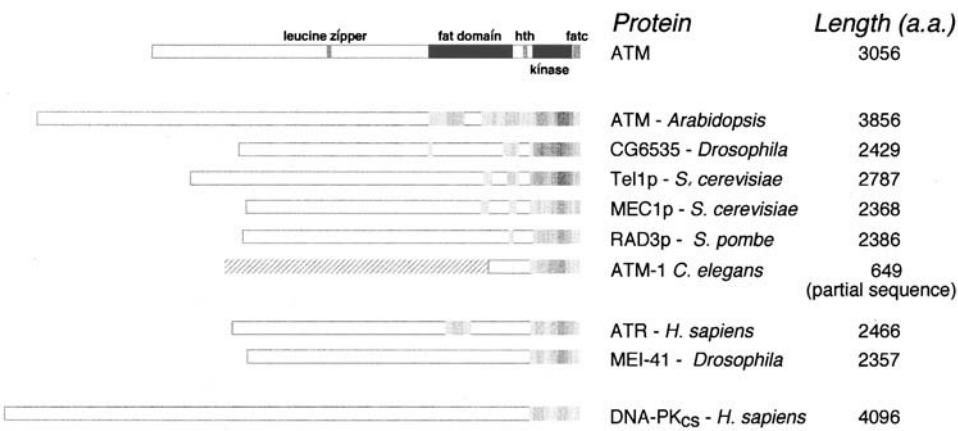

Figure 3 Block alignment of the ATM protein and its homologues. The 3056 amino acid ATM protein is shown with several of its predicted sequence features: leucine zipper (residues 1216–1239), fat domain (residues 1970–2056), helicase-turn-helicase (hth) domain (residues 2256–2277), kinase domain (residues 2713–3011), and fatc domain (residues 3024–2055). For other members of the ATM family and related PI-3 kinases, regions of similarity to the ATM protein are indicated by increasing degrees of shading. (Alignments were generated using sequential BLAST searches of 100 amino acid fragment blocks of ATM, shifted 25 amino acids at a time. Regions with BLAST scores less than 29 are white, while region with BLAST scores ranging from 29 to 147 are represented by light and dark grey, respectively.)

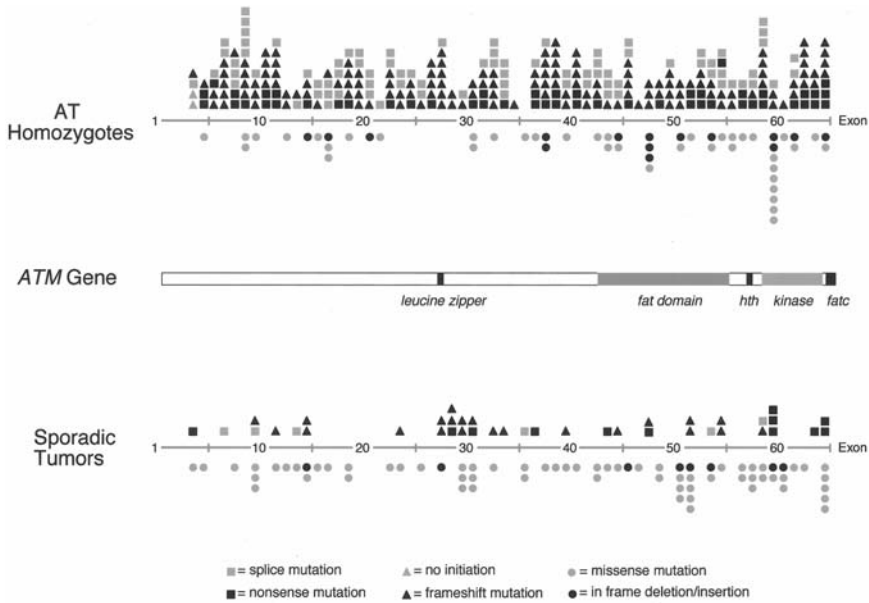

Figure 4 Spectra of *ATM* mutations found in AT patients and sporadic tumors (based on data from the ataxia-telangiectasia database at http://www.vmmc.org/vmrc/atm.htm). Only 55/353 (16%) of reported mutations in AT patients code for missense mutations or in-frame deletions in the *ATM* coding sequence, compared to 40/111 (64%) of mutations in sporadic tumors.

shares many phenotypic similarities with the genes that code for these proteins, including x-ray sensitivity, cell cycle checkpoint defects, genetic instability, and meiotic abnormalities (4,31,114,117–122).

The AT complementation groups A/B, C, D, and E previously defined by somatic cell fusion studies (123) are all due to mutations in the *ATM* gene (116), as is the variant, AT *Fresno* (124). In retrospect, difficulties with using radioresistant DNA synthesis as a measure of the S phase checkpoint for analysis of heterodikaryons may have been responsible for the complementation study results (125). Although AT is inherited in an autosomal recessive manner, almost all patients from nonconsanguineous families are compound heterozygotes. Over 300 different *ATM* mutations have been described (for a list, see http://www.vmmc.org/vmrc/atm.htm). While there are founder mutations in certain ethnic populations (126,127), mutations in outbred populations are typically private. As seen in Figure 4, nonsense and splice mutations that result in the production of unstable truncated proteins account for >85% of mutations in AT patients.

IV. *ATM* PROTEIN

The ~360-kD ATM protein is expressed in multiple embryonic and adult tissues (128). Expression is particularly high in the spleen, thymus, and testis (128), tissues in which active gene rearrangements occur. *ATM* expression is constant throughout the cell cycle in cultured cells (129). ATM is primarily a nuclear protein; however, it has been found in cytoplasmic vesicles from lymphocytes and in the cytoplasm of postmitotic neurons (129–131). ATM is highly expressed in the developing nervous system, including fetal human and murine Purkinje cells, but postnatal expression in the Purkinje cells of mice is low (131–133). ATM forms nuclear foci of unknown significance in undamaged mitotic cells (129) and is a member of the BASC super complex of BRCA1-associated proteins (134). In pachytene spermatocytes and oocytes, foci of ATM localize to synaptonemal complexes (135). Purified ATM protein has been found in both monomeric and tetrameric forms and binds to double-stranded DNA in vitro, with a distinct preference for DNA ends and x-irradiated DNA (136,137).

Initial sequence analysis of the ATM protein indicated that it has a phosphatidylinositol 3-kinase (PI3-kinase) domain (116), but there is no evidence that phosphoinositols are the biologically relevant targets for its phosphotransferase activity. On the contrary, ATM has been shown to be a Mn^{+2}-dependent protein kinase that phosphorylates serine and threonine residues of target proteins with a consensus sequence of LSQE (138–140).

X-irradiation increases ATM kinase activity (141) and may also cause severalfold increases in intracellular concentrations of ATM protein (142,143). This effect appears to be restricted to efficient inducers of DSBs, as ultraviolet (UV) irradiation does not affect ATM expression (144). Following x-irradiation, a small fraction of ATM becomes resistant to detergent extraction. This fraction forms nuclear foci which colocalize with foci of NBS1, suggesting that the detergent-resistant ATM may be localizing to DSBs (145). This possibility is supported by recent observations that ATM associates with genomic loci undergoing VDJ recombination (146), and that it localizes to subnuclear regions containing laser microbeam-induced DSBs (Fig. 5) (147).

V. PHENOTYPE OF *ATM* KNOCKOUT MICE

Several different strains of $Atm^{-/-}$ mice have been generated since the cloning of the *ATM* gene (96,99,148). The mice all have a similar AT-like phenotypes in that they express neurological abnormalities, immune defects, genetic instability, radiation sensitivity, infertility, and a high incidence of lymphoma. Despite these phenotypic similarities with AT patients, there are differences, including severe growth retardation, universal sterility, and death from lymphoma by age 6 months in $Atm^{-/-}$ mice (96,99,148). The neurological phenotype of the mice is notable.

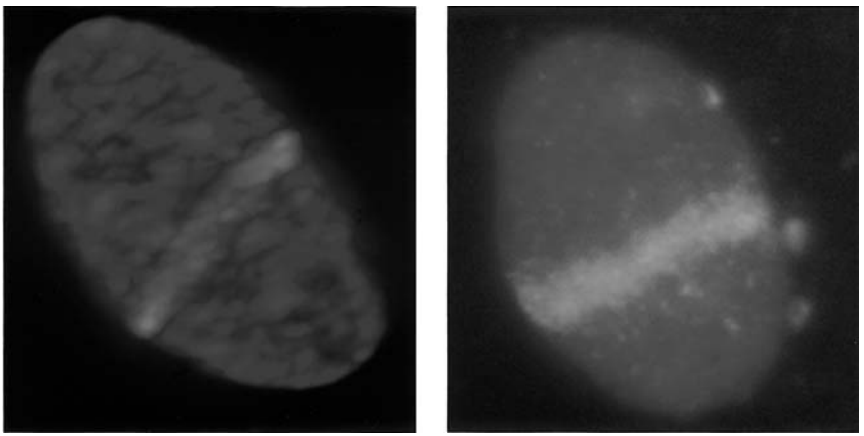

Figure 5 ATM rapidly localizes to DSBs in the nuclei of human fibroblasts. Left panel: DSBs form in the path of a laser microbeam. Right panel: ATM protein localizes to regions of laser-induced DSBs within 15 min following irradiation. [For these images, DSBs were introduced in genomic DNA of human fibroblasts by briefly exposing the cells to Hoechst dye and then irradiating them with a 1 micron diameter beam of 390nm laser light (Ref. 147). DSBs were detected by end labeling with Cy3-tagged dTCP. ATM was identified by immunohistochemical staining using an anti-ATM primary antibody and an FITC-labeled secondary antibody. Both cells were counterstained with DAPI to detect nuclear DNA.] (See color insert.)

Like AT patients, *Atm*$^{-/-}$ mice have impaired coordination and they share some histopathological abnormalities, including an increased frequency of ectopic Purkinje cells and selective deficiencies of dopaminergic neurons (149,150). However, the cerebellar dysfunction does not appear to be progressive in the *Atm*$^{-/-}$ mice (151). *Atm*$^{-/-}$ Purkinje cells appear to be normal histologically, and there is not the ongoing, large-scale loss of Purkinje cells from the cerebellum seen in AT patients (152). An electrophysiological study of *Atm*$^{-/-}$ mice did find an age-dependent decrease in calcium firing, which was proposed to be an early sign of impending degeneration (152). Nevertheless, the lack of ongoing losses of Purkinje cells and other neurons in the *Atm*$^{-/-}$ mice has cast a shadow on the suitability of the mice as animal models for the degenerative neurologic disease seen in AT patients.

The essentially universal occurrence of lymphomas in *Atm*$^{-/-}$ mice in the first 6 months of life appears to be a consequence of aberrant V(D)J recombination, as RAG-1– and RAG-2–deficient mice, which cannot undergo V(DJ) recombination, do not express this early wave of oncogenesis (153,154). However, dysfunctional V(D)J recombination is not the only source of genetic instability leading to lymphomas in ATM-deficient mice, as some *Rag2*$^{-/-}$ *Atm*$^{-/-}$ mice develop lymphomas later in life (154).

VI. DSN MODEL AS A WORKING HYPOTHESIS FOR *ATM* FUNCTION

In the past, individual deficits in DNA repair, genetic recombination, chromatin structure, and cell cycle checkpoint control all have been proposed as the underlying pathological defect in AT (reviewed in Ref. see 155). However, it now clear that much of the AT phenotype is caused by defects in signaling pathways that activate multiple cellular responses to DNA damage (155–160).

Prior to the cloning of the *ATM* gene, we proposed a model for AT in which the AT defect results in an inability to coordinately activate a group of diverse cellular functions in response to DNA damage (83,155). Figure 6 depicts a current version of this model, in which the detection of DSBs in DNA triggers an *ATM*-dependent signal transduction network, resulting in a kinase cascade that activates cellular functions that promote genetic stability by temporarily arresting the cell cycle and enhancing homologous recombination and other forms of DSB repair. At same time, the network modulates cellular survival by inhibiting execution of damage-induced programmed cell death in some cells while promoting it in others. These ATM-dependent responses allow cells to react to intentional DSBs created during meiosis and immune gene rearrangement as well as unintentional DSBs that result from stalled replication forks, aberrant telomeres, endogenous oxidative DNA damage, and environmental mutagens. In addition to the responses illustrated in Figure 6, there are likely to be other, as yet undefined, ATM-dependent functions. ATM-dependent pathways are presumed to function in meiotic, mitotic, and postmitotic cells.

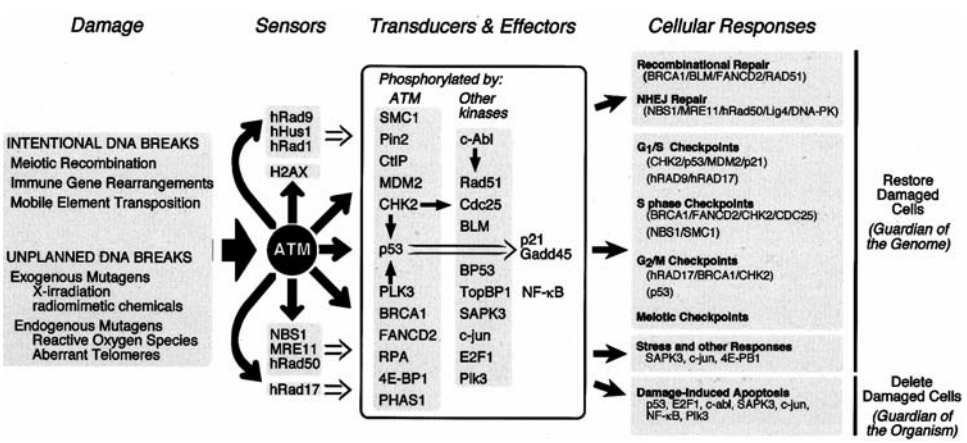

Figure 6 A DNA Damage Surveillance Network. As part of this signal transduction network, the ATM protein activates multiple cellular functions in response to the detection of spontaneous or induced DNA damage.

The primary abnormality in AT homozygotes presumably creates a defect in this network that prevents the activation of these cellular functions in response to DSBs, aberrant telomeres, and related DNA anomalies. This inability to respond to spontaneous and induced DNA damage can result in increased genomic instability. At the same time, this defect allows the triggering of apoptosis by otherwise nonlethal DNA damage in some cells but blocks damage-induced apoptosis in others. These abnormalities contribute, in turn, to the multiple in vivo and in vitro abnormalities seen in AT homozygotes.

The ATM network appears to deal specifically with DSBs in genomic DNA, as AT cells are not particularly sensitive to other forms of DNA damage such as UV-induced photoproducts (161). Strand breaks containing modified 3′ termini may be particularly good inducers of the ATM network, given the sensitivity of AT homozygotes to agents which induce strand breaks containing 3′ phosphoglycolates (42–44). Short or aberrant telomeres also may activate the ATM network (103), suggesting that the ends of abnormally short telomeres may be perceived by the cell as DSBs. ATM-dependent functions also appear to respond to DSBs generated during meiotic synapsis and recombination, as meoisis ends in apopotic death during pachynema in $Atm^{-/-}$ mice (96,97).

How far upstream ATM protein functions in the DSB surveillance network is not certain, but there is experimental support for the idea that ATM acts at an early stage. AT cells lack the majority of cellular responses to DSBs, thereby placing ATM at a point in the network prior to the branching of DNA repair, cell survival, and cell cycle checkpoint pathways. The identification of multiple protein targets for ATM-dependent phosphorylation also suggests an early role for ATM (see below).

Could ATM could also act as a DNA damage sensor? Indirect support for this possibility has been provided by studies showing that ATM has an affinity for binding to DSBs in vitro (136) and our own findings that ATM localizes to sites of DSBs in mouse spermatocytes and oocytes (135,162) and to regions of induced DSBs in human fibroblasts (see Fig. 5) (147). However, if ATM is a direct sensor of DSBs, then it does not appear to activate damage surveillance pathways alone, as a functional NBS1 is required for optimal radiation-induced ATM kinase activity in vivo (163–165).

The ATM surveillance network may use multiple sensors. Other candidate sensors include H2AX, the NBS1/Mre11/Rad50 complex, hRad17, and the hRad9/hHus1/hRad1 (9-1-1) complex. Molecules of the histone H2AX located at sites of DSBs undergo phosphorylation at serine 139 within 1 min of DSB induction (166), the most rapid known association of a protein with DSBs. The NBS1/Mre11/Rad50 complex rapidly associates with DSBs in vivo (167) and cells from patients defective in Mre11 (ATLD syndrome) and NBS1 (Nijmegen breakage syndrome) share similar cellular defects to AT (168,169), suggesting a role in early surveillance network events. The hRad17 protein and the hRad9/hHus1/hRad11 (9-1-1) complex, whose yeast homologues function as detectors of DNA damage, also are likely to serve as sensors of DSBs for the

dependent damage surveillance network, as hRad17 and hRad9 mutants are both cell cycle checkpoint defective and radiosensitive (170–172).

Regardless of whether ATM functions as a direct sensor of DNA damage, it is clear that a major function of the ATM protein is signal transduction. Our growing understanding of ATM biochemistry supports this hypothesis. As summarized in Table 1, 25 proteins are known to undergo ATM-dependent phosphorylation following induction of DSBs, and ATM has been shown to directly phosphorylate 15 of these proteins in vitro. These results support the idea that ATM is an upstream component of a kinase cascade that operates via phosphorylation and dephosphylation of signaling molecules that links detection of DNA damage to modulation of the enzymatic machinery of cell cycle progression, genetic recombination, and apoptosis (see Fig. 6).

For ATM-dependent cell cycle checkpoints we now know the control pathways in some detail (see Fig. 6). ATM controls the G1/S cell cycle checkpoint through direct and indirect phosphorylation of p53 and mdm2, thereby modulating the activity of p21/waf1, which in turn controls the G1/S checkpoint by inhibiting the formation of cdc2/cyclinE complexes (see Fig. 6). Following induction of DSBs, ATM triggers the S phase checkpoint through parallel pathways dependent on NBS1 and Chk2 (173) and activates the G2/M checkpoint through p53 and BRCA1-dependent pathways (174,175). Exactly how ATM exerts control over DNA repair, homologous recombination, apoptosis, and stress responses are not as clear, although some individual steps have been identified (see Table 1 and Fig. 6).

Although ATM controls most cellular responses to DSBs, there are both early and late responses that are ATM independent. DSB-dependent induction of BLM protein does not require ATM (176), and the DSB-induced late G2/M checkpoint still functions in AT cells (67). Following induction of DSBs, many proteins involved in the ATM-dependent damage surveillance network form nuclear foci that are thought to be involved in DNA repair and other damage responses. The involvement of ATM in focus formation is variable. Optimal formation of foci of H2AX, Mre11, Rad50, and Chk2 appear to require functional ATM, whereas formation of Rad51, TopBP1, and FANCD2 appear to be independent of ATM function (147,177–181).

Eukaryotes from yeast to humans practice a certain economy in their damage-response networks. As a result, networks that respond to different types of DNA damage share some common components and end functions. For example, although the DSB and UV surveillance pathways use separate PI-3 kinases, ATM and ATR, to respond to different forms of DNA damage, they both use BRCA1 to control cell cycle checkpoints (182). Network components also can serve multiple biological roles. For example, BRCA1 is involved in the S phase and G2/M checkpoints as well as homologous recombination (67,183), whereas p53 activates cell cycle checkpoints, modulates cell survival, and plays a role in DNA repair (156,174,184,185). Differential phosphorylation may allow a single surveil-

lance network protein to respond differentially to different upstream transducers and to activate multiple downstream functions. For example, both ionizing and UV-irradiation induce phosphorylation of BRCA1 at multiple serine residues sites. However, phosphorylation of BRCA1 at serine 1387 is x-irradiation specific, whereas serine 1457 is predominately phosphorylated following UV-irradiation (182). Serine 1387 phosphorylation is a required step in BRCA1-dependent triggering of the S phase checkpoint by ionizing radiation, whereas phosphorylation at serine1423 is necessary for BRCA1 to activate the G2/M checkpoint following irradiation (186).

A. Alternative Functions for *ATM*

Models such as the one presented in Figure 6 can account for much of the clinical and molecular phenotype of AT (84). However, ATM may have other functions as well. For example, it has been suggested that ATM is involved in oxidative stress responses in addition to reacting to DNA damage (187). ATM protein interacts with beta-adaptin in cytoplasmic vesicles (188), and ATM protein is primarily cytoplasmic in Purkinje cells (131), suggesting that ATM may have different, perhaps cytoplasmic, functions in neurons. Finally, ATM may have structural as well as signaling functions. In $Atm^{-/-}$ pachytene spermatocytes, fragmentation of synaptonemal complexes occurs at sites of putative DSBs and is coincident with the first appearance of ATM foci along the axes of newly paired bivalents (189), suggesting that ATM might be required to maintain the physical integrity of DSBs so as to prevent their conversion into chromosome breaks and subsequent activation of apoptosis in meiotic cells.

VII. DIAGNOSIS AND TREATMENT

The diagnosis of AT in index cases is typically delayed until telangiectasias appear at 6 or 7 years of age (6). However, the absence of telangiectasias, immune deficits, and/or elevated serum AFP do not rule out AT as a diagnosis for the toddler or young child with slowly progressive ataxia. The size of the *ATM* gene and the large number and even distribution of mutations along the *ATM* gene make molecular diagnosis difficult (see Fig. 3). As a result, sequential evaluation of children suspected of having AT is recommended, beginning with a clinical assessment of neurologic and immunologic deficits accompanied by serum AFP determination and karyotype. Further investigations include protein truncation testing and Western blot analyses of ATM protein, measurement of ATM kinase activity, colony-survival assays using lymphocytes and SSCP analysis of the *ATM* gene. This combined approach is thought to yield a false-negative rate of $<1\%$ (190).

Improvements in supportive care have increased the quality of life for AT patients and extended their expected life span. However, despite major strides in

our understanding of the *ATM* gene and the molecular basis of AT, therapy still focuses on symptomatic relief of neurologic and immunologic problems rather than treatment of underlying deficits (191). Although antioxidants, L-dopa, gene therapy, and neuronal transplants all have been considered as potential therapies, effective treatments have yet to emerge from our new knowledge of AT pathobiology. The recent establishment of an Ataxia-Telangiectasia Clinical Center at Johns Hopkins in Baltimore has already led to large-scale studies of the course of the disease and, in the future, may facilitate clinical trials that could lead to the development of new ways to treat this devastating condition (http://ww2.med.jhu.edu/ataxia/clinicar.htm).

VIII. ETIOLOGY OF NEURODEGENERATION IN AT

The one facet of the AT phenotype with the greatest clinical impact is neurodegeneration due to the gradual but inexorable loss of Purkinje cells, and, to a lesser extent, other neurons in the brain and the peripheral nervous system. Why mutations in a gene that controls cell cycle checkpoints should affect the fate of postmitotic neurons remains a major unanswered question in AT research. Several hypotheses have been proposed over the years. Building on histopathologic analyses of cerebella from AT patients, Vinters et al. (192) proposed that AT Purkinje cells are placed at risk for cell death by abnormal positioning within the cerebellum during embryonic development. Although this hypothesis accounts for the fate of Purkinje cells, it does not provide a ready explanation for the ongoing loss of other neurons in AT patients (4). Meyn (84), noted that AT cerebella contain a high frequency of abnormal Purkinje and granule cells that exhibit the highly condensed, pyknotic nuclei expected from programmed cell death in neurons (193–195) and hypothesized that neuronal loss in AT is due to a low threshold for triggering apoptosis in response to spontaneous DSBs occurring in the genome of postmitotic neurons. Rotman and Shiloh (187) have suggested that neurons that lack ATM are under constant stress from reactive oxygen species which damage DNA and other macromolecules, leading to degeneration and death.

Studies with $Atm^{-/-}$ mice have provided some indirect support for these hypotheses: The mice express ectopic placement of Purkinje cells, elevated frequencies of DNA strand breaks in brain tissue, and increased numbers of reactive oxygen species in the brain (150,196–198). However, at present, there is no strong experimental support for one hypothesis versus another. An alternative possibility as to how ATM, a protein that controls cell cycle checkpoints, might affect survival of postmitotic neurons is suggested by recent work on Alzheimer disease. Yang et al. (199) have found that a significant fraction of neurons in Alzheimer disease initiate an abortive cell cycle, replicating their genome but then failing to enter mitosis. They suggest this abortive cell cycle ends in death, accounting for

the ongoing loss of neurons in Alzheimer's disease. A similar situation may occur in AT neurons. Further investigation should help clarify this key issue.

IX. DO *ATM* MUTATIONS PLAY A ROLE IN COMMON CANCERS?

BRCA1, p53, NBS1, Mre11, and Chk2 can all undergo mutational inactivation in sporadic and/or familial cancers (200–206). As these proteins are downstream of ATM in DNA-damage surveillance pathways, it would not be surprising if germline and/or somatic *ATM* mutations were involved in the development of common malignancies such as breast cancer. However, this issue has been a persistently controversial aspect of AT research. Based on studies of AT families, it has been proposed that ~7% of all breast cancers occur in women who are *ATM* heterozygotes (207). If true, then *ATM* mutations contribute as much to the overall incidence of breast cancer as BRCA1 and BRCA2 mutations combined (208). However, several early surveys failed to find frequent *ATM* mutations in breast cancer patients (209,210), casting doubt on possibility that cancer-associated *ATM* mutations represent a significant public health issue. While this important issue awaits definitive resolution, consideration of several different areas of research may clarify the current situation.

X. EVIDENCE FROM STUDIES OF AT FAMILIES

An increased incidence of cancer among relatives of AT patients first was noted by Reed et al. (211). Subsequent family studies have provided consistent support for an increased risk of breast cancer in female AT heterozygotes (212–218). In a 1994 meta-analysis, Easton (219) estimated this risk to be 3.9-fold (95% confidence interval of 2.1–7.2), about the same as the increased risk associated with having a first-degree relative with breast cancer. These same family studies suggest that there is little increased risk for AT heterozygotes developing other tumors (219).

The case for an increased risk of breast cancer in AT heterozygotes has been strengthened by recent work in which polymorphic markers linked to the *ATM* locus were used retrospectively to identify heterozygotes among breast cancer patients within AT families (207,220,221). This approach avoids ascertainment bias problems inherent in previous work. In these studies, an excess of breast cancers were found in female heterozygotes. For example, of the 33 AT family members with breast cancer in one study, 25 were found to be AT heterozygotes when only 14.9 were expected, an odds ratio of 3.8 with a $P < .0001$ (207).

Taken as a whole, the family studies support the conclusion that AT heterozygotes who are relatives of AT patients are a subpopulation of individuals at

increased risk to develop breast and perhaps other cancers. Given this conclusion, the frequency of mutant *ATM* alleles in the general population becomes an important consideration. Estimates of the frequency of AT heterozygotes in the general population range from 0.34 to 2.42%, with a reasonable average of about 1% (reviewed in Ref. 3). A 1% carrier frequency, together with the data summarized above that suggest that carriers of *ATM* mutations are at an approximately fourfold increased risk for breast cancer, leads to the prediction that 3–5% of women with breast cancer are germline heterozygotes for mutations in the *ATM* gene. This estimate is similar to that of Easton (219), and suggests that, in North America, ~7500 female heterozygote carriers of *ATM* mutations develop breast cancer each year.

XI. DO GERMLINE *ATM* MUTATIONS PLAY A ROLE IN FAMILIAL AND SPORADIC BREAST CANCERS?

Since surveys of AT families consistently show an increased risk of breast cancer in AT heterozygotes, one might expect that mutations in the *ATM* gene would play a role in familial breast cancer. Yet, studies of breast cancer families have not demonstrated linkage to the *ATM* locus on 11q23 (e.g., see Refs. 222, and 223). This apparent contradiction can be resolved if one considers the penetrance of heterozygous *ATM* mutations and the type of *ATM* mutations that might predispose to cancer in *ATM* heterozygotes.

The risk of AT heterozygotes developing breast cancer has been calculated to be 11% by age 50 years and 30% by age 70 years (224). As a result of this relatively low penetrance, first-degree relatives of AT heterozygotes should have only a modest increase in their relative risk of developing breast cancer. Hence, *ATM* mutations should account for relatively few "breast cancer families." However, because *ATM* mutations are more frequent in the general population than mutations in the *BRCA1 and BRCA2* genes, germline *ATM* mutations may give rise to many more "sporadic" tumors. The net result is consistent with the idea that *ATM* mutations may be responsible for at least as many cases of breast cancer in the general population as germline mutations in *BRCA1* and *BRCA2*.

If this last prediction is true, then screening large groups of breast cancer patients should reveal excessive numbers of germline *ATM* mutations. Early studies focused on identifying truncating *ATM* mutations. One such study found truncating *ATM* mutations in 7 of 82 early-onset breast cancer patients, suggesting that these *ATM* mutations conferred a nine-fold increase in the relative risk of breast cancer (225). However, most surveys have not yielded statistically significant increases in the frequency of germline truncating *ATM* mutations among breast cancer patients. For example, FitzGerald et al. found only two truncating *ATM* mutations in 401 women who developed breast cancer under age 40 years (209). In a

focused study of the truncating mutations that cause >80% of AT in Norway, only one mutation was found in 483 Norwegian breast cancer patients (226). No truncating *ATM* mutations were reported in a surveys of 38 randomly chosen breast tumors (227), 100 breast cancer patients under age 40 years (228), 100 breast cancer patients with family histories of breast cancer (210), 47 patients with late-onset breast cancer (229), and 57 breast cancer patients with bilateral cancer (230).

Individually, these early surveys lacked sufficient statistical power to detect the four- to sixfold increased cancer risks predicted by studies of AT families. In addition, two of the studies used protein truncation assays (PTT) to detect mutations, a relatively insensitive technique that can miss >35% of the *ATM* mutations that occur in AT patients (127). Nonetheless, taken as a group, the published surveys suggest that germline truncating *ATM* mutations do not play a major role in breast cancer in the general population. Recent reports support a biological explanation for these epidemiological findings: Missense mutations in *ATM* may be a more important factor in breast cancer than truncating *ATM* mutations (see below).

Several researchers have documented an elevated frequency of germline *ATM* missense mutations in breast cancer patients. In a Seattle-based case-control study, 11 of 142 breast cancer patients had missense *ATM* mutations compared to only 1 of 81 controls (231). Another survey found novel missense *ATM* mutations in 6 of 52 breast cancer patients, including four nonconservative changes (232). A German study of 1000 breast cancer patients and 500 controls also found an excess of rare missense *ATM* mutations in the breast cancer cohort with one missense substitution being five times more frequent in patients with bilateral disease than in controls (233).

These studies also highlight a difficult practical issue: distinguishing neutral from deleterious mutations in such a large gene. For example, in the work published by Atencio et al., exon sequencing of the *ATM* gene found 142 common polymorphisms and apparently neutral mutations in 42 of 52 breast cancer patients, whereas apparently deleterious missense mutations were found in 6 individuals (232).

XII. DO SOMATIC MUTATIONS IN THE *ATM* GENE PLAY A ROLE IN SPORADIC TUMOR DEVELOPMENT?

Loss of normal control of genomic stability is thought to be a necessary step in carcinogenesis that allows clonal populations of cells to accumulate a sufficient number of mutations to acquire a fully malignant phenotype (234,235). Given that genetic instability is a cardinal feature of AT, one might expect that the *ATM* gene would, like *p53* and the DNA mismatch repair genes, play a key role as a guardian of the genome. If so, then inactivation of the *ATM* gene may be a frequent event in the development of certain sporadic common cancers. This is supported by loss

of heterozygosity studies and by the demonstration of decreased ATM expression in breast cancers and in leukemias.

XIII. *ATM* STRUCTURE AND FUNCTION IN SPORADIC BREAST TUMORS

Loss of heterozygosity (LOH) at 11q22-23 loci is a frequent event in many solid tumors, and loss of 11q23 heterozygosity occurs in 40–50% of sporadic breast cancers (reviewed in Ref. 3). Although initial loss of heterozygosity (LOH) studies provided only a very rough molecular estimate as to the location of the relevant 11q23 loci that are lost during tumor development, recent work indicates that a 5- to 10-cm region of 11q23 that includes the *ATM* locus is deleted in approximately one-third of breast cancers (236–241).

LOH studies demonstrating 11q23 deletions provide broad, but indirect, support for the idea that loss of ATM function plays a role in the development of cancer. However, there are other oncogenes in the 11q23 region besides *ATM*; for example, *DDX10, RDX, NCAM, FDX1*, and *PLZF*. If *ATM* is a true target for inactivation in breast cancers, then one might expect those tumors which have lost one *ATM* allele also will have inactivated the remaining allele by mutation. Although this has been proven to be consistently true for leukemias and lymphomas (see below), initial tests of this prediction in breast cancer have been mixed. For example, Izatt et al. found loss of the remaining wild-type *ATM* allele in breast cancers from five of seven individuals with germline missense *ATM* mutations (228). However, in another small series, only one-twenty-fifth of breast cancers with LOH at the *ATM* locus was found to have undergone a somatic mutation in the remaining *ATM* allele (242).

Another prediction of the tumor-suppressor hypothesis would be that *ATM* expression should be decreased in breast cancers. Initial reports tend to bear this out. In an analysis of 39 breast carcinomas, Waha et al. (243) found that *ATM* mRNA levels were reduced by approximately sixfold in tumors when compared to normal breast tissue. Angele et al. reported a significant decrease in ATM immunoreactivity in 10 of 17 sporadic breast cancers (244). Kairouz et al. (245) found that metastatic tumors were more likely to have reduced expression: 10 of 14 metastatic tumors had decreased or absent protein expression compared to 14 of 42 nonmetastatic tumors, a highly significant difference. On the other hand, Kovalev et al. (246) analyzed 89 random breast cancers by reverse transcriptase–polymerase chain reaction (RT-PCR) and found that none of the tumors had lost *ATM* expression.

With further studies, a more accurate picture of the role of somatic and germline *ATM* gene inactivation in breast cancer should emerge. Large-scale con-

sortium studies examining breast cancer in AT families and *ATM* mutations in breast cancer patients are currently underway and should help resolve these important public health issues (247).

XIV. FREQUENT INACTIVATION OF *ATM* IN SPORADIC LEUKEMIAS

Given that the creation of DSBs is an obligatory step in lymphocyte development, ATM protein associates with chromatin undergoing V(D)J recombination and loss of *ATM* function suppresses DNA damage–induced apoptosis in lymphocytes, it is not surprising that AT patients and $Atm^{-/-}$ mice are highly susceptible to leukemia and lymphoma. These same observations suggest that *ATM* mutations might occur frequently in sporadic lymphoid tumors. Multiple clinical studies support this prediction. In 1997, Vorechovsky et al. (248) reported the occurrence of frequent *ATM* mutations in T-cell prolymphocytic leukemias (T-PLL) and in non-Hodgkin lymphomas, a finding that has since been confirmed by others (249,250).

Disruptions of both *ATM* alleles also have been found in B-cell chronic lymphocytic leukemia (CLL), the most common form of adult leukemia (251–254). *ATM* mutations may also be involved in adult-onset acute lymphocytic leukemia (ALL) and B-cell non-Hodgkin's lymphoma, as LOH involving the *ATM* locus has been found in these tumors (255–257). Lichter's group has reported frequent deletion of *ATM* locus with point mutations in the remaining *ATM* alleles in mantle cell lymphomas (258,259). Although ATM mutations are found in many hemopoietic tumors, they do not appear to be frequent in chronic myeloid leukemia (260).

As might be expected for a tumor-suppressor gene, when LOH involving the *ATM* locus has been observed in a T-PLL or mantle cell lymphoma, the remaining allele is frequently mutated (248–250,261). Similar results have been reported for B-CLL (253,254). The *ATM* mutations found in T-PLL were primarily missense mutations and complex intragenic rearrangements rarely seen in AT patients (248,262). Mantle cell lymphoma mutations included missense mutations in the PI-3 kinase domain of the protein (261).

Alterations in *ATM* gene structure or expression appear to be a poor prognostic sign in B-CLL. Starostik et al. (263) reported that the 34% of 111 B-CLLs analyzed that had decreased expression of ATM protein also had an aggressive clinical course. Cuneo et al. found that loss of the *ATM* locus was associated with decreased survival in patients with B-CLL (264). A similar association has also been reported for B-cell non-Hodgkins lymphomas (256) but not ALL (255).

Given that *ATM* is frequently inactivated in sporadic hemopoietic tumors, one might expect to find *ATM* germline mutations in familial leukemias and lymphomas. Several studies of small numbers of sporadic B-CLL patients have documented the presence of germline *ATM* mutations (251,253), However, surveys of AT families have not found yielded consistent results: Swift's study of American AT families found a statistically significant elevation in the risk for CLL among AT heterozygotes (265), but differences in the frequency of leukemias in AT heterozygotes versus controls were not statistically significant in a more recent French survey (221), and a Norwegian study found no increases in risks (218) In addition, a study of 24 families with CLL found no linkage between CLL and the *ATM* locus (266). It would appear from these surveys that germline *ATM* mutations play, at most, a minor role in familial CLL; however, further work will be required to assess fully the involvement of *ATM* in inherited forms of this common adult leukemia.

XV. ETIOLOGY OF GERMLINE CARRIERS' CANCER RISK

The vast majority of *ATM* mutations seen in AT families are functionally null (see above). As a result, AT heterozygotes could be at increased risk to develop cancer solely because they have only one functional *ATM* allele to inactivate before they lose ATM-dependent damage responses and become genetically unstable. Alternatively, their increased cancer risk could in part be because cells with only one functional *ATM* allele may have mild phenotypic abnormalities that directly facilitate the accumulation of somatic mutations necessary for tumor development.

The latter possibility is supported by multiple studies that have found that cells from some, but not all, heterozygote members of AT families respond abnormally to DNA damage. These abnormalities are mild compared to those seen in AT patients, but include increased sensitivity to the cytotoxic and clastogenic effects of ionizing radiation and radiomimetic drugs, persistence of radiation-induced chromosome breaks, postirradiation DNA synthesis abnormalities, and prolonged G2 arrest (reviewed in Ref. 3). *ATM* heterozygotes also are more likely than normal individuals to suffer from late in vivo side effects of radiation therapy (267,268). Data from $Atm^{+/-}$ mice support the conclusion that individuals with one functional ATM allele have impaired responses to DSBs. Fibroblasts and lymphocytes from $Atm^{+/-}$ mice have cell cycle checkpoint abnormalities and are sensitive to the killing effects of ionizing radiation (100,269), $Atm^{+/-}$ mouse embryonic fibroblasts (MEFs) are more readily transformed than normal MEFs (270), and $Atm^{+/-}$ mice show increased susceptibility to radiation-induced mammary ductal hyperplasia (271).

XVI. ARE THERE TWO CLINICALLY SIGNIFICANT CLASSES OF *ATM* MUTATION?

As summarized in Figure 3, the majority of *ATM* mutations found in AT patients code for truncating mutations that result in the expression of unstable mutant proteins (272,273). In contrast, most *ATM* mutations found in patients with breast cancer and leukemia code for missense mutations.

These differences led Gatti (190) and Meyn (274) to raise the possibility that there may be two distinctive classes of *ATM* mutation: (a) mutations that block expression of ATM protein or code for mutant polypeptides that are so unstable that they are present in insignificant amounts within a cell and (b) mutations that code for stable ATM proteins that are present at normal intracellular levels but function abnormally. Because class 1 mutations act as nulls, heterozygote carriers of class 1 mutations would have cells with 50% of wild-type ATM activity and a normal or nearly normal phenotype. Class 1 homozygotes or compound heterozygotes would be expected to have AT, as they would have no functional ATM protein.

Overexpression of an ATM polypeptide that lacks the PI-3 kinase domain has been shown to increase genetic instability in normal cells grown in culture (275). This suggests that cells that are heterozygous for class 2 mutations may have less functional ATM activity than cells that are heterozygous for class 1 mutations. One possible mechanism by which this might occur is if ATM polypeptides form a functional complex with itself and/or other proteins and class 2 mutations code for stable mutant polypeptides that competes with normal ATM polypeptides in complex formation. Because they are functionally abnormal, class 2 polypeptides would inactivate the complex and perturb the cell's ability to respond to DNA damage. The result is a dominant negative cellular phenotype.

Strong experimental support for this hypothesis has recently been provided by Lavin and colleagues (276). By introducing *ATM* cDNAs containing defined mutations into normal and AT cells in culture, they were able to demonstrate that three missense *ATM* mutants and an in frame deletion not only failed to complement the phenotype of AT cells but also acted as dominant negatives that impaired the kinase activity of the endogenous ATM protein in wild-type cells leading to increased chromosomal instability and reduced cell survival following irradiation.

The majority of *ATM* mutations that have been identified in AT patients would fit class 1, as ~85% of these mutations result in the production of truncated, unstable proteins. Many class 2 mutations would not be found in AT patients, because the function of mutant proteins coded by these class 2 alleles would not be sufficiently compromised to give the full AT clinical phenotype when paired with either themselves or other mutant *ATM* alleles. Yet, because loss of ATM function would be more severe in class 2 than in class 1 heterozygotes, class 2 heterozygotes would be expected to have a greater cancer risk than class 1 heterozygotes.

A prototypic example of a class 2 mutation may a 7271G → T missense mutation that codes for wild-type levels of a mutant ATM protein and gives rise to very mild AT in homozygotes and a high breast cancer risk in heterozygotes (277). Another example of a class 2 mutation may be the 1066-T → G mutation found by Dork et al. in 7 of 1000 breast cancer patients (233). This mutation causes skipping of exon 11 and has been shown by Chenevix-Trench et al. (278) to act as a dominant negative in cells in culture.

Those missense *ATM* mutations found in cancer patients but not in either AT patients or normal controls also are likely to be class 2 mutations (e.g., the missense mutations seen in T-PLL [227]). These cancer patients may not have a strong family history or a particularly early age of onset, given that the penetrance of class 2 *ATM* mutations may still be low compared to that of BRCA1 and BRCA2. In addition, because class 2 mutations are missense mutations, they would be missed by the protein truncation test (PTT) screening assays frequently used in initial surveys of *ATM* mutations in cancer patients (209,210).

XVII. LESSONS FOR CANCER SCREENING

As discussed above, heterozygote carriers of *ATM* mutations may represent a group of individuals whose risks for developing cancer differ from that of the general population. Much needs to be learned before cancer screening issues in *ATM* carriers can be fully resolved. However, our knowledge has progressed to the point where several issues can begin to be addressed.

A. Identification of AT Heterozygotes

Cloning the *ATM* gene has opened up the possibility of developing molecular screening tests for *ATM* heterozygotes. Although molecular analyses can identify heterozygotes within AT families, the large size of the *ATM* cDNA and the existence of more than 300 "private" mutations scattered throughout the gene make it difficult to develop a practical screening test for identifying *ATM* heterozygotes in the general population. At the same time, functional assays are not yet specific enough to be of use in population-based screening (reviewed in Ref. 3).

B. Mammography

ATM heterozygotes would appear to be a high-risk group that could benefit from mammographic screening. However, it has been suggested that physicians consider alternatives to mammography in *ATM* heterozygotes (214,279). This issue has provoked much discussion, both pro and con (e.g., see Refs. 280–284). Typical radiation exposures to the breasts from mammography average 2–3 mGy

(280,285) compared to the 1–2 mGy/yr exposure from background radiation sources (286). Based on these figures, it has been estimated that the additional lifetime risk for breast cancer resulting from annual mammography is about 1.5% for the general population. The sensitivity of cells from *ATM* heterozygotes to radiation-induced genetic alterations averages about 25–60% higher than that of controls (e.g., see Ref. 287). This suggests that the lifetime risk of breast cancer in *ATM* heterozygotes from mammography is less than twofold higher than controls; perhaps 2–3% (288). This 2–3% risk compares to an estimated lifetime risk of developing breast cancer in *ATM* heterozygotes of >30% (224). Given that annual mammography reduces the mortality of breast cancer through early detection by ~30% or more (289), the benefits of mammographic screening would appear to outweigh the risk of mammography-induced cancer in *ATM* heterozygotes by age 50 years if not earlier.

C. Cancer Therapy

AT patients respond poorly to treatment regimens that include ionizing radiation, radiomimetic drugs, and topoisomerase inhibitors. Care must be taken to modify standard protocols so as to minimize iatrogenic damage, but reduced therapeutic regimens can be tolerated for these individuals (e.g., see Refs. 15, 36, and 290). *ATM* heterozygotes could, in theory, represent a significant fraction of cancer patients who have adverse responses to radiation and chemotherapy. Experimental evidence for this possibility is mixed (reviewed in Ref. 3). Many studies have demonstrated a group correlation between in vitro radiosensitivity and adverse reactions to radiotherapy (287,291–294). However, several small PTT-based surveys of breast cancer patients with adverse reactions to radiotherapy have failed to detect high frequencies of *ATM* mutations (295–297).

XVIII. CONCLUSIONS

A great deal of progress has made in the last few years toward understanding the molecular pathology of AT and the critical role that the *ATM* gene plays in mediating cellular responses to DNA damage, maintaining genetic instability, delaying aging, and preventing cancer. We now are entering a new era of intense study that will focus on the pathobiology of AT as well as the role played by the ATM protein in DNA metabolism, cell cycle checkpoints, genetic instability, telomere maintenance, senescence, and programmed cell death. These studies should test the predictions of our current models for ATM function, further our understanding of basic biological processes, shed light on the mechanism of aging, and improve our diagnosis and treatment of this rare, but formidable, disease.

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14
Nijmegen Breakage Syndrome

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I. INTRODUCTION

Nijmegen breakage syndrome (NBS, MIM 251260) is extremely rare. Inheritance follows an autosomal recessive modus with complete penetrance. We are aware of some 70 families worldwide, the majority of which are in central and eastern Europe. The observed carrier frequency in Poland, Ukraine, and the Czech Republic is 1:177; however, considerably fewer than the expected 1:95,000 patients have been currently ascertained (1).

Historically, NBS belongs to the chromosomal instability syndromes. Based on the clinical and cellular similarity to ataxia-telangiectasia (AT) (2,3), NBS has long been regarded as an AT variant. However, early complementation experiments using cell fusion among AT and NBS cells suggested (that) different genetic defects underlie AT and NBS. The genetic localization and subsequent cloning of the gene defective in patients with NBS ultimately separated the two genetic entities AT and NBS. Recent molecular data, however, demonstrate that the *ATM* and *NBS1* gene products may be closely, even directly, linked in the cellular response to DNA damage following ionizing radiation. Thus, this chapter will include some limited information on AT (see Chapter 13) for comparative reasons. In addition to reviewing the phenotypic NBS characteristics as well as the cloning of *NBS1*, recent advances in the characterization of the *NBS1* gene product and its presumed role in various cellular pathways will be summarized.

Some chromosomal instability syndromes are clinically characterized by premature aging. According to the "disposable soma theory" (4,5), aging can be regarded as the process of accumulating somatic damage. Cellular and organismic aging also has been shown to correlate with the telomere metabolism (6) (see Chapter 13). Therefore, this chapter will include information on the known and putative roles of the *NBS1* gene product in repairing cellular damage (i.e., DNA damage and the telomere metabolism), as well as the possible implications for aging processes at the cellular and organismal levels.

II. NBS PHENOTYPES

Classic patients with NBS are characterized by a pleiotropic phenotype reminiscent of and overlapping with AT (2,3,7–10). For both syndromes, a defect in DNA repair, in particular repair of DNA double-strand breaks, has been postulated to explain the pleiotropic clinical and cellular features of these disorders (11,12). However, there are clear distinctions between NBS and AT in clinical parameters, whereas the characteristics of cultured patient cells are remarkably similar. An overview of the clinical and cellular phenotypes in NBS and AT is presented in Table 1.

Table 1 Clinical and Cellular Characteristics of Nijmegen Breakage Syndrome (NBS) and Ataxia-telangiectasia (AT)

Characteristic	NBS	AT
Clinical		
Predisposition to malignancy	++	+
Radiosensitivity	+	+
Immunodeficiency	+	+
Ovarian dysgenesis	+	+
Hyperpigmentation	+	(+)
Growth retardation	+	(+)
Microcephaly	+	−
Distinctive face	+	−
Ataxia	−	+
Cerebellar degeneration	−	+
Ocular telangiectases	−	+
Cellular		
Chromosome breakage	++	+
Radiosensitvity	+	+
Radioresistant DNA synthesis	+	+
Defective cell cycle checkpoints	+	+
Reduced p53 response	+	+
Telomeric shortening	n.d.	+

A. Clinical Phenotypes

1. Craniofacial Features

All patients with NBS present with a characteristic face that was often previously called "birdlike." This facial appearance can be better described as a combination of receding forehead and receding mandible together with a prominent midface. Although these features become more pronounced with age, the lengthened philtrum is obvious even in newborns. In addition, the majority of patients have epicanthal folds, large ears, and sparse hair (2,13).

The facial features are perhaps even more striking, since all patients with NBS are also microcephalic. Most patients are born with microcephaly, but in 25% of patients, this becomes apparent only after a few months. Cranial magnetic resonance imaging (MRI) of 10 patients showed decreased size of the frontal lobes and narrowing of the frontal horns of the lateral ventricles (14,15). Reduced brain growth without cerebellar degeneration is the probable cause of microcephaly in NBS; however, there is also premature fusion of the cranial sutures. Mental retardation is generally mild, with most patients having an IQ within the normal range during early childhood, but with progressive retardation as they grow older. Severe mental retardation is not found, and IQ in the normal range can clearly be found in these microcephalic patients (16).

There are a variety of cutaneous manifestations, including café au lait spots, vitiligo spots, and even cutaneous telangiectasia in a minority of patients. Limited scleral telangiectasia, more characteristic for AT, is observed in a few individuals.

2. Growth Retardation

Patients with NBS are small. Growth retardation is seen in all patients, with the majority in the 10th percentile, even though birth weight and size are generally within normal limits. Short stature is apparent by 2 years of age, and has been reported as trunk shortening rather than lower extremity shortening. Body weight is proportional to body height (9).

3. Immunodeficiency

Owing to a deficiency in the cellular and humoral immune systems, patients with NBS are infection prone. There is, however, considerable variation in the immunological status among patients. Some 10% of patients have normal levels of immunoglobulins, but the majority have deficiencies in humoral immunity. Selective deficiencies of IgA or IgG4, alone of in combination, are common. Agammaglobulinemia has been reported for about one-third of patients with NBS.

Cellular immunity is more consistently deficient in patients with NBS, and reduced proportions of $CD3^+$ and $CD4^+$ T cells are found in most patients. Consequently, the $CD4^+/CD8^+$ ratio is reduced, since levels of $CD8^+$ cells are gen-

erally within the normal range. T-lymphocyte proliferation in response to mitogenic stimuli is reduced in more than 90% of patients (17).

4. Ovarian Dysgenesis

Although ovarian dysgenesis, as evidenced by primary amenorrhea and elevated gonadotropin levels, has been reported in several patients with NBS, the small numbers make the significance of this finding more difficult to assess (7). In AT, gonad dysplasia is also found and correlates with the involvement of the AT gene in meiosis. The same also may be true for the *NBS1* gene (see Sect. IV).

5. Malignancy

Patients with NBS are cancer prone (8). Approximately 40% of patients with NBS develop a malignancy prior to the age of 20 years. In the Polish NBS registry, 18 of 48 patients developed lymphoma by the age of 15 years; thus the risk for this malignancy is increased 1000-fold in this age group (M. Dura, personal communication). The majority of lymphomas are non-Hodgkin's lymphomas (NHLs), primarily diffuse large B-cell lymphomas (DLBLs), which are not characteristic for childhood malignancy (18) (M. Dura, personal communication). DLBL is most frequent after the age of 60 years: Immunoblastic B-cell lymphoma occurs in more than 50% of patients after the age of 65 years; centroblastic B-cell lymphoma has a broad range (19–88 years), but the majority of patients are more than 60 years old. Peripheral T-cell lymphoma (angioimmunoblastic lymphodenopathy with dysproteinemia or AILD) has been described in NBS and, again, is a malignancy of adulthood with a range of 16–84 years and median age of 61 years. The only typical childhood malignancies reported in patients with NBS are three patients with lymphoblastic precursor T-cell lymphoma (T-cell lymphoblastic lymphoma/acute lymphoblastic leukemia, or T-LBL/ALL) and one patient with a medulloblastoma.

Generally speaking, therefore, children with NBS suffer from malignancies otherwise rarely observed in childhood, and this shift toward an adult pattern of malignancy must be an expression of the defective pathway in NBS. Chromosomal instability is a hallmark of NBS, with somatic cells displaying chromatid breaks and chromosomal recombination figures; in T lymphocytes, these often involve chromosomes 7 and 14 with the breakpoints at the loci of immunoglobulin and T-cell receptor genes. Presumably aberrant lymphocyte clones arise from chromosomal reunions, expand, and undergo transformation to produce the lymphomas of patients with NBS. Lymphomas from patients with NBS have not yet been characterized to this extent, but rearrangements involving breakpoints in chromosome 14q32 have been found in patients with T-cell prolymphocytic leukemia with the closely related autosomal recessive disease, ataxia-telangiectasia, in which reunions between chromosomes 14 and 7 are also frequent (19). The particular malignancies found in NBS also can reflect the immunodeficiency of

patients with NBS. Treatment of rheumatism by immunosuppression or immunosuppressive therapy following organ transplantation has been associated with an increased risk of NHL (20,21).

In mice, aging is associated with changes in the antibody response and also with clonal B-cell expansions that can lead to B-cell neoplasia (22), and various changes in the human immune system with age have been reported (23,24). Possibly, the immunodeficiency of patients with NBS is comparable to this age-related compromise in the immune system, thus explaining their pattern of malignancy.

6. Malignancy in Heterozygotes

Based on the frequency of malignancy among relatives of patients with NBS, there may be an increased cancer risk in NBS heterozygotes (25). To examine this possibility, the *NBS1* gene has been examined for mutations in patient DNA extracted from tumors. No increased incidence of *NBS1* mutations was observed in malignant B- or T-cell lymphomas (26–28); however, these analyses looked at children with NHL, and as discussed above, these patients show a different spectrum of NHL subtypes. Several amino acid substitutions have been observed in patients with ALL, indicating a possible role of *NBS1* in the pathogenesis of this malignancy (29). Interestingly, allelic imbalance is frequent at the *NBS1* locus in colorectal carcinoma, a malignancy with an occurence peak after 70 years of age (30). There is no association between *NBS1* mutation and breast cancer (31).

B. NBS Cellular Phenotypes

1. Radiosensitivity

The documented clinical radiosensitivity of patients with NBS is reflected by at least a two-fold increase in cellular hypersensitivity to ionizing radiation (IR) of primary and immortalized NBS cells (2,10). This hypersensitivity is also evident following exposure of NBS cells to bleomycin, streptonigrin, etoposide, camptothecin, and the cross-linking agent mitomycin C, but not to ultraviolet wavelength C (UV-C) (32–34), indicating that DNA double-strand breaks (DSBs), produced through different mechanisms by these agents, are the crucial molecular lesions for cell killing in NBS. However, the mechanism leading to NBS cell death following induction of DSBs has not been determined (e.g., no evidence for increased apoptosis has been described). Indeed, one report even showed diminished IR-induced apoptosis in leukocytes from a patient with NBS (35).

Hypersensitivity to DSB-producing agents and the concomitant observation of increased spontaneous and IR-induced chromosomal aberrations resulted early on in the concept that NBS cells are deficient in either repair and/or signaling of DSB-damaged genomic DNA or DNA-damage–dependent cell cycle regulation.

2. Cell Cycle Checkpoint Deficiencies

Following the induction of DSBs by IR, replicating cells are transiently arrested in cell cycle progression upon reaching cell cycle checkpoints in the G1, S, or G2 phase. It is widely believed that the transient cell cycle arrest serves to repair the DSBs prior to replication of damaged DNA templates and mitosis. Thus, cell cycle checkpoints may play crucial roles in determining the fate of damaged cells, as is exemplified in certain yeast mutants (36). In accordance with this hypothesis, cell cycle checkpoint deficiencies in G1, S, and G2 have been demonstrated conclusively in radiosensitive AT cells. The G1 arrest following IR is mainly dependent on the ataxia-telangiectasia mutated (ATM)/p53 pathway (37). Upon IR, p53 is phosphorylated by the kinase activity of ATM and thus stabilized in the G1 phase. Accumulation of the p53 protein, in turn, induces p21 transcription and subsequent G1 arrest. Several studies have shown that, similar to AT cells, NBS cells exhibit delayed and reduced accumulation of p53 and reduced p53-dependent activation of p21 and other genes in response to IR (38–42). However, although the accumulation of p53 is clearly blunted following IR, a normal G1 arrest has been observed in all NBS fibroblast lines tested (40,42). In contrast, the G1 arrest was defective in one NBS lymphoblastoid line (33) and intermediate among normal and AT cells in other lymphoblastoid NBS cell lines (41).

Inhibition of DNA replication (i.e., the S phase arrest) is a very fast response in actively replicating cells treated with IR (43,44). This decrease in the rate of DNA synthesis typically follows a biphasic kinetic curve, with a first steep component that presumably reflects blockage of initiation by preventing firing at origins of replication, and a second shallow component due to inhibition of chain elongation. In NBS cells, inhibition of DNA synthesis following IR is greatly reduced, a phenomenon termed *radioresistant DNA synthesis* (RDS) (45,46). RDS has been observed in virtually all NBS cell lines tested so far (for one exception, see ref. 42) and is a distinctive phenotype of cells from patients with NBS, AT, and ataxia-telangiectasia–like disorder (ATLD)—the latter being attributable to mutations in the *hMRE11* gene (47). Molecular analyses have shown that besides *NBS1*, *hMRE11*, and *ATM*, other genes may be involved in the regulation of RDS, such as an unknown gene on human chromosome 4 (48), and components of a calmodulin-dependent regulatory cascade (49). Recovery from radiation-induced DNA synthesis inhibition is supported by the catalytic subunit of the DNA-dependent protein kinase (DNA-PKcs) but not Ku80, DNA ligase 5, or XRCC4 (50), illustrating the high complexity of pathways controlling DNA synthesis in response to IR.

As for the G1 arrest, data on IR-induced G2 arrest are somewhat controversial. Whereas Yamazaki (40) and Girard (42) found no obvious defect in IR-induced G2 arrest in NBS fibroblasts, Ito (51) has described a prolonged G2 phase compared with control cells. Antoccia (52) also has found differences in the IR-induced G2 arrest among NBS and AT as well as normal lymphoblastoid cell lines.

There may be several reasons for the inconsistencies in experimental data relating to the IR-induced G1 and G2 arrest in NBS cells. Different experimental results may depend on the particular cell type used, on their different genetic background, with respect to the *NBS1* or secondary modifying mutations, as well as on different experimental setups. Clearly, however, RDS is a major hallmark and key diagnostic feature of the cellular NBS phenotype.

3. Genomic Instability

Spontaneous chromosomal instability in cells from patients with NBS is evident in T lymphocytes, with frequent rearrangements being observed at the loci of immunoglobulin and T-cell receptor genes on chromosomes 7 and 14 (2,9). In cultured (and immortalized) NBS lymphoblast and fibroblast cell lines, spontaneous chromosomal instability is characterized mainly by an elevated frequency of chromatid breaks occurring at random sites (41,42). In addition, telomeric fusions leading to dicentric chromosomes have been observed with increased frequency in metaphases from NBS cells.

Following irradiation in the G1 phase, the frequency of IR-induced aberrations is drastically increased in cultured NBS cells compared with normal cells. For example, following 2-Gy IR, the frequency of total chromosomal aberrations shows a 2.4- to 3.9-fold increase in NBS cells as compared with normal cells (41). Interestingly, the spectrum of aberration types is different in NBS. Whereas normal cells display mainly chromosome-type aberrations following IR, metaphases from NBS cells are characterized by up to an 8- to 13-fold higher amount of chromatid-type aberrations compared with normal cells (17,41,45,52,53). Concomitant with the increased aberration frequencies, the percentage of damaged metaphases also is increased about two fold in NBS versus normal cells (41).

4. DNA Repair Defect

In contrast to the obviously increased numbers of spontaneous and IR-induced chromosomal aberrations, using pulsed-field gel electrophoresis (PFGE) demonstrates no (34,54), or at best very little (42), deficiency in NBS cells regarding the kinetics and efficiency of DSB rejoining following high doses of IR. The same holds true for AT cells.

Premature chromosomal condensation (PCC) has been used to detect nonrepaired DNA damage that leads to chromosomal breaks. In this assay, two primary NBS fibroblast lines showed slightly elevated frequencies of extrachromosomal fragments 24 hrs following IR compared with normal cells, indicating aberrant repair in NBS (42). In addition, the formation of micronuclei that arises when acentric fragments are generated was found to be slightly higher in untreated NBS (as well as AT) cells compared with normal cells. Following IR, hypersensitivity in micronuclei formation was significant only at higher doses (42). It has

been reported that dinucleotide repeat sequences are stable over time in NBS cells, indicative of normal DNA mismatch repair systems and normal replicative fidelity in NBS (33). Also, normal DNA-PKcs and Ku levels, as well as normal DNA-PKcs activity in vitro have been reported in NBS protein extracts (33), suggesting that this pathway of DNA repair was normal in cells from a NBS patient.

Collectively, the data reflect an obvious inconsistency between apparently normal, or at best slightly deficient, DNA DSB repair and high levels of chromosomal aberrations in NBS. This may be due to limitations in the method used, since small numbers of nonrepaired DSBs, almost undetectable in PFGE, ultimately may lead to chromosomal aberrations and resultant IR hypersensitivity. In this regard, it has been reported that few or even one unrepaired DSB may be lethal for an individual cell (55). Alternatively, this disparity may reflect the possibility that the efficiency of DSB rejoining, per se, is not affected in NBS but rather the fidelity of DSB joining may be error prone. Finally, multiple independent pathways of nonhomologous DSB repair may have redundant and overlapping functions, thus masking the deficiency of the *NBS1* pathway in terms of DSB-rejoining efficiency.

5. Phenotypic Correlations

Although cell cycle deficiency is expected to play a causative role in chromosomal instability and radiation sensitivity, recent experimental data suggest that cell cycle checkpoints may not be a crucial determinant of this phenotype in AT (56–58) and NBS. Girard et al. (42) have demonstrated that primary fibroblasts from different patients with NBS, all of which share the same common founder mutation, display different RDS deficiencies and IR-induced cell cycle checkpoints, while retaining the hypersensitivity to IR regarding cell survival and DNA repair assays. Moreover, an increase in chromosomal aberrations and decreased DNA repair following IR have been demonstrated in noncycling cells, thus ruling out the possibility that cell cycle deficiencies may contribute to IR-sensitive phenotypes. Likewise, it has been demonstrated that the RDS phenotype is not solely responsible for IR sensitivity and chromosomal instability in NBS and AT cells (48,57). Some investigators therefore have suggested that despite their limited detection, defects in DNA repair rather than in cell cycle checkpoints may be the primary reason for IR sensitivity in NBS and AT cells (56).

III. NBS1 GENE

A. Cloning and Structure of *NBS1*

The gene mutated in patients with NBS was mapped to chromosome 8q21 by linkage analysis (59) and complementation studies (60) and was finally cloned by the positional approach (61–63). The gene was termed *NBS1*. It consists of 16 exons

and spans approximately 50 kb of genomic DNA (2). The *NBS1* mRNA may be polyadenylated at two different sites. Owing to this, two mRNA species approximately 2.4 kb and 4.4 kb in size were detected that were ubiquitously expressed in all tissues analyzed (61,64). Increased levels of the 2.4-kb mRNA species have been detected in testes and ovary (61,62,64), indicating a potential role of *NBS1* in cells undergoing meiotic recombination. The *NBS1* cDNA encodes a protein of 754 amino acids, termed *nibrin*. Sequence analysis of nibrin reveals no obvious enzymatic motifs. In the N-terminus, a breast cancer carboxy-terminal domain (BRCT), first described in the *BRCA1* gene, and a fork-head–associated domain (FHA), named according to the transcription factor family, were detected (Fig. 1A). The lack of enzymatic motifs and the presence of BRCT and FHA domains (expected to be involved in protein–protein interactions) points to a putative signaling function for nibrin.

In addition to the identification of *NBS1* by positional cloning, simultaneous identification was made by Carney and colleagues (64) during a search for the human homologues of yeast genes involved in DSB repair. A human protein, p95, was isolated as a member of a trimeric protein complex including the human homologues of yeast Mre11 and Rad50 proteins. The yeast Mre11, Rad50, and Xrs2 proteins had been implicated previously in DSB repair, suggesting that p95 represented the human functional orthologue of Xrs2. A role for hRad50 and hMre11 in DSB repair in human cells was elegantly shown by the visualization of repair

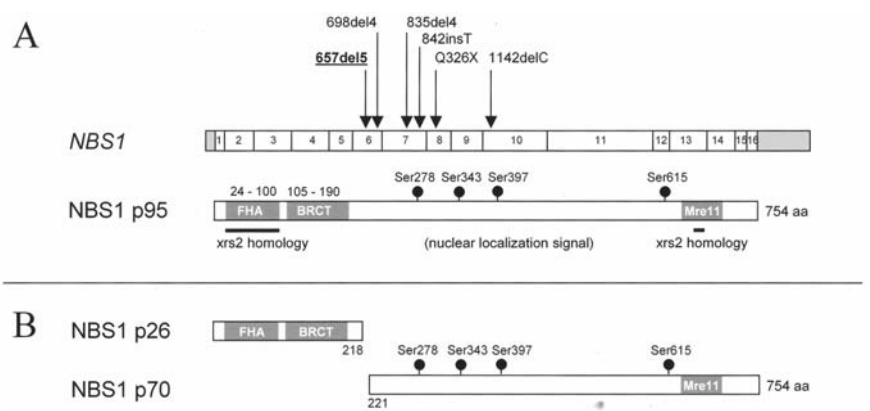

Figure 1 Structure of the *NBS1* gene and protein(s). (A) The *NBS1* gene and mutations are depicted, as well as the derived wild-type p95 protein and its regions homologous to yeast Xrs2. Functional domains of p95 include the FHA-, BRCT-, and Mre11-binding domains. Serine residues described as being potential phosphorylation targets are shown. (B) Truncated p95 proteins are shown that arise in patients carrying the 657del5 mutation (According to and adapted from Ref. 69.)

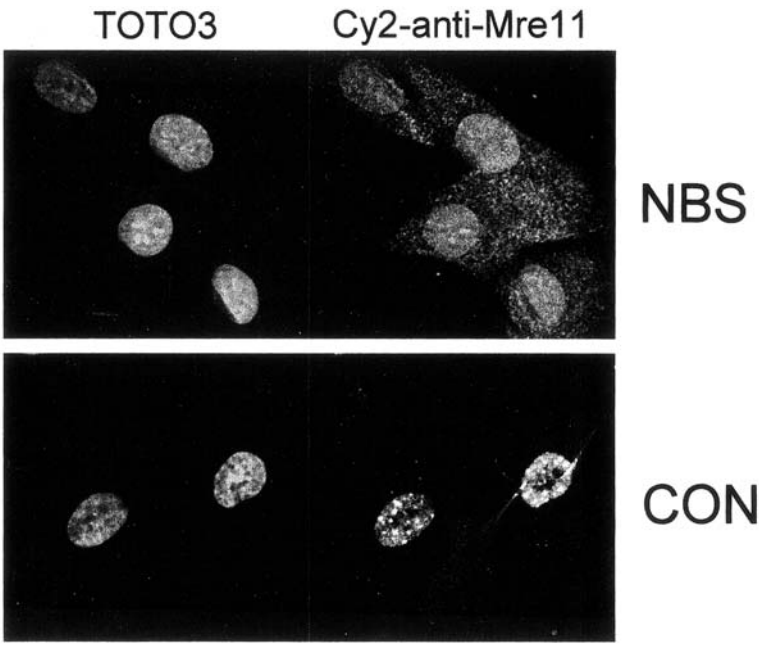

Figure 2 IRIF in primary fibroblasts detected by anti-Mre11. Control and NBS cells were irradiated with 12 Gy and 8 hrs later fixed and stained with primary rabbit anti-Mre11 and secondary Cy2 conjugated, antirabbit Ig antibodies. Cells were counterstained with the DNA stain TOTO3. Irradiated control cells show discrete, bright nuclear foci, whereas NBS cells show a diffuse cytoplasmic staining.

as nuclear foci, termed IRIF (ionizing radiation-induced foci), formed in response to treatment with DSB-inducing agents (65–67). Nuclear hMre11/hRad50 foci formation was absent in cells from patients with NBS (Fig. 2), strongly supporting a functional role of p95 in the relocalization of hMre11 and hRad50 to the nucleus and formation of the trimeric DSB repair complex, thus demonstrating the equivalence of p95 and nibrin.

The formal proof for *NBS1* being the defective gene that causes NBS syndrome has been accomplished by complementing NBS cells with an intact *NBS1* expression cDNA. Reintroduction of intact nibrin into NBS cells rescued nuclear hRad51/hMre1/nibrin foci formation and cellular radiation sensitivity (51,68).

B. *NBS1* Mutations

Patient analyses have revealed a 5-bp truncating mutation, 657del5, in over 90% of all patients with NBS analyzed to date, all of whom carry a conserved marker

haplotype (13,61). Apart from this common founder mutation, five additional truncating mutations have been identified in patients with other distinct haplotypes (see Fig. 1A). These rare mutations are clustered between nucleotides 657 and 1147, and all known mutations are predicted to truncate the nibrin protein downstream of the N-terminal BRCT and FHA domains (69).

Initial analyses of NBS patient cells pointed to the notion that the naturally occurring *NBS1* mutations represent a "null" phenotype, since no nibrin protein or truncated nibrin versions had been detectable with the available antibodies in Western analyses. However, a recent report has demonstrated that the *NBS1* 657del5 mutation is a hypomorphic defect (70). Cells from patients with NBS carrying this founder mutation were found to express a truncated N-terminal nibrin variant of 26 kD (see Fig. 1B). The truncated p26 protein arises upon termination of translation at a stop codon 15 amino acids downstream of the deletion, which is encoded by the alternative reading frame. Even more surprisingly, an additional 70-kD protein was detected by immunoprecipitation analyses using antinibrin antisera, representing the C-terminal part of nibrin (see Fig. 1B). The p70 protein was detected only in lymphoblasts, but not in fibroblasts, from patients carrying the 657del5 founder mutation. Translation of the 70-kD protein is initiated from two cryptic ATG start codons upstream of the deletion locus that are brought into frame following the deletion and frame shift event. A similar mode of expression may explain the fact that in protein extracts from cells of another NBS mutation, 835del4, a 60-kD N-terminally truncated nibrin protein species was detectable.

In summary, it turns out that at least some cells from patients with NBS may express a short N-terminal nibrin fragment as well as a longer C-terminal nibrin fragment. Partial functional activity of these truncated mutant nibrin species may diminish the severity of the clinical phenotype in patients with NBS (70), whereas mice or cell lines completely inactivated for *NBS1* (71), or the genes encoding its functional interacting partners *MRE11* (72,73) and *RAD50* (74), are not viable. It might be expected that the nibrin fragments also are produced in the cells of heterozygous carriers and disturb the function of the intact protein by competition in a dominant-negative fashion. This may have consequences for the health of heterozygous carriers.

Recent clinical cases that are identical or very similar to NBS have been described that show no apparent mutations in the *NBS1* gene (75–77). This situation has provoked the possibility that mutation of other genes, presumably located in the *NBS1* pathway, might impose an NBS phenotype on respective patients.

C. Animal Models

Several attempts to generate mice deficient in the *NBS1* homologue *Nbn1* by targeted disruption failed owing to early embryonic lethality (71). It seems plausible that nibrin may mediate functions apart from DSB repair that are essential during

cell proliferation (71). Such nibrin functions are supported by the finding that expression of nibrin in murine development is enhanced in highly proliferating tissues (see Sec. III.F). The generation of conditionally targeted Nbn1 knockout mice should ultimately clarify the role of nibrin in cell proliferation.

The lack of viability of nibrin-deficient mice supports the hypothesis that patients with NBS and their derived cell lines may not result from complete inactivation of *NBS1*. Instead, and in accordance with the detection of truncated nibrin proteins in patients with NBS, hypomorphic truncation mutations may allow viability.

D. Regulation of *NBS1* Expression and Activity

NBS1 expression seems to be indispensable for viability, and nibrin certainly plays a crucial role in cell survival following DNA damage, implying that regulation of *NBS1* expression and activity is an important issue.

On the RNA level, *NBS1* may be regulated by several mechanisms. Increased *NBS1* mRNA levels have been detected in testes and ovary (61,62,64), suggesting that tissue-specific regulation of the basal RNA level may occur. Through the use of different polyadenylation sites, two specific mRNA species of 2.4 and 4.4 kb have been described. Interestingly, the larger mRNA predominated in most tissues, whereas the smaller 2.4-kb transcript was more common in high expressing testes (61), suggesting differential tissue-specific expression of the two transcript sizes. In the course of cloning an *NBS1* cDNA, Paul and Gellert (78) have found an *NBS1* mRNA species lacking exon 13, suggesting that alternative splicing may contribute to regulation of specific *NBS1* activities.

Basic *NBS1* mRNA expression thus may be regulated by several mechanisms; however, no accumulation of *NBS1* mRNA has been observed following 5-Gy irradiation (61), implying that *NBS1* expression and activity in response to DNA damage may not be induced on the transcriptional level.

In Western analyses, no changes of nibrin protein levels have been observed either in specific cell cycle phases or following irradiation of cells. Taken together, it thus seems plausible that regulation of nibrin function is preferentially exerted on the protein level through modulation of activity.

E. Nibrin Phosphorylation by *ATM*

Recent reports have independently demonstrated IR-induced phosphorylation of nibrin in an ATM-dependent manner (79–82). IR-induced phosphorylation of nibrin is a rapid process carried out by the kinase activity of ATM that specifically occurs in response to IR but not following treatment of cells with other DNA-damaging (UV light) or cell cycle perturbing (hydroxyurea) agents. The latter responses may well be carried out by the ATM homolog ATR (ATM-and Rad3-re-

lated). It is thus conceivable that DSBs, generated following IR, are the lesions recognized by some molecular sensor that lead to ATM activation and subsequent nibrin phosphorylation. Nibrin itself, either alone or in its trimeric complex with hMre11 and hRad50, is not required to activate the ATM kinase upon IR. This rules out the possibility that nibrin or the trimeric complex is the sensor for DSBs that might lead to activation of ATM and, in a feedback loop, phosphorylation of nibrin.

Although different research groups have shown at least four different serine residues that can be phosphorylated by ATM in response to IR, all of the groups demonstrated phosphorylation of Ser343 (see Fig. 1A). It is not known yet whether the reported phosphorylation sites are collectively involved in regulation of the same phenotypes, or whether phosphorylation of specific residues, or combinations thereof, might contribute to specific functional aspects of nibrin. In functional analyses, NBS cells were reconstituted with mutant forms of *NBS1* that cannot be phosphorylated at ATM-dependent serine residues. Lack of phenotypic complementation in these cells has demonstrated that Ser343 phosphorylation is necessary for regulation of RDS, IRIF formation, and cellular radiosensitivity. In particular, phosphorylation of Ser343 is necessary for inhibition of DNA synthesis following IR, and its absence leads to the RDS phenotype in NBS (80,81). The preceding data confirm earlier suggestions of ATM-nibrin interaction, which were based on the fact that IRIF formation is reduced in AT cells (64). Biochemical experiments have shown that in addition to ATM-dependent phosphorylation of nibrin, hMre11 and hRad50 are phosphorylated in response to DNA damage independent of specific cell cycle phases (83). In two independent NBS cell lines, phosphorylation of hMre11 was not detected, suggesting that nibrin is essential for this modification, which may precede and trigger IRIF formation.

Nibrin phosphorylation by ATM thus represents a (presumably direct) molecular relationship among the ATM and nibrin proteins that trigger common molecular mechanisms responsible for the overlapping clinical and cellular characteristics of AT (ATLD) and NBS.

F. Expression of *Nbn1* in Murine Development

In a different experimental approach to try to understand the pleiotropic phenotype in patients with NBS, expression of the *NBS1* homologue *Nbn1* has been analyzed during murine development (84,85). During all stages of development, the whole embryo showed low *Nbn1* expression. Upon completion of organogenesis between 12.5 and 14.5 days post conception, *Nbn1*expression becomes more distinct and is pronounced in regions of high proliferative activity (i.e., brain, liver, kidney, and gut). Interestingly, there is complete overlap for expression of *Nbn1*and *Atm* in these tissues. *Nbn1*expression, but no *Atm* expression, also has been found in muscle and connective tissue sheets.

In adult animals, the proposed function of *Nbn1* in DNA repair and/or recombinational processes is supported by its high expression in mature testis, spleen, and thymus. Surprisingly, the highest *Nbn1* expression has been detected in the large Purkinje cells of the cerebellum, although patients with NBS do not develop ataxia as do patients with AT due to presumed Purkinje cell death. In the brain, *Nbn1* and *Atm* expression in the cerebral cortex, hippocampus, and cerebellum is very similar to the expression pattern of rag1 (86) and DNA-PKcs (87), two other genes involved in DSB processing during DNA repair or recombination. In testis, the timing of *Atm* and *Nbn1* expression during subsequent stages of spermatogenesis is identical. Since female patients with AT and NBS develop ovarian dysgenesis and male patients with AT prove to be infertile, it is highly probably that *Atm* and *Nbn1* play a role in meiotic processes. High *Nbn1* expression in the mature spleen might easily be explained by its potential involvement in repair or recombinational processes for V(D)J recombination and isotype class switching of B lymphocytes, since both processes require the processing of DSBs. However, the exact localization of *Nbn1* expression signals within the spleen is not confined to the germinal centers of the white pulp, the site of B lymphocyte maturation, but to a few highly positive cells within the red pulp. Interestingly, this unexpected expression pattern also has been observed for DNA-PKcs, the enzyme responsible for V(D)J recombination (87). In the adult thymus, the organ of T-lymphocyte differentiation, prominent *Nbn1* expression has been found to be restricted to a few highly positive cells of the medulla, where final maturation and negative selection of lymphocytes occur.

In summary, the expression patterns of *Nbn1* in tissues of developing as well as adult mice support a role of *Nbn1* in the processing of DSBs. Apart from sites of physiological DSB occurrence, like the testis, thymus, and spleen, *Nbn1* also has been detected in tissues and organs that are not known to undergo DSB repair or recombination. We thus suggest that, in accordance with the inviability of *Nbn1*-deficient mice, *Nbn1* may have additional functions during (murine) development, such as a role in cellular proliferation of the brain, muscle, and intestine, as well as unknown functions in mature Purkinje cells of the cerebellum.

G. Homology Lessons from Yeast, Chicken, and *Xenopus*

The nibrin protein shares weak sequence homology with Xrs2 from *Saccharomyces cerevisiae,* a protein that forms functional trimeric complexes with the highly homologous yeast counterparts of hRad50 and hMre11 (66). Overall, 87 residues of the N-terminus (amino acids [aa] 24–111) show 46% similarity and 28% identity of amino acids (64); an additional small region of high homology to yeast Xrs2 has been found at aa 678–694 of the human protein (88). Despite its relatively weak sequence homology, nibrin is regarded as being the functional ortholog of Xrs2 based on the trimeric complex formation with hMre11 and hRad50.

Analyses of yeast mutants have demonstrated that the trimeric Rad50/Mre11/Xrs2 (M/R/X) complex plays a crucial role in mitotic and meiotic homologous recombination and recombinational repair (89–95), repair by nonhomologous end joining (NHEJ) (96–101), maintenance of the telomere length (102–104), and cell cycle regulation (105,106).

Apart from the fact that similar trimeric complexes form in yeast and human cells, a significant overlap in functional properties of its components exists in respective human and yeast mutants. Deletion of *xrs2*, *mre11*, or *rad50* imposes a slow growth rate on the cells, supporting a functional role in DNA replication. *Xrs2*-deficient yeast cells are hyperrecombinant and display IR hypersensitivity (107). In an assay for detection of gross chromosomal rearrangements, yeast strains deficient in either *xrs2*, *mre1*, or *rad50* show approximately a 600-fold increase in the rate of accumulating chromosomal rearrangements (108), a phenotype correlating with the chromosomal instability seen in cells from patients with NBS. Concomitant with IR hypersensitivity and hyperrecombination, two DSB repair pathways are partially defective in *Xrs2*-deficient yeast cells, namely, gene conversion and single-strand annealing, the latter using microhomologies of DNA strands (109). Xrs2 is indispensable for meiotic recombination in yeast, since the creation and resection of meiotic DSBs depend on the M/R/X complex as well as a few other proteins (110). Interestingly, Xrs2 is a target for in vitro phosphorylation by Tel1 (111), a structural and functional homolog of the ATM kinase (112–114). This closely resembles the situation in human cells where nibrin is phosphorylated by ATM.

The vertebrate M/R/N complex seems also to be involved in homologous recombination-based DSB repair, as analyzed in chicken cells (73). Targeted disruption of endogenous *Mre11* and subsequent conditional expression of chicken *Mre11* in DT40 cells allowed the functional analysis of this protein with regard to different DSB repair mechanisms. Chicken cells devoid of Mre11 protein show increased IR sensitivity and accumulated chromosomal aberrations within a few days followed by G2/M arrest and cell death. Although NHEJ, and specifically Ku70-dependent DSB repair, seemed unaltered after *Mre11* disruption, the capacity to repair both IR-induced as well as spontaneous DSBs by homologous recombination is reduced in these cells. Based on these data, the Mre11 protein is clearly involved in HR-mediated DSB repair in chicken cells, presumably together with its functional interacting partners nibrin and Rad50.

Cell-free extracts of *Xenopus* oocytes, which can carry out DNA replication and mitosis in vitro, show dramatic accumulation of DSBs in genomic DNA when immunodepleted of the *Xenopus* Mre11 homolog (115). The ATM-dependent S phase checkpoint was unaffected, suggesting that Mre11 is particularly invovled in the repair of DSBs occurring during DNA replication. Such a central role in the maintenance of genomic integrity could explain the requirement for the M/R/N complex for cell viability (see Sec. III.C).

IV. MOLECULAR ANALYSES OF NIBRIN FUNCTION

A. Nibrin Is Associated in a Trimeric Complex with hMre11 and hRad50

Similar to the situation in yeast, the hMre11 and hRad50 proteins are found tightly associated in human cells, presumably interacting directly and independently of nibrin (65,66,116). hRad50, a coiled-coil structural maintenance of chromosomes (SMC) protein, has ATP-dependent DNA-binding activity (117). hMre11 is expected to have structural DNA-binding activities as well as catalytic activities including single-stranded endonuclease and DNA double-stranded 3′–5′ exonuclease activity (110,116,118–120). In normal human cells, nibrin is localized in the nucleus in tight physical association with hMre11 and hRad50 (67).

Following induction of DSBs in IR-treated cells, hMre11 rapidly binds to damaged DNA within 30 min following irradiation (121). At later times (8–12 hr) following irradiation, immunocytological staining reveals very bright IRIF foci containing hMre11, hRad50, and nibrin in most of the irradiated fibroblasts (64,67). Foci containing this complex are also occasionally visible in untreated cells. However, following induction of DSB by IR, the average number of foci per cell and the frequency of foci-positive cells clearly increase. These late persisting foci may represent sites of ongoing DNA DSB repair or of unresolved DNA breaks.

In NBS cells, which lack the intact wild-type nibrin but may still contain N-terminal and C-terminal nibrin protein fragments, the interaction of hMre11 and hRad50 is still occurring, but the dimeric complexes are largely confined to the cytoplasm and cannot form nuclear IRIF foci. IRIF formation depends on the Mre11 and FHA/BRCT domains as well as the Ser273 and Ser343 phosphorylation sites (see Sec. IV.C). However, IRIF formation seems not to be essential for cellular survival following IR.

B. In vitro Enzymatic Activities of the hMre11/hRad50/Nibrin (M/R/N) Complex

In vitro, nibrin binds cooperatively with hMre11 and hRad50 to DNA and forms distinctive DNA/protein complexes (78). hMre11 possesses 3′–5′ dsDNA exonuclease activity as well as endonuclease activity in vitro (116,118), and both activities are greatly increased in the presence of hRad50 (118). The preferred substrate specificity of the hMre11/hRad50 endonuclease complex is extended from hairpin loops (containing mismatched nucleotides) to fully paired hairpins when nibrin is added, releasing products with 3′ overhangs of one or two nucleotides (78). In addition to the nuclease activities, the trimeric complex is able to partially unwind duplex DNA from a 3′ overhang in an ATP-dependent manner. ATP is presumably bound to the trimeric complex via the hRad50 protein,

since mutation of the Walker A and B ATP-binding motifs in hRad50 abolishes the ATP-dependent reactions.

Taken together, biochemical analyses suggest that within the trimeric complex, hMre11 is the only catalytically active nuclease, and that hRad50 and nibrin serve as signaling and/or modifying factors of this enzyme. These in vitro data might argue for an enzymatic function of the M/R/N complex in processing of DSBs rather than plain structural binding. However, this has not been demonstrated yet in vivo. Surprisingly, experimental data from yeast show that nuclease-negative mutations of yeast Mre11 are still proficient in end joining of DSBs (110,120) and homologous recombination-based DSB repair (93), although their survival after DNA damage is reduced. Thus, the in vivo functional relevance of the enzymatic properties of M/R/N, which have been highly conserved in evolution, remains to be conclusively demonstrated.

C. Functional Domains of Nibrin

1. hMre11-Binding Domain

The C-terminal portion of nibrin has been shown to contain the hMre11-binding domain by various approaches. Tauchi et al. (88) and Desai-Mehta et al. (122) have demonstrated that codons 665–693 or 653–669, respectively, of nibrin are essential for hMre11 binding by yeast two-hybrid analyses and expression of the respective *NBS1* cDNA mutants in NBS cells. Following expression of mutant cDNAs, immunoprecipitation of nibrin mutants with hRad50 only occurred when the hMre11-binding domain was present. Thus, expression of the hMre11-binding domain of nibrin seems to be crucial for formation of the triple complex. Concomitantly, expression of full-length *NBS1* or mutants containing the hMre11-binding domain was accompanied by nuclear localization of hMre11 and hRad50 in NBS cells, whereas expression of mutants lacking the hMre11-binding domain led to predominantly, if not exclusively, cytoplasmic localization of hMre11 and hRad50 (88,122). Confounding the experimental data above, the amino acid stretches of nibrin described to bind hMre11 have also been shown to share sequence homology between human, mouse, and chicken homologs, a smaller part of the sequence is also identical to yeast Xrs2 (88).

Following IR, nuclear IRIF containing all three proteins were only observed when the hMre11 domain as well as the FHA/BRCT domains were expressed in NBS cells. However, formation of nuclear nibrin foci without hMre11 has also been reported in a C-terminally truncated nibrin mutant following IR (122). NBS cells expressing the Mre11-binding domain of nibrin show restoration of radiation resistance in colony survival assays, suggesting that the nuclear localization of all three proteins—nibrin, hMre11, and hRad50—is essential for radiation resistance.

Taken together, it seems that physical binding of hMre11 through the binding domain of nibrin is essential for recruiting hMre11 to the nucleus, for IRIF formation, and for normal survival following radiation exposure.

2. BRCT and FHA Domains

BRCT domains are expected to mediate protein–protein interactions, and they are found in many proteins that are involved in the cellular response to DNA damage. For example, BRCA1, XRCC1, DNA ligase 4, PARP, and 53BP1 share a common BRCT domain (123). Interestingly, the p53-binding protein 53BP1 also forms nuclear foci in response to induction of DSBs that colocalize to M/R/N IRIF. However, 53BP1 foci formation seems to be independent of ATM and nibrin, since they are readily found following IR in respective cell mutants (124).

Expression of *NBS1* mutants lacking the FHA and/or BRCT domain also resulted in the nuclear localization of the triple complex. However, upon IR, no foci formation was observed in these cells (88). Since these cells were also complemented in their radiation sensitivity, the FHA and BRCT domains might not be essential for radiation resistance or DNA repair (88), implying that nuclear localization of the triple complex but not foci formation following IR may be a crucial determinant in radiation resistance.

3. Nuclear Localization Domains

In studies using overexpression of N-terminally and C-terminally truncated *NBS1* cDNAs, it has been shown that despite deletion of the BRCT/FHA or hMre11-binding domains, the mutant nibrin protein itself still localizes to the nucleus. It thus has been postulated that in the middle part of the protein, nuclear localization signals may occur that direct nibrin, independent of its functional binding partner hMre11, to the nucleus. Indeed, two potential nuclear localization signals were found in the middle part of nibrin, one of which is highly conserved in the mouse as well the *Drosophila* homolog of nibrin (122).

D. Association of Nibrin with BRCA1

Another participant in the functional cellular response to IR seems to be BRCA1. Zhong et al (125) have demonstrated that BRCA1 is associated with the M/R/N complex based on its temporal and spatial colocalization in IRIF. The formation of IRIF is dramatically reduced in breast cancer cells carrying a homozygous BRCA1 mutation, whereas ectopic expression of wild-type, but not mutated, BRCA1 restores IRIF formation. Restoration of IRIF formation by wild-type BRCA1 renders the cells less sensitive to the DNA-damaging agent methyl methanesulfonate (MMS). Taken together the data suggest that BRCA1 is involved in mediating the cellular responses of M/R/N to DNA damage.

In another study, BRCA1-associated proteins were biochemically characterized following immunoprecipitation and mass spectrometry (126). BRCA1 was found to associate with numerous DNA-repair proteins including ATM, BLM, MSH2, MSH6, MLH1, RCF1, -2,-4, and the M/R/N trimeric complex, forming a large multiprotein complex termed *BASC* (BRCA1-associated genome surveillance complex). In agreement with the study by Zhong (125), BRCA1 was found to colocalize with the M/R/N complex to large nuclear foci; however, these investigators demonstrated that M/R/N foci could form despite the absence of BRCA1. The investigators suggest that the BASC multiprotein complex may represent a molecular sensor of abnormal DNA structures and/or be a regulator of the postreplication repair process (126). Indeed, such a role has been demonstrated for BRCA1 itself. BRCA1 strongly binds to branched DNA structures and inhibits the exonuclease activity of M/R/N in vitro, presumably by competing for binding sites at the DNA (127).

E. Nibrin and V(D)J Recombination

V(D)J recombination is the event leading to the generation of IgG and T-cell receptor diversity through site-specific recombinational processes. It is initiated by the recombination activating gene 1 (RAG1) and RAG2 proteins, which introduce DSBs among immunoglobulin and T-cell receptor (TCR) coding gene segments and flanking recombination signal sequences. RAG-mediated DNA cleavage thus generates two blunt signal ends and two covalently closed coding hairpin ends (128). Resolution of the V(D)J ends is accomplished by components of the DSB repair machinery (129).

Three independent aspects suggest the possible involvement of nibrin in V(D)J recombination. First, the M/R/N triple complex has been shown to cleave fully paired DNA hairpins in vitro by an endonuclease activity of hMre11. This reaction requires ATP-binding by hRad50 and is dramatically increased by the presence of nibrin. Interestingly, fully paired hairpin structures occur as necessary intermediates during V(D)J recombination. Second, patients with NBS suffer from elevated levels of chromosomal translocations apparent in isolated lymphoblastic cells, in particular those involving V(D)J recombination sites of the IgG and TCR genes on chromosomes 7 and 14. These common chromosomal translocations may be regarded as the consequences of inaccurate V(D)J recombination events. Third, the *S. cerevisiae* homologs of the trimeric complex, Mre11, Rad50, and Xrs2 are involved in nonhomologous end joining (NHEJ) in yeast. So far, all known DNA-repair components that are essential for NHEJ also have been found to be necessary for the DNA-joining step in V(D)J recombination.

In three recent reports, the possible involvement of nibrin in V(D)J recombination has been addressed. Chen et al. (130) have shown by immunofluorescence analysis that 20% of freshly isolated thymocytes displayed intense staining with

nibrin in addition to γ-H2A-X. The majority or fresh thymocytes showed one discrete immunofluorescent spot, whereas IR of cells produced multiple foci dispersed throughout the nucleus. The nibrin foci were restricted to immature $CD4^+CD8^+$ double-positive thymocytes, a fraction of which actively undergoes rearrangement of TCR genes. In contrast, mature single-positive $CD4^+$ populations showed diffuse nuclear staining of nibrin and H2A-X. Formation of nibrin foci in immature thymocytes required RAG cleavage of DNA and was reduced upon termination of RAG expression. Furthermore, nibrin foci were located to the TCRα alleles, the sites of active V(D)J recombination. Since V(D)J coding-end intermediates are extremely short lived and are rare in wild-type mice (131), it is assumed that nibrin foci may form within the postcleavage complex and remain associated with unresolved signal ends before they are joined (132,133). Alternatively, nibrin foci may persist as "footprints" on already completed recombination sites.

Despite the clear nibrin foci localization studies, the analyses of V(D)J recombination in NBS patient cells detected no deficiency in NBS. Sequencing revealed normal immunoglobulin heavy chain rearrangements in an NBS lymphoblastoid cell line (134). Using extrachromosomal V(D)J recombination substrates, the V(D)J recombination frequencies and the quality of signal and coding joining have been found to be normal in cells from a patient with NBS (54). In addition, no differences were detected in the CD3 sequences of endogenous Ig λL and κL chain gene loci that had been cloned from peripheral blood lymphocytes of a patient with NBS. In summary, it appears as if nibrin specifically localizes to V(D)J recombination sites in immature thymocytes, but the molecular mechanism of V(D)J recombination seems to be normal in patients with NBS.

F. Nibrin and Telomeres

Proper maintenance of the duplex array of telomeric TTAGGG repeats at mammalian chromosome ends requires counteraction of the loss of terminal sequences during DNA replication, the so-called "end replication problem" (135). The addition of telomere sequences in replicating cells may be accomplished through either the enzyme telomerase (136) or an alternative mechanism termed ALT (alternative lengthening of telomeres) (137). Failure of proper telomere maintenance may contribute to aging and cancer (6). However, telomere ends must be masked in the cells to be distinguishable from random (or mutagen-induced) DNA breaks that activate ATM/p53-dependent DNA damage checkpoints and the DNA repair machinery. Such capping of telomere ends has been described as occurring through binding of the TRF1 and TRF2 proteins to duplex TTAGGG telomeric repeats (138–140). The TRF1- and TRF2-dependent formation of large duplex t loops, involving strand invasion of the G strand overhang, provides an attractive model for the architecture of telomere ends (141).

Circumstantial evidence points to an involvement of nibrin in telomere metabolism. First, cytogenetic findings in NBS as well as AT cells demonstrate frequent telomeric fusions of metaphase chromosomes that reflect abnormal telomere function. Based on biochemical properties of the M/R/N complex, models have been proposed in which the Rad50 complex is involved in the processing of telomere ends for presentation to telomerase (142). Second, yeast cells lacking the functional ortholog Xrs2 are deficient in telomere length regulation, presumably acting in the same pathway as Tel1p, a yeast homolog of human ATM (105). The applicability of this observation to the situation in human cells is confounded by the fact that, similar to findings in yeast, nibrin is phosphorylated by ATM, which itself has been shown to be involved in telomere metabolism (114,143). However, unlike AT cells—in which abnormally shortened telomere have been described (144–147)—no such data are available for NBS cells.

Several recent reports have described the possible involvement of nibrin in telomere metabolism based on immunofluorescence and colocalization studies. In human meiotic cells, nibrin has been found to localize specifically to telomeric chromosome ends as well as to TRF1 during late leptonema, zygonema, and early pachynema of male spermatogenesis (148). This observation is remarkable, as nibrin would represent the first protein found specifically to bind to telomeres in human meiotic cells, and surprising when compared with the situation in yeast. In yeast meiosis, the M/R/N complex is tightly associated to DSBs that initiate homologous recombination. In some cases, changes of the chromatin structure at meiotic DSBs, dependent on Xrs2, Mre11, and Rad50, have been observed (149). Zhu et al. (150) have demonstrated that a small fraction of nibrin, hRad50, and hMre11 are associated with TRF2. In immunofluorescent staining experiments, a small subset of cells displayed nibrin association with TRF2 and telomere ends in S phase, whereas hRad50 and hMre11 were present at TRF2 and telomeres throughout the cell cycle. The investigators suggest that a facilitating and/or stabilizing function of hMre11 and hRad50 complexes occurrs in t-loop formation of human telomeres, whereas nibrin might be specifically involved in telomere replication. In addition, loss of TRF2 may initiate nonhomologous end-joining pathways, resulting in dicentric chromosomes and anaphase bridges (151). Interestingly, this type of otherwise rare chromosomal aberration is seen frequently in AT as well as in NBS cells, supporting a potential functional interaction between nibrin and TRF2. Nibrin also has been shown to be locally concentrated together with TRF1 in promyelocytic leukemia (PML) nuclear bodies of cells undergoing alternative lengthening of telomeres, or ALT (152). Since telomeric repeat sequences have been found in TRF1-containing PML bodies (153), and the association of nibrin with TRF1 in PML bodies occurs specifically in the late S/G2 phase of the cell cycle, a nibrin-dependent replicative mechanism of telomere elongation has been proposed to occur in PML bodies of ALT cells (152).

Taken together, several independent observations suggest the involvement of nibrin in telomere metabolism. Given the facts that many recombination proteins like hRad51 and hRad52 localize to PML nuclear bodies (153) and that telomeric t loops show structural resemblance to recombination intermediates, a role of nibrin in telomere metabolism may involve recombination or recombinationlike DNA structures.

V. MODELS FOR NIBRIN FUNCTION

Collectively, the preceding data demonstrate several roles of human nibrin: in processing and/or repair of DNA-DSBs, in mediating cell cycle responses following IR, potentially in telomere metabolism, and potentially in replicative processes.

In general, two important and interrelated questions arise. What are the general mechanisms and pathways of nibrin function, and what is its mode of action? The molecular mechanisms of nibrin function have not been determined and may depend on the specific cellular context and phenotype examined. No matter which phenotype, nibrin is found almost exclusively in tight nuclear association with hMre11 and hRad50, suggesting that the molecular mechanisms depend on the interplay between the three, or even more, associated partners. Although homologous recombination as well as NHEJ have been shown to be exerted by the Mre11/Rad50/Xrs2 complex in yeast, and Mre11 regulates homologous recombination-dependent DSB repair in chicken DT40 cells, no such data are as yet available for nibrin or the M/R/N complex in human cells.

Concerning the mode of action, it is possible that nibrin in its trimeric complex with hMre11 and hRad50 serves strictly structural functions at DSBs. Alternatively, if the hMre11-encoded catalytic sequences and in vitro activities are relevant in vivo, they may be modulated by its interacting partners nibrin and hRad50. Data from yeast as well as human systems argue in favor of structural functions of the trimeric complex since the nuclease activity encoded by Mre11 has been found to be dispensable for certain functions. The trimeric complex may thus be necessary for tethering the loose ends of DSBs together and also for allowing resection of single-stranded overhangs by specific nucleases to proceed. Alternatively, this nuclease activity may well be exerted by Mre11, assigning the complex both structural and enzymatic functions.

Apart from modulating the structure of DNA DSBs, nibrin must be involved in signaling processes following IR, presumably in concert with, or dependent on, ATM, since NBS and AT cells show a loss of p53 activation and cell cycle checkpoint(s). Following IR, ATM activates nibrin via phosphorylation, which may allow signaling by nibrin to occur, presumably as part of the M/R/N complex and through protein binding at the BRCT domain.

VI. CONCLUSION: ROLE OF *NBS1* IN CHROMOSOMAL INSTABILITY AND AGING

Cells from patients with NBS are clearly characterized by an increase in spontaneous and IR-induced chromosomal instability due to defective repair and/or cell cycle response to DSBs. In comparative studies, increased DNA repair capacities (154) and poly(ADP)polymerase levels, an enzyme involved in maintenance of genome integrity (155) has been shown to correlate with increased mammalian life span. In addition, genetic or environmental manipulations conferring extended life spans in various organisms imposed increased resistance to cellular stress, including DNA damage (156). The phenotype of NBS cells thus partly meets aging criteria according to Kirkwood (154). On the cellular level, a role of the *NBS1* gene product in telomere metabolism seems likely, but definitive proof is missing. However, a causative role of telomere shortening in aging of the whole organism has been ruled out for mice (157). Taken together, several cellular features point to a potential role of nibrin in cellular aging.

On the clinical level, the extreme genetic homogeneity of patients with NBS may impact the clinical picture of NBS with regard to aging. In particular, the possibility of partial functional activity of truncated nibrin proteins in the hypomorphic founder mutations may diminish clinical aging phenotypes that might be expected based on putative cellular roles of nibrin in aging. The chromosomal instability may be responsible for the development of lymphomas in patients with NBS. In general, children with NBS, who are highly cancer prone, show a shift toward the adult pattern of malignancy dependent on defective cellular nibrin pathways. A compromise of the human immune system and altered antibody responses in mice have been shown to occur during aging; again suggesting that patients with NBS, who generally develop early immunodeficiency, may show aging phenomena.

In conclusion, *NBS1* mutations clearly cause genomic instability, but, unlike the situation for other genomic instability syndromes—for example, Werner syndrome (see Chapter 9)—this genomic instability is only poorly reflected in accelerated aging on the cellular and clinical levels. Animal models completely devoid of nibrin function and further molecular analyses of the hMre11/hRad50/nibrin complex may ultimately clarify this issue.

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15
Dyskeratosis Congenita

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I. INTRODUCTION

Dyskeratosis congenita (DKC), or Zinsser–Engman–Cole syndrome, is a congenital, multisystem disorder first described by Zinsser in 1906, followed by Engman in 1926, and Cole in 1930. DKC was originally described as a rare skin disease characterized in the early stages by reticulate skin pigmentation, nail dystrophy, and leukoplakias of the tongue. With an accumulation of clinical reports, it is now apparent that DKC is a bone marrow failure syndrome. This syndrome initially manifests as severe anemia and is the consequence of a progressive deterioration of the hemopoietic system. It may be accompanied by signs of immunosuppression, and the cellular and humoral immune systems can be affected. Most patients with DKC die in their early teens as the result of bone marrow failure. Chromosomal aberrations are observed in skin fibroblasts and bone marrow cells of some patients with DKC, and although these are not increased by treatment with chemical and physical DNA-damaging agents, DKC has been referred to as a chromosomal instability disorder. This is congruent with the increased risk of patients with DKC for developing a range of malignancies. There is extreme heterogeneity in the phenotypic manifestation, severity, age at onset, and clinical course of the disease. Based largely on the phenotype and modes of inheritance (as far as these can be traced), X-linked recessive, autosomal dominant, and recessive forms of the disease exist. A severe allelic variant of the X-linked form of the disease, Hoyeraal–Hreidarsson syndrome (HHS), also has been identified. Even though the majority of patients are male, female patients with DKC and HHS have been described. Although the gene for X-linked DKC/HHS has been identified, the ge-

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netic etiology of the other forms of DKC/HHS remains to be elucidated. Predominantly missense mutations have been identified in the *DKC1* gene, and although these are distributed throughout the gene, regions of mutation hot spots have become apparent.

The identification of the X-linked *DKC1* gene, together with a functional characterization of the protein dyskerin, has prompted the coining of an increased number of molecular terms for the disease. Dyskerin is a nucleolar protein and functions in a number of processes related to regulating and/or maintaining cellular proliferation and survival. Dyskerin is a pseudouridine synthase and is thought to function in the pseudouridylation of specific uridine residues in precursor rRNA (pre-rRNA) as well as in the cleavage of pre-rRNA into the smaller mature rRNA forms. An extended role for dyskerin in ribosomal biogenesis has therefore been implied. This is supported by clues that dyskerin may function in the intranuclear and nucleocytoplasmic trafficking of proteins, which is suggestive of its involvement in the maturation and packaging of ribosomes. Although rRNA processing and ribosomal biogenesis appeared to be normal in cultured cells derived from two patients with DKC, substantial evidence that would exclude a subtle defect in rRNA and ribosomal biogenesis as a pathophysiological mechanism of the disease is still lacking. Seemingly unrelated to its role in rRNA and ribosomal biosynthesis, there is some evidence to support the notion that dyskerin function in the processing of the RNA component of the telomerase complex (hTR) and thereby regulates the activity of telomerase. In light of the function of the telomerase enzyme in replacing the terminal repeats at the ends of chromosomes that are lost during replication, it is intriguing that patients with DKC exhibit significantly shortened telomeric ends. It has therefore been inferred that DKC is a telomeric maintenance disorder. The question of whether this is central to the pathomechanism of the disease nonetheless demands verification. Unequivocal proof will be derived by establishing a reproducible link between the presence of mutant dyskerin, a decrease in telomerase activity, and a reduction in the lengths of telomeres in patients with DKC. In this context, it is interesting that accelerated telomeric shortening is one molecular characteristic of premature aging, and that this agrees with the recognition that some of the clinical signs of DKC resemble features of premature aging. Despite an abundance of critical research that has ensued since identification of the DKC1 gene, extensive analyses of the various functions of dyskerin will be required before we can fully elucidate the molecular pathomechanism(s) of the disease.

II. HETEROGENEITY OF THE DKC PHENOTYPE

A. Description of the Phenotype

DKC is a progressive, multisystem disorder characterized by heterogeneous phenotypes that cover a broad spectrum of clinical signs. These should be viewed as

being part of a spectrum of anomalies that occur in various combinations rather than as distinct features (1). One hallmark of DKC is the manifestation of the abnormalities largely in tissues consisting of cells with a high proliferative capacity. This includes the cutaneous and mucocutaneous epithelia and their derivatives, as well as the stem cells of the hemopoietic system. The progressive deterioration of the hemopoietic system is one of the most severe complications of the disease and ultimately leads to fatal bone marrow failure. Generally, the disease shows a resemblance to features of premature aging at the phenotypic and molecular levels. Based largely on differences in the severity and age at onset of the clinical signs, X-linked recessive (MIM 305000), autosomal dominant (MIM 127550), and recessive (MIM 224230) forms of the disease are recognized. The DKC1 gene that is responsible for causing X-linked DKC has been identified and characterized (2). Consequently, even though the different forms may not always be distinguished by phenotype alone, it is now possible to differentiate unambiguously the X-linked form from other forms of the disease. As the majority of patients with DKC are males (86% recruited by the DC registry), the X-linked recessive form of DKC is considered to be the predominant form of the disease (1,3,4).

In the early stages of the disease, the majority of patients with DKC display a triad of clinical signs that includes nail dystrophy, mucosal leukoplakia, and abnormal reticulate skin pigmentation. Other abnormalities include the premature graying and/or loss of hair (alopecia), hypodontia, atresia of the lacrimal ducts, osteoporosis, short stature, dysplastic lesions of the gastrointestinal and gastrourinary tracts, testicular atrophy, and neurological problems such as ataxia and microcephaly in some cases. Progressive pancytopenia develops in the majority of patients with DKC, and this may affect one or more hemopoietic lineages. In the majority of patients, there is a progressive decline of all hemopoietic progenitors, including the erythroid, myeloid, and megakaryocytic lineages (76.3%), although there are also examples of patients who exhibit only thrompbocytopenia (6.6%) or only leukopenia (2.6%), whereas others exhibit no cytopenia (14.5%) (1). As is exemplified by the hypocellular bone marrows of some patients, the bone marrow failure is thought to be a consequence of the reduced proliferative potential of the hemopoietic stem cells (1,3). Bone marrow failure leads to death in 90% of the cases, and this occurs at a median age of 16 years. Another less frequent cause of untimely death is the increased prevalence of developing pulmonary disease. This occurs as a result of a reduction in the diffusion capacity of the pulmonary epithelia and is presumably linked to the failure of the epithelial cells to regenerate (4). Death from pulmonary disease is often the result of complications that develop following bone marrow transplantation, but this is nevertheless a problem inherent to DKC, as it also occurs in the absence of such palliative interventions (2–4).

Generally, the X-linked form of the disease is considered to be more severe with a greater likelihood of early death from the bone marrow failure compared with other forms of DKC. Male and female patients with milder forms of DKC

have been described. These patients exhibit a different phenotypic spectrum, which in part overlaps with the more severe clinical forms, but in addition, includes abnormalities that are not necessarily observed in patients with a poor clinical prognosis. This may be explained in part by the longer life spans of patients with milder phenotypes who survive until their third or even fourth decade of life. One fairly consistent observation is that these patients are predisposed toward acquiring opportunistic infections that can cause death. There is now increasing awareness that the tendency to succumb to opportunistic infections may be attributable to an immunodeficiency arising from disturbances in the cellular and humoral immune systems (1,5). Evidence for this derives from observations that abnormal ratios of distinct immunoglobulin classes have been observed in some patients. This is reflected in the decline of specific T and B cells, although the progressive degeneration of the hemopoietic system overall would also be expected to contribute to the immunosuppression (1,5). A problem that may be related to the immunosuppressed state is that patients who survive beyond the second decade of life have an increased risk of developing a range of malignancies. Prevalence in the occurrence of a specific type of cancer does not exist. Dokal (1) cites four cases of myelodysplasia, one of Hodgkin lymphoma, seven cases of different carcinomas (bronchus, colon, larynx, esophagus, pancreas, skin, tongue), and one case of Hodgkin lymphoma. Of 13 cases with malignancies, 3 were in female patients (1). The increased cancer risk may be linked to the propensity of DKC cells to spontaneously accumulate chromosomal aberrations, the incidence of which was shown to increase with increasing age of the patient (6).

B. Variants of DKC

Subsequent to the identification of the DKC1 gene, DKC1 mutations were found in children with Hoyeraal–Hreidarsson syndrome (HHS, MIM 600545) (7,8). HHS is a severe bone marrow failure syndrome that previously had not been recognized as an allelic variant of DKC (9–12). Although one reason for this may have been the failure to recognize similarities in the clinical course of the disease, another explanation lies in the rareness of HHS. To date, only nine cases of HHS have been described of which six were boys and three were girls (12). Children with HHS do not develop the characteristic skin and nail changes typical of the early stages of DKC. In contrast to patients with DKC who develop the symptoms postnatally, children with HHS exhibit growth retardation and severe microcephaly with prenatal onset. Further, children with HHS show severe aplastic anemia and pronounced immunodeficiency with death usually resulting from bone marrow failure within the first four years of life. There is one example of a girl with HHS, however, who is alive at age 7 (12). In addition to the growth retardation and microcephaly, children with HHS suffer from severe mental retardation with spastic paresis, ataxia, and pancerebellar hypoplasia.

As is the case for DKC, children with HHS show a clinically diverse spectrum of phenotypes. It is interesting that the affected female cases of both DKC and HHS exhibit heterogeneous phenotypes ranging from mild to severe. Heterozygous female carriers of the X-linked recessive form of DKC, for example, may exhibit mild clinical features ranging from very discrete forms of nail dystrophy on isolated nails to patches of abnormal skin pigmentation (4). In addition, although most affected females with DKC generally exhibit mild phenotypes, there are a few examples of female patients with a severe DKC phenotype who died of bone marrow failure at a very early age (1). The situation may be similar in HHS. To date, no female patients with DKC or HHS have been found to harbor mutations in the DKC1 gene. Further, it is apparent that approximately 60% of male patients with DKC who were thought to represent the X-linked form of the disease do not carry DKC1 mutations. This heterogeneity in the phenotypic spectrum, together with the absence of DKC1 mutations in male and female patients, suggests that one or more autosomal or X-linked DKC/HHS disease loci exist. It is further conceivable that there are other allelic variants and/or phenocopies of X-linked DKC. One such group may be represented by the unexplained severe idiopathic aplastic anemias (SAAs) that may be caused either by mutations in the *DKC1* gene or by the genes responsible for the autosomal forms of DKC/HHS (1).

III. PROPERTIES OF DKC CELLS

A. Cell Viability

The premature aging of the blood and skin cells of patients with DKC indicates that DKC1 mutations lead to a reduction in the cellular proliferation and/or survival capacity, especially of cells with a high turnover. This is further exemplified by the severe skewing of X chromosome inactivation consistently observed in peripheral blood cells of heterozygous female carriers of X-linked DKC, and is believed to be the consequence of the selective survival of cells expressing wild-type dyskerin on one hand accompanied by a preferential loss of cells expressing mutant dyskerin on the other (13,14). Further support for the role of mutant dyskerin in impeding cellular proliferation and survival derives from observations that primary skin fibroblasts from patients with DKC exhibit defective division rates and senesce rapidly in culture (15). The in vitro mitotic index of these cells is two to five times lower compared with normal cells. This is accompanied by an abnormal morphology (15). Unlike the spindle-shaped appearance of healthy fibroblasts, fibroblasts from patients with DKC are polygonal in shape, undergo ballooning, and develop extensions reminiscent of dendritic cells (15). Similarly, lymphoblastoid cells from patients with DKC generally fail to thrive in culture and undergo rapid senescence. For this reason, it is very difficult to establish DKC cell lines. In the case of lymphoblastoid cell cultures, this is further compounded

by the fact that peripheral blood cells from patients with DKC are resistent to immortalization by Ebstein–Barr virus (EBV) transformation (15).

B. Chromosomal Instability

Coupled to the reduced proliferative potential of DKC cells, there is an increased tendency toward an accumulation of chromosomal aberrations that may reflect an inherent chromosomal instability. The type of chromosomal aberrations observed in cultured fibroblasts, peripheral blood lymphocytes, and bone marrow metaphases of patients with DKC predominantly represent unbalanced rearrangements occurring in the form of dicentrics, tricentrics, and translocations. Occasionally, inversions and isochromosomes are detected (15,16). There is a high incidence of hypodiploid metaphases resulting from the random loss of chromosomes, and this is thought to be the consequence of increased rates of premature centromeric disjunction, as there is no preferential loss of specific chromosomes or pairs of chromosomes. The pulverization of single chromosomes also has been observed and is likely to be a consequence of premature chromosomal condensation (17).

Because chromosomal abnormalities have been observed by some investigators but not by others, there is extensive controversy regarding the question of whether DKC is a chromosomal instability disorder or not. The classic chromosomal instability disorders exemplified by Bloom syndrome, xeroderma pigmentosum, ataxia-telangiectasia, and Fanconi anaemia (FA) are typified by two distinct characteristics: One is that these syndromes accumulate specific types of chromosomal aberrations that may virtually be diagnostic; the other is that there is a pronounced sensitivity of the chromosomes to treatments with DNA cross-linking agents (mitomycin C, diepoxybutane), ionizing (x-rays, γ-rays), and non-ionizing radiation (ultraviolet [UV] rays). This contrasts with DKC, where neither the chromosomal instability nor an increased sensitivity to clastogenic agents can be consistently demonstrated. This underscores the fact that the molecular basis underlying the chromosomal instability is unique to DKC, and that the sensitivity to clastogenic agents may not be a unifying feature of chromosomal instability disorders.

IV. CHARACTERISTICS OF THE DKC1 GENE

In 1986, it was established that the X-linked form of DKC is linked to the telomeric band of the long arm of the X chromosome, Xq28. With the establishment of the Dyskeratosis Congenita Registry (DCR, London, Hammersmith Hospital) in 1995, which has now recruited over 80 DKC families (1,3,4), and with the use of the many genetic markers in Xq28, refined genetic linkage analysis on multiplex

pedigrees has been possible. As a result, the DKC candidate interval was reduced from 9.0 to 1.4 megabases, which faciltated the screening of 28 candidate genes for mutations in patients with DKC (18,19). Detection of a partial gene deletion at the 3′ end of one of these genes in one patient led to the identification of the DKC1 gene (2). Subsequently, a number of different, mainly missense-type, mutations have been identified (2,20).

A. cDNA and Northern Blot Expression Data

The DKC1 transcript is highly conserved with significant homologies to a large number of ESTs (expressed sequence tags) and cDNAs derived from vertebrate and invertebrate species, as well as from various plants, protozoans, fungi, yeast, and bacteria. The full-length DKC1 cDNA shows a high degree of identity ranging in ascending order from 52 to 75% to the full-length cDNA sequences of the bacterium (*Methanococcus jannaschii*), yeasts (*Kluyveromyces lactis, Saccharomyces cerevisiae, Schizosaccharomyces pombe*), nematode (*Caenorhabditis elegans*), fruitfly (*Drosophila melanogaster*), rat (*Rattus norvegicus*), and mouse (*Mus musculus*) (2).

The major 2.6-kb transcript is expressed ubiquitously in adult and fetal tissues. In addition, a larger 4.5-kb transcript is expressed selectively in testis, cerebellum, and peripheral blood lymphocytes, although at much lower levels than the major transcript (21). The situation is similar in the mouse, except that the larger transcript is also detected in adult liver and kidney, which appears not to be the case in humans (21) (Fig. 1). In the mouse, whereas the 2.6-kb transcript is expressed very early and right throughout embryonic development, the 4.5-kb transcript is only expressed in 7-day mouse embryos after which embryonic expression ceases (21) (Fig. 1). The differential expression pattern of the larger transcript strongly suggests that the DKC1 gene may perform functions that are tissue-specific and/or specific to different embryonic stages of development. In *Drosophila,* two ubiquitously expressed 1.8- and 2-kb mRNA subforms exist. These two transcripts share the same coding region and differ in length owing to the use of alternative polyadenylation signals, which leads to a variable number of 3′UTR exons. It is conceivable that these two transcripts correspond to the human and mouse 2.6-kb transcript, and that they were not resolvable on the Northern filters. In addition, the *Drosophila* ortholog weakly expresses a ~4-kb transcript, and although no sequence data exist, this more than likely corresponds to the larger mammalian transcript (22). Currently, the origin of the alternative transcript is unknown, and it remains to be established whether it arises through alternative splicing or through alternative polyadenylation. Clearly, aspects relating to the origin of the multiple transcripts and their functional implications demand further investigation and require consideration when constructing mouse models of the disease. Further, it should be borne in mind that less than 40% of patients with DKC carry

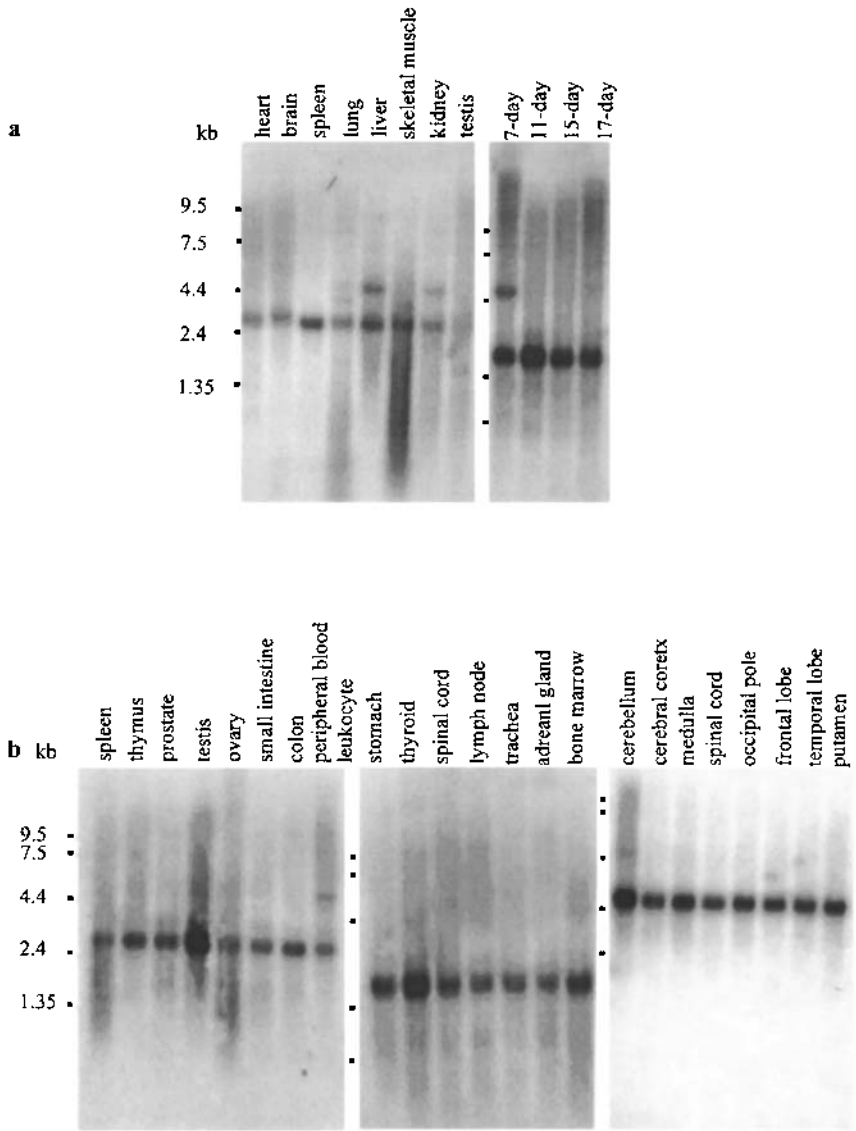


Figure 1 Northern hybridizations of blots containing (a) mouse and (b) human mRNA showing ubiquitous expression of the 2.6 kb transcript and weak tissue-specific expression of the 4.5-kb transcript. Filters were hybridized with the full-length human DKC1 probe as opposed to the shorter probes used previously, which had identified solely the 2.6 kb transcript. (From Ref. 21.)

mutations in the major DKC1 transcript (1). Even though additional autosomal DKC loci may explain the genetic etiology of the other forms of DKC, mutations within alternative transcripts represent equally plausible alternatives.

B. In Situ Expression of *Dkc1* in Embryological Development: How Does This Relate to the Clinical Course of the Disease?

The ubiquitous *Dkc1* gene expression pattern detected on Northern blots is reflected in the ubiquitous in situ hybridization signals observed on mouse tissue sections and whole mounts. The latter technique has provided a deeper insight into the possible functional meaning of variable mouse *Dkc1* expression levels in specific cells and tissues. Elevated levels of *Dkc1* expression occur in a number of embryonic epithelia such as the skin, the outer root sheath of the hair bulb, the nasal epithelia, and epithelial lining of the gut (21). In the 10.5 days post coitum (dpc) mouse embryo, stronger *Dkc1* expression has been observed in distinct cell islands of the yolk sac, which is the tissue where hematopoietic activity first becomes visible in the mouse (23). It may be of functional relevance that this expression pattern concurs with tissues that remain proliferatively active in adults, and that these are the tissues most prevalently affected in patients with DKC. Clinical manifestations include altered skin pigmentation, alopecia, mucosal leukoplakias, gastrointestinal complications, and pancytopenia.

Increased levels of *Dkc1* gene expression, however, are not confined to tissues that maintain a high proliferative capacity throughout life. In the adult testis, for example, higher levels of *Dkc1* expression is restricted to the Leydig cells, which produce the testosterone necessary for normal development of the testes (24). There may be a functional connection between the expression of *Dkc1* in Leydig cells and the hypogonadism and undescended testes observed in patients with DKC (3). In the adult brain, *Dkc1* expression was found to be ubiquitous with higher expression confined specifically to the Purkinje cells of the cerebellum and the mitral cells of the olfactory bulb (21). This suggests that the Purkinje and mitral cells produce high levels of the protein dyskerin even though they are expected to be fully differentiated and quiescent. The Purkinje cells are the largest neurons of the cerebral outer gray matter and are part of the complex neuronal circuit where they control motor coordination by modulating the size of the excitation signals by sending out inhibitory signals. Although it will be necessary to verify whether the increased levels of *Dkc1* transcript correspond to increased levels of the protein at the immunohistochemical level, the expression studies indicate that some functions of dyskerin are crucial to these cells. It is interesting that a number of patients with DKC show moderate mental deficiency and that a certain degree of microcephaly has been reported (1,3,4). Further, brain abnormalities are more pronounced in children with HHS who exhibit severe microcephaly, mental

retardation with spastic paresis and ataxia, and pancerebellar hypoplasia (9–12). This seems to underline the functional significance of *Dkc1* expression in the Purkinje cells. It is interesting that there are examples of diseases such as spinocerebellar ataxia types 1 (SCA1) (25) and 6 (SCA6) (26) and ataxia telangiectasia (30), where the degeneration or loss of Purkinje cells is associated with the progressive loss of coordination. Although a brain magnetic resonance imaging (MRI) study of one girl with HHS showed delayed myelination of the cerebral white matter and a hypoplastic corpus callosum, the resolution of an MRI was not sufficient to analyze structural abnormalities in single cell layers, such as the Purkinje cell layer (12). Nevertheless, there may be a functional connection between the pronounced neurological findings in children with HHS and the elevated *Dkc1* expression levels in embryonic tissues of neuroectodermal origin, including the telencephalon, spinal ganglia, and the spinal cord (24). In general, this raises the question of whether DKC is a disease with prenatal and neonatal onset, and that this was not recognized previously because the symptoms are less pronounced in patients with the more classic DKC phenotype.

Understanding the role of dyskerin in specific differentiated cells, such as those of the brain and testis, remains a challenge. Certain pertinent functions associated with dyskerin may be more tissue-specific than previously anticipated and appear not to be limited to cells that maintain a proliferative capacity throughout life (21). The identification of tissue-specific transcription factors and an understanding of the tissue-specific functions of dyskerin will be of great value. It is also without question that the analysis of mouse models will be indispensable to complement our understanding on *Dkc1* gene expression and disease progression.

C. *DKC1* Gene Structure

At the genomic DNA level, the DKC1 gene consists of 15 exons spanning close to 15 kb (20) (Fig. 2; Genbank accession numbers: AJ0101395, AJ0101396). The internal exons range in size from 65 to 185 bp. The 5′ and 3′ exons are 108 and 908 bp in size, and there are no introns in the untranslated regions (UTRs) (Fig. 2). Overall, the introns are small and range in size from 194 to 2251 bp. In total, the larger introns contain two medium reiteration frequency repeats (MERs), six Alus, and three long interspersed repetitive elements (LINEs) (Fig. 2). Intron 1 contains the following simple repeat sequence:
$(TA)_6AATA(TG)_4(TA)_3TG(TA)_2T(TA)_3(TGTA)_2T(TA)_2(TGTA)_2TAT(TA)_6$.
Intron 11 contains a SINE-R retroposon insertion derived from the human endogenous retrovirus HERV-K10 long terminal repeats (LTRs). As is characteristic for SINE-R elements, the DKC1 retroposon has a 5′ to 3′ direction and lies in a reverse orientation to the DKC1 gene. It consists of a glucocorticoid-rich stretch of 40-bp tandem repeats followed by a polypurine tract, a glucocorticoid-respon-

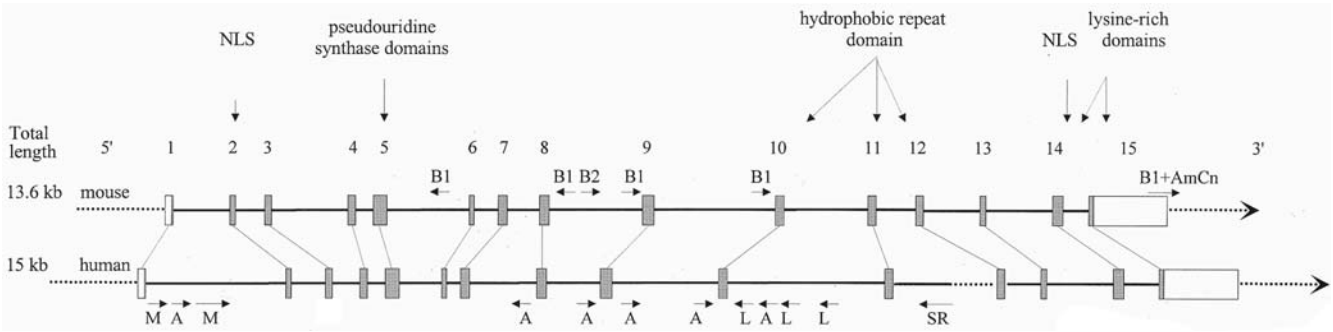


Figure 2 Line diagram of the genomic structure of mouse *Dkc1* compared with human DKC1. The coding exons are shown in shaded boxes and are numbered above; the 5′ and 3′ untranslated regions (UTRs) are unshaded; thin connecting lines indicate the orthologous exons. The schematic representation of the exons and introns reflects their relative sizes and is not completely accurate to scale. The gap in human DKC1 intron 11 is indicated by a partially dashed line. The direction of the repetitive elements, with respect to the genes, is shown by the small arrows; B1, B2 = Alu-like mouse-specific repetitive elements, B1 + AmCn = B1 element in 3′ UTR flanked by variable number of AmCn blocks, A = Alu, M = Mer, L = LINE, SR = SINE-R11. (From Ref. 21.)

sive element (GRE), a polyA signal, and a polyA tail (20). The potential functional relevance of this element has not been investigated.

Comparison of the human and mouse gene structures indicate that the number and length of the exons is conserved with some variation in the lengths of the introns (see Fig. 2). The mouse gene is marginally smaller and spans 13.6 kb versus the 15 kb covered by the human gene (20) (see Fig. 2). The distribution, type, and orientation of repetitive elements differ (see Fig. 2). As is the case for the human gene, the splice sites of mouse *Dkc1* conform to the consensus sequences (21). One exception in the human and the mouse genes is the 3′ splice acceptor site of the intron 2, which consists of the rare sequence AAG, which corresponds to the position of the putative splice site mutation detected in one DKC patient (20,21). Sequence comparison of the 680 bp sequence 5′ of the ATG of the human gene (28) with the equivalent sequence in the mouse, revealed a homology of 65%. This suggests that the 5′ UTR and part of the putative promoter sequences are contained within this sequence, and that they contain functionally conserved regulatory elements. Further, various transcription factor–binding sites have been identified using promoter prediction programs (21). Clearly, functional verification of the putative regulatory promoter elements would be an important contribution toward unraveling the functional implications of the differential and tissue-specific expression of the DKC1 gene.

V. MUTATION SPECTRA AND LACK OF PHENOTYPE–GENOTYPE CORRELATIONS

To date, DNA samples from patients with DKC belonging to over 80 families have been screened for mutations in the DKC1 gene. These are mostly missense mutations that are scattered across the gene in the exons 1, 3, 4, 5, 9, 10, 11, and 12. The spectrum of mutations includes an in-frame 3-bp (single amino acid) deletion in exon 3, a 2-bp missense substitution in exon 4, a putative splice site mutation in intron 2, a cryptic splice site mutation in intron 1, a putative promoter mutation, and a 2-kb deletion that leads to the removal of the last exon, exon 15 (1,2,7,8,20,29–31). To date, no premature stop codon mutations, whole gene deletions, or frameshift mutations have been identified, indicating that complete loss-of-function mutations would not sustain life. There is a clustering of mutations, especially in the exons 3 and 11, and a certain degree of clustering in exons 4 and 10. Although most mutations identified so far are unique, there are at least two examples of different mutations affecting the same codon and at least three examples of recurrent mutations. The recurrent mutations lie in exons 3, 4, and 11 and are indicative of mutation hot spots. The exon 11 A353V mutation is particularly interesting, because it is the most frequently detected recurrent mutation that occurs in over a third of patients with DKC harboring mutations in the DKC1 gene

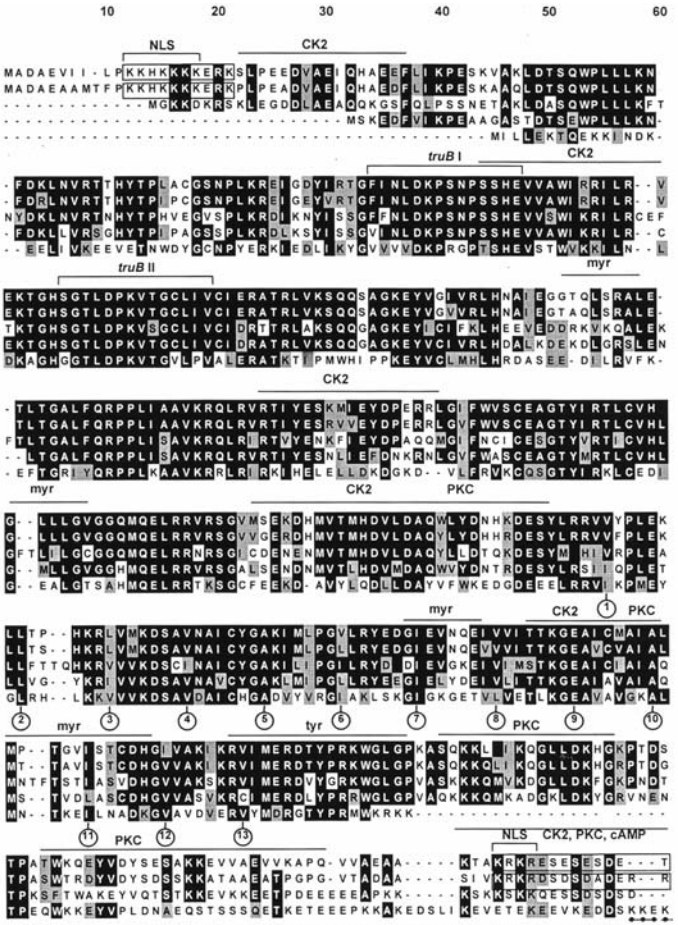
(20). Further, it is so far the only mutation described to have arisen de novo in numerous unrelated probands (20).

There is a great deal of phenotypic heterogeneity, and to date no phenotype–genotype correlations have been possible. This problem is exemplified particularly well by the fact that the recurrent mutations in the exons 3 (T49M) and 11 (A353V) have been identified in patients with DKC and in children with HHS. It is intriguing that these mutations should give rise to the mild as well as the severe forms of this disorder, and emphasizes our lack of understanding on how one missense mutation in the same gene can give rise to such a variable phenotype. This is reminiscent of three other allelic syndromes in Xq28, X-linked hydrocephalus, spastic paraplegia type I, and MASA (mental retardation, adducted thumbs, shuffling gait, aphasia) syndrome, which are all caused by mutations in the *L1CAM* gene (31,32). Of the large mutation spectrum in *L1CAM,* two mutations were found that give rise to all three phenotypes in different generations of the same family (33). As is the case of the L1CAM syndromes, even though DKC is a monogenic disorder, strictly speaking, there appear to be other genetic and/or environmental factors that modulate the phenotype. Conceivably, HHS may turn out to be another example of digenic inheritance as is the case for Antley–Bixler syndrome (31,34) and Waardenburg syndrome type 2 with ocular albinism (31,35). As the DKC1 gene itself is highly conserved and only one polymorphism altering the peptide sequence has been identified (20,28), a plausible alternative explanation could also be the modulation of the HHS phenotype by functionally significant polymorphisms in a second DKC gene. The complexity of this problem and the lack of a relationship between the phenotype and genotype are further reflected in findings that only about 40% of male and no female patients with DKC bear DKC1 mutations (1). Although most of these patients are likely to represent one of the autosomal forms of the disease, the possibility cannot be excluded that the mutations lie in one of the other transcripts or in noncoding and presumably nonfunctional regions of the gene. It will be of diagnostic and prognostic value to establish correlations between the DKC1 mutations and clinical variability. In the long term, this will undoubtedly be of importance for improving existing therapeutic options and for designing optimal curative strategies.

VI. FUNCTIONS OF DYSKERIN

A. Properties of Dyskerin

Dyskerin consists of 514 amino acids and has a predicted molecular weight of 57 kD. It is a highly charged peptide with an isolelectric point (pI) of 10.2. Of the 173 charged residues, 105 are basic. This is largely owing to the many lysine residues (K) that make up 12% of the peptide and are especially abundant at the C-terminus. In agreement with the high degree of conservation of the DKC1 transcript at the nu-


10 20 30 40 50 60
NLS
CK2
H. sapiens (dyskerin)
R. norvegicus (NAP57)
C. elegans
S. cerevisiae (Cbf5p)
M. jannaschii
truB I
CK2
truB II
myr
CK2
myr
CK2
PKC
myr
CK2
PKC
tyr
PKC
NLS
CK2, PKC, cAMP
CK2, PKC, cAMP
myr, CK2, PKC

cleotide level, the protein dyskerin exhibits regions of high homology and regions of identity with orthologous proteins from diverse organisms (2). The high degree of conservation of dyskerin to the rat NAP57 (87% identity, 94% similarity) (36) and yeast Cbf5p peptides (69% identity, 84% similarity) (37,38), and to the peptides from *C. elegans* (65% identity, 79% similarity) and *M. jannaschii* (41% identity, 60% similarity) is exemplified in the CLUSTAL alignment (Fig. 3). In addition, low homologies of dyskerin, NAP57, and Cbf5p to members of the family of tRNA pseudouridine (Ψ) 55 synthases (TruB) isolated from various bacterial species and from *S. cerevisiae* (PUS4) also have been observed (39,40). Although the homologies are restricted to two short motifs in dyskerin, NAP57, Cbf5p and in *Escherichia coli* TruB, the *S. cerevisiae* PUS4 peptide (although shown to possess tRNA: Ψ55 synthase activity) only has one such motif (2) (Fig. 3).

Eight putative casein kinase II, seven protein kinase C (PKC), and two cAMP phosphorylation sites have been identified by PROSITE, suggesting that dyskerin is a phosphoprotein (2) (see Fig. 3). In addition, one putative C-terminal myristylation site and two nuclear localization signals (NLSs) at the amino- (KKHKKK) and carboxy-termini (KRKR) also conserved in the rat sequence were found (2) (see Fig. 3). Although putative NLSs can be discerned at similar positions in *C. elegans* (KKDK and KSKK), these are absent in yeast. As expected, no NLSs are present in the *M. jannaschii* orthologue. A conserved repeat unit with a hydrophobic residue at every seventh amino acid (except for the ninth and tenth hydrophobic residue) was detected in all organisms (2) (see Fig. 3). Overall, the dyskerin peptide is hydrophilic and does not contain hydrophobic domains of sufficient length to span membranes, although the hydrophobic repeat domain can be expected to facilitate the crossing of membranes (2).

Characteristic repeat motifs similar in amino acid composition but different in their primary sequence have been noted in the C-termini of dyskerin, NAP57,

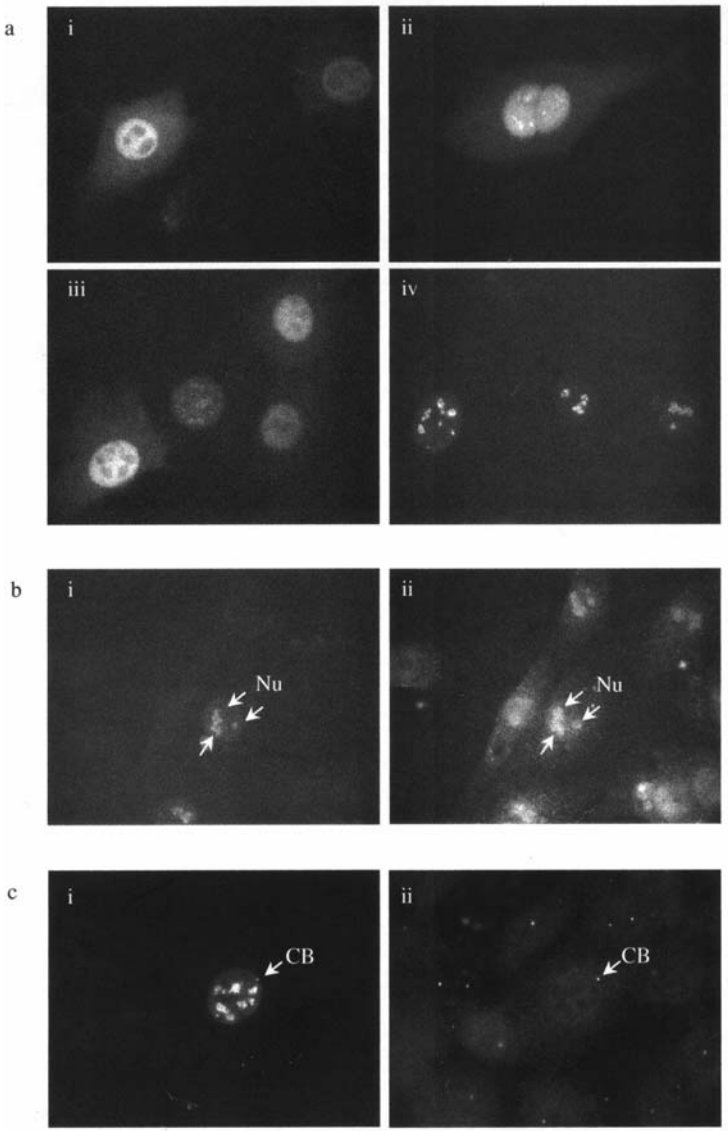
Figure 3 CLUSTAL/BOXSHADE alignment showing the high degree of conservation of dyskerin with proteins from other species. White letters on a black background highlight identical amino acids; black letters on a gray background highlight different but conserved amino acids; black letters on a white background highlight different and nonconserved amino acids. Amino acids forming a hydrophobic repeat domain are marked below the sequence by circled numbers. The nonhydrophobic ninth and tenth amino acids in this motif are shown in stippled circles. The putative amino and carboxy-terminal nuclear localization signals (NLS) and the associated highly charged regions are framed by stippled boxes. The TruB Ψ55 synthase domains are indicated above the sequence by brackets; the corresponding domains of the *E. coli* TruB and *S. cerevisiae* PUS4 proteins are shown below the alignment as boxed excerpts. Lines above the sequence marked with CK2, PKC, and cAMP indicate putative casein kinase II, protein kinase C, and cAMP phosphorylation sites, respectively; myr indicates a putative myristylation site. The C-terminal lysine-rich triplet repeat domains are underscored with dots joined by lines. Positions of the five missense mutations are highlighted by asterisks above the sequence. (From Ref. 2.)

and Cbf5p (2) (see Fig. 3). As a result, the high degree of homology breaks off (2) (see Fig. 3). The rat carboxy-terminal sequence contains the KKE/KKK/KKE/KA and RKK/KKK/KA repeat motifs that show similarity to the KKE/KKK/S/KKD/ KKA and KKK/KKK/KKA motifs in dyskerin (see Fig. 3). By contrast, the carboxy-terminus of *C. elegans* appears to lack such tandem repeat motifs (see Fig. 3). Although *S. cerevisiae* Cbf5p exhibits a perfect tandem KKE/KKD repeat domain also found in *S. pombe* and *K. lactis* (data not shown), the imperfect lysine-rich motifs in dyskerin and NAP57 are reminiscent of the KKE/KKK/SD/KRK/ KDK/EKK/EKK/KKA/KKA domain found at the C-terminus of the Treacher Collins syndrome protein Treacle (2,41).

B. Dyskerin Localizes to the Nucleoli and Coiled Bodies

The predominant intracellular localization of dyskerin is the nucleolus (42) (Fig. 4a, b). A more specific localization of rat NAP57 to the dense fibrillar component (DFC) of the nucleolus has been shown (36), and studies on *Drosophila* Nop60B indicate that a nucleolar localization occurs in the early stages of embryonic development (43). In eukaryotes, the nucleolus is the site of rRNA synthesis and ribosomal subunit assembly. The transport of proteins into the nucleus is a complex process requiring the cooperative effects of several protein factors and signal sequences that regulate import, retention, and export. Many nuclear proteins do not passively diffuse into the nucleus, but are actively transported from the cytoplasm into the nucleus across the nuclear pore complex (NPC). This requires protein–protein interactions, which are mediated by short clusters of sequences, the NLSs. Most NLSs consist of basic amino acid residues, especially lysine and arginine. Also, the spatial distribution of acidic and neutral amino acids as well as other three-dimensional properties of the protein is important. As a result, although there is an NLS consensus sequence, a universal NLS does not appear to exist. Targeting of proteins to the nucleoli is similarly thought to be controlled by

Figure 4 (a) Representative cells showing the localization of full-length dyskerin tagged to EGFP at the N- or C-terminal end. (i) Nucleoplasmic localization observed in all cells 2 hr after microinjection; (ii) nucleoplasmic and nucleolar localization observed in most cells 4, 6, and 8 hr after microinjection, in very few cells 24 hr after microinjection; (iii) mixed population of cells observed 4, 6, and 8 hr after microinjection, sometimes 24 hr after microinjection; (iv) sole nucleolar localization observed in a few cells 4, 6, and 8 hr after microinjection and representing the majority of cells after 24 hr. (b) (i) Nucleolar localization of dyskerin; (ii) indirect immunofluorescence of the same cells stained with the B23 (nucleophosmin) antibody verifying the nucleoli. (c) (i) Localization of dyskerin to the nucleoli and a coiled body; (ii) indirect immunofluorescence of the same cells stained with the p80coilin antibody verifying the position of the coiled body. Nu = nucleolus; CB = coiled body. (From Ref. 42.)


a
i
ii
iii
iv
b
i
Nu
ii
Nu
c
i
CB
ii
CB

nucleolar localization signals (NuLSs), which are unique to specific proteins. Dyskerin harbors a number of putative NLSs at the N- and C-termini, which consist of the lysine-rich motifs, KKHKKKKERKS and KRKR(X)17KKEKKK SKKDKKAK(X)17KKKKKKKKAKEVELVSE, respectively (42). Especially the lysine-rich cluster is reminiscent of overlapping bipartite NLS motifs that have been described for a number of nuclear proteins. A functional delineation of these motifs has shown that the C-terminal KRKR sequence is primarily responsible for the nuclear import of dyskerin, and that the lysine-rich clusters situated at the furthest C-terminal end are responsible for influencing the rate of nucleoplasmic and nucleolar accumulation. NuLSs appear to lie in the N-terminal sequence KKHKKKKERK, although minor NuLSs also are contained within the C-terminal NLS motifs. The dynamics of the intracellular distribution of dyskerin are thus determined by the synergistic and additive effects of a number of NLSs and NuLSs (42).

The nucleus is a highly compartmentalized structure. In addition to the nucleoli, it contains a variety of other small subnuclear structures with a punctate distribution, such as the coiled bodies. The coiled bodies share a number of protein components that include p80 coilin, Cbf5p/NAP57/dyskerin, and Nopp140. Although the exact functions of the coiled bodies remain to be elucidated, there is evidence that they play a role in the storage, maturation, and transport of proteins necessary for rRNA processing, and that they are physically, dynamically, and functionally linked with the nucleoli (44–46). Rat NAP57 and human dyskerin have been shown to localize to the nucleoli and to the coiled bodies, suggesting that dyskerin is dynamically associated with these structures (36,42); (see Fig. 4b,c). Indeed, a dynamic interaction with the nucleoli and coiled bodies has been shown for rat Nopp140, a protein that interacts with NAP57. This led to suggestions that the functional path of Nopp140 to the coiled bodies leads through the nucleoli, and more specifically derives from findings that there is a temporal lag in the targeting of Nopp140 from the nucleoli to the coiled bodies (47). A similar scenario can be envisaged for NAP57/dyskerin. Although nuclear transport kinetics must be demonstrated definitively for NAP57 and dyskerin, indirect evidence exists for human dyskerin to support this hypothesis. By following a time course of expression of recombinant dyskerin, it has been observed that newly synthesized dyskerin initially localizes to the nucleoplasm, followed by a sequential translocation to the nucleoli and to the coiled bodies (42) (see Fig. 4a). Colocalization to the coiled bodies has been detected only in those cells where dyskerin had already accumulated in the nucleoli. Further, there is evidence that Nopp140 shuttles not only between the nucleoli and the coiled bodies but also between the nucleus and the cytoplasm (47,48). A role for Nopp140 in the transport of ribosomal proteins and/or rRNA therefore has been implied (48). Presumably, NAP57/dyskerin is escorted along the same pathways. A similar chaperoning and/or protein-transporting role of dyskerin is therefore conceivable (42).

C. Dyskerin and Its Role in rRNA and Ribosomal Biogenesis

The nucleolar localization of dyskerin is in agreement with accumulating evidence that dyskerin is involved in at least two steps in the pathways that lead to fully functional rRNA and ribosomes. In the nucleolus, precusor rRNA transcripts (pre-rRNA) undergo a number of chemical covalent posttranscriptional modifications and are then cleaved into smaller mature rRNAs prior to their packaging with ribsosomal proteins. Three main types of covalent modifications occur on specific nucleotides of primary precursor rRNA transcripts. These include base methylation, methylation of the 2′-hydroxyl group of sugar residues (2′-O-methylation), and conversion of the uridine residues to pseudouridine by base rotation (52). Each of these modifications is carried out by a specific set of enzymes. From yeast and *Drosophila* studies, there are now several lines of evidence that dyskerin possesses pseudouridine synthase activity. The two conserved TruB pseudouridine synthase motifs that are characteristic of bacterial and yeast tRNA pseudouridine synthases are thought to represent the uridine-binding motif and the catalytic site for enzymatic activity (2,39,40). Investigations on yeast null mutants and *Drosophila* mutants expressing very low levels of Nop60B indicate that the absence of Cbf5p/Nop60B is associated with a reduction in the levels of pseudouridylated rRNA (22,50). Similarly, yeast cells expressing Cbf5p mutants harboring amino acid substitutions in the pseudouridine synthase domains tend to accumulate pseudouridylated pre-rRNA (51). Although a function for Cbf5p/Nop60B as a pseudouridine enzyme is more than likely, explicit proof that the enzymatic activity is directly contributed by Cbf5p is still required (51). It is intriguing that yeast and *Drosophila* null mutants, in addition, exhibit a dramatic accumulation of the large pre-rRNA at the expense of the smaller 28S, 18S, and 5.8S rRNAs, but that this has not been observed in yeasts expressing the mutant Cbf5p TruB isoforms (22,50,51). This implies that the function of the pseudouridine synthase motif is uncoupled from the rRNA cleavage function. Even though true pseudouridine synthase activity remains to be demonstrated for dyskerin, the yeast and *Drosophila* data, together with the extended biocomputational investigations of residues critical to pseudouridine synthase domains, provide convincing evidence that dyskerin represents the first mammalian rRNA pseudouridine synthase (2,39,52).

The site specificity of the pseudouridylation is guided by a class of small nucleolar RNAs (snoRNAs) called the box H + ACA snoRNAs (53). The box H + ACA snoRNAs are associated with a number of protein components to form the box H + ACA snoRNP (ribonucleoprotein) complex. In yeast, close scrutiny of the RNA–protein box H + ACA snoRNP complex has led to dissection of the individual protein components that includes Cbf5p (50,54). That HA-tagged dyskerin coimmunoprecipitates with the box H + ACA snoRNAs now provides

direct evidence of an interaction of dyskerin with these snoRNA molecules in humans (58). As yeast Cbf5p and *Drosophila* Nop60B have been shown physically to stabilize the RNA and protein components of the box H + ACA snoRNP complex (50,54), by analogy it can be assumed that dyskerin likewise functions as the core component of this complex.

Despite the undisputed role of dyskerin in ribosome biogenesis, the conjecture that a defect in rRNA processing represents a mechanism of the disease remains hypothetical. So far, only one DKC1 mutation lies within the pseudouridine synthase domain, and this mutation has not been functionally characterized (7). In DKC cell lines harboring two different missense mutations situated outside of the TruB motifs, no defects, either in the levels of pseudouridylated rRNA or in pre-rRNA cleavage, have been detected (55). Although these experiments may not reflect the in vivo situation, the results do suggest that a major abnormality in rRNA processing may not underlie the etiology of the disease. The idea that DKC is a ribosomal biogenesis disorder should not be entirely abandoned, however, as mutant dyskerin may subtly impact on rRNA functioning, which may reduce the efficiency and fine tuning of ribosomal biogenesis.

D. Dyskerin and Its Role in Telomeric Maintenance

The nucleolus does not solely act as the depot for rRNA processing, but rather is a plurifunctional structure that also provides the environment for the processing and trafficking of non-rRNAs (46,56). One example of a non-rRNA that may be processed in the nucleolus is the RNA component (hTR) of the telomerase ribonucleoprotein complex (RNP). This assumption is based on findings that a portion of the hTR cofractionates with purified nucleoli (57). Further, the hTR carries a stable motif at its 3′ end that resembles the H + ACA motif found in the nucleolar box H + ACA snoRNAs (57,58). Like the box H + ACA snoRNAs that associate with proteins to form the box H + ACA snoRNP complex, telomerase is a ribonucleoprotein (RNP) consisting of the hTR and protein components, one of which is the telomerase reverse transcriptase (TERT) (58). Exciting findings have indicated that dyskerin interacts not only with the box H + ACA snoRNAs but also with the hTR, thereby providing evidence that dyskerin represents a shared protein component of both the box H + ACA and telomerase RNP (55). Not surprisingly, this has fueled interest to investigate the functions that may be shared by these two complexes and serves to underscore the functional and dynamic complexity that connects the intranuclear structures.

It is a well-accepted fact that the primary role of telomerase is to maintain the lengths of telomeres, and that telomerase is required for the indefinite proliferation of primary cells. The telomeres serve as the insulating or protective caps at the ends of chromosomes, but as their structure precludes a complete replication by the conventional DNA replication machinery, portions of the telomere are

whittled away after each DNA replication cycle. This occurs especially in tissues lacking telomerase. Although most normal differentiated tissues consisting of quiescent cells do not express telomerase, telomerase is expressed at low levels in rapidly dividing tissues such as in the blood, skin, and epithelia of the gastrointestinal tract (59,60). Despite this, there is in vivo and in vitro evidence of progressive telomeric attrition with age (61). Telomeric shortening can therefore be used as an indicator of aging, and accelerated telomeric shortening has indeed been used as a marker of premature aging. Accelerated erosion of the telomeres is associated with a number of monogenic disorders such as the two bone marrow failure syndromes, Fanconi anemia (62) and aplastic anaemia (61), and the chromosomal instability disorder, ataxia-telangiectasia (63). Two independent studies have now shown that the presence of abnormally short tracts of telomeres is also a feature of DKC (55,64). In the first study, shortened telomeres were detected in cell cultures derived from patients with DKC carrying two independent mutations (55). In the second study, the telomeric lengths of chromosomes in primary mononuclear cells of patients with DKC were investigated in 37 affected males representing 10 different DKC1 mutations, and in a second group of 17 patients who represented a mixture of male and female patients whose genetic etiology remains unknown (64). In all these cases, the telomeres were significantly reduced by an average of >3 kb (64). This demonstrates that the products of the DKC1 gene, and of possible other, as yet unidentified DKC genes, could play a role in telomeric maintenance (64).

The question remains how telomeric shortening is related to levels of telomerase expression and activity. Limited evidence that mutant dyskerin coincides with reduced telomerase activity is derived from fibroblast cell cultures (taken from patients with DKC) transfected with hTERT. These cell lines displayed an approximately five-fold reduction in the steady-state levels of the endogenous hTR and an equivalently curtailed activity of exogenously expressed TERT (58). This was not verifiable in primary mononuclear cells derived from patients with DKC, which exhibited normal telomerase activity levels despite the significantly shortened telomeres (64). Although the results from these two studies may not be comparable, because transfected cultured cells were used in the one case, whereas primary cells were used in the other, another explanation could lie in the fact that the majority of accelerated telomeric shortening occurs during embryological development of patients with DKC when normally high levels of telomerase would be required. The comparably normal telomerase activity detected in DKC mononuclear cells may reflect remnants of telomerase activity comparable to that observed in normal cells (64). That the disease-related telomeric shortening occurs in early development is in line with the finding that there is no direct relationship between telomeric reduction and the increasing age of patients with DKC. Further, the extent of telomeric shortening does not appear to correlate with the severity of the DKC phenotype (64). By contrast, in Fanconi anemia, there is

an association between progressive telomeric shortening and clinical severity (62). Moreover, in Fanconi anemia, the progressive telomeric shortening concurs with an activation of telomerase rather than a reduction in activity (62). There are a number of disorders characterized by progressive telomeric shortening, and this may or may not be associated with altered levels of telomerase activity (65). In some cases, such as in chronic lymphocytic leukemia (CLL), a periodic decrease in telomerase expression followed by an increase in telomerase expression and activity has been observed (65). This indicates that neither enhanced nor reduced telomerase expression levels consistently correlate with abnormally short telomeres. Regardless of telomerase levels and telomerase activity, telomeres may shorten with progression of these diseases owing to an overall lack of efficient telomeric maintenance.

Even though dyskerin's role in causing accelerated telomeric shortening in DKC remains elusive, it is interesting to dwell on the notion that wild-type dyskerin may promote the interaction of the telomerase complex with the nucleolus, and that this might facilitate hRNA processing or ribonucleoprotein (RNP) assembly required for full telomerase activity (66). An analysis of the DKC variants should provide a potential means not only to identify other components of the telomerase complex or hTR processing factors but also to investigate whether mutations in dyskerin interfere with a binding to the telomerase complex.

VII. WHAT CAN BE GLEANED FROM THE MODEL SYSTEMS?

A. Yeast

The failure to detect DKC1 null mutations in patients suggests that the elimination of dyskerin is lethal in embryological development and agrees with findings that yeast Cbf5p and *Drosophila* Nop60B null mutants are nonviable. Studies on yeast temperature-sensitive mutants have facilitated insight into specific functions of Cbf5p in the context of a unicellular organism. As mutant dyskerin causes perturbations in cellular proliferation that eventually lead to premature cell senescence (notably of the epithelia and blood), investigations into deciphering the role of Cbf5p in the cell cycle should be of relevance to dyskerin as well. Yeast cells deprived of Cbf5p arrest at the G1/S boundary of the cell cycle prior to DNA replication. This indicates that Cbf5p is primarily required for the G1/S transition (37). Further, while cells with a Cbf5p null background are not rescued by constructs bearing a complete C-terminal truncation of the 10 tandemly arranged KKE/KKD repeats, those retaining three of the 10 KKE/KKD repeats delay with replicated genomes at G2/M phase of the cell cycle (37). It is interesting that the 3′ end deletion mutation (that so far has been

detected in two patients with DKC) leads to a similar truncation of the lysine-rich clusters (1, 2). In the broad context of dyskerin's role in maintaining cellular proliferation and in maintaining the lengths of telomeres, it is interesting that other studies seeking to understand how the cell cycle relates to telomeric elongation in yeast suggest that there is a coupling between the in vivo telomerase activity and conventional DNA replication. In fact, telomeric elongation initiates in late S phase, which is the level of the cell cycle at which Cbf5p null mutants stall (67). This raises the question whether a defect in dyskerin leads to accelerated telomeric attrition by a mechanism that delays DNA replication rather than by a direct impairment of telomerase function.

B. *Drosophila*

The diversity of DKC phenotypes reflects the effects of dyskerin impaired largely by missense mutations, but fails to provide insight into how a reduction or absence in expression of the DKC1 gene would alter the phenotype. Research on *Drosophila* strains carrying partial knockout mutations that reduce the Nop60B expression levels to variable degrees has extended our understanding of the phenotypic effects of quantitative rather than qualitative alterations. Expression of only residual levels of Nop60B is usually lethal in the very early larval stages, although depending on the genetic cross, some flies survive until first instar stage, whereas others survive to the second or early third instar stage. This reflects the effects of low but variable levels of gene expression (22). Flies bearing partial loss-of-function mutations exhibit modest reductions in gene expression and survive the larval stages, although development is delayed. They exhibit pleiotropic defects that include a markedly reduced body size, a reduction in the length and thickness of abdominal bristles, reduced fertility, and apoptotoic cells in ovaries (22). In a similar study of Nop60B loss-of-function mutations, polyphasic lethality with a variability in the severity of impairment during embryonic development was observed. This was attributed to the large contribution of Nop60B RNA from the maternal oocyte, which allows zygotic homozygotes to develop for varying lengths of time with death ensuing after the maternally provided functions have been depleted (43). At the molecular level, the embryological defect appears to lie in the insufficient pseudouridylation and cleavage of precursor rRNA in the absence of Nop60B. In summary, by extending these results, it is interesting to speculate whether redundancy in the genome may also compensate for modest loss-of-function dyskerin mutations in mammals. Although a thorough comprehension of DKC disease mechanisms remains somewhat open ended, the *Drosophila* mutants currently provide the only multicellular animal model for further in vivo investigations into the involvement of mutant dyskerin in ribosomal biogenesis and telomeric maintenance.

C. Short Telomeres in hTR Knockout Mice, Normal Human Aging, and DKC

Telomeric shortening is thought to contribute to the physiological decline that occurs with aging (68). Since accelerated telomeric erosion is one molecular hallmark shared by patients with DKC and hTR knockout mice, and is also a process that accompanies normal human aging, a comparison of the corresponding phenotypes is relevant, especially since *Dkc1* mouse models are currently unavailable for analysis (reviewed in ref. 68). The phenotypes of DKC and mTR knockout mice are characterized by hematological abnormalities, and in both cases there is a decline in cells derived from one or more blood cell lineages. Although this is not a common characteristic of human aging, the increased risk of acquiring opportunistic infections due to an overall reduction in the capacity of the immune system to respond to stress is a feature shared by normal aging. Other features shared by all three phenotypes include the poor wound healing, premature loss and graying of hair, gut defects, and an increased cancer incidence. In all cases, this appears to be directly related to the accelerated telomeric shortening. In view of suggestions that the overly short telomeres in tumor cells are at least in part responsible for the chromosomal havoc in tumor tissues, it is reasonable to assume that the exposed telomeric ends of chromosomes in patients with DKC are directly related to increased chromosomal instability. Further, it has been proposed that when telomeres shorten below a critical length, a DNA-damage response pathway is activated and induces cell cycle arrest. Indeed, the low proliferation rates and early senescence of DKC cells is reminiscent of a DNA-damage–induced cell cycle arrest, which concurs with the increased incidence of chromosomal aberrations.

VIII. CONCLUSION

DKC is a bone marrow failure syndrome with features of chromosomal instability, accelerated telomeric shortening, and premature aging. The identification of the DKC1 gene, and knowledge that dyskerin is a nucleolar protein involved in ribosomal biogenesis, has opened avenues for investigating the molecular mechanisms underlying the pathophysiology of DKC. In particular, yeast and *Drosophila* models have been invaluable in deciphering the more specific involvement of dyskerin in the pseudouridylation and cleavage of precuror rRNA. Further, dyskerin is either directly or indirectly involved in maintaining the lengths of chromosomes, although the mechanism remains unknown. Despite the significant shortening of telomeres in patients with DKC, there is no convincing evidence that this is associated with altered levels of telomerase expression. Another question that remains unanswered is why the majority of telomeric shorten-

ing has occurred at birth and does not progress with increasing age of the patients even though the disease itself is progressive. If it holds true that accelerated telomeric shortening in DKC is largely complete at birth, it remains equally difficult to explain why cancers do not more consistently develop in patients with DKC, and why high cancer incidence and chromosomal instability occur largely in patients who survive longer. This is more analogous to the acquisition of cancers as an accompaniment of the slow but progressive process of normal aging. It also remains unresolved why a defect in telomeric maintenance should lead to such a severe phenotype and early death of children with HHS but not of patients with DKC who may share the same DKC1 mutation. In conclusion, it remains to be elucidated whether there are one or more mechanisms central to the pathophysiology of DKC. Similarly, it remains open whether DKC is a ribosomal biogenesis and/or a telomeric maintenance disorder. Mouse models of the disease should shed light on the functional connections between mutant dyskerin, rRNA biogenesis, telomerase activity, and telomeric shortening. From a clinical point of view, although establishing phenotype–genotype correlations has not been possible largely because of the broad spectrum of phenotypes and mutations, the identification of the DKC1 gene is of great diagnostic value and has already improved counseling of affected families. Unfortunately, with rare exceptions of patients with DKC who have successfully undergone bone marrow transplantations, treatment of most individuals remains palliative. The identification and functional characterization of the DKC1 gene has immensely contributed toward a deeper understanding of the disease pathomechanism. In the future, this will greatly improve the chances of developing better treatment options for patients with DKC.

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16

Genetic Instability and Fanconi Anemia

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I. INTRODUCTION

Fanconi anemia (FA) is an autosomal recessive genetic condition that was first described by the Swiss pediatrician Guido Fanconi in 1927 (1). FA is caused by homozygous or by compound heterozygous mutations in a number of genes that maintain genomic stability and thus are important for normal aging. The phenotypic features of FA consist of a combination of developmental and somatic abnormalities, including alterations that are reminiscent of premature aging. Developmental defects dominate the clinical phenotype in 70% of patients with FA (2,3). Truly progeroid features are less conspicuous in FA than in the classic progeroid syndromes, and decline of bone marrow function is a much more progressive and devastating event in FA than in normal aging. This may be the reason why Martin and Oshima (4) did not include FA in their tabulation of human genetic instability syndromes with progeroid manifestations. Features such as radial ray defects, pigmentary changes, urogenital malformations, and short stature clearly belong to the category of developmental defects, whereas endocrinopathies, myelodysplastic syndrome, increased incidence, and early onset of squamous cell carcinomas may reflect premature aging. As in normal aging, endocrine abnormalities occur in more

than 80% of patients with FA, including hyperinsulinemia, growth hormone insufficiency, and hypothyroidism (5). Less than 50% of patients survive to adulthood, and reproduction is severely impaired due to spermatogenic failure in males and a low pregnancy rate in females (6). The key feature that justifies the inclusion of FA in a discussion of "premature aging syndromes" is genetic instability that is correlated in these patients with a sharply increased risk of neoplasia. There is a 15,000-fold increased risk for acute myelogenous leukemia, but squamous cell carcinomas of the female genital tract and of the oral cavity (in both male and female patients) are typical cancers of advanced age that occur 30–40 years earlier in patients with FA (7).

Since this chapter focuses on genetic instability in FA within the context of aging, other important aspects of the disease will receive less attention. There are a number of phenotypic manifestations in FA that are poorly understood and may not be a primary reflection of genetic instability. For example, patients with FA exhibit elevated levels of the proinflammatory cytokine tumor necrosis factor-α (TNF-α) (8,9), increased sensitivity toward interferon-γ (10), and elevated levels of α-fetoprotein (11). Even though bone marrow failure is the single most life-threatening feature in patients with FA, it is not known whether the loss of bone marrow function is a direct consequence of the mutant FA proteins, or whether it results from precipitous decline of bone marrow stem cells because of excessive accumulation and/or defective repair of DNA damage. There is some evidence that the FA proteins may play a direct or indirect role in the regulation of hematopoiesis (12–14), but the fact that FA knockout mice have normal bone marrow function (unless exposed to alkylating agents) argues in favor of a protective, rather than a regulatory, function of the FA proteins (15,16).

II. COMPLEMENTATION GROUPS

Genetic heterogeneity was recognized as early as 1980 as a typical feature of FA when cell fusion studies by Sperling and colleagues provided the first evidence for the existence of at least two complementation groups (17). In 1992, Buchwald and coworkers in Toronto, using an expression cloning strategy, cloned the first FA gene (*FANCC*) and increased the number of complementation groups to four (18). In the following years, Joenje and associates in Amsterdam defined additional complementation groups by fusing genetically marked lymphoid cell lines from patients with FA and looking for reversal of mitomycin C (MMC) sensitivity, raising the total number of definitive FA genes to eight (19,20). One of these, complementation group H, was reassigned to group A in further studies (21). Since a number of primary cells and cell lines derived from patients with FA cannot currently be assigned to any of the known complementation groups, either by cell fusion or by transduction studies, it is very likely that additional complementation

groups and genes will be found. The clinical course of the disease appears to be much more strongly determined by the type of mutation and ethnicity rather than by belonging to a given complementation group (22,23). For example, the IVS4+4A>T mutation in the complementation group C gene causes early onset of hematological symptoms and consistent short stature combined with radial ray defects, whereas the 322delG mutation in the same gene usually causes fewer somatic abnormalities and a much later onset of aplastic anemia (24). There are some indications that patients belonging to complementation group G and patients homozygous for null mutations in the complementation group A gene may have a more unfavorable clinical course (25), but definitive genotype–phenotype correlations require prospective studies.

III. GENES

To date, seven of the eight genes predicted by complementation analyses have been cloned (26). Since 1999, the internationally accepted nomenclature denotes FA genes by using the prefix "FANC" in combination with alphabetical labeling of the respective complementation groups. Table 1 summarizes the current information of the FA genes, including map location, transcript size, and molecular weights of the predicted proteins. Several points emerge from this tabulation: With the exception of chromosome 9 that harbors two of the FA genes (*FANCC* and *FANCG*), the other FA genes are distributed throughout several chromosomes

Table 1 Characteristics of Cloned FA genes and Their Predicted Proteins

Gene	%	Chromosome	Exons	mRNA	Amino acids	Protein	Motifs
FANCA	70	16q24.3	43	5.5 kb	1456	163 kDa	NLS,LZ
FANCC	8	9q22.3	14	4.7	558	63	LZ (?) p53 binding (?)
FANCD2	<5	3p25.3	44	4.4	1451	166	HMG-like
FANCE	<5	6p21.3	10	2.6	536	60	2 NLS
FANCF	<5	11p15	1	1.2	374	42	ROM?
FANCG	12	9p13	14	2.7	622	68	(=XRCC9), LZ

% Refers to the average percentage of Caucasian patients belonging to the respective complementation groups. NLS, nuclear localization signal (nuclear import signal); LZ, leucine zipper (mediates protein–protein interaction); HMG, high-mobility-group proteins (DNA-binding motif shared by nonhistone components of chromatin and transcriptional regulators; ROM, RNA 1 modulator (determines copy number in plasmid pBR322); XRCC9, putative human DNA-repair gene that complements the mitomycin C–sensitive Chinese hamster cell mutant *UV40*. Question marks denote incomplete/uncertain motifs. The genes for complementation groups B and D1 (FANCB and FANCD1) were recently shown to be identical with BRCA2 (157).
Source: Modified from Ref. 26.

of the human genome. It is intriguing that two of the FA genes are syntenic on human chromosome 9, since this chromosome is a highly conserved ortholog of the avian Z chromosome, whose dosage is thought to be instrumental in avian sex determination (27). *FANCC* and *FANCG* are highly expressed in testicular tissues. Their conservation on human chromosome 9 within segments that are orthologous to the avian Z chromosome may indicate a function of these genes in meiosis and recombination. Additional evidence for a putative function of the FA genes in gametogenesis derives from the observation of impaired fertility of patients with FA (6), failure of spermatogenesis in *FANCC* and *FANCA* knockout mice (28,29), expression of FA genes in testicular tissue (30), and association of FA proteins with the synaptonemal complex (31). Of the few conserved motifs that seem to be present in some of the FA proteins (NLS predicts import into the nucleus), the HMG-like domain may indicate a role of *FANCD2* in transcriptional regulation during embryogenesis (32), and partial or complete Leucine-Zipper motifs predict interaction of the FA proteins either with themselves or with other proteins (33, 34). FANCG has been shown to be a phosphoprotein (35), and phosphorylation is thought to occur at multiple sites of the FANCA protein prior to nuclear import (36). It remains to be seen whether two putative p53 consensus sequences in FANCC have functional significance (37).

A. Gene Functions

Of the presently known genes whose defects cause genetic instability and a propensity toward cancer in humans, the FA genes have remained the most enigmatic. This is because the genes responsible for the helicase defects (Bloom, Werner, and Rothmund–Thompson syndromes), as well as the genes causing ataxia-telangiectasia and Nijmegen breakage syndrome, have turned out to have close homologs in lower species such that their likely functions could be deduced by analogy (4). In contrast, with the exception of the newly described *FANCD2* gene, the FA family of genes seems to have emerged only recently in evolution. The most distantly related species that show partial sequence homologies are ray-finned fish including *Tetraodon nigroviridis, Takifugu rubripes,* and *Danio rerio* (26). There are no apparent homologies in nonvertebrate, yeast, or bacterial sequences. This lack of homology, and the relative absence of DNA and/or protein motifs with clear-cut functional significance, has severely hampered the elucidation of the function of the FA genes. It should be noted, however, that the developmental expression pattern of FA genes in mice correlates remarkably well with the developmental (skeletal) defects seen in patients with FA (38). This has led Joenje and Patel (26) to speculate that the emergence of the FA genes (with the exception of *FANCD2*) may have coincided with the evolutionary emergence of an internal skeleton.

Following the cloning of the six presently known FA genes, immunofluorescence, immunoprecipitation, and yeast two-hybrid studies by a number of lab-

oratories suggest functional interaction, between the respective proteins, most likely via protein complex formation (39–42), nonexclusive but preferential nuclear localization of the gene products (40,43), and a role in the cellular response to DNA damage (31,44–46). The first direct connection between DNA repair and one of the FA genes emerged with the cloning of the *FANCG* gene that turned out to be identical to a previously isolated human gene that complements the MMC-sensitive Chinese hamster cell mutant *UV40* (47). An excellent review by Joenje and Patel (26) summarizes what is currently known about the FA genes, their mutations, their proteins, and putative functions.

As Joenje and Patel point out, a major conceptual breakthrough was the recent cloning of the *FANCD2* gene. *FANCD2* is the only FA gene to date that appears to be highly conserved, having orthologs in *Arabidopsis thaliana*, *Caenorhabitis elegans,* and *Drosophila* (48). D'Andrea and colleagues suggest that all the other FA genes, following their recruitment into the nucleus, activation via phosphorylation, and multimeric association, function as an enzymatically active protein complex that monoubiquinates the evolutionarily ancient and exclusively nuclear *FANCD2* gene product at its lysine 561 position. In this view, activation of the D2 protein via monoubiquination would occur in response to DNA damage and would serve to target the D2 protein to nuclear foci that contain other caretaker and DNA repair proteins, notably, BRCA1, BRCA2, and Rad 51 (31). The observation that activated FANCD2 protein appears to colocalize with BRCA1 in radiation-induced nuclear foci and in synaptonemal complexes of meiotic chromosomes argues strongly for a role of the FA proteins in the maintenance of DNA integrity. This key observation connects the FA pathway to the cancer field and to known constituents of the nuclear DNA-damage defense system. A summary of these most current concepts of FA gene product interaction and function is presented in Figure 1.

Since FA is an autosomal recessive disease in which heterozygotes exhibit only subtle, if any, manifestations, an enzymatic function of some kind (like ubiquination) is expected of the FA genes. Moreover, the *FANCD2*- "effector-gene" hypothesis that emerges from the discovery of the two isoforms of the downstream *FANCD2* gene (reflecting differences in ubiquination status) would explain why mutations in any of the upstream FA genes causes a similar phenotype. What remains difficult to explain is why at least five—and probably more FA genes and their assembly into a multiprotein complex—are needed to carry out a relatively simple task like the monoubiquination of FANCD2. It would come as no surprise if additional functions of the FA genes were to emerge in future studies. Such alternative functions might not be limited to the cell nucleus but may, for example, involve major detoxification pathways within the cytoplasm of mammalian cells. Such a scenario has been suggested by the observation of interaction of the FANCC protein with NADPH cytochrome P450 reductase (49). There are some indications that the *FANCC* gene may have special functions that set it apart

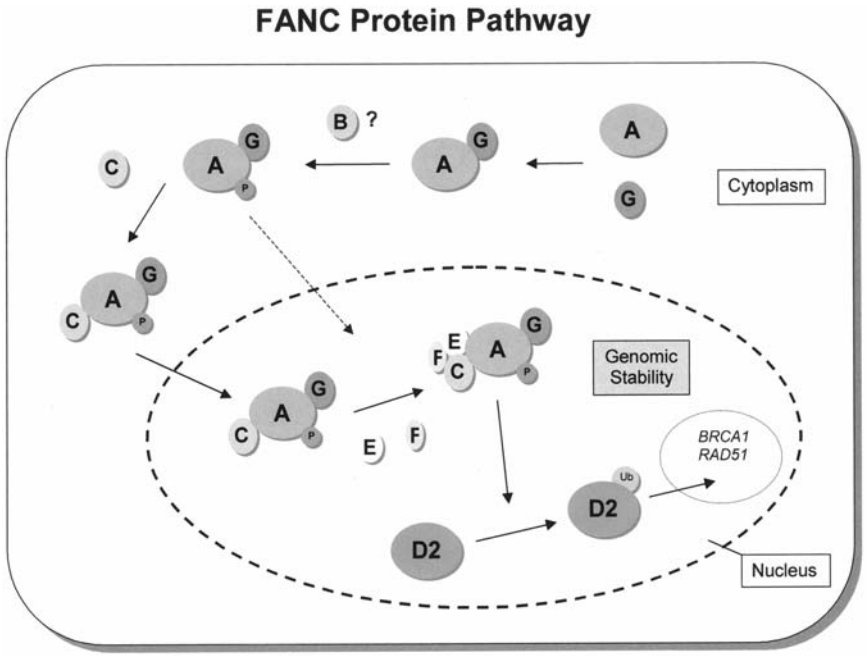

Figure 1 Current concept of cytoplasmatic assembly, nuclear transport, and nuclear function of the Fanconi anemia protein complex. Size of symbols reflects size of proteins. (Based on concepts discussed in Refs. 26, 31, 42, and 53.)

from the other FA proteins. For example, it has been estimated that 90% of the FANCC protein resides in the cytoplasm (50); overexpression localizes the FANCC protein to the cytoplasm where it corrects MMC sensitivity in mutated cells (43,51); and in the yeast two-hybrid system, FANCC interacts much more weakly, if at all, with the other FA proteins (52,53).

IV. GENETIC INSTABILITY AND DNA REPAIR

Elevated levels of spontaneous chromosomal aberrations are generally thought to reflect inadequate repair of DNA damage. A very convincing causal link between the emergence of chromosomal aberrations and the absent or impaired function of a gene product that is involved in the maintenance of DNA integrity has been established for the Werner and Bloom syndrome helicases (4). Early cytogenetic studies in FA cells demonstrated elevated levels of spontaneous aberrations and a prevalence of chromatid-type alterations, including chromatid exchange figures (reviewed in Ref. 54). Increased levels of such unstable types of chromosomal

aberrations are typically found in senescing cell cultures, and a manifestation of genetic instability that links FA fibroblasts to senescing cells is the relative abundance of extrachromosomal, small polydisperse circular DNA in FA fibroblasts (55). A key question that remains is whether the function of the FA genes is limited to sensing and recognition of DNA damage, or whether these genes play a direct and active role in DNA repair. There is no question that the preservation of chromosomal stability depends on mechanisms that eliminate chromosomal lesions prior to cell division (56). These mechanisms include G2/M checkpoints (to halt damaged cells from progression into mitosis) and double-strand break (DSB) repair via homologous recombination. A number of studies have suggested DNA repair deficiencies in FA cells (46,47,57–61), but a systematic repair defect involving each and every complementation group has been difficult to prove. A most intriguing observation in light of the recent FANCD2–BRCA connection is the reported decreased fidelity of nonhomologous end joining in FA cells, which suggests a defect associated with one of the DSB repair pathways (26).

Proteins involved in homologous recombinational repair and in nonhomologous end joining have been shown to accumulate in nuclear foci following treatment with DNA-damaging agents. Some of these proteins, like Mre11, NBS1, and Rad50, colocalize at sites of DSBs within 30 min (62) and are thought to mediate the direct joining of broken DNA ends. Others, including Rad51, Rad52, and replication protein A (RPA) appear at single-stranded DNA lesions (63,64). During homologous recombinational repair, 3′ overhanging single-stranded DNA (ssDNA) strands are formed that subsequently associate with Rad51, which in turn appears to cooperate with Rad52 in response to DNA damage (65). There are at least five Rad51 paralogs involved in homologous recombinational repair (66) whose potential importance with respect to FA is highlighted by the Rad51–BRCA– FANCD2 connection. Rad51 and replication protein A (RPA) are never found in foci containing the Mre11/NBS/Rad50 complex and vice versa, indicating a cellular commitment to either homologous recombination or nonhomologous end joining. In contrast, the BRCA1 protein can be detected in nuclear foci in both types of DNA lesions. Owing to their rapid appearance at sites of DNA damage, nuclear foci are thought to represent sites of DSB repair. However, there is increasing evidence that foci also might be associated with apoptotic events whose pathways appear to be altered in FA (13,67–69). DSB repair usually occurs within no longer than 5 hr following exposure to DNA-damaging agents, but nuclear foci of any kind can be seen as late as 24–48 hrs following the damaging event. Rad51 foci have been shown to coalesce 1 to 2 days following irradiation and are removed into micronuclei where DNA is subject to apoptotic degradation (70). Likewise, radiation-induced micronuclei display RPA-positive foci, suggesting an association with cell death after x-ray treatment. It thus remains an open question whether nuclear foci actually represent sites of DSB repair or whether they reflect sites of irreparable DNA lesions. What is firmly established is that nuclear

foci represent accumulations of multifunctional protein complexes that assume variable positions within the nuclear protein scaffold in response to cellular insults.

A. Genetic Instability and Susceptibility to Clastogens

The feature of increased genetic instability and the putative role of the FA genes in the cellular response to DNA damage justifies including FA in the category of human caretaker gene syndromes as defined by Kinzler and Vogelstein (71). Genetic instability in FA was first described in 1964 by Schroeder and coworkers as increased spontaneous chromosomal breakage rates in 72-hr peripheral blood lymphocytic cultures (72). Subsequently, a number of investigators found that FA cells were hypersensitive toward bifunctional alkylating agents such as MMC, nitrogen mustard (NM), and diepoxybutane (DEB). Exposure to these agents causes a significant increase in the rate of chromosomal breakage in FA versus control cells, such that this hypersensitivity could be exploited for diagnostic testing. Whereas induction of chromosomal breakage by DEB has become the gold standard in the diagnosis of FA (73), many laboratories use the less toxic compound MMC for diagnostic purposes. The sensitivity toward bifunctional alkylating agents holds for all complementation groups (74), but the mechanism underlying this sensitivity is not understood. FA cells are not hypersensitive to monofunctional agents such as MMS or EMS, nor are they unusually sensitive toward ultraviolet (UV) irridiation. Much of the early literature reporting extensive cytogenetic clastogen and dose-response studies has been summarized in a 1989 monograph on FA (54).

An issue that has been hotly debated in the literature is whether FA cells are sensitive toward ionizing radiation (reviewed recently in Ref. 75). Within the context of the conditioning regimen for bone marrow transplantation, there has been a longstanding clinical impression of increased radiosensitivity of patients with FA who also seem to tolerate cyclophosphamide less well than patients who do not have FA. However, in vitro studies with FA cells have yielded conflicting results. An unpublished study from our laboratory shows that FA fibroblasts of all known complementation groups respond to increasing x-ray doses in the same way as control cells (Fig. 2). Moreover, when fibroblasts from patients with ataxia-telangiectasia (which are known to be highly radiosensitive) are taken as positive controls, studies from our laboratory provide no evidence for increased radiosensitivity of FA fibroblasts under in vitro conditions. A surprising result has been obtained when primary mononuclear blood cells were exposed to x-rays and analyzed via the single-cell electrophoresis (comet) assay (Fig. 3). Homozygous and heterozygous FA cells deviated significantly from control cells with respect to several parameters of this assay (75). The current interpretation of these unexpected findings is that nuclei carrying mutations in the FA genes release more fragmented DNA following x-ray exposure than control nuclei do. It remains to

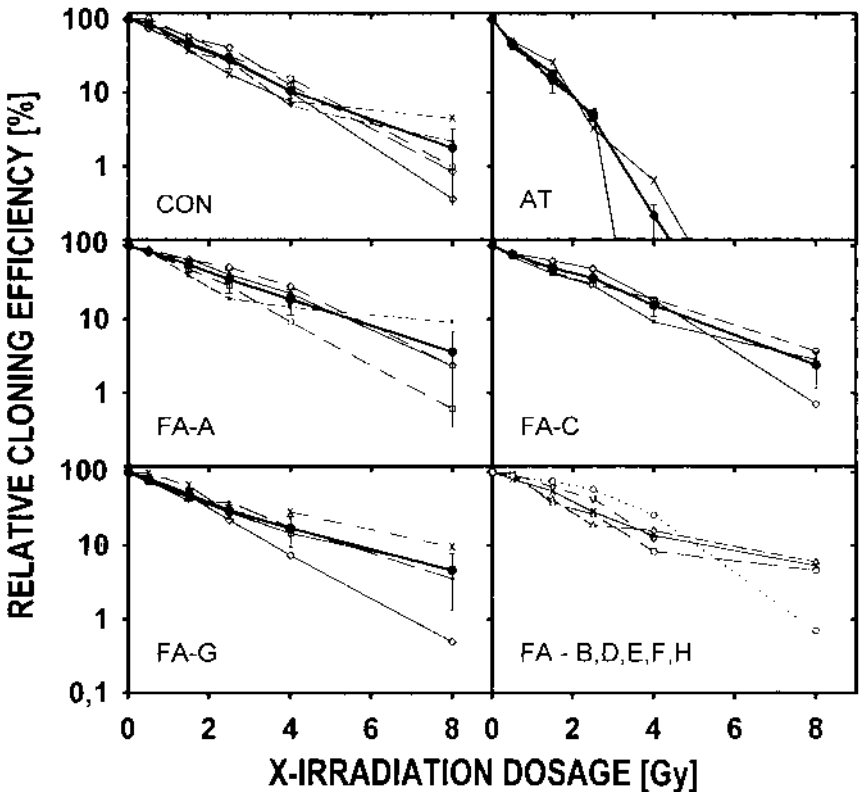

Figure 2 Unpublished study of cloning efficiencies of passage four to six fibroblast cultures from multiple FA patients belonging to complementation groups A, C, and G and single patients belonging to the remaining groups. CON, control fibroblast cultures; AT, fibroblast cultures derived from patients with ataxia-telangiectasia (AT). Note similar kinetics of decline of cloning efficiencies as a function of x-ray dose among the various FA and control cultures, whereas the cloning ability of AT cultures declines much more rapidly.

be seen whether this aberrant behavior can be explained, as postulated by the investigators, by an altered chromatin architecture in cells that carry one or two mutations in one of the FA genes.

B. Genetic Instability and Chromatin Structure

The hypothesis of FA being a "chromatin disease" was first postulated by Tanja Reuter, who, in yeast two-hybrid studies, found evidence for a consistent interaction between FA proteins and a number of proteins known to be involved in chro-

matin assembly and chromatin remodeling (76,77). It is quite plausible that a change in chromatin structure should accompany DSB repair to permit repair proteins access to damaged DNA (78). Multisubunit protein complexes with partially overlapping and shared, but also antagonistic, functions are required for the disruption and reformation of nucleosomal arrays during transcription, DNA replication, and DNA repair (for review, see Ref. 79). It is conceivable that the FA proteins participate in some yet unknown way in these changes of higher order chromatin structure after DNA damage has occurred. Since BRCA1 is known to be associated with the SWI/SNF-related complex that appears to play a central role in chromatin remodeling (80), the recently discovered FANCD2–BRCA1 connection provides additional support for a chromatin-associated function of the FA proteins.

One candidate for interaction with the FANCC protein that fits nicely into the chromatin hypothesis has been isolated by Hoatlin (81). Named FAZF, this interactor is homologous to the promyelocytic leukemia zinc finger protein (PLZF) and contains a BTB/POZ domain implicated in oncogenesis, limb morphogenesis, hematopoiesis, and cell proliferation. Wild-type FANCC protein and the interactor FAZF have been shown to colocalize in nuclear foci and may be involved in

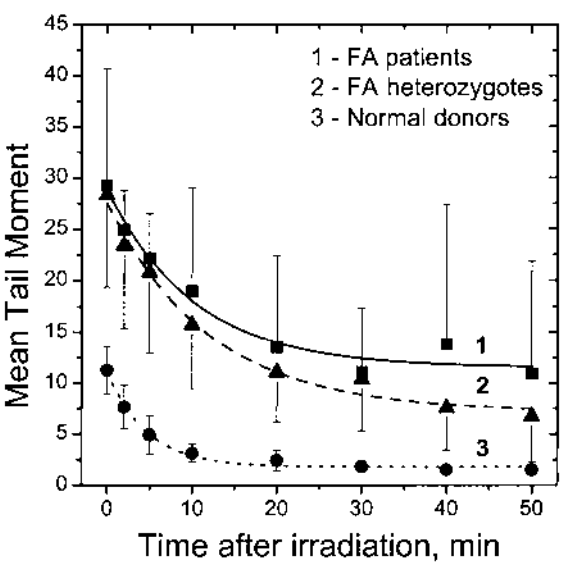

Figure 3 Results of single-cell electrophoresis assays of 8 patients with clinical suspicion of FA, 36 heterozygous obligate carriers (parents of FA patients), and 10 clinically normal blood donors. There is a highly significant difference in the initial tail moment parameter between FA genotypes and controls, indicating differences in nuclear chromatin response to x-ray exposure. (See Ref. 75 for details.)

transcriptional repression via chromatin remodeling (81). This observation agrees with the idea that local chromatin organization has to be changed in order to permit the DNA repair machinery to gain access to damaged DNA (82).

The most direct evidence yet for an intimate connection between FA and chromatin proteins derives from biochemical fractionation and immunocytochemical studies by Kupfer and coworkers demonstrating a physical association among FA proteins in nuclear matrix and interphase chromatin. This association appears to be enhanced by exposure toward MMC but is lost during the metaphase stage (83). Given that an altered chromatin architecture is a hallmark of leukemic cells, the finding by Joenje and colleagues of aberrant profiles of FA proteins in leukemic cells would fit nicely into the picture of a chromatin maintenance, assembly and/or a remodeling function of the FA family of proteins (84). However, one should not lose sight of the fact that the known FA proteins seem to lack features such as paired helical domain (PHD) fingers, bromo domains, or heterochromatin localization domains that characterize proteins involved in the regulation of chromatin structure.

C. Genetic Instability and Mosaicism

As shown in Figure 4A, there is a dose-dependent increase of chromosomal breakage in 72-hr FA lymphocytic cultures following exposure to 50 and 100 ng/mL MMC. Pathognomonic for FA is the emergence of metaphases with breakage rates in excess of 10 breaks/cell (see Fig. 4D). Such highly susceptible cells are not, as a rule, seen in control cultures. There are, however, a number of patients with FA in whom chromosomal breakage studies yield an intermediate and clearly bimodal distribution of breakage rates (see Fig. 4B, C). Such patients are considered to be mosaics for defective and self-corrected cells (85). The emergence of subpopulations of lymphocytes that have lost their MMC or DEB sensitivity is a rather frequent event in FA. For example, between 20 and 40% of B-cell–derived lymphoblastoid cell lines from these patients convert to MMC resistance following variable times in culture. Detection of mosaicism in short-term peripheral blood lymphocytic cultures requires extensive cytogenetic screening and might be frequently missed. On the other hand, completely normal chromosomal breakage studies might obscure the diagnosis of FA in cases of complete lymphocytic mosaicism (86). By separately analyzing and comparing the causal FA mutations in cell colonies from peripheral T cells, granulocyte–macrophage, and erythroid precursors of a single individual, Gregory and coworkers (87) have provided evidence for a genotypic reversion in the *FANCA* gene that must have taken place in a lymphohematopoietic stem cell. Self-correction via intragenic crossover in patients with FA was proved by genetic marker analysis for a compound heterozygote *FANCC* patient (85). Since compound heterozygosity is the rule rather than the exception for *FANCA* patients, mosaicism via intragenic crossover may arise rather frequently in this patient group. Moreover, being a highly polymorphic

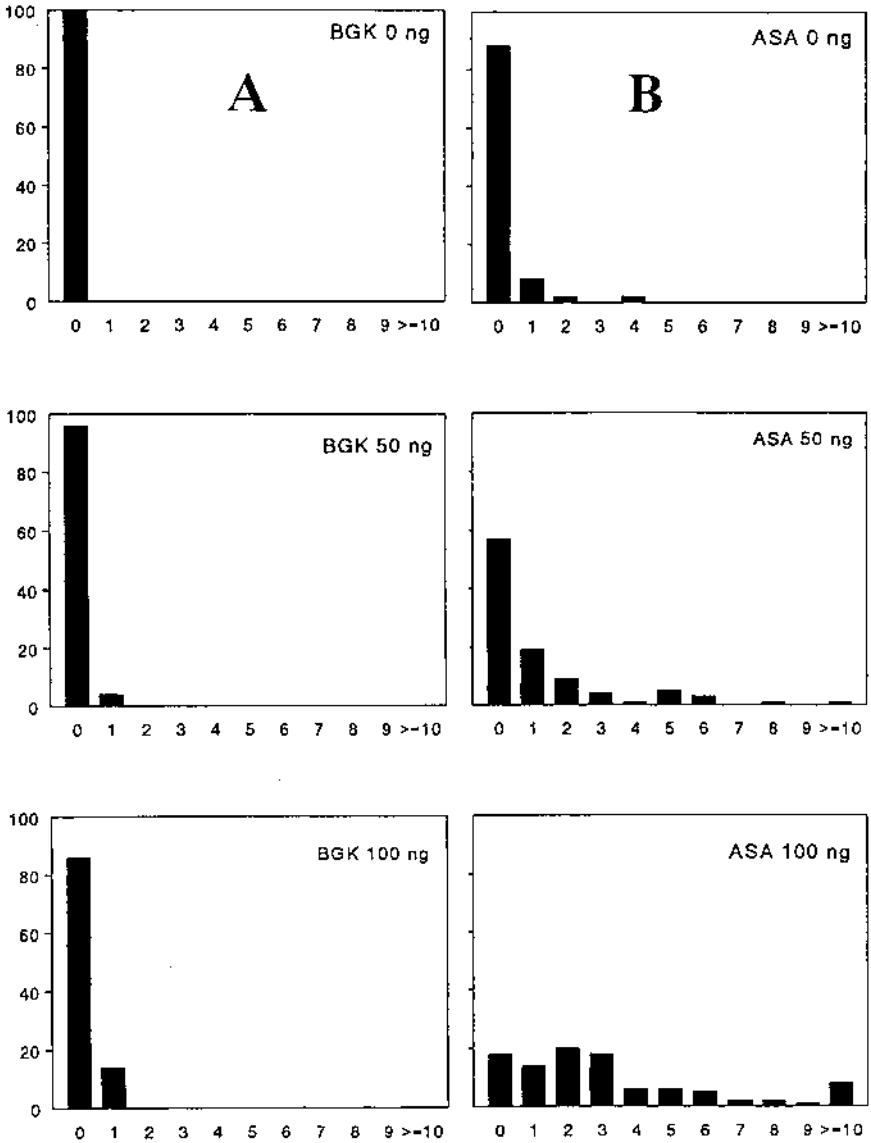

Figure 4 Chromosomal breakage studies in 72-hr blood cultures following exposure to 0, 50, and 100 ng/mL MMC. A, clinically normal blood donor showing MMC resistance and unimodal, low breakage rates; B and C, mosaic FA patients exhibiting bimodal breakage distributions after MMC exposure; D, nonmosaic FA patient, showing unimodal and uniformly high breakage rates. Horizontal axis denotes breakage events per cell; vertical axis denotes percentage of distribution of breakage rates per 50 metaphases analyzed at each MMC concentration.

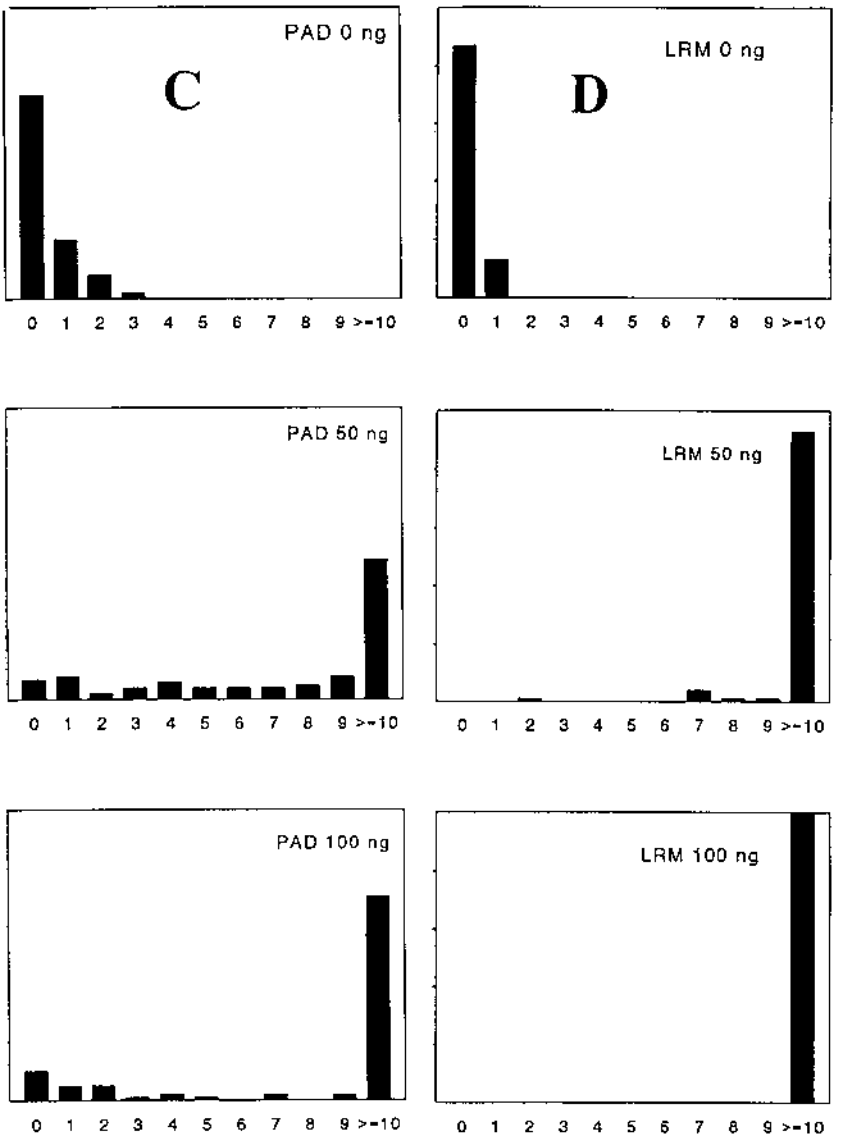

Figure 4 *Continued.*

gene that harbors a variety of repeat structures (88,89), it is not surprising that the *FANCA* gene is predestined to undergo replicative and transcriptional errors that may lead to a correction of the primary mutation and/or introduce a second mutation that compensates the ill effect of the primary lesion. Waisfisz and colleagues (90) have documented very instructive examples of this type of "natural gene therapy" through compensating somatic alterations in *cis* that partially restore the loss of function caused by the constitutional, inherited mutation.

A second type of mosaicism that is prevalent in FA affects the bone marrow. It is quite common to encounter cytogenetically aberrant clones in bone marrow aspirates of young patients with FA several years prior to the onset of a clear-cut myelodysplastic syndrome (91). There usually is a gradual increase of clonal chromosomal changes in bone marrow specimens over many years, but in contrast to patients who do not have FA, the appearance of aberrant clones in FA seems to be less worrisome with respect to the emergence of leukemia (92). Alter and coworkers have concluded that a morphologically defined myelodysplastic syndrome (MDS) may be more important than cytogenetics in predicting an adverse outcome (93). There is, however, increasing evidence that certain types of clonal aberrations may be connected with a poor prognosis (H. Neitzel, personal communication). One such aberration, monosomy 7, is nonspecific for FA but a frequent clonal change in FA bone marrow aspirates. Since benzene metabolites such as hydroquinone and phenol have been reported to increase the level CD34-positive blood progenitor cells with monosomy 7 (95), McDonald and colleagues (96) have pointed out that the endogenous presence of these metabolites (derived from diet and gastrointestinal flora) may be a causal factor in the emergence of leukemias. If patients with FA are more sensitive toward these nutrionally derived and/or endogenously formed metabolites as they are toward bifunctional alkylating agents, one could explain their high freqency of monosomy 7 bone marrow clones. Other fairly frequent aberrations in FA bone marrow involve triplications of 1q23q42, monosomy 20, and trisomy 13 (94). Even though accelerated telomeric shortening and 4.8-fold higher telomerase activity have been reported in FA mononuclear blood cells, there was no apparent correlation between telomeric length and the presence of bone marrow clonal abnormalities (97). If the size of the telomeric repeat is taken as a measure of biological age, patients with FA appear to be 20–30 years older than controls. Accelerated telomeric shortening is also seen in non-FA aplastic anemia and may generally reflect clonal hematopoiesis involving a reduced and/or defective stem cell pool (98).

In the context of aging, the phenomenon of mosaicism clearly deserves special attention. The emergence of mosaicism may be viewed as a direct consequence of genetic instability of dividing somatic cells for which FA is a remarkably instructive model disease. Mosaicism provides the basis for in vivo selection and as such is at the heart of clonal proliferation and clonal attenuation that characterize aging as well as neoplastic cell populations (99–101). Forward mosaicism

as defined by Foe and collegues (85) may give rise to local pathologies in heterozygous individuals during their lifetimes. As such, mosaicism may underlie defects in proliferative homeostasis and organ pathology that accompany the aging process. For the patient with FA, genetic instability can be seen as a double-edged sword. On the one hand genetic instability implies increased cancer risk, but on the other hand, genetic instability might lead to self-correction and thereby improved bone marrow function. The fact that self-corrected cells emerge in these mosaic patients is highly encouraging with respect to the prospects of gene therapy, since these "experiments of nature" demonstrate quite clearly that corrected cells can attain an in vivo growth advantage.

D. Genetic Instability and the Cell Cycle

Poor growth due to prolonged generation times has been noted in FA primary fibroblast cultures in early studies (102,103), but Dutrillaux and coworkers were the first to show that this prolongation arises mainly during the G2 phase of the cell cycle (104). Using high-resolution, bivariate bromodeoxyuridine (BrdUrd)/Hoechst flow cytometry and modified Smith-Martin exit kinetics, Kubbies and associates (105) dissected the cell cycle behavior of 72-hr peripheral blood mononuclear cell cultures from three patients with FA, showing that cell cycle arrest occurs in FA cells during the S and the G2/M phases of the cell cycle, whereas prolonged transit times were found mostly for the G2/M phases of three consecutive cell cycles. An example of the characteristic cell cycle disturbance seen in FA cells is shown in Fig. 5. With the exception of leukemic cells, it could be shown that typical G2 phase accumulations of FA cells and their DEB- or NM-induced chromosomal breakage rates were highly correlated, such that a number of laboratories now use flow cytometry as a primary screening tool for differentiation between FA and non-FA forms of childhood aplastic anemias (106,107). The impaired cell cycle of FA cells would be difficult to reconcile with normal lymphocytic counts and function that characterize the majority of patients with FA prior to the pancytopenic stage. Kubbies and coworkers have shown that the prolongation of G2/M phase transit is compensated to a certain degree by a shortening of the following G1 phase transit, such that the overall cell cycle transit time of FA cells remains near normal (105).

There are two formal possibilities to explain the cell cycle alterations observed in FA cells: They are a normal consequence of excessive or unrepaired DNA damage or they represent a defective cell cycle checkpoint function. When Kruyt and colleagues (108) and Kupfer and colleagues (109) independently found that the activity of the cyclin B1/cdc2 kinase complex might be modulated by the product of the *FANCC* gene, a direct influence of the FA proteins on cell cycle checkpoints was suggested. However, two recent studies show that cross-linking agents cause S phase arrest in normal cells, whereas FA cells apparently undergo

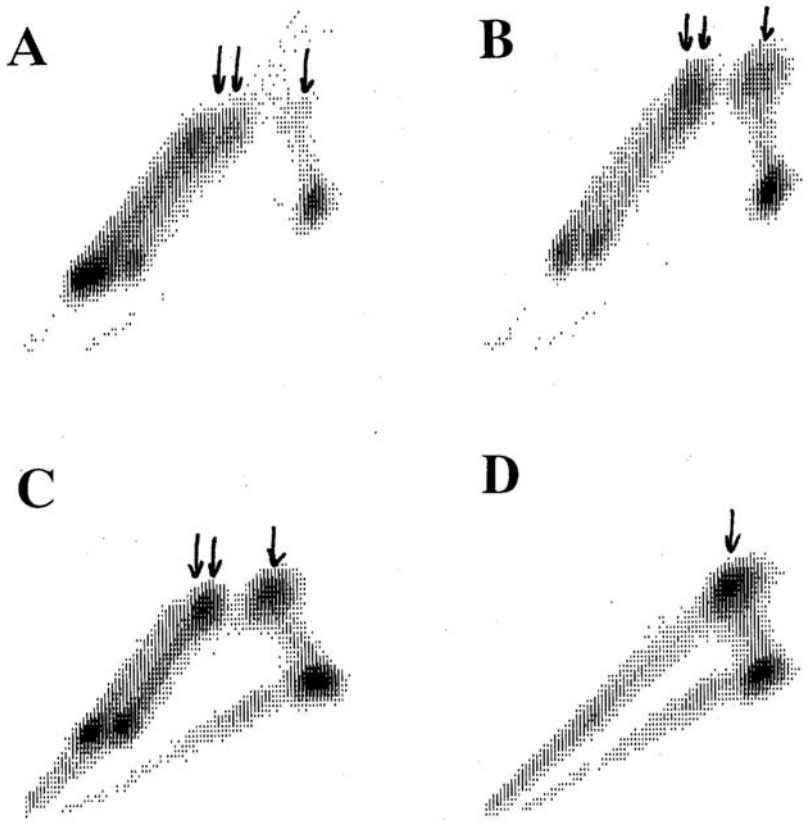

Figure 5 Bivariate BrdUrd flow histograms of 72-hr peripheral blood mononuclear cell cultures stimulated by phytohemagglutinin. Horizontal axis denotes 22358 Hoechst fluorescence, vertical axis denotes ethidium bromide fluorescence (arbitrary units). The first cycle G2 phase is marked by a single arrow. The second cycle G2 phase is marked by a double arrow. A, control culture showing cells distributed throughout three consecutive cell cycles, with most cells being in the G1 phase of the third cell cycle. B, control culture treated with 50 ng/mL MMC. Note arrest of cells in the G2 phases of the first and second cell cycles. C, FA patient. Note spontaneous accumulations of cells in the G2 phases of the first and second cell cycles. D, FA patient cells treated with 50 ng/mL MMC. Note complete absence of proliferating cells beyond the first cell cycle, G2 phase accumulation, and apoptosis of G2 phase and G1/G0 phase cells. (For details, see Refs. 105, 107, 113, and 116.)

damage-resistant DNA synthesis (110,111) and accumulate in G2. Activation of cdc2 by treatment with the G2 checkpoint inhibitor caffeine normalizes the duration of the G2 phase in FA lymphocytic cultures (112,113), but these "rescued" cells accumulate in the subsequent G1 phase, because their DNA lesions have not been removed. These and other studies (114,115) suggest that cell cycle checkpoints function normally in FA cells, and that delay and accumulation during the G2 phase reflects the existence of excessive and/or unrepaired DNA damage in these cells.

E. Genetic Instability and Oxidative Stress

One of the leading theories of aging is the free radical theory of aging. Many aspects of the aging process can be explained by a gradual loss of endogenous oxygen homeostasis (116). Interspecies comparisons, particularly among mammals and birds, emphasize the importance of the rate of free radical production in longevity (117,118). In humans, FA represents a unique example of genetically determined hypersensitivity toward oxygen. This was first shown by Joenje, who exposed primary lymphocytic cultures to oxygen concentrations ranging from 2 to 45% (v/v). He found a striking oxygen dependence in the rate of chromosomal aberrations in patients with FA (119). Since standard cell culture conditions provide oxygen at ambient air concentrations (20% v/v), it is very likely that the elevated "spontaneous" chromosomal breakage rates observed in untreated FA lymphocytic cultures simply reflect the genetically determined oxygen sensivity of these cells. Support for this notion came from a long-term study of fibroblast cultures, comparing growth and cloning of FA, obligate heterozygote, and control cells at 5 versus 20% (v/v) oxygen (120). This study showed that growth capacity and cloning efficiency of FA fibroblasts are severely impaired under ambient oxygen conditions but can be restored to near normal conditions under hypoxic (5% v/v) culture conditions (Fig. 6). On the cell cycle level, improved growth at low oxygen was paralleled by a reduction of G2 phase accumulations. The disproportionate and beneficial effect of hypoxic culture conditions on FA fibroblasts was confirmed by Saito and coworkers (121), who in addition compared the growth behavior of primary versus simian virus 40 (SV40)–transformed FA fibroblasts at ambient and reduced oxygen tension. Since there was no effect of oxygen concentration on the growth performance of the SV40-transformed FA cell line, the investigators considered hypersensitivity toward oxygen a "uniform but secondary defect" in FA cells. However, in a subsequent paper (122), they themselves showed that SV40-transformed cells generally lose their oxygen-dependent growth pattern, and that this is in no way limited to FA cells. Poot and colleagues (123) extended the observation of oxygen hypersensitivity to lymphoblastoid cell lines derived from patients with FA. They have suggested that this unique cellular phenotype may be mediated by the amount of iron in the culture media, impli-

cating a reduced capacity of FA cells to cope with the products of Fenton-type reactions; that is, reactive oxygen species. This may be relevant for hematopoietic stem cells, since these cells appear to be especially sensitive toward reactive oxygen species, which is indicated by their improved cloning efficiencies under conditions of reduced oxygen tension (124,125).

Pagano and Korkina (126) have reviewed and summarized the numerous efforts to prove the adverse effects of reactive oxygen species on the genome of FA cells. These proponents of the "oxidative stress theory in FA" believe that in vivo evidence points to the fact that oxidative stress characterizes the metabolic situation of different types of peripheral blood cells from patients with FA (127). By overexpressing thioredoxin, an ubiquitous intracellular antioxidant, in FA fibroblasts, Ruppitsch and colleagues (128) claim to prevent the clastogenic effects of MMC and DEB, which would argue strongly in favor of an oxygen-related path-

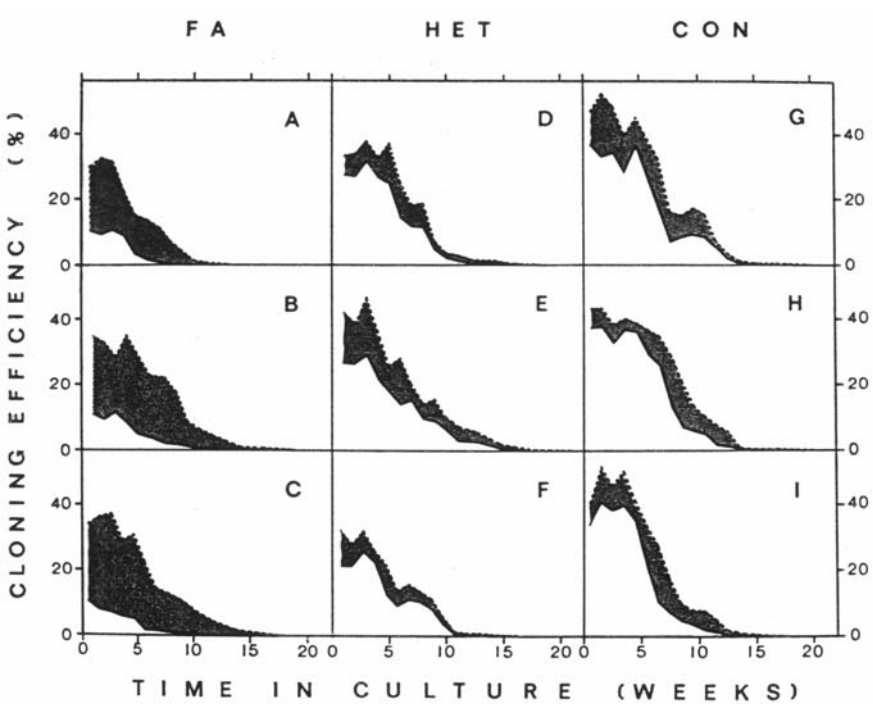

Figure 6 Serial cloning efficiencies of 3 FA, obligate heterozygote and control fibroblast cultures, each at ambient (20% v/v oxygen; lower margin of the blackened areas) and hypoxic (5% v/v oxygen; upper margin of the blacken areas) cell culture conditions. Note that the blackened areas are much wider in the FA fibroblast cultures, indicating a disproportionate gain in cloning ability at 5% vs 20% oxygen. (See Ref. 121 for details.)

way of the cytotoxic function of these agents. Based on complementation studies, the same group also reported evidence for a direct involvement of the FANCC protein in the repair of oxidatively damaged DNA (129). Using a classic shuttle vector system, Liebetrau and coworkers (130) reported increased rates and specific types of mutations in the plasmid pZ189 that was passaged through FA cells at 20 versus 5% (v/v) oxygen conditions. Most recently, Kelley and colleagues (131) showed that the *Drosophila* S3 multifunctional DNA repair/ribosomal protein protects FANCC cells against oxidative DNA-damaging agents. On the cytogenetic and cell cycle levels, DL-α-tocopherol (as a potent lipophilic antioxidant) has been reported to decrease the frequency of chromosomal damage and the duration of the G2 phase in 72-hr cultures of primary FA lymphocytes (132). It is obvious from these studies that the oxidative stress theory provides an attractive explanation for a variety of phenotypic features and cellular behaviors that are commonly encountered in patients with FA. These features include the early and frequent occurrence of MDS (133), bone marrow failure, pigmentary chances, and the emergence of squamous cell carcinomas at locations of typically high oxygen exposure (126,128). The strongly elevated cancer risk persists after successful bone marrow transplantation (see Fig. 7).

Many of the studies attempting to provide evidence in favor of the oxidative stress theory have not been confirmed, await confirmation, or have yielded contradictory results. For example, if the theory were correct, one would not expect FA cells to be relatively resistant to H_2O_2-induced DNA damage (134). Much of the speculation about oxidative stress in FA has been fueled by the idea that the striking hypersensitivity of FA cells toward agents like MMC and DEB may be caused by the property of these agents (that need to be metabolically activated) to generate oxygen radicals under in vitro conditions (135, 136). If oxidative stress is a major part of the FA cellular phenotype, one should expect to find evidence for increased baseline levels of oxygen-related DNA base modifications, notably 8-OHdG. Evidence in that direction has been provided by Degan and colleagues (137), who found significantly elevated 8-OHdG levels in patients with FA and to a lesser extent in FA heterozygotes. In a subsequent paper, these investigators modified their findings showing that induction of 8-OHdG by H_20_2 was higher in FA compared with control cell lines, but that the removal of 8-OHdG was reduced only in cells belonging to complementation group E (138). Quite opposite results were obtained by Will and coworkers (139), who in careful studies found only normal steady-state levels and normal levels of repair of oxidative DNA base modifications sensitive to Fpg protein (including 8-OHdG) in FA lymphoblastoid cell lines from various complementation groups. There also were no differences among FA fibroblasts and control cells regarding the detection of single-stranded breaks by the alkaline elution assay following short-term exposure to very high (50–95% v/v) oxygen concentrations (140). Additionally, Joenje and coworkers (141) and Tower and coworkers (142) reported negative results with respect to the

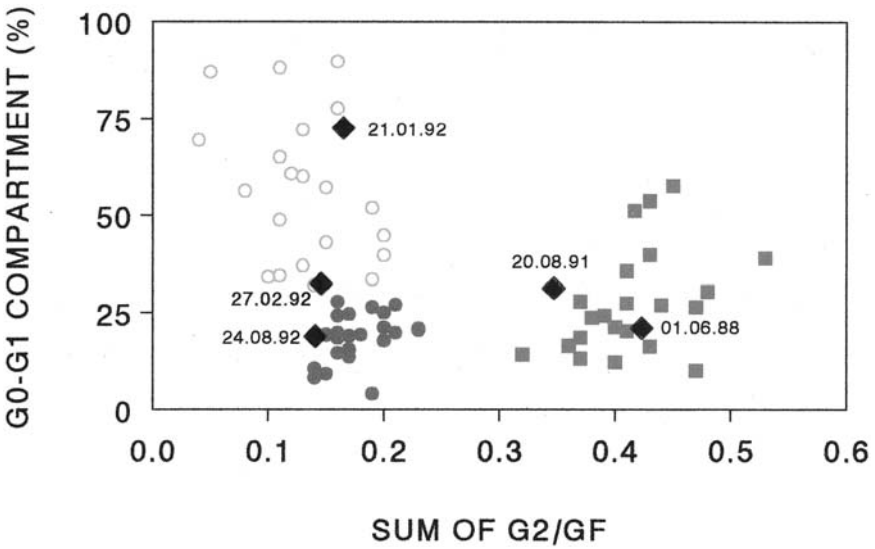

Figure 7 Time course of successful bone marrow transplantation (BMT) of an 8-year-old FA patient monitored by analysis of G2 phase accumulations in 72-h peripheral blood mononuclear cultures. The patient′s blood was analyzed on two occasions prior to BMT on May 1, 1992, each time showing a sum of G2/GF values typical for other FA patients (straight squares). There was no evidence for G2 phase accumulations at 2 weeks after transplant, but the fraction of noncycling (G0-G1) cells was as high as usually seen in patients with non-Fanconi aplastic anemia (outlined circles). One month later, mitogen response was much improved, as indicated by the lower G0-G1 value, and at 8 months after transplant, the patient's cells could not no longer be differentiated from control cells (solid circles) indicating complete repopulation of his bone marrow by donor cells. The patient died 8 years after transplant from carcinoma of the tongue.

hypothetical involvement of FA proteins in the oxidative stress response. In these studies, neither oxygen tension of the cell culture environment nor clastogens and oxidative stress were found to influence the expression of the *FANCC* gene that appears to be constitutively expressed at rather stable levels throughout the cell cycle.

V. PUZZLES AND PERSPECTIVES

Despite an unclear role of oxidative stress in FA, the recent finding by D'Andrea and colleagues (40,157) that the FA proteins and BRCA L-proteins may cooperate in a common damage-recognition and/or damage-response pathway opens a new and exciting direction for future research. It would not be surprising if the FA proteins turned out to be part of a major protein complex, such as the BASC

complex, which consists of BRCA-associated proteins that are involved in the recognition and repair of defective DNA (143). Le Page and coworkers (144) have shown that the products of the *BRCA1* and *BRCA2* genes are required for transcription-coupled repair of oxidative 8-OHdG lesions in human cells, and BRCA1 nullizygous embryonal cells are unable to perform transcription-coupled repair of oxidative DNA lesions (145). These observations would be consistent with the suggestion (from a single laboratory at the time of this writing) that the FANCC protein plays a direct role in the repair of oxidatively damaged DNA (129). The putative FA–BRCA connection should therefore shed new light on the oxidative stress hypothesis in FA and perhaps clarify some of the controversy surrounding the idea that the FA family of proteins represents a new class of proteins with direct or indirect antioxidant functions. It is quite obvious that warm-blooded and long-lived mammals that generate high endogenous levels of reactive oxygen species during normal cell functions (e.g., oxidative phosphorylation) require special protection against oxidative DNA damage. The evolutionarily recent family of FA proteins might well serve this purpose. Given the paucity of solid experimental evidence, many of the ideas focusing on the oxidative stress theory have still to be considered within the realm of speculation. What remains established beyond doubt is that primary FA fibroblasts are hypersensitive toward ambient oxygen, but this hypersensitivity also is seen in lymphoid cell lines and in colony-forming unit (CFU) assays of bone marrow cells (125). Moreover, hypersensitivity toward oxygen appears not to be due to primary defects in any of the classic free radical scavenging systems (146). Since hypersensitivity toward oxygen can be reversed by growing FA cells at reduced rather than ambient oxygen levels, it is tempting to conclude that the proper function of the FA family of proteins requires a cellular milieu of low oxidative stress. What remains unclear is whether the in vitro hypersensitivity toward oxygen causes greater accumulations of DNA lesions in FA as opposed to non-FA cells, or whether the removal of these lesions is less effective or somehow impaired in FA cells. Some of these open questions might be resolved by the availability of a double knockout mouse model with deficiencies of the Fancc and Cu/Zn superoxide dismutase genes. In addition to liver abnormalities, these mice exhibit impaired hematopoiesis, which suggests that incomplete protection against endogenously generated free radical species may cause bone marrow failure in FA (147)

From a phenotypic view, many of the adolescent and adult patients with FA offer the impression of accelerated aging. These patients mostly are underweight individuals with short stature, a delicate body build, pale appearance, and a hoarse voice. They appear older than their biological age. Survival to age 50 year occurs only in rare instances, and many of the patients surviving to adulthood without bone marrow transplantation may in fact owe their relative longevity to self-correction of bone marrow cells manifesting as mosaicism. There is, however, the puzzle of rare patients with FA who appear phenotypically and clinically normal until adolescence and/or adulthood, and who may only be diagnosed when their

fibroblast cultures are tested with MMC (see Fig. 8). Whether these patients represent complete bone marrow stem cell mosaics or whether they harbor certain types of "mild" or compensatory mutations with sufficient residual protein function remains unclear. If a matching sibling donor is available, bone marrow transplantation (BMT) using a modified conditioning regimen is the therapy of choice (148). More than 80% of the transplanted patients survive BMT for more than 5 years, but squamous cell carcinomas of the tongue and genitals remain a serious problem (149). Since less than 25% of all patients with FA have a matching sibling donor, improvements of unrelated donor BMT are urgently needed. Modification of BMT protocols, including use of cord blood cells and cytoreduction with low-dose cyclophosphamide and fludarabine, raise hope for improved survival of unrelated donor transplants (150,151). Because of the relatively high risk of unrelated donor transplants, in the future, more couples are likely to opt for preimplantation genetic diagnosis to select progeny who are both unaffected by FA and who may be a compatible donor for an affected child (152).

Symptomatic therapies include steroids and androgens, granulocyte colony-stimulating factor (G-CSF), transfusions, and antioxidant supplementation (153). Gene therapy trials have been conducted with several *FANCC* and *FANCA* patients, but success has been difficult to prove (154). Protein replacement by receptor-mediated endocytosis is currently being explored (155). FA therefore remains a challenge at the therapeutic and the basic science level. Progress must be made in understanding how the FA family of proteins interact with other members of nuclear matrix and chromatin-associated proteins and which role, if any, oxidative stress plays in the regulation and function of the FA genes. It would come as no surprise if FA genes were to play a part in the regulation of hematopoiesis through formation of multimeric complexes in the chromatin akin to what is known from the *Drosophila* polycomb group genes (156). There also is little doubt that FA qualifies as a premature aging syndrome, since there is ample evidence, in vitro and in vivo, of disturbed proliferative homeostasis, which has long been recognized as a fundamental feature of the aging process (157). If it should turn out that the FA family of proteins, in fact, protects us from the ill effects of oxidative stress, the FA genes truly deserve to be called antiaging genes.

NOTE ADDED IN PROOF, JULY 2002

It was recently discovered that biallelic mutations in the early onset breast cancer 2 gene (BRCA2) cause Fanconi anemia in a small number of patients belonging to complementation groups FA-D1 and FA-B (157) in whom impaired fidelity of double-strand break (DSB) processing had been previously shown (158). This exciting discovery highlights the important role of FA proteins in the maintenance of genomic stability via the homologous recombination pathway of DSB recogni-

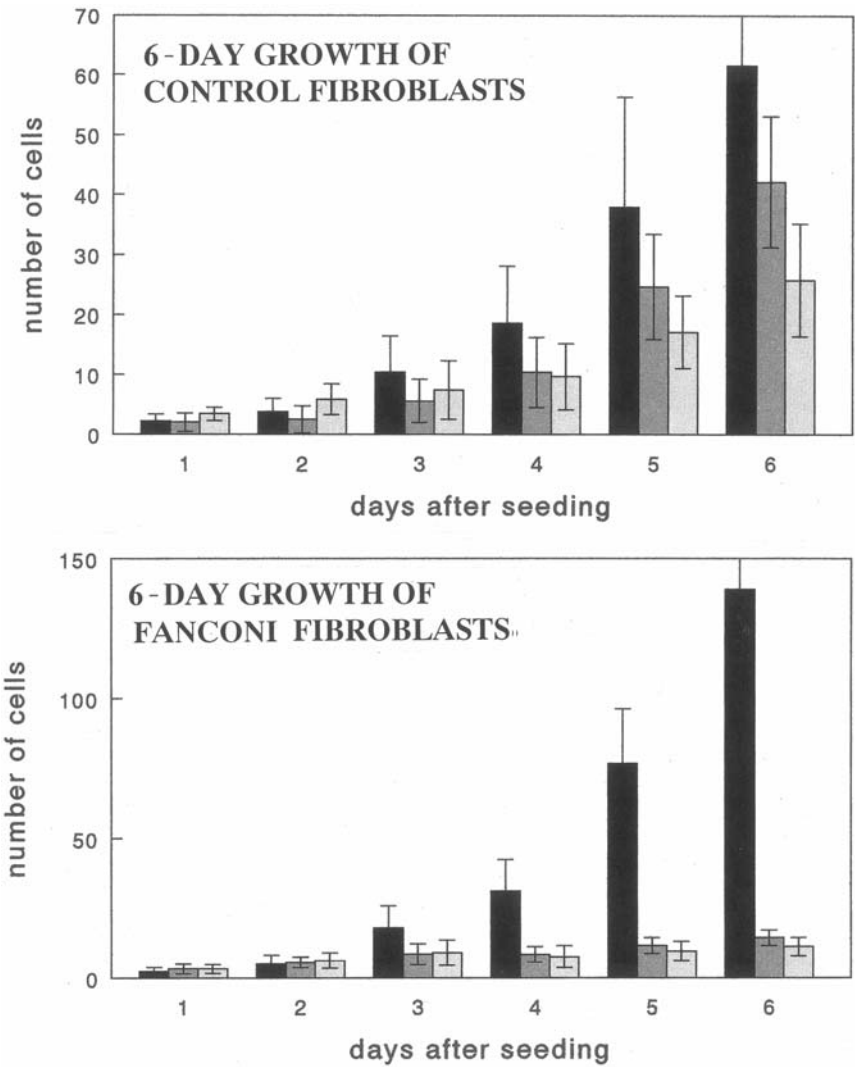

Figure 8 Six-day growth performance (cell counts) of control and FA fibroblasts with (solid black bars) and treated for 24 hr with 1 ng/mL (dark gray bar) and 2 ng/mL (light gray bar) MMC. Even though the untreated FA fibroblast culture grew better than the untreated control culture, exposure to MMC abolished growth completely in the fibroblast culture from a mosaic FA patient (lower panel) but only partially in the fibroblast culture from a control donor (upper panel).

tion and repair. Assuming a BRCA2 carrier frequency of 1/100, the Hardy-Weinberg rule predicts a frequency of biallelic mutation carriers in the order of 1/40,000 which is much bigger higher than actually observed. This implies that, akin to observations in the BRCA2 −/− mouse model, most biallelic BRCA2 mutations are embryonic lethals. Apparently only "mild" biallelic mutations with residual protein function are compatible with survival, accounting for the very rare category of BRCA2/FA-patients (157).

Another important discovery linking the FA-family of genes to the maintenance of genomic stability is that the FANCD2 protein belongs to large groups of proteins that are phosphorylated by ATM (ataxia-telangiectasia, mutated) protein after X-irradiation (159). In fact, FANCD2 patients may show a phenotype intermediate between Fanconi anemia and ataxia-telangiectasia patients, although the radiosensitivity of FANCD2 −/− cells, appears to be far less than that of ATM −/− cells (159). Both the recognition of BRCA2 as a bona fide FA-gene and the recognition of FANCD2 as a target of phophorylation by ATM support the notion of a close interaction and shared functions of the many proteins that protect our genome from DNA damage. Reactive oxygen species clearly are a major source of endogenous DNA damage against which long-lived species mammalian species need additional protection, apparently provided by the FA-family of proteins (160). Rapid progress is also being made in deciphering the functions of the individual members of the FA-protein complex. For example, it could be shown that FANCE binds to both FANCC and FANCD2, thereby providing a critical bridge between the FA protein complex and the downstream "effector" protein FANCD2 (161).

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17
Xeroderma Pigmentosum

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I. INTRODUCTION

Among the most prominent DNA-damaging agents, the ultraviolet (UV) spectrum of sunlight induces lesions in DNA that may alter the genetic information and lead to genomic instability and carcinogenesis. To eliminate DNA damage, an extremely accurate system of complementary DNA-repair mechanisms has evolved (1). A defect in any of these repair pathways reveals their biological importance in counteracting DNA damage. The consequences of defective repair of UV-induced DNA damage are depicted in three rare autosomal recessive photosensitive syndromes: xeroderma pigmentosum (XP), Cockayne syndrome (CS), and the photosensitive form of the brittle hair disease trichothiodystrophy (TTD) (2).

XP is a human hereditary disorder with a cancer-prone phenotype that is genetically heterogeneous, and in most cases (but not all) results from defective nucleotide excision repair (NER) of DNA damage (2,3). Cell fusion experiments have defined seven different genetic XP complementation groups that are defective in NER and are referred to as XP-A through XP-G. The eighth group, called the XP-V (variant) group, is deficient in the ability to replicate DNA containing UV photoproducts. With the exception of XP-V, all the proteins defective in groups XP-A to XP-G are components of the NER pathway and include three damage-recognition proteins (XPA, XPC, and XPE), two helicases (XPB and XPD), and two nucleases (XPG and XPF). XPB and XPD are part of the basal transcription factor TFIIH that is required for NER and initiation of transcription by RNA polymerase

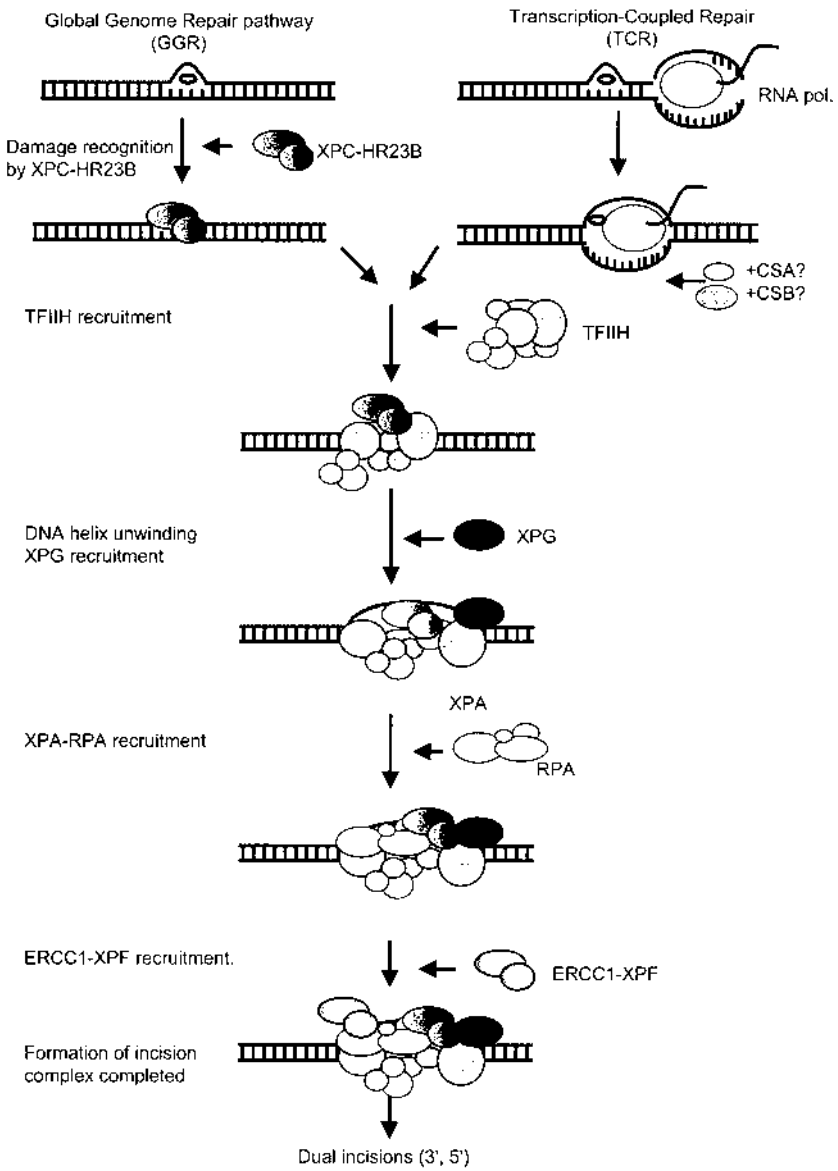

Figure 1 Involvement of different XP proteins in NER. The figure shows the role of each XP protein in the different steps of NER. Damage is recognized by XPC in GGR, or stalled transcription complexes and the CSA and CSB proteins in TCR. TFIIH is recruited and forms an open complex. XPG and XPA/RPA are then recruited and position XPG and XPF/ERCC1 on either side of the damage, resulting in incision of the DNA on both sides of the damage. (Modified from Ref. 27.)

II (4). Because of this dual role of TFIIH, mutations in the two helicase subunits can affect both NER and transcription and thereby give rise to XP-associated syndromes, such as the combined clinical phenotype of XP/CS and TTD. The features of CS and TTD are quite different from those of XP and are thought to result from impaired transcription activity (4). In contrast to the other seven XP complementation groups, XP-V cells show normal NER activity, but they are deficient in a postreplication repair–translesion synthesis pathway (5,6). Cloning of the *XPV* gene has shown that it encodes a novel DNA polymerase, polη, which is able to synthesize DNA past UV damage (7,8).

NER is a major DNA defense mechanism against the carcinogenic effects of sunlight. It removes a wide range of helix-distorting lesions, such as UV-induced cyclobutane pyrimidine dimers (CPDs) and pyrimidine (6–4) photoproducts (6-4 PPs), as well as bulky adducts induced by chemicals such as N-acetoxy-2-acetylaminofluorene (AAAF) and benzo[a]pyrene-diol epoxide.

NER requires the function of at least 30 polypeptides and consists of several sequential steps: (a) recognition of DNA damage, (b) local opening of the DNA helix around the lesion, (c) single-stranded incision on either side of the lesion, (d) excision of the damaged portion of the DNA strand, (e) DNA repair synthesis for filling in the resulting gap using the intact complementary strand as a template, and (f) ligation of the newly synthesized strand.

Two partly overlapping subpathways (Fig. 1) have been described for NER. The first subpathway, known as *global genome repair* (GGR), involves repair activity on DNA lesions across the whole genome; for the majority of the lesions, this activity is relatively slow. The second subpathway is coupled to active transcription and eliminates lesions from the transcribed strand of active genes that block ongoing transcription. The latter pathway is termed *transcription-coupled repair* (TCR). As shown in Figure 1, it is the initial damage-recognition step that differs between the two pathways, whereas subsequent steps use the same mechanism. For GGR, the XPC/HR23B complex detects the damage and recruits the repair machinery, whereas in TCR, stalled RNA polymerase II and possibly the CSA and CSB proteins (specifically required for TCR and defective in patients with CS) are the damage-recognition signal for the recruitment of the NER machinery.

This chapter will describe the variable clinical features of XP and the roles of the XP gene products in the NER and postreplication repair processes.

II. CLINICAL PICTURE

XP is inherited as an autosomal recessive disorder. It is distributed worldwide with a population incidence of about 1 per 250,000 in Europe and the United States to 1 in 40,000 in Japan (9). A review of 830 published cases was provided by Kraemer and colleagues in 1987 (10).

Patients with XP are extremely photosensitive, with the appearance of the first symptoms of freckling and progressive cell degeneration of the skin and eyes following sun exposure appearing around age 1 or 2 years (Fig. 2). Patients with XP show different types of pigmentation changes and an increased incidence of developing cancers in sun-exposed areas that is about 1000-fold greater than it is in the general population. The most frequent skin cancers are basal and squamous cell carcinomas and to a lesser extent melanomas. The eyelid, conjunctiva, and cornea are the main affected areas of the eyes, since they are exposed mostly to UV irradiation from sunlight. In addition, the appearance of squamous cell carcinoma on the tip of the tongue is 20,000 times greater in patients with XP than in healthy people. The mean age of onset for skin cancers in patients with XP is 8 years old—about 50 years earlier than for the general population. This reduction in the age of appearance of neoplasia may be one of the most dramatic for a recessive hereditary disease (11). Patients with XP under the age of 20 years show a 10- to 20-fold increased risk of developing internal tumors (12).

A significant proportion of patients with XP (18–20%) shows progressive degeneration of normally predeveloped neurons, which results in cortical and spinal atrophy, cochlear degeneration, and a mixed distal axonal neuropathy. Microcephaly, mental retardation, deteriorating hearing, and loss of tendon reflexes

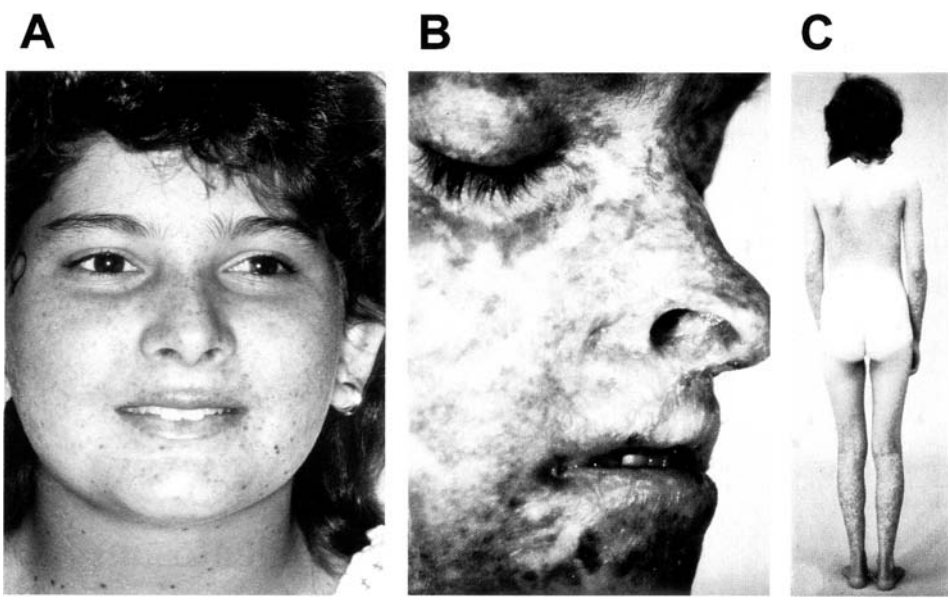

Figure 2 Patients with XP. (A) Mildly affected patient. (B) Face of 16-year-old patient showing severe skin lesions. (C) Posterior view of same patient showing absence of skin damage on unexposed areas. (From Refs. 145 and 146.)

are other results of the premature neuronal death. Unlike several other chromosomal instability syndromes (e.g., CS, TTD, ataxia-telangiectasia, and Werner syndrome), patients with XP do not show signs of premature aging except for the accelerated neuronal degeneration in those individuals with neurological abnormalities. Patients with XP without neuronal abnormalities who survive to relatively advanced ages have no unusual aging indications. Likewise, neither the Xpa nor Xpc mouse models discussed below have premature aging features.

The severity of the repair defect and of the clinical symptoms is quite heterogeneous among the different XP complementation groups (Table 1). The most severely affected patients are XP-A, XP-B, XP-D, and XP-G individuals. The severity of the NER deficiency of the above groups seems also to be associated with neurological problems, since XP-F, XP-E, and XP-C patients, who show moderate UV sensitivity and have a partially impaired NER, do not develop neuronal degeneration.

III. CELLULAR FEATURES AND DIAGNOSIS

The cellular features of NER-defective XPs include increased sensitivity to the killing and mutagenic actions of UV light and chemical carcinogens that produce bulky DNA lesions, which would normally be removed by NER. All steps of NER are defective. The NER process involves the removal of the damaged section of the DNA, followed by repair synthesis to fill in the remaining gap. This repair synthesis step is easy to measure in the laboratory and provides a useful diagnostic test for XP. Replicative DNA synthesis is confined to the S phase of the cell cycle, whereas repair synthesis takes place in all phases of the cell cycle, even in nondividing cells, and in the presence of the inhibitor hydroxyurea. This unscheduled DNA synthesis (UDS) can be measured either autoradiographically or by liquid scintillation spectrometry following incubation of UV-irradiated cells in the presence of [^{3}H]thymidine.

UV damage also leads to the inhibition of RNA synthesis, which subsequently recovers rapidly in normal cells, because lesions are rapidly removed from transcribing regions of the DNA by transcription-coupled repair. This recovery does not take place in most XP cells, because the lesions are not removed. The exception is XP-C cells, because, as discussed below, the XPC protein is not required for TCR. Lack of RNA synthesis recovery is another simple cellular diagnostic test that can be measured in a uridine incorporation assay.

Complementation group assignment is achieved by fusing the unknown XP cells with tester strains from known complementation groups. The fused culture is then irradiated and UDS is measured autoradiographically in binucleate cells. Heterokaryons can be distinguished from homokaryons by preincubating each donor cell population in the presence of different-sized latex beads, which the cells in-

Table 1 Human XP Genes, Chromosome Location, Function, and Clinical Features

Gene	Chromosome	Protein/function	Specificity	Frequency	Clinical features	Mice
XPA	9q34.1	Damage verification	NER	Frequent in Japan and the West	Very severe skin and neurological abnormalities	Enhanced incidence of skin cancer on exposure to UV. No neurological abnormalities
XPB	2q21	3′–5′ Helicase—Part of TFIIH/open complex formation	NER, essential for transcription initiation	Very rare	XP in combination with CST TTD	
XPC	3p25.1	Damage recognition	GGR	Frequent in West	Severe skin abnormalities	Skin and eye cancer on exposure to UV
XPD	19q13.2	5′-3′ Helicase–TFIIH component/open complex formation	NER, essential for transcription initiation	Frequent in West	XP-D: Mild to severe with neurological defects. TTD: varying severity XP/CS: 2 severe cases	Knockout mice not viable "TTD mice": brittle hair, premature aging
XPE (*DDB2*)	11p11-p12	DNA damage–binding protein/damage verification?	GGR of CPDs, chromatin remodeling?	Rare	Mild symptoms	
XPF	16p13.3	Heterodimeric complex with ERCC1/5′ structure specific endonuclease	NER, recombination	Rare	Mild symptoms	ERCC1 mice: extremely sick, premature death
XPG	13q32-33	3′ Structure-specific endonuclease	NER, TCR of oxidative damage	Rare	XP-G: mild symptoms XP/CS: very severe	Postnatal growth failure, pre mature death
XPV	6p12-21.1	Polymerase η/bypass of UV-induced photolesions	Translesion synthesis	Intermediate	Skin abnormalities with varying severity	

gest prior to fusion. After fusion, all the beads in homokaryons are of the same size, whereas heterokaryons are identified, because they contain beads of both sizes. Restoration of UDS in heterokaryons indicates that the donor cells are in different complementation groups.

Molecular diagnoses for XP have not been widely used, because most patients with XP have different mutations. The exception is patients with XP-A in Japan, 95% of whom have the identical mutation. This mutation generates an *Aln*WI cleavage site and is therefore readily identified using restriction fragment length polymorphism (RFLP) techniques (13).

XP-V patients have normal NER and cells from XP-V patients are only marginally sensitive to killing by UV. The cellular defect, discussed in detail in section IV.H, is in the ability to synthesize intact daughter DNA strands after UV irradiation; a process referred to as postreplication repair (5). This defect results, as it does with NER-deficient XPs, in hypermutability by UV light (14). The deficiency in postreplication repair in XP-V cells is exacerbated in the presence of caffeine, and as a consequence, XP-V cells are specifically sensitive to UV-induced killing by incubation in the presence of caffeine following UV irradiation. This has enabled a cellular diagnostic test to be developed, whereby cells are UV-irradiated and incubated in the presence of caffeine. Viability is measured either several hours (15) or days (our unpublished data) later by the ability of the culture to synthesize DNA. (Note: This assay measures replicative DNA synthesis and should not be confused with the UDS assay, which is specifically designed to measure repair synthesis in nonreplicating cells.)

IV. MOLECULAR AND BIOCHEMICAL ANALYSES

The genes corresponding to all eight known XP complementation groups were cloned in the decade from 1989 to 1999, and mutations have been identified in affected patients in all groups. A summary of all identified mutations was published in 1999 by Cleaver and colleagues (16). This section describes the functions of the individual XP proteins in the NER process (see Fig. 1), followed by the XPV protein used in translesion synthesis. A summary of the genes, proteins, their functions, and the phenotypes of affected patients and knockout mice is presented in Table 1.

A. XPC

The XPC protein carries out the initial damage-recognition step of NER. The XP-C complementation group is one of the most frequent in the Western population, but it is rare in Japan. Although XP-C patients have severe skin problems, they rarely suffer from neurological abnormalities (see Table 1). Cells derived from

this complementation group are totally deficient in global genome repair of UV-induced DNA damage but maintain the ability to remove photoproducts from the transcribed strand of transcriptionally active genes (17,18). Consequently, RNA synthesis recovers with kinetics similar to those of normal cells following UV irradiation. The XPC protein, in contrast to XPA, XPB, XPD, XPF, and XPG proteins, is therefore involved in only one of the NER subpathways. This likely accounts for the milder phenotype of XP-C patients compared with those from completely NER-deficient complementation groups. Since XP-C patients can repair damage in transcribing DNA, these crucial regions of DNA, which are in active use, are repaired normally.

The human *XPC* gene, located on chromosome 3p25.1, encodes a protein of 125 kD, which forms a tight complex with the 58-kD HR23B protein and is required for the initial steps of NER. HR23B and HR23A are two human homologs of the yeast NER protein Rad23 (19). HR23B harbors an XPC-binding domain of 54 amino acids, which is sufficient to stimulate the catalytic activity of XPC in vitro (20). Both HR23 proteins are more abundant than XPC in mammalian cells, and they contain a ubiquitin-like region at their amino-terminus (19) that, at least in their yeast homolog, is essential for its repair function (21,22). Recent studies have shown that the XPC-HR23B complex contains a third subunit, the centrosomal component centrin2/caltractin1 (CEN2), which stabilizes the complex and stimulates XPC activity (23). The significance of this association is not yet clear.

Although the involvement of XPC in an early step of GGR has been known for a long time (24), its role as the first protein to recognize the damage has been recognized only recently (25–27). This function was for a long time attributed to XPA—the first NER protein shown to have preferential affinity for DNA lesions. It has now been demonstrated both in vitro and in vivo (25,27) that XPA is involved in a later step of the NER pathway (see Sec. IV.C and Fig. 1).

XPC–HR23B possesses binding affinity for both single- and double-stranded DNA, and its affinity for damaged DNA is at least 10 times greater than it is for undamaged DNA (19,24,25). By using plasmids of different sizes that contained known NER-substrate lesions in an in vitro damage-recognition competition assay, Sugasawa and coworkers (25) showed that repair is detected only when the damaged plasmids are incubated with purified XPC–HR23B complex or with $XPC^{+/+}$ extracts, before XPA, but not vice-versa. Agreeing with the above findings are the results from an in vivo study (27), in which irradiation through a filter was used to introduce damage to specific sites within the nucleus. Immunofluorescence was then used to show the migration of different NER components to the site of the damage. This work showed that assembly of the NER components at the site of the damage is sequential. Localization of XPC to the damage was independent of all other components, whereas migration of any of the other components did not occur in XP-C cells. These data strongly suggest that the XPC–HR23B protein complex is the prerequisite factor for the initial step of dam-

age recognition of GGR by NER and for the recruitment of all subsequent NER factors in the preincision complex.

XPC–HR23B binds to small bubble structures, irrespective of whether they contain damage, but incisions by the NER nucleases (see Sec. IV.F and G) will only be effected if damage is present in the bubble. This is consistent with a two-step mechanism for damage recognition. First, the helix distortion is recognized by XPC–HR23B, and the presence of damaged bases is verified subsequently by other factors (28). XPC interacts with TFIIH, and this interaction is independent of XPA (26). As described in Sec. IV.D and E, TFIIH contains the XPB and XPD proteins and is used to open up the DNA structure at the site of the damage. It is likely that recruitment of TFIIH to damaged sites by XPC is the next step in the NER process.

Two independently generated knockout mouse models for Xpc are viable and develop normally, indicating that the XPC protein is not essential for life (29–31). Although age-dependent spontaneous mutagenesis is increased in Xpc-defective mice, there is no increased incidence of spontaneous tumorigenesis or premature aging at least until 1 year of age (29,30,32). Xpc mice are, however, sensitive to UVB irradiation with a high predisposition to skin and eye tumors compared with wild-type mice (29,30,33,34).

B. XPE

The XPE protein binds to CPDs and is involved in GGR of CPDs. Patients in the XP-E complementation group have mild dermatological symptoms and no neurological malfunctions. In most cases, cells derived from XP-E individuals have an NER level between 40 and 60% of normal cells and only slight UV sensitivity. The role of XPE in NER remained unclear until recently, as it was not required for in vitro systems that were dependent on all the other known NER proteins.

Band shift assays using UV-irradiated plasmids indicate that in some XP-E cell lines, a damaged DNA-binding protein is absent (35). This DNA damage–binding activity (UV-DDB) has been purified as a protein complex with two subunits of 128 and 48 kD (36). Sequence analysis of the p48 subunit has revealed mutations in the p48 gene in several XP-E patients (37,38), indicating that p48 is the *XPE* gene. However, other XP-E cell lines show normal levels of DDB activity, and they have no mutations in the p48 or the p127 subunits. It is quite likely that the relatively high levels of NER in these patients has led to incorrect assignment to the XP-E group.

Hwang and coworkers (39) have shown that the p48 *XPE* gene is UV inducible, and that this induction is dependent on p53. Furthermore, they showed that XPE is specifically involved in global genome repair of cyclobutane pyrimidine dimmers (CPDs), but it is not required for the removal of 6-4 PPs or for the transcription-coupled repair of CPDs. This accounts for the mild cellular and clinical features of XP-E patients.

Recent data indicate that DDB also has a possible transcription-stimulatory activity (40,41), and that it could function as a transcriptional partner of E2F1. Datta et al. (42) have shown that the p48 subunit of DDB (damaged DNA binding) protein associates with the CBP/p300 family of histone acetyltransferase in vivo and in vitro, implying a possible involvement of DDB in chromatin remodeling for recruitment of the NER machinery at the injured sites.

C. XPA

XPA verifies the DNA damage and positions the excision nucleases. Patients from the XP-A complementation group exhibit the most severe phenotypes with multiple skin cancers and neurological abnormalities (10). XP-A is one of the most common complementation groups, with the highest prevalence in Japan (43). Cells from most XP-A patients have no NER activity, and they are defective in TCR and GGR pathways, suggesting that XP-A plays a central role in NER.

The XPA gene product is a 31-kD metalloprotein that contains a zinc finger motif indicating DNA binding activity (44). There are several reports that show the affinity of XPA for NER-specific DNA lesions, including 6-4 PPs and CPDs (45–47). In addition, XPA interacts with the single-stranded DNA binding protein, RPA, another essential NER protein factor that is also necessary for recombination and replication. Even though XPA has a higher preference for binding damaged over undamaged DNA, this preference increases significantly upon interaction of XPA with RPA (48,49). A short region of 122 amino acids has been identified as the minimal region of XPA required for its DNA-binding activity (50), which is also essential for its function (47,51). Nuclear magnetic resonance (NMR) studies have revealed two subdomains in this region: an N-terminal zinc-binding core and a C-terminal loop-rich subdomain linked together with a short sequence (52–54). Chemical shift perturbation studies have shown that even though the zinc motif is essential for the NER function of XPA, it is the loop-rich subdomain that binds directly to DNA (52–54).

XPA interacts with several of the NER proteins, such as ERCC1 (see Sec. IV.G) (55,56), TFIIH (57,58), CSB protein, and the p34 subunit of the basal transcription factor TFIIE (57,59). Because of this apparent central role of XPA and the severe cellular and clinical phenotype of XP-A patients, it had been accepted dogma for many years that XPA was the factor responsible for damage detection and recruitment of the NER components. However, recent data described above have shown that this is not the case, and that XPC–HR23B is the initial sensor of DNA damage (25,27,60). Using the local UV irradiation technique described above, Volker and coworkers have demonstrated that the assembly of the core NER factors, TFIIH, and XPG depends on the previous binding of the XPC–HR23B protein complex at the site of the damage, but it does not require a functional XPA protein (27). In contrast, recruitment and positioning of the XPF/ERCC1 complex

at the 5′ side of the UV-induced DNA lesion and the incision activity of XPF/ERCC1 and XPG at the 5′ and 3′ side of the damage, respectively (see Sec. IV.F and G), are completely dependent on the XPA protein, in cooperation with RPA (61–63). It is likely that RPA binds to the undamaged strand and protects it from cleavage (63). The association of RPA with XPA generates a double-check sensor that detects simultaneously single-stranded regions (RPA) and backbone distortion (XPA) (64). The above findings place XPA in a later step after damage recognition, possibly in the verification of NER specific lesions and in positioning the incision factors correctly around the damage. Since verification of the existing damage is necessary in GGR and TCR, it also explains the deficiency of XP-A cells in both pathways.

Many different mutations have been identified in XP-A patients (65). Most of them are truncating mutations, but several missense mutations also have been detected. XP-A is relatively common in Japan and analysis of the mutations reveals a founder effect, with the majority of mutations being a G to C transition at the acceptor site of intron 3, which results in missplicing and a truncated protein (13).

Two different groups have generated NER-deficient Xpa mice. The mice develop normally, are fertile, and they do not show any enhanced lethality at up to 1.5 years. A small increased incidence of liver carcinomas and lymphomas was detected in older mice, but no spontaneous neurological abnormalities were observed. Xpa mice are extremely sensitive to skin carcinogenesis induced by UVB or dimethylbenzanthracene (66,67). Also, UVB-induced immunosuppression is greatly enhanced (68,69), implying that impairment of the immune system after UV exposure might contribute to the high incidence of skin cancer in XP-A patients (see Sec. V).

D. XPB

XPB is a subunit of TFIIH with 3′–5′ helicase activity and is essential for transcription initiation and for formation of open complexes during NER. The clinical phenotypes of patients with XP in the XP-B and XP-D complementation groups are diverse and complex, because the XPB and XPD proteins are both components of the dual-function TFIIH, which has critical roles in transcription initiation and NER. Mutations in these genes can affect either or both of these functions with differing clinical outcomes. Patients in the XP-B complementation group are extremely rare, and only three families have been described so far (9,70,71). Their clinical symptoms vary from very severe to mild, with two families having combined features of XP and CS and a third with TTD.

The *XPB* gene, originally known as *ERCC3* and located on chromosome 2q21, encodes an 89-kD protein with 3′→5′ helicase (72) and DNA-dependent ATPase activities (73–75). XPB is the largest subunit of the core basal transcrip-

tion factor TFIIH, and together with the XPD protein, another subunit of TFIIH (see Sec. IV.E), is part of the preincision complex of NER. XPB and XPD are the helicases responsible for formation of an open complex around the injured DNA (76). TFIIH is an essential component of transcription initiation by both RNA polymerase II and NER. In both processes, TFIIH is required for unwinding the DNA double helix (76–78), but the requirements for promoter melting and open complex formation during NER are different. The XPB helicase is part of the core of TFIIH, and its activity is absolutely required for transcription initiation. Promoter footprinting studies demonstrate that promoter melting, which is necessary to activate the RNA pol II initiation complex, requires the XPB helicase (77,79,80). Using reconstituted TFIIH, Tirode and coworkers have shown that in the absence of the XPB subunit, no transcription initiation can take place (81). If, however, the transcription substrate contains an artificially open promoter, then the XPB protein is not needed.

In addition to its involvement in promoter melting and open complex formation, the XPB helicase also plays a vital role in promoter escape (82). The central role of XPB in the essential process of transcription initiation, in contrast to XPD (see following section), probably renders it intolerant of structural alterations that might result from mutational changes. This could explain the paucity of XP-B patients. Most mutations in this gene are likely to be incompatible with life.

Recent work suggests that apart from the role of the XPB protein in open complex formation during NER, it has an additional role in a later step of NER—the 5′ incision reaction. An XPB construct carrying mutations in the carboxy-terminal domain of XPB has been shown to unwind DNA and allow a 3′ incision, but not a 5′ incision, showing that XPB facilitates the 5′ incision by XPF–ERCC1 (76) (see Sec. IV.G).

E. XPD

XPD is a subunit of TFIIH with 5′-3′ helicase activity, which is essential for NER but not for transcription initiation. The *XPD* gene, located on chromosome 19q13.2, is the most complex and most interesting of the XP genes in terms of genotype–phenotype relationships. Mutations in this gene can result in a bewildering array of clinical outcomes, including XP (usually with neurological abnormalities), TTD, and also rare cases of combined features of XP and Cockayne syndrome (XP/CS), the CS-related disorder cerebro-oculofacioskeletal syndrome (COFS), and individuals with some features of both XP and TTD.

XPD, like XPB, is a subunit of TFIIH with helicase activity, although in the case of XPD, it is a 5′-3′ helicase (83,84). Unlike XPB, XPD is not part of the central core of TFIIH, but it is somewhat more loosely associated and forms a link between the core and another three-component subcomplex, designated the CAK complex (85,86). Also in contrast to XPB, the helicase activity is not required for

the transcription function of TFIIH. Destruction of the helicase activity by site-directed mutagenesis of the ATP-binding domain abolishes NER but has no effect on transcription (87). In assays for in vitro transcriptional activity of TFIIH, XPD had a stimulatory effect but was not essential (82,86). It thus appears that for the transcriptional function of TFIIH, the role of XPD is largely structural, being required to maintain the conformation and stability of the TFIIH complex. As a consequence, a large number of mutations are compatible with life and can result in the different clinical features depending on exactly how NER and transcription are differentially affected. More than 50 *XPD* mutations have been identified so far (Fig. 3). In contrast to its "supporting role" in transcription initiation, active XPD is absolutely required for NER. The helicase activity is needed for formation of the open complex by TFIIH (76,87).

Within the TFIIH complex, the C-terminal half of XPD interacts with the p44 subunit. This interaction with p44 results in a 10-fold stimulation of the helicase activity of XPD. Several mutations (located close to the C-terminus of XPD) identified in patients interfere with the interaction with p44. These mutations do not affect the basal helicase activity of XPD, but they abolish the p44-mediated stimulation (88). Structural studies using electron microscopy have revealed that the TFIIH core, together with XPD, form a ringlike structure with a central cavity, which could accommodate double-stranded DNA. XPD and XPB flank the p44 subunit, and the CAK proteins form a protuberance from the ring in the region of XPD/p44/XPB (89,90).

When the involvement of XPB and XPD in TFIIH was discovered, it was proposed that mutations in *XPD* that affect only NER might result in the clinical features of XP, which is considered to be a repair syndrome. On the other hand, if the mutations subtly affect the transcriptional activity, then the multisystem disorder TTD would result; that is, TTD is proposed to be a transcription syndrome (91). A prediction of these proposals is that mutations resulting in different clinical phenotypes would be located at different sites in the gene. Analysis of the different mutations found in the *XPD* gene in different patients has been difficult, because many patients are compound heterozygotes (92). However, the results of these analyses have supported the idea that each mutational site is disease specific; that is, the site of the mutation largely determines the clinical outcome (92) (see Fig. 3). Most of the mutations are located in the C-terminal third of the protein (92–95). Molecular modeling of the XPD protein on the structure of the *Escherichia coli* UvrB helicase shows that the C-terminal region that contains the majority of the mutations forms a separate structural domain (96).

Proof of the hypothesis that the site of the mutation in the *XPD* gene determines the phenotype comes from a mutant mouse generated by de Boer et al. (97). The mouse contained a single mutation in the *XPD* gene, which generated the change arg722trp—a mutation that had been found in several patients with TTD (see Fig. 3) but not in any with XP. The phenotype of the mouse was similar to

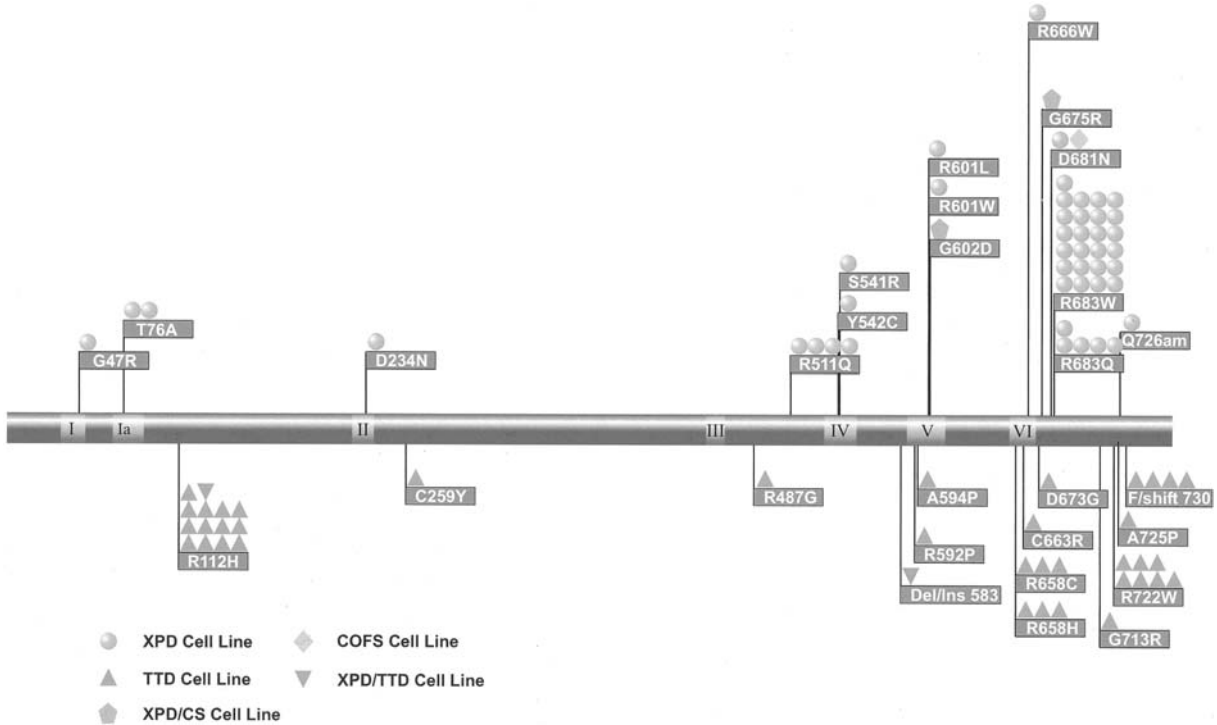

Figure 3 Spectrum of mutations found in the *XPD* gene. The XPD protein is shown as a bar with the seven helicase domains indicated. The flags denote the amino acid changes in different patients, with the number of symbols indicating the number of times that the particular allele has been identified in patients (i.e., two symbols for homozygotes, one for compound heterozygotes). Circles, XP; triangles, TTD; pentagons, XP/CS. Alterations considered to be completely inactivating (Ref. 92) are not shown. (Modified from Ref. 94.)

that of TTD in humans; namely, the hair was brittle and deficient in sulfur-rich matrix proteins. It went through cycles of loss and regrowth. Furthermore, like TTD individuals, the mice showed growth retardation, skin abnormalities, premature aging, and early death. To date, this is the only mouse with mutations in the *XPD* gene. Others are eagerly awaited.

Whereas the XP phenotype in XP-D patients is thought to result from reduction or abolition of helicase activity, the abnormal transcription thought to result in TTD appears to be caused by decreased stability of the TFIIH complex. Measurements of the levels of TFIIH in TTD cells show a substantial reduction in cellular TFIIH content in cultured fibroblasts (M. Stefanini, personal communication). In TTD patients with one specific mutation, arg658cys, the XPD protein appears to be temperature sensitive, with greatly decreased NER and transcriptional activity in cells cultured at elevated temperature (98). This correlates with the clinical phenotype, in that these patients tend to show hair loss in association with high temperatures due to fever.

Two severely affected XP-D individuals with unique mutations have the combined features of XP and CS. Cells from these patients are extremely sensitive to UV-induced lethality. On exposure to UV, these cells not only are unable to carry out NER, but they introduce aberrant breaks into the genomic DNA at sites distant from the UV-induced damage (99).

In general, the clinical features of XP and TTD are exclusive. Despite the very similar levels of NER, TTD patients do not develop the sunlight-induced skin pigmentation abnormalities and skin cancers characteristic of XP, and XP-D patients do not have sulfur-deficient brittle hair and other developmental abnormalities typical of TTD. The latter is explained relatively easily if the mutation affects only the repair capacity but not the transcription function of TFIIH. To account for the former, we have proposed that critical steps in the development of cancer and pigmentation abnormalities require fully active transcription. If transcription is impaired as a result of the *XPD* mutations, cancer does not ensue (100).

Recently, two XP-D patients have been identified that unusually do have some, but not all, of the features of XP and TTD, adding a further level of complexity to the range of clinical phenotypes (101). Finally, one patient with COFS, a disorder related to CS, has been found to be NER defective and to have a mutation in the *XPD* gene (102).

F. XPG

The XPG nuclease cleaves the DNA on the 3′ side of the damage; it also has a role in the repair of oxidative damage. Patients from the XP-G complementation group with heterogeneous clinical features are quite rare. Using sequence analysis, Nouspikel et al. have shown that patients who bear missense mutations in the *XPG* gene have a milder phenotype than those with mutations resulting in truncated

forms of the protein (103). The latter usually develop severe combined characteristics of both XP and CS.

Analysis of the *XPG* gene has revealed that its product is a member of the FEN-1 family of structure-specific endonucleases. Members of this family incise always with the same polarity at junctions of duplex and single-stranded DNA (ssDNA). XPG cleaves a variety of artificial DNA substrates, and it is required for the incision at the 3′ side of the open complex during NER (104,105). Positioning of the XPG protein specifically on the damaged strand seems to be coordinated upon its interaction with TFIIH and possibly RPA, whereas its nuclease activity in NER is dependent on functional XPA protein (48,62,63).

Several lines of evidence indicate that XPG has a second role in the cell, and that this might be an involvement in the repair of oxidative damage. Cells from XP-G/CS patients are deficient in the transcription-coupled repair of thymine glycols, whereas cells from patients that exhibit only XP characteristics are normal (106). This is consistent with the idea that mutations that result in severely truncated forms of XPG protein abolish all of its functions and are responsible for the severe clinical picture observed in XP-G/CS patients. In contrast, missense mutations in the *XPG* gene result in loss of the nuclease activity but retention of its function in TCR of oxidative damage. This could lead to the milder picture of XP-G patients. Further insight into a possible role for XPG in repair of oxidative damage has come from the finding that XPG stimulates the binding of the hNth1 protein to DNA and increases its activity. hNth1 is a glycosylase/AP lyase, which removes thymine glycol and related lesions from DNA (107,108). The stimulatory activity of XPG is retained in mutants inactive in NER (108).

The viability of Xpg knockout mice suggests that XPG is not essential for life. However, these mice are extremely sick, with severe growth retardation and early death by 23 days postpartum (109). The greater severity of the features of Xpg compared with Xpa mice, even though both are completely defective in NER, is consistent with a role for XPG in addition to its involvement in NER.

G. XPF

The XPF nuclease cleaves DNA on the 5′ side of the damage. XP-F patients show mild clinical characteristics with skin sensitivity and no neurological abnormalities. Mutation analysis in several XP-F patients has identified only missense mutations in the responsible gene (110).

A heterodimer protein complex between the XPF protein and the ERCC1 gene product functions as the second structure-specific nuclease involved in the incision of the damaged DNA during NER. This complex formation between the XPF and ERCC1 proteins has been shown, both in vitro and in vivo, to be quite stable (111–115). XPF/ERCC1 cleaves the DNA 5′ to the damage after XPG has

incised on the 3′ side (114). Recruitment of the XPF/ERCC1 protein complex to the 5′ side of the damage is totally dependent on the XPA protein, since no relocalization of the complex is observed at the damaged sites in locally irradiated XP-A cells (27). This is also consistent with in vitro observations that show specific interactions between XPF/ERCC1 and XPA (116,117). Positioning of the XPF/ERCC1 nuclease at the damage strand seems to be coordinated by RPA (63), possibly via interactions with the XPF protein (61,117).

As yet, there are no Xpf knockout mice, but Ercc1 knockout mice have been generated. These mice are viable, but they survive only for few weeks before dying of liver failure. They are extremely sick, having a severe phenotype resembling more of a syndrome of cell cycle arrest or premature aging than of a DNA-repair deficiency disorder (118,119). Cells from Ercc1 mice are characterized by increased levels of unrepaired lesions and double-stranded breaks leading to genomic instability. Since Xpa mice do not show any of these features, these findings imply that the ERCC1 protein—and most likely its protein partner, XPF—have an additional role. It has been proposed that this second role is an essential S phase–mediated recombination mechanism (120). This second role of XPF/ERCC1 protein complex might be the reason that the only mutations that have been identified so far are missense mutations in XPF, and there are no known patients that carry mutations that result in truncated form of either of the two proteins.

H. XP Variants

The XPV *gene encodes DNA polymerase* η*, which can replicate DNA containing different types of damage.* About 20% of patients with XP have no defect in NER, and their cells are only marginally sensitive to the killing action of UV. This class is designated XP variants. They have classic XP skin symptoms but no neurological degeneration. Despite the near-normal sensitivity to killing by UV, they are extremely hypermutable by UV (14). The defect in XP-V cells is in the ability to synthesize high molecular weight DNA during DNA replication following UV irradiation (5). This damage tolerance process is sometimes referred to as *postreplication repair* or *daughter-strand repair*. The molecular basis for this defect has been established only recently. In vitro studies using cell extracts have shown that the defect is in the process of translesion synthesis (TLS), the ability to synthesize DNA directly past damaged nucleotides (6). This in vitro defect allows the protein defective in XP-V cells to be purified (121) and the gene cloned (7,8). The protein turns out to be a member of a new family of DNA polymerases and has been designated *DNA polymerase* η *(pol*η*)* and the gene variously designated *POLH, XPV*, or *hRAD30A* (the latter by virtue of its sequence homology to *Saccharomyces cerevisiae RAD30*).

The members of this family of new polymerases (the Y-family) are related in sequence to each other but unrelated to classic DNA polymerases. They differ

from classic polymerases in that they are distributive (unlike the highly processive replicative polymerases), and they have low fidelity when replicating undamaged templates (122,123). This low fidelity confers on them the ability to replicate past different types of DNA damage, in contrast to the highly faithful replicative polymerases, which are completely blocked at sites of damage.

The ability of polη to replicate past different damaged structures has been examined in several laboratories. It can replicate a template containing CPDs with the same efficiency as undamaged templates (124). It also can carry out TLS past (in order of decreasing efficiency) 8-oxoguanine, AAF lesions, cisplatin adducts, and AP sites (124–127). Polη is not able to carry out TLS past a 6-4PP (124,128). Remarkably, when polη carries out TLS past lesions, in most cases, it inserts the correct bases (AA opposite the TT-CPD, C opposite AAF-G adducts, and CC opposite cisplatin-GG intrastrand cross links). This "accurate" replication is achieved first by a strong preference for the polymerase to insert the correct nucleotide, and second, by an inability to extend strands in which "incorrect" bases have been introduced. This double selection process provides a high degree of accuracy in copying damaged bases (124,129). It also most likely accounts for the elevated UV mutability of XP-V cells, which lack polη. In these cells another polymerase with lower accuracy must carry out TLS.

The ability of polη to insert AA opposite TT-CPDs also can account for the different UV mutation spectra seen when comparing XP-V and normal cells. In normal and excision-defective XP cells, the majority of the induced mutations are C to T transitions, probably arising at sites of 6-4 PPs. In XP-V cells, however, the majority of the mutations are transversions and different mutations at A:T sites (130). These results indicate that, in the absence of polη, a polymerase with decreased fidelity and different specificity carries out TLS at the sites of photoproducts.

The DNA polymerase Y-family shows a high degree of sequence conservation in the N-terminal half of the protein, which contains the active site. With polη, the N-terminal 511 amino acids (aa) of the 713-aa protein are sufficient for full activity in vitro (124). The C-terminal part of the protein is involved in localization and presumably protein–protein interactions. Following UV irradiation, polη accumulates at foci in the nuclei, which represent replication factories where the replication forks are stalled at sites of DNA damage (131). The C-terminal 70 aa are required for nuclear localization, and a further 40 aa are required for relocalization into replication foci (131). These relocalization sequences contain a putative C_2H_2 zinc finger and a putative proliferating cell nuclear antigen (PCNA) binding site.

Mutations in the *POLH* gene have been detected in XP-V patients. Many of these mutations result in severe truncation of the protein (7,8), but several missense mutations have been identified in the conserved N-terminal region (131a). In addition, three patients have truncations close to the C-terminus, which leave

the TLS activity of the protein intact but prevent the protein from being localized in the nucleus (131).

The somatic hypermutation that is part of the generation of diversity in the immune response is thought to involve an error-prone DNA polymerase. Evidence for polη involvement in somatic hypermutation has come from an examination of the spectrum of mutations generated by somatic hypermutation in XP-V patients. Although the frequency of mutations in an immunoglobulin variable chain gene has been found to be unaltered, a significant change in the mutation spectrum has been shown, with a decrease in mutations at A:T sites and a concomitant rise at G:C sites (132). These data again suggest that functions of polη can be substituted by other polymerases with different specificity.

V. RELATIONSHIP OF MOLECULAR AND CELLULAR DEFECTS TO CLINICAL FEATURES

The work described above has shown that all patients with XP have defects either in NER or in TLS. Although only a few mutagenesis studies have been carried out, all XP cells examined are hypermutable by UV light. In NER-defective XPs, mutation spectra studies have shown that the majority of the mutations are C to T transitions and occur at the sites of dipyrimidines. A particular signature of UV-induced mutations is the occurrence of CC to TT tandem mutations. A series of papers by Sarasin and coworkers has shown a high level of mutations in the *p53* gene in all types of tumors excised from patients with XP (133,134). Tandem CC to TT transitions have been found in many of the mutated *p53* genes, indicating that they arise as a direct consequence of sunlight-induced photoproducts. Likewise, similar mutations in the tumor suppressor gene *patched* have been found in basal cell carcinomas from patients with XP (135). There is therefore overwhelming evidence that the defect in NER or TLS results in an elevated level of sunlight-induced mutations in the skin cells of patients with XP. Mutations in critical tumor suppressor genes like *p53* or *patched* will increase the probability of the mutated cell generating a tumor. It is likely that other factors also contribute to the enormous increase in skin cancer incidence in XP. Increased UV-induced immunosuppression has been proposed as a further contributory factor, and there are several lines of evidence, none of them as yet conclusive, to support this contention.

The neurological abnormalities found in the more severely affected patients with XP are less readily explained. One possibility is that free radicals, which are abundant in neurons, produce lesions in the DNA that are normally repaired by NER. In XP cells, these lesions accumulate, resulting in premature neuronal death (136). Recently, it has been suggested that 8,5′-(S)-cyclo-2′-deoxyadenosine (cyclo-dA), a free radical–induced bulky lesion that is repaired extremely poorly in

XP-A cells, is a candidate for an endogenous DNA lesion that might contribute to neurodegeneration in XP (137,138).

VI. PREVENTION AND TREATMENT

As yet, there is no cure for XP. Prevention can take various forms. In families with individuals who suffer from XP, prenatal diagnosis can be carried out by measuring UDS in amniocytes or chorionic villus tissue. Early diagnosis of affected patients followed by strict sun avoidance and screening can prevent most of the skin abnormalities. In trials using Xpa mice, skin tumors were reduced by a factor of 13 after pretreatment with factor 60 sunscreen (139). Face masks made from UV-resistant materials enable affected children to go outdoors in daylight. There is unfortunately no preventive treatment that can alleviate the progressive neurological degeneration in those XP individuals that have neurological abnormalities.

The retinoid isotretinoin has been used in trials with patients with XP. During the course of one of these trials, the incidence of new skin cancers decreased substantially, but increased back to the initial rates when the treatment was withdrawn because of adverse side effects (140). The role of the retinoids is thought to be one of keeping tumors in check.

Preliminary results on a therapeutic regimen using T4 endonuclease V applied as a liposome lotion are promising (141). T4 endonuclease V is a microbial enzyme that is able to circumvent the defects in NER when introduced into cultured XP cells (142,143). When applied to a cohort of patients with XP in a 1-year regimen, there was a significant decrease in the appearance of new basal cell carcinomas in the treated population as compared with controls, and a substantial reduction in the number of new actinic keratoses at the end of the treatment (141). No adverse side effects were reported. These results are encouraging. Since the T4 endonuclease V removes the initiating damage rather than the precarcinogenic cells, major effects on skin cancer incidence might be anticipated only during the years following treatment.

Gene therapy for XP cells or XP mice has been attempted using liposomes containing an XPA-complementing gene applied to the skin of Xpa mice. UDS levels after UV exposure were restored almost to normal levels (144).

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18
Down Syndrome

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I. INTRODUCTION

Aging is a very complex phenomenon. Martin (1) has proposed that Down syndrome (DS) is a disorder of premature aging, because the syndrome scored highly in the author's review of syndromes with several age-related pathologies. However, few studies have attempted to verify this assertion.

This chapter will assess the utility of DS as a model for premature aging. To do this, we review the health disorders of a population of aging individuals with DS in British Columbia and compare them with other recent studies in the literature as well as with general population statistics.

In the second part of this chapter, we review what is known about the biology of Alzheimer disease (AD) and the hypotheses of how aneuploidy could contribute to the excess of early Alzheimer-like pathologies described in DS, concentrating mainly on the amyloid precursor protein and the oxidative stress hypothesis.

II. REVIEW OF MEDICAL PROBLEMS IN ADULTS WITH DS

This review is based on a survey of health disorders in adults with DS carried out in an institution in British Columbia, Canada (2). The survey used a chart review of 38 individuals with comprehensive yearly medical assessments from

1981 to 1992 living in one of the provincial residential centers for individuals with DS. Charts were divided into two groups: adults with DS younger than 50 years (younger group) and adults with DS older than 50 years (older group). All individuals had developmental testing around the time of admission and were categorized as severely or profoundly retarded. Table 1 summarizes the demographic information of the surveyed individuals. Thirteen of the 38 adults were deceased, and autopsies were carried out on three of the nine older adults with DS.

The results will first be described in a system-by-system review and then tabulated and discussed in more general terms.

A. System-by-System Review

1. Central Nervous System Disorders

The primary disorders noted in aging individuals with DS are epilepsy and generalized decline in cognitive abilities often attributed to AD.

a. Epilepsy

The prevalence of seizures in people with DS appears to be highest in infancy and late in life. Seizures occur in 5–10% of children with DS (3). Different seizure types have been reported, including infantile spasms, tonic-clonic, and myoclonic, generalized seizures. In the studied population, seizures occurred in 50% (9/18) of patients with DS in the younger group and in 55% (11/20) of pa-

Table 1 Demographic Information on Down Syndrome Adults (DS-A) Residing at Woodlands

Demographic feature	DS-A < 50 years (n = 18)	DS-A > 50 years (n = 20)
Birth	1949–1963	1917–1940
Mean age at time of chart review	36.2 (30–43) years	59.7 (47–68) years
Mean age at admission	7.3 years (2 months to 13 years)	32.5 years (6–54 years)
Mean length of stay	31 (20–41) years	25.8 (1–49) years
Sex	15 men and 3 women	13 men and 7 women
Deceased by 1992	4/18	9/20
Severe–profound mental retardation	18/18	20/20
Karyotypes	16/18 47, N, +21 2/18 47, N, +21/46, N	20/20 47, N, +21

tients with DS in the older group. Three of these individuals have a history of onset of seizures in childhood, one of whom is reported to have had infantile spasms. All other individuals with DS who had seizures had seizure onset in adulthood. Seizures can be associated with the onset and progression of Alzheimer-type changes in the brain (3), being largely responsible for the increase in epilepsy among older individuals with DS.

b. Alzheimer's Disease

The literature states that almost all individuals with DS aged 40 years and older exhibit loss of abilities in activities of daily living and evidence of AD changes on magnetic resonance imaging (MRI) scan, electroencephalography (EEG), and neuropathological brain examination. Clinically, dementia secondary to AD appears to be less frequent. In a study of 53 individuals with DS living in institutions, dementia was present in 8% (2/25) of patients 35–49 years old, 55% (11/20) of patients 50–59 years, and 75% (6/8) of those aged 60 years and older (4). Eighty-four percent of the reported patients with dementia and DS have seizures, and 20% have symptoms of Parkinson's disease. Men with DS are three times more likely than women to develop AD symptoms (5). All adults with DS with apolipoprotein E 3/4 and 4/4 genotypes are four times more likely than those with the 3/3 genotype to develop AD symptoms (5). Thus, the same protective effect of the E3 allele is seen as in the general population.

Visser et al. (6) have prospectively evaluated clinical signs, cognitive functioning, and EEGs in 307 adults with DS living in institutions. Progressive mental and physical deterioration was found in 18% (56/307), with a mean age of onset of dementia of 56 years. The prevalence is 11% among adults aged 40–49 years, rising to 77% in those aged 60–69.

MRI findings in adults with DS with dementia demonstrate reduced total brain mass, particularly in the hippocampal and amygdala regions, and increased ventricular size (7). Hippocampal size is also reduced in normal adults with DS, so that reduction in the amygdala size is more specific for DS (8). Prasher and coworkers (9) have suggested using MRI as a tool for diagnosis of AD in adults with DS, particularly when standardized intellectual assessments are not possible.

Even without dementia, adults with DS are known to have declining cognitive function (10–12). Depression (13,14) and other psychiatric disorders (15) also can interfere with their ability to carry out normal skills of daily living.

Declining cognitive function in adults with DS is observed in the studied population and has been reported by others. The reason for admission of all 13 adults with DS admitted to Woodlands in adulthood is because of declining abilities and behavioral problems limiting their ability to remain in a home setting. Significant factors contributing to decline in adaptive behavior are aging, dementia, and severity of mental retardation (16), as well as major illnesses. Absence of

medical illness is a positive predictor of a higher level of adaptive behavior in older adults with DS (16).

The prior lack of objective and quantitative assessment tools for individuals with moderate to profound mental retardation has limited the our ability to document cognitive changes in the Woodlands adults with DS. Witts and Elders (17) have reported on the reliability of the "Severe Impairment Battery" for assessing adults with DS. Das and coworkers (12) have confirmed that older individuals with DS perform more poorly when assessed by the Das-Haglieri cognitive assessment system and other tests of intellectual functioning than individuals of comparable mental handicap without DS. Use of one of these tools is recommended for periodically evaluating adults with DS as part of the routine health care screening.

Known confounding factors for diagnosing dementia in DS include depression, thyroid dysfunction, sensory deficits (e.g., progression of cataracts and hearing loss), neuroleptic-induced dementia (18), and medication overdosage, particularly involving anticonvulsants and antipsychotics. Although only one of the individuals with DS in the younger group in the current study has a diagnosis of early dementia, the clinical notes on other individuals are suggestive of declining abilities. In several of the individuals in the older group, stressful life events, such as an operation or a change in residence, precipitated a sudden and lasting global deterioration. One such individual died within a year of admission following the death of his mother and relocation from living at home to our institution.

2. Eye Disorders

Previous reports have confirmed the high frequency of eye abnormalities in individuals with DS of all age groups (19). Normal ocular findings in adults with DS are considered to be an exception. Diagnosis of early cataracts and keratoconus may be difficult in marginally cooperative patients. In childhood, the chief concerns for individuals with DS are congenital cataracts, refractive errors, strabismus, and amblyopia. With increasing age, adult-onset cataracts are common (20,21), and the current study found that refractive errors (usually myopia and strabismus) are common: Adult-onset cataracts were present in 33% (6/18) of individuals in the younger group and in 65% (13/20) of individuals in the older group.

Keratoconus is another condition frequently reported in adults with DS. The etiology is not known. Hestnes and coworkers (19) have reported nine patients with DS with keratoconus, two of whom developed phthisis bulbi. In the current study, keratoconus is more common in individuals with DS in the older group (20%) than it is in individuals in the younger group (11%). One person has been reported with an earlier keratoplasty for keratoconus who subsequently developed phthisis bulbi. Frantz and coworkers (22) report favorable results of keratoplasty for individuals with DS.

3. Hearing Disorders

Recurrent and chronic ear infections in noncommunicative adults often go undiagnosed and can lead to mastoiditis and hearing loss. Almost half of the studied adults with DS in the current study have hearing disorders commonly from recurrent ear infections, with 18% having a history of mastoiditis, usually resulting in mastoidectomy. Significant hearing loss was present in at least 25% of individuals older than 50 years in the current study, and we assume that difficulties in testing and failure to assess led to an underestimation of less severe hearing disorders. Balkany and coworkers (23) have reported significant hearing loss in 78% of 107 individuals with DS aged 2 months to 60 years who were tested with otoscopy, audiometry, and impedance tympanometry. Evenhuis and coworkers (24) have reported the most complete study available on hearing disorders in adults with DS in residential centers in 1991. Thirty-five adults with DS aged 35–62 years were evaluated with otoscopy, pure tone audiometry, impedance audiometry, and auditory evoked responses. A loss of greater than 20 db was present in 59 ears tested. The documented hearing losses were conductive, presumed to be secondary to middle ear disease, and sensorineural.

4. Cardiovascular Diseases

Congenital heart anomalies are a common structural anomaly in DS, being identified in 42% of newborns evaluated by echocardiography (24). The incidence in surviving adults is unknown. In the current study, 15.8% of DS diagnosed with untreated congenital heart anomalies, including three with ventricular septal defects, two with atrioventricular canal defects, and one with a patent ductus arteriosus.

An additional 26% (10/38) individuals with DS in this population have been reported with significant cardiac murmurs without cardiac symptomatology. Hamada and coworkers (25) have reported a high incidence of valvular heart disease in 30 institutional adults with DS with no history of congenital heart anomalies and no cardiac symptoms. Echocardiograms on these adults with DS revealed that 26.7% had mitral valve prolapse with a 20% increase in echo brightness of the mitral valve, 16.75% had mitral valve regurgitation, and 13.3% had aortic valve regurgitation.

Rheumatic heart disease is documented in only one man, who died of aortic insufficiency and congestive heart failure at age 35 years.

In the current study, 3 of 38 (7.8%) individuals have documented myocardial infarctions: a 65-year-old woman, a 61-year-old man, and a 35-year-old man with a history of a nonhypertensive stroke. Atherosclerotic changes are reported in 75% (6/8) of individuals upon whom autopsies were performed. Routine investigation for lipid disorders is not part of the yearly assessment at Woodlands. Interestingly, hypertension is not a finding in any of the assessed individuals with DS in the current study.

5. Respiratory Tract Diseases

Acute and chronic respiratory tract problems occur frequently in the current population. Three of the individuals with documented congenital heart anomalies also have pulmonary hypertension. Pulmonary hypertension—which occurs earlier and is often the cause of death when left untreated in individuals with DS—is well reported in the literature (25a,25b). In 1982, Thase reported that pneumonia is common in institutionalized DS populations (25c). In our group, 55.2% succumbed to pneumonia during their stay. In the current study, recurrent pneumonia with incomplete recovery was seen more often as mobility decreases. In addition, six of the individuals with DS older than 50 years have chronic interstitial changes of the lungs (documented by radiography) with insidious onset attributed to chronic, recurrent aspiration. Respiratory problems related to aspiration are known to be associated with lower esophageal sphincter incompetence and gastroesophageal reflux disease in the general population, in patients with neurogenic dysphagia, obesity, and a sedentary lifestyle, and in handicapped persons without overt neurological deficits (26).

6. Gastrointestinal Disorders

The most common gastrointestinal problems in infants with DS, duodenal atresia, and Hirschprung's disease are not reported in the current study population. Although aspiration pneumonitis and chronic interstitial changes from presumed gastroesophageal reflux are apparent, in our study population only one individual with DS has a diagnosis of gastroesophageal reflux, recurrent esophagitis, and stricture requiring surgical treatment. Hiatal, ventral, postoperative incisional, and inguinal hernias are common in the Woodlands study population: occurring in 20% of elderly individuals with DS.

Obesity is a common disorder among individuals with DS: occurring in 70% of men and 95% of women (27) compared with 50% in individuals with other mental disabilities. In the current study, obesity was present in 77% (10/13) of individuals who were institutionalized as adults and appeared to improve with dietary restrictions. Obesity resulting in hypoventilation syndrome was documented in 2.6% (1/38), presenting clinically as hypersomnia. Normal weight tables for adults with DS are not available.

Basal metabolic rate in healthy individuals with DS is comparable to normal controls (28), implying that dietary interventions can be effective. Constipation is a common complaint, particularly among inactive elderly individuals with DS. Rectal prolapse was found in 37.5% (3/8) of the adults in the current study with chronic constipation.

There is controversy in the medical literature regarding whether or not celiac disease occurs more frequently in DS compared with the general population (29). None of the adults in the current study were diagnosed with celiac disease.

7. Urinary Tract Disorders

Recurrent urinary tract infections (UTIs) are of particular concern. In the current study population, UTIs are documented in over 25% of individuals with DS, particularly those who are bedridden. Bladder neck resection was performed in 20% (4/20) of individuals with DS in the current study in the older group and in none of the individuals in the younger group. Mesangiocapillary glomerulonephritis, focal segmental glomerulosclerosis with hyalinosis, acute glomerulosclerosis, minimal glomerular change disease, and membranous nephropathy causing renal failure have been reported in DS (30,31).

Among the adults in the current study, renal failure is present in one. This is attributed to chronic, recurrent pyelonephritis; however, results of the biopsy are not available in the patient's chart. Another man who is a hepatitis B carrier has persistent proteinuria and hematuria with normal blood pressure. His clinical picture is attributed to minimal glomerular change disease associated with being a hepatitis B carrier. Documented hydronephrosis has been reported in one person. Other structural renal anomalies are not reported in the current population; however, systematic renal ultrasounds are not part of the routine yearly medical evaluations.

Review of adults with DS for structural anomalies with renal ultrasound is recommended along with routine monitoring for renal and especially glomerular disease (31).

8. Musculoskeletal Disorders

Premature degenerative bone and joint disease in adults with DS has been reported in the orthopedic literature (32,33). Pain and limitation of movement related to degenerative osteoarthritis is a frequent concern in the current population. Among individuals with DS in the younger group, osteoarthritis of the spine has been reported in 22% (4/18) and none with osteoarthritis of other joints. In the older group, 40% (8/20) have osteoarthritis of the spine with 62.5% (5/8) having osteoarthritis in other joints. The radiological presentation of spinal osteoarthritis in adult individuals with DS is similar to that reported in other geriatric patients; although in DS, the symptoms appear to occur at a younger age (34).

Common symptoms related to spinal degeneration, paresthesia, numbness, weakness, and pain may go unreported in less communicative adults with DS. Carpal tunnel disease frequently occurs among adults with DS and needs to be considered in the differential diagnosis (35). Yearly evaluations should include careful neurological examinations. Early loss of ambulation among older individuals with DS is attributable to osteoarthritis as well as to AD. Diagnostic investigations are indicated, since symptoms related to osteoarthritis are treatable (36).

Fractures secondary to osteoporosis and trauma are common in the current population. In the older group, 55% (11/20) had long bone fractures, 30% (6/20) had fractures of other bones, and 30% (6/20) had documented collapse of vertebral

bodies. Paraplegia secondary to a compression fracture of the spine was reported in one of the individuals with DS in the younger group who was injured when he jumped out of a window. Another older man with DS had a compression fracture secondary to osteoporosis. Decreased exercise, inadequate dietary calcium, and limited exposure to sunlight in the Vancouver region are all contributing factors for increased osteoporosis in the current population. Other metabolic factors related to the underlying aneuploidy also may contribute to this clinical problem.

Atlantoaxial subluxation is a serious but infrequent complication of DS (29,37,38). There is suggestion that the radiological gap decreases with age, presumably as ligamentous laxity decreases, reducing the risk for spinal cord trauma (29). One of the individuals in the younger group in the current study has paraplegia secondary to atlanto-occipital instability. Two elderly adults have atlanto-occipital instability without neurological symptoms.

9. Cancer

Cancer, including leukemias, has not been reported in any of the adults in the current study. Baird and Sadovnick (39) have reported on the attributable causes of death from certificate data of 324 individuals with DS, 9 of whom died of unspecified tumors. Overall, the rate of leukemia in DS is estimated to be 1 in 150, which represents a 20-fold increased risk over the general population (40). This risk increase is thought to extend to adulthood. Testicular germ cell tumors may be more frequent in DS adult males compared with the general population (41). Satge and coworkers (42) have reviewed cancers in patients with DS, finding that most malignant solid tumors occur less frequently compared with the general population. Those solid tumors Satge and coworkers (42) identify as occurring in excess in patients with DS include lymphoma, extragonadal germ cell tumors, testicular germ cell tumors, and a possible increase in retinoblastoma, pancreatic, and bone tumors. Life style cancer risks, such as lung and cervical cancers, appear to be decreased in individuals with DS.

10. Infections

Problems with cell-mediated and humoral immunity are well documented in individuals with DS (reviewed in Ref. 29). Recurrent infections, particularly upper respiratory tract, middle ear, and skin infections are common in the Woodlands population. However, it is difficult to determine whether or not the frequency of infections in individuals with DS is increased compared with other residents.

Chichon and coworkers (44a) hypothesize that the increase in periodontitis associated with DS is secondary to an exaggerated immune inflammatory response. The prevalence of hepatitis B surface antigen is higher in individuals with DS independent of whether they are in institutional care or living with their family (43,44). Hepatitis B antibodies are documented in 44% (8/18) individuals in

the younger group and in 25% (5/20) in the older group. Seventy-seven percent (10/13) are considered to be chronic hepatitis B carriers. Screening of Woodlands residents in 1985 identified 7% of residents to be hepatitis B carriers. Fifty-three percent (20/38) of these carriers have DS. This rate is far in excess of DS representation among Woodland's residents (7.7% [42/543] in 1985). Immunization of nonimmune adults with DS against hepatitis B, as well as hepatitis A and other common immunizable disorders, is strongly recommended.

B. Other Health Disorders

1. Thyroid Disorders

Thyroid disorders and other autoimmune disorders are common in individuals with DS of all ages (45,45a,46). In a survey of 106 adults with DS, more than 30% had elevated microsomal autoantibodies; however, only 5% are hypothyroid, and an additional 4% have elevated thyroid-stimulating (TSH) levels with normal T3 and T4 levels (47). A subsequent longitudinal study by Karlsson and coworkers (46) of 85 individuals with DS followed up to 25 years found a much higher rate of thyroid abnormalities. Thirty-five percent (30/85) had hypothyroidism and 2.4% (2/85) had hyperthyroidism. In the Woodlands population, hypothyroidism is being treated in 22.2% (4/18) of the individuals in the younger group and in 35% (7/20) of the individuals in the older group. Owing to the high suspicion of hypothyroidism, yearly screening of TSH and T4 is recommended for early detection (48,29).

One of the individuals with DS in the current study has discoid lupus erythematosus. Vitiligo and alopecia areata also are mentioned in the records of several patients. However, there is inconsistency in reporting this disorder.

III. IS DS A SYNDROME OF PREMATURE AGING?

Aging studies have tended to look at a variety of levels ranging from the molecule to the entire organism. Causes of senescence at the molecular or cellular level often are difficult to correlate with aging at the organismal level. The following discussion examines aging at the level of the organism.

The life expectancy of individuals with DS has increased markedly in North America during the last 50 years (49–51). There is some evidence of variability within races and socioeconomic groups (52). Median age of death for Caucasian individuals with DS rose from 2 years in 1968 to 50 years in 1997, whereas for African-American individuals with DS during the same period the median age of death increased from 0 to 25 years. It is therefore important to be aware that most studies on individuals with DS have examined the Caucasian population. The life

expectancy of all individuals with DS, on average, is at least 10–20 years shorter than for the general population.

Much of this change in life expectancy can likely be attributable to changes in the aggressiveness of treatment practices, such as correction of congenital heart defects, which is a standard of care, and improvement in the quality of treatments and therapies. In addition, many individuals with DS are now cared for in the community and are no longer in institutions.

These changes can be expected to have a large impact on the health of younger individuals with DS as they age. One would expect to see some consistent genetic effects as well.

Table 2 compares the rates of health disorders in the current study population with rates in the general population and seeks to ascertain whether these disorders increase to a greater degree with advancing age in the DS population than in the general aging population. Table 2 reveals the following:

1. Conditions such as osteoarthritis, hearing loss, and blindness occur earlier in the DS population, but apparently at about the same rates as in the general population.
2. Frequencies of asthma, bronchitis, and UTIs occur at about the same rates in the general population as in the DS population. Asthma and bronchitis do not vary more with age. UTIs become more frequent with age, but not significantly more in the DS population.
3. Adult-onset cataracts and keratoconus, pneumonia, congenital heart disease, abdominal hernia, and AD appear to occur considerably more frequently in the DS population than expected and at an earlier age. In addition, it is accepted in the literature that thyroid problems and hepatitis B are more common in DS. In the current study population, one individual had vitiligo and alopecia areata; however, general population rates of these disorders are not readily available.
4. Hypertension and certain solid malignancies occur less often in the aging DS population.
5. As would be expected, certain aspects of health are significantly different for individuals with DS. For example, it is more appropriate to compare the prevalence of behavioral problems and seizures in a DS population with the prevalence in a population of individuals with mental retardation or cerebral palsy than to make comparisons with the general population.

Table 2 suggests that for many of these conditions, the rates for the DS population are similar or follow a similar pattern but occur 10–20 years earlier than in the general population. These findings support the progeroid model of DS, but not for all systems. This supports others studies of comparative aging for DS (1). Nakamura and Tanaka (53) suggest that individuals with DS age about two times

Table 2 Health Concerns in DS Compared with General Aging Population in United States[a]

Concerns	General pop. young old (%)	General pop. older old (%)	Middle-aged DS <50 (%)	Elderly DS >50 (%)	All DS (%)
Visual					
Adult-onset cataracts	3.9–10	39–46	33	65	65
Blindness	11.0	25.0	16	25	21
Strabismus	[b]	[b]	33	40	36
Keratoconus	0.2–8[c]		11	20	16
Refractive errors	[b]	[b]	22	45	34
Hearing					
Hearing loss	12.0	24–39	–	25	45–84
Respiratory					
Pulmonary hypertension	[b]	[b]	11	5	8
Pneumonia	3.5–6	6.5–17	50	60	55
Asthma/bronchitis	Doesn't change with age. 5–8%		5	10	8
Aspiration with lung changes	Common in patients with swallowing difficulties from esophageal problems and neurological deficits		NI	30	30
Cardiovascular					
Congenital heart disease	<% 1 at birth				42
Arteriosclerotic heart disease		5–23	5.5	20	13
Cerebral vascular accidents (incidence)	<1	3–9	0	10	2.6
Hypertension	7–19	25–33	0	0	0
Genitourinary					
Recurrent UTI	5–10	15–55	0	15	7.9
Renal failure	<0.06	—	0	5	2.6
Gastrointestinal					
Abdominal hernia	2.7	5.9	0	35	15
Gastroesophageal reflux	[b]; Gallup poll shows no age or sex difference. 20% of the population report weekly symptoms.		5.5	30	18
Prolapsed rectum	[b]	[b]	0	15	8
Musculoskeletal					
Osteoarthritis	23	48	22	40	32
Fractures	[b]	[b]	0	55	85
Paralysis	[b]	[b]	11	5	7.8

(continues)

Table 2 *Continued.*

Concerns	General pop. young old (%)	General pop. older old (%)	Middle-aged DS <50 (%)	Elderly DS >50 (%)	All DS (%)
Central nervous system					
Seizures	0.55 increase substan tially with age		50	56	53
Alzheimer disease	10% >65 yr; 50% >85 yr		0	75	42
Parkinsonian symptoms	Variable in different populations < 0.2%, increases with age		0	6	3
Behavioral problems	[b]	[b]	67	35	50
Miscellaneous					
Thyroid	2	4	22	35	29

NI, Not investigated

[a] General population figures come from either (a) Hazzard et al. 1999 or (b) Grimley Evans et al. 2000; where possible they are divided into <65 and >65. However figures were not always available in this format.

[b] Figures not available in the general population.

[c] Krachmer JH, Feder RS, Belin MW. Keratoconus and related noninflammatory corneal thinning disorders Surv Ophthalmol 1984;28:293–322. Rabinowitz YS. Keratoconus. Surv Ophthalmol 1998:42:297–319.

faster than patients with cerebral palsy based on studies of hematological and blood characteristics. An early study by Brugge and coworkers (54) purports to find evidence for accelerated aging of the skin in DS.

Alternatively, the current study did not find evidence for an increased level of degenerative vascular disease as suggested by Martin (1) except as a complication of congenital heart disease. It is unclear if this is because it was not routinely looked for in the clinical evaluations.

Of those conditions that occur more frequently in the DS population, complications of congenital heart disease may relate to the underlying dysmorphology of DS. An increase in frequency in abdominal hernia could be attributed to hypotonia and connective tissue laxity that are congenital. This joint laxity is the attributable cause for early degenerative bone disease as well, but other factors also may be important.

Other conditions, like more frequent and earlier onset of cataracts, may relate to an advanced aging mechanism or to an underlying abnormality at the molecular level in the same way the "cause" of decreased rates of solid tumors probably reflects a combination of life style differences and an underlying genetic difference in individuals with DS. This remains to be clarified.

The medical problems that pose the greatest risks to adults with DS include pulmonary hypertension associated with untreated congenital heart anomalies, ac-

quired cardiac diseases, recurrent respiratory infections and aspiration, complications attributable to presenile dementia and Alzheimer-type disease, adult-onset epilepsy, osteoarthritic degeneration of the spine, osteoporosis and resultant fractures of the long bones and vertebral bodies and untreated atlanto-occipital instability, hypothyroidism, and arthritis. Acquired sensory deficits can be significant problems both with respect to delayed diagnosis as well as with long-term morbidity resulting in decreased enjoyment in daily activities. Of particular concern, clinically, are loss of vision due to early onset of cataracts, recurrent keratitis and keratoconus, and loss of hearing resulting from chronic middle ear infections and presbycusis. Behavioral problems related to the underlying cognitive deficits, loss of abilities with advancing age, and onset of symptoms of AD pose ongoing challenges in everyday care.

IV. HIGHLIGHTS OF EMERGING GENE INFORMATION ON CHROMOSOME 21

Chromosome 21 has now been sequenced and found to contain about 1–1.5% of the human genome. Twenty-four disease loci have been mapped according to OMIM (54a). With the completion of mapping and sequencing, chromosome 21 has been found to carry the smallest number of genes of all the chromosomes, with only 225 genes, about half as many as on chromosome 22, although the two chromosomes are roughly equivalent in size.

It is not clear how three copies of a gene, instead of the normal two, result in the precise developmental abnormalities that we recognize as DS. Theoretically, three copies may result in a dosage imbalance, with half as much again of the gene product being expressed as in the normal individual. This effect has been demonstrated for some gene products such as *superoxide dismutase-1* (54b) and the *GARS-AIRS-GART* gene (54c). The type of function of the gene product would be expected to determine the degree of effect of dosage imbalance. For example, transcription regulators might be expected to have a relatively greater effect than enzymes.

Assessing gene dosage effects requires an interesting shift in thinking from our usual concern with "absence" of effect in recessive biochemical disorders, or "presence" of effect in dominant disorders of aberrant structural development. Much work appears to be being undertaken now to try to elucidate the role that various genes play in bringing about specific characteristics, such as congenital heart malformations and abnormal brain development. From a clinical perspective, there are intriguing parallels between observed characteristics and some of the genes now being characterized on chromosome 21.

For example, *DFNB10* and *Claudin 14* have both been linked to genetic forms of deafness. *NCAM* (cell adhesion molecule, neural 2) and Purkinje cell

protein have both been proposed as important genes in brain development. Crystalline α is linked to both a dominant and recessive form of cataract. Interferon-α and β, ω receptors 1 and 2, γ receptor 2 (integrin β-$_2$), and an autoimmune regulator responsible for autoimmune polyglandular disease are all also found on chromosome 21. How might these genes interact to bring about immune suppression or the excess of thyroid disease seen in DS? The answers are not yet available in the literature.

Other genes, such as *MX1* now thought to also play a role in alopecia areata, or *TAM* proposed to play a role in transient leukemia of DS, have been proposed to contribute to specific conditions seen at higher levels in the DS population.

With respect to aging, the discovery of the amyloid precursor protein (*APP*) gene on chromosome 21 ignited a flurry of work to try to elucidate the biology of AD, as DS exhibits one of the clearest examples of premature age-related pathology. The next section reviews this work in more detail.

V. BIOLOGY OF ALZHEIMER-TYPE DEMENTIA (AD) IN DS

Clinical findings in the aged DS population were summarized in the first part of this chapter. In the U.S. population, it has been estimated that 1 in 6 individuals older than 65 years is clinically demented, and 1 in10 has AD (55). By age 85, the prevalence of AD is nearly 50% (56). AD is a diagnosis of exclusion that requires an insidious, progressive decline in abilities in conjunction with neuropathology, and where other causative factors have been ruled out.

Changes in the brains of individuals with AD include diffuse atrophy of the cortex and the hippocampus, neuronal loss, degeneration, and a reduction in the efficiency of synapses. In addition, hallmark structures called senile plaques (SPs) and neurofibrillary tangles (NFTs) are necessary for diagnosis of the disease.

Senile AD (SAD) is diagnosed in individuals older than 65 years. The disease typically progresses over 20–30 years. Early-onset AD is considered when the diagnosis is made in persons younger than 65 years. Generally these individuals have a more rapid course of disease. Studies of families where multiple individuals develop early-onset AD and of individuals with DS who exhibit premature AD pathology have provided the first clues to the biology of AD. Several investigators have recently reviewed the biology of AD (57,58).

The main constituent of SPs is Amyloid-beta, and a major component of the NFTs is tau.

The A-beta in these SPs is in a fibrillar form. There are multiple other constituents of these structures in the brain, including α-1-antichymotrypsin (ACT), proteoglycans, and apolipoprotein-E (ApoE). A-beta appears also to be deposited in cerebral blood vessels. SPs are extracellular structures made up of a core of A-beta, a protein derived from APP by proteolytic processing. Other neural cells,

such as microglia, fibrillary astrocytes, and dystrophic neurites, surround the SP cores.

APP is a common protein in the body and is not unique to neurons. The biological function of APP is still unknown. APP is expressed broadly in various tissues in the body and can be present in a variety of different forms, as it is transcribed alternately. It can be cleaved at three different sites—alpha, beta, and gamma. The APP695 form is the form that is primarily present in neural tissue. It has a transmembrane region. Another form, APP770, is heparin binding and present in platelets. APP processing and secretion are regulated by a number of hormones and growth factors, as well as in response to injury and during development.

A-beta also is produced in several forms. A-beta 42, the longer form, can aggregate more easily than A-beta 40 in vitro. There is also a soluble form, which appears to have neurotrophic effects. Aggregated, fibrillar A-beta has been shown to be neurotoxic in vitro. It appears to disrupt calcium homeostasis in the neurons.

It is not yet clear from the literature whether A-beta is a cause of AD or a marker of disease. There appear to be two camps.

NFTs are produced later than SPs. They also are considered to be necessary for the diagnosis of AD. Disease progression in AD correlates better with NFT number and density than it does with SP formation. NFTs are made up of paired helical filaments (PHFs) that are made up of the microglial associate protein tau, which binds microtubules and promotes their assembly.

In addition, tau has been linked to several other neurodegenerative diseases, called the tauopathies, which include progressive supranuclear palsy, frontotemporal dementia (FTD), and Pick's disease. Tau also is believed to reduce microtubular instability, assist in maintaining neuronal integrity, and play a key role in axonal transport and axonal polarity.

Within the PHFs, tau is hyperphosphorylated. PIN1 can restore the ability of Tau to bind microtubules and their function. Decreased levels of PIN1 in vitro have been shown to induce mitotic arrest and apoptotic cell death. Heutink (59) has recently reviewed tau biology.

VI. ADDITIONAL GENETIC PLAYERS IN THE AD STORY

Studies of families with individuals who have early-onset AD have demonstrated a linkage to several genetic loci, including one on chromosome 21, which is known to code for APP. However, these families account for only a small subset (5%) of early-onset families. Other genes that have been shown to play a role in AD include *ApoE* and *presenilin-1* and *-2*.

Presenilin-1 mutations occur in almost half of families with early-onset AD, and *presenilin-2* accounts for a small fraction. It is unclear exactly how these

membrane-spanning proteins interact with A-beta. *Presenilin-1* is now thought to be a γ secretase. It localizes to endoplasmic reticulum and the Golgi compartments. Mutations in *presenilin-1* result in increased production of A-beta 42–43 (59a).

ApoE occurs in three common forms—*ApoE2*, *3*, and *4*. *ApoE4* is linked to an increased risk for onset of AD in both early-onset groups and the general AD population; *ApoE2* appears to have a protective effect. Again, the exact mechanism for the interactions of these proteins is unclear. *ApoE* is an intracellular protein, which appears to play a role in neurite growth and in myelin and cell membrane growth during development or after injury.

In addition, it appears that the gene for one of the enzymes that cleaves A-beta from the APP is also located on the long arm of chromosome 21. It is called *BACE* (beta amyloid site cleaving enzyme). What the effects of an additional copy of BACE may have for individuals with DS has yet to be evaluated.

S100beta is a calcium-binding protein synthesized by the central nervous system (CNS) astrocytes and is believed to have a trophic effect on neurons and to promote neurite extension and proliferation. It is expressed in excess (up to 20 times normal) in brain regions with severe neurodegeneration (59b). S100beta is coded for by a gene on chromosome 21.

Part of the evidence for an important role for APP or A-beta in the pathogenesis of AD comes from the excess in the DS population. Interestingly Prasher and coworkers (60) have reported a 78-year-old woman with partial trisomy 21 with only two copies of APP who had no clinical or pathological evidence of AD.

A-beta messenger RNA is elevated in the brains of individuals with DS. Some investigators suggest that it is as much as seven times higher (60a,60b). It is deposited in cells, vessels, and SPs in an age-dependent manner, and it has been suggested that it may play a normal role in myelination of neurons. Increased levels are detectable from the second decade of life. A-beta 42, the longer version that is more prone to form fibrillar structures, is the predominant form in DS brains.

The density of A-beta deposits is slightly different in DS than in SAD. The deposits tend to start in the more superficial layers but move to the deeper layers of the brain with age, such as the frontal and entorhinal cortex. SAD accumulation of A-beta tends to be more superficial (61).

It remains unclear from the literature, however, exactly how these proteins interact to bring about AD in DS patients or in the general population. At present, although they are considered to be necessary for the development of AD, it is not clear whether they are sufficient to bring about AD without additional contributing factors.

There are several hypotheses being actively investigated seeking to elucidate the mechanisms that cause AD pathology. Van Leeuwen and colleagues (62) have proposed a novel mechanism whereby misreading mutations are generated in a clonal manner in individual cells because of misprocessing of dinucleotide re-

peats. They have demonstrated aberrant forms of APP and ubiquitin (a common "clean-up" protein) in the brains of individuals with DS and AD. They suggest that this misreading results because of higher rates of protein production and can occur particularly frequently in individuals with DS. Abnormal proteins produced in this manner are difficult to degrade and result in accumulation of these forms in plaques and tangles.

Very recent work by two groups (63,64) appears to show that A-beta and tau interact to increase the amount of tangle formation, and that either APP or A-beta influences the formation of tau tangles. This work was undertaken in mouse models carrying mutant APP or mutant tau. Neither experiment exactly replicates the AD pathology.

Another popular theory to explain the early event initiating a cascade of downstream effects is the oxidative stress hypothesis, which also has been suggested to account for some other more generalized progeroid effects in DS.

VII. OXIDATIVE STRESS HYPOTHESIS AND AGING IN DS

The oxidative stress hypothesis of aging was proposed several decades ago. The chemistry is fairly well established. The suggestion that oxidative stress at the cellular level can contribute to aging at the level of the organism has been gaining support among the general and scientific public.

Essentially, free radicals (reactive oxygen species [ROS]) are produced as a by-product of metabolism of ATP to ADP in the mitochondria. If not "tidied up" by biological "housekeepers," these rogue chemicals can act as destructive agents in the cell, because they are highly effective at reducing chemicals that can induce damage to other larger molecules within the cell. The main ROS are superoxide anion O^{-}_{2} and hydrogen peroxide H_2O_2. In the presence of iron, additional substances such as OH^{-} and nitric oxide (NO) are also formed.

In their turn, ROS act upon the membrane of the cell, of the endoplasmic reticulum, and the mitochondria and interfere with normal functions, such as membrane polarization, calcium and potassium gradient creation, and maintenance and glucose transport. Lipid peroxidation appears to be one route by which free oxide radicals bring about alteration of the cell membranes (64).

Certain substances can tidy up these ROS. Superoxide dismutase (SOD) acts to reduce the superoxide anion to hydrogen peroxide. This in turn is reduced by catalase in the peroxisome and by glutathione peroxidase in the mitochondria and cytosol. There are several types of SOD which differ in their subcellular localization: SOD1 is in the cytosol, SOD2 is in the mitochondria, and SOD3 is in the extracellular space.

The details of how ROS can contribute to aging are not completely worked out yet, and experimental data do not seem to be consistent across species. How-

ever, there seems to be a general consensus that ROS can contribute to cell damage, and that unchecked they can bring about features of cellular senescence (66).

It appears that DNA and protein damage can also occur. This damage is repairable by the cellular machinery. Left unrepaired, however, these alterations may initiate the apoptosis cascade, killing the damaged cells. Raji and coworkers (67) have documented lower DNA-repair efficiency and accelerated decline in DNA-repair capacity with age.

A biological model of the importance of these antioxidants occurs naturally in a rare and occasionally inherited disorder, familial amyotrophic lateral sclerosis (ALS), where a small proportion of families carry inactive copies of their *SOD1* gene. The chronic oxidative damage to motor neurons leads to the onset of progressive paralysis and a shortened life span. It appears to preferentially affect specific motor neurons in the anterior horn.

Experiments with transgenic *Drosophila* show that increases in the expression of *SOD1* and *catalase* can increase the life span by 30%. Reduction in their expression can decrease life span by 40%, and rescue can be achieved by replacing the *SOD1* with human *SOD1*. However, similar experiments in mice show mild phenotype to the inhibition of *SOD1* and *SOD3*, but a more striking phenotype with *SOD2* inhibition, thus cautioning against overgeneralization of the *Drosophila* data to other species.

Individuals with DS show increased levels of *SOD1* and a general imbalance of the antioxidant enzymes, which is thought to lead to accumulation of reactive oxygen species and oxidative stress to the cells (68). In addition, individuals with DS and cell lines from individuals with DS have been shown to be impaired in their ability to repair oxidative damage to mitochondrial DNA compared with age-matched control cells. Oxidative damage is thought to contribute to some other parts of the phenotype of aging in DS; for example, the increased rates of adult-onset cataract formation is hypothesized by some to occur in response to increased oxidative stress.

VIII. CONCLUSION

DS appears to be a segmental progeroid syndrome. In individuals with DS, certain systems undergo aging faster than in the general population. Some disorders occur at a higher rate in individuals with DS, whereas others occur at rates similar to the general population but earlier in life. In other respects individuals with DS age appropriately or even better than the general population; specifically, individuals with DS have a lower incidence of certain solid tumors and hypertension as they age.

There are many genes that are present in three copies in this aneuploidy syndrome. A triplicate copy is not expected to be detrimental in all cases. Much work

remains to be done to elucidate the specific genetic contributions to the phenotype. Using AD as a model of one specific premature aging condition, we see that many different genes play a role in the pathology. Several are present on chromosome 21, but other genes likely also impact the ultimate phenotype.

In this context, the progeroid effects in DS are likely multifactorial in origin. Initial developmental differences combined with differential expression of certain genes and differential exposure to life style factors combine together, and so as our knowledge increases, we appear to end up with more questions than answers.

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19
Genomic Instability and Yeast Aging

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I. INTRODUCTION

Genetically tractable model organisms have been utilized in the search for novel genes involved in the aging process. Among these organisms is the budding yeast *Saccharomyces cerevisiae*. Features of yeast that make it attractive for study include the ease and speed with which genetic manipulations can be performed and the short time required to determine the life span.

A number of techniques have been used to identify genes that influence the life span in yeast. As described below, genes that modulate yeast the life span have been identified in genetic screens using stress resistance as a surrogate. Out of the analyses of these, and of other mutations that the affect life span, a novel form of genetic instability that is a major determinant of longevity in yeast has been identified: the accumulation of extrachromosomal ribosomal DNA circles (ERCs).

There are undoubtedly multiple causes of yeast aging. Although the focus of this chapter will be the ERC model of replicative aging in yeast, there are alternative models of aging in yeast. For example, yeast can also age chronologically as a population of nondividing (stationary phase) cells. The survival of nondividing yeast is associated with stress resistance (to heat shock or paraquat), and may be prolonged by overexpression of the antiapoptotic protein Bcl-2 (1) or by mutations in adenylate cyclase or Sch9, which is homologous to the AGC family of serine/threonine kinases, which includes the Akt ortholog involved in the daf-16–dependent pathway of life span extension in the roundworm (2).

II. BACKGROUND

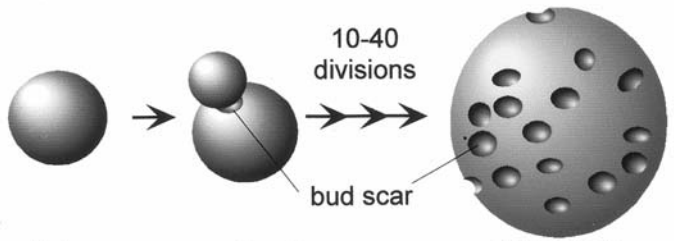
Although yeast cultures are immortal, individual yeast cells display a finite life span (3,4). *S. cerevisiae* divides asymmetrically, giving rise to a larger mother cell and a smaller daughter cell with each cell division. A circular bud scar on the mother cell's surface is left at the site of division, serving as a physical marker of the number of cell divisions a yeast cell has undergone. The cell wall and many other constituents of daughter cells are synthesized de novo, and most daughters have the potential for a full life span. Thus, the yeast population is immortal. The number of cell divisions that the mother cell undergoes varies somewhat between yeast strains. By a labor-intensive process, the number of divisions a mother cell undergoes can be determined by microscopic observation of individual large mother cells and manual separation by micromanipulation of the smaller daughter cells. Yeast aging has most often been defined as the number of times the yeast mother cell divides. Although the median life span varies among different laboratory yeast strains (in the range of 18–30 cells divisions), it remains relatively constant for a given strain, thus supporting a strong genetic influence on the life span (4–6). The mortality rate for mother cells increases exponentially with the number of cell divisions (7,8). These characteristics have allowed for the use of yeast as a simple system for the genetic study of aging and senescence.

Additional features that make a yeast an attractive experimental model include its relatively uncomplicated and short life cycle; its small genome size (about 6000 genes), many of which have been shown to have orthologs in the human genome; its well-developed system of homologous recombination allowing for relatively simple knockout or knockin of individual genes; and its economy of cell culture—it is inexpensive to grow in large quantities on simple medium.

The ease with which mutants in nearly any biologically interesting phenomenon can be isolated in yeast has been difficult to translate directly to the study of yeast replicative aging. In any growing culture of yeast cells in logarithmic phase, approximately one-half the cells will be virgin cells, one-quarter will have completed one division, one-eighth will have completed two divisions, and so on. In a growing culture, cells near the end of their replicative life span (~20 divisions) are present as a minute fraction, and direct genetic approaches based on the isolation of mutant colonies are not applicable.

III. CHANGES IN AGING YEAST

It is useful to characterize changes that occur during normal aging in yeast, as it helps to distinguish between mutations that decrease the life span owing to a generalized decrease in viability from those that decrease the life span through accelerated senescence. The analysis of the cause of phenotypic changes that occur as yeast age is also one of the paths of investigation that has led to the implication of ERC accumulation as a causative factor in yeast aging.



	virgin mother	mother + small daughter	old mother
Size:		small	large
Cell surface:		smooth	wrinkled
Cell cycle:		1.5 hr	2 - 3 hr
Cell division:		asymmetrical	symmetric
Nucleolus:		20% of nucleus	60% of nucleus
Nucleolus:		intact	fragmented
Mating:		fertile	sterile
Sir3p:		telomeres and HM loci	nucleolus
Daughters:		normal life span	reduced life span
Arrest:			G1
ATP, NAD^+:			increased

Figure 1 Biomarkers of yeast aging. *S. cerevisiae* undergoes asymmetric division for most of its life span, producing a mother cell and a smaller daughter cell with each division. The cell wall of the daughter cell is synthesized de novo, and a permanent chitinous bud scar is left on the mother cell's surface at the site of attachment of the bud. (Adapted from Ref. 101.)

Aging yeast cells grow larger (4), become sterile (9,10), the cell cycle period elongates (4), and the nucleolus becomes larger and fragmented (9,10) (Fig. 1). Although mother cells produce small daughters throughout most of their lifetime, in the final divisions before mitotic senescence, this asymmetry breaks down, and daughter cells are produced that are similar in size to the mother cells.

IV. DNA DAMAGE AND TELOMERIC LOSS IN YEAST AGING

Two forms of genetic instability that likely play an important role in aging in higher organisms are unlikely to play a significant role in yeast aging: the accumulation of unrepaired DNA damage and telomeric sequence loss.

Two lines of evidence argue against the accumulation of DNA damage as a limiting factor for replicative capacity in yeast. First, cells heterozygous for nutritional marker genes do not display increased rates of auxotrophy when clones are

derived from old cells (6). If DNA damage accumulated during yeast aging, then an increased rate of auxotrophy as the single wild-type allele accumulated mutations would be expected. Second, if replicative capacity in yeast resulted from DNA damage, then all the daughter cells from old mother cells should stably inherit this trait and should exhibit a shortened life span. The majority of daughter cells produced by a yeast mother cell exhibit a normal life span. As the asymmetry in cell division begins to break down in the final divisions before replication ceases, some of the daughter cells show a diminished life span (5,11). The descendents of these prematurely senescent "daughters of old mothers" again regain a normal life span within a few generations, indicating that this effect is not due to a permanent genetic alteration.

It has been proposed that telomeric shortening contributes to the physiological decline in human aging. The limited replicative capacity of cultured human fibroblasts, which lack telomerase, is termed replicative senescence and is caused by telomeric shortening (12). Telomeric sequence loss with cell division does not contribute to the limited replicative capacity of yeast mother cells. Yeast cells are normally telomerase positive (13), and no change in telomeric length has been observed during replicative senescence of yeast mother cells (14,15). In fact, when the relationship between telomeric length and replicative life span in yeast strains of differing telomeric lengths was examined, a weak, inverse correlation between telomeric length and replicative life span was found (16).

V. TRANSCRIPTIONAL SILENCING, THE NUCLEOLUS, AND YEAST AGING

Three lines of investigation revealed the importance of the nucleolus to yeast aging: First, the analysis of the cause of sterility in aging yeast mother cells has revealed a movement of silencing factors from the telomeres and silenced mating-type loci to the nucleolus. Second, a genetic screen performed to find mutations capable of increasing yeast life span uncovered a mutant of *SIR4*, a yeast protein involved in silencing. Third, the analysis of the function of the *SGS1*, the yeast homolog of the genes mutated in the human progeroid disorders Werner syndrome and Rothmund–Thomson syndrome uncovered marked nucleolar fragmentation and accumulation of ERCs in aging yeast mother cells.

VI. SILENCING AND YEAST AGING

Yeast cells can be of two "sexes"—referred to as *MAT***a** and *MAT*α mating types. All yeast cells contain the genetic information for both mating types. Yeast cells are normally fertile because the two repositories of mating-type information, *HML*

and *HMR*, are maintained in a transcriptionally silenced state. Yeast are capable of switching between *MAT***a** and *MAT*α mating types. To switch between the two mating types, a cell must transpose the opposite, silenced, information into the mating type locus where it is then expressed. The maintenance of silencing at *HML* and *HMR* requires the activity of many genes, including *SIR2*, *SIR3* and *SIR4* (17,18).

The first phenotype of old yeast cells to be explained on a molecular level was sterility. That sterility is caused by a loss of silencing at the silenced mating type loci *HML* and *HMR* was demonstrated when the α*1* mRNA was detected by reverse transcriptase–polymerase chain reaction (RT-PCR) in old cells of *MAT***a** mating type (10). The α*1* mRNA is not normally expressed in *MAT***a** strains; expression of both **a** and α mating type information leads to sterility in yeast. That sterility is caused by loss of silencing also has been demonstrated directly by the failure of old *MAT***a** cells to respond to α mating factor. The response of old *MAT***a** cells to α mating factor was restored by deletion of *HML*α, indicating the derepression of the *HM* loci is the causal agent in the age-specific phenotype of sterility (10). In addition to the loss of silencing at the silenced mating type loci, a loss of silencing at telomeres also occurs in old cells (19).

The importance of silencing in yeast aging was independently indicated when a SIR protein was identified in a screen for long-lived mutants. Genetic screens for mutations that extend life span in *S. cerevisiae* have been limited by the technical obstacle that the life span can only be determined for individual cells and only by micromanipulation. The performance of an unbiased aging screen is impractical. To circumvent this limitation, Kennedy et al. (20) capitalized on the well-established correlation between stress resistance and longevity (reviewed in ref. 21). A genetic screen for starvation resistance mutations was performed, and each mutant was independently tested for increased longevity. Four complementation groups were obtained (*UTH1–4*) that increased the life span by 20–55%. Two of these, *UTH2* and *UTH4*, were further characterized in detail. Both of these genes have been shown to play a role in transcriptional silencing.

To facilitate subsequent life span determinations, the strain used in the genetic screen for stress resistance was chosen for its short life span. The shorter life span was due to a frame-shift mutation in the *UTH4* gene that truncated the gene product after 207 residues (22) (designated the *uth4-14c* allele). *UTH2* was found to be an allele-specific suppressor of *uth4-14c* mutation and is an allele of *SIR4*. This mutation, *SIR4-42*, was created by the generation of a stop codon that removes 121 residues from the C-terminus of Sir4p. This region of Sir4p has been shown to bind to Rap1p (23,24), a protein that is present at telomeres (25,26) and at mating-type loci (27). Overexpression of the terminal 154 residues of Sir4p not only eliminated silencing at the silent mating type loci and at telomeres, but also extended the life span only in strains expressing wild-type *SIR4* (20).

The *SIR4-42* mutation behaved like a null allele with respect to silencing at the mating-type loci and telomeres; silencing was abolished. However, only the

SIR4-42 allele, and not *SIR4* deletion, extended the life span. *SIR4-42* was dominant to *SIR4* for life span extension. A null mutation in *SIR4* actually shortened the life span. The extension of the life span by the *SIR4-42* allele required *SIR2* and *SIR3*, indicating that this was a property of the SIR complex (20).

VII. THE "AGING LOCUS"

These results were interpreted as *SIR4-42* strains having a longer life span, because *SIR4*-mediated transcriptional silencing is redirected from silenced mating-type loci and telomeres to an (at that time) unidentified "aging locus" (Fig. 2). This model is consistent with experiments that demonstrated a competition between *HM* loci and telomeres for recruitment of SIR proteins (28,29). The aging locus would define a third locus (or loci) with an affinity for SIR proteins. In young *SIR4-42* cells, increased silencing at the aging locus would allow an increase in the number of cell divisions before replicative senescence occurs. In old wild-type cells, the decreased silencing at *HM* loci and telomeres was predicted to cause increased silencing at the aging locus (30).

Using indirect immunofluorescence microscopy, it was possible directly to determine the subnuclear localization of SIR proteins in wild-type cells and

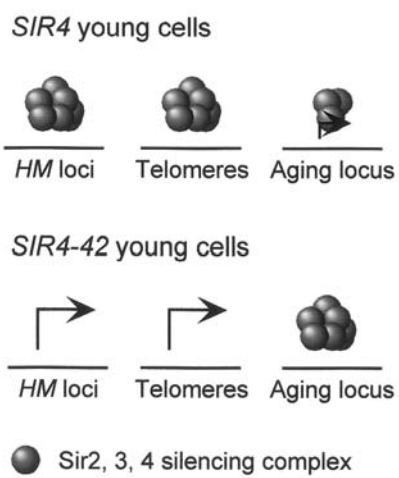


Figure 2 Model for extension of life span by *SIR4-42*. In wild-type young cells, the majority of *SIR2*, *3,* and *4* transcriptional silencing occurs at *HM* loci and telomeres and to a lesser extent the aging locus. In *SIR4-42* cells, transcriptional silencing is redirected from old *HM* loci and telomeres to the aging locus. The activity of the *SIR* proteins at the aging locus allows for increased number of cell divisions in the *SIR4-42* cells resulting in an increased life span. Arrows indicate transcriptional start sites. (Adapted from Refs. 20 and 30.)

thereby determine the identity of the proposed "aging locus" (31). In *SIR4-42* cells, Sir3p and Sir4p were localized to the nucleolus, as evidenced by colocalization with the abundant nucleolar protein Nop1p. In wild-type *SIR4* cells, Sir3 and Sir4 proteins are in perinuclear, punctate foci that colocalize with telomeric DNA (23,32). The function of the relocalization of Sir3p and Sir4p is an open question. In old wild-type cells, Sir3p was also seen to localize to the nucleolus (31). However, with respect to localization of SIR proteins, the nucleolus exhibits the behavior predicted for the "aging locus."

In accord with the finding of SIR proteins in the nucleolus, Sir2p has been shown to limit rDNA recombination (33) and to mediate silencing in the nucleolus (34,35).

A. Werner Syndrome, Rothmund–Thomson, Bloom Syndrome, and *SGS1*

The molecular mechanism by which decreased silencing and increased rDNA recombination function to limit yeast replicative life span came out of the analysis of the molecular phenotype of yeast *SGS1* mutants, a protein that is homologous to the proteins mutated in the human progeroid disorders Werner syndrome (WS) and Rothmund–Thomson (RTS) syndrome.

Inherited human segmental progeroid disorders such as WS, RTS, and Hutchinson–Guilford syndrome (HGS) are characterized by symptoms that resemble premature aging (36). The genes responsible for a subset of WS (37) and RTS (38) have been cloned, and in both cases the genes encoded are homologous to *Eschericia coli* RecQ DNA helicase (39). WS cultured fibroblasts senesce prematurely and shorten their telomeres at a faster rate per population doubling (40); studies of replicative senescence in RTS and Bloom syndrome (BS) have not been reported. *E. coli* RecQ functions in recombination involving ssDNA gaps (41,42) and in the repair of ultraviolet-disrupted replication forks (43). The in vivo functions of the eukaryotic RecQ family members are currently unknown.

Whereas there are five RecQ homologs in humans, there is only one RecQ homologue in yeast: *SGS1*. *SGS1* was first identified as a suppressor of the slow-growth phenotype that is produced by mutation of the topoisomerase III protein (44,45). Sgs1p also interacts with the topoisomerase II protein in a yeast two-hybrid screen (46). Deletion of *SGS1* increases mitotic and meiotic chromosomal missegregation (46–48) and decreases sporulation efficiency (47). Mitotic recombination is also elevated in *sgs1* mutants, as assayed by marker loss from the rDNA locus (44,48), subtelomeric Y elements, and other loci (48).

Given the accelerated replicative senescence exhibited by WS cells and the progeroid features of all the human RecQ helicase diseases, the effect of mutation of *SGS1* on yeast aging has been determined (49). The life span of an *sgs1* mutant strain is 40% shorter than the wild-type. To support that the *sgs1* mutant yeast

were dying because of accelerated aging and not dying from other causes of decreased viability, three phenotypes of yeast aging have been assessed: (a) Old *sgs1* mutant cells show accelerated rates of sterility, a phenotype shared by old yeast cells during normal aging (8) (see Fig. 1). As a demonstration that all short-lived mutations do not show increased sterility with age, mutations in neither transcriptional activator *hap5* nor in the coactivator *ada1* (which result in a markedly decreased life span) result in increased sterility with age (49). (b) Old *sgs1* mutant cells show the age-associated relocalization of Sir3p from telomeres to nucleoli. (c) Old *sgs1* mutant cells showed the age-associated nucleolar fragmentation and nucleolar enlargement. The finding of these phenotypes at 9 generations in *sgs1* cells (phenotypes that do not normally occur until greater than 20 generations in wild-type cells) supports that the *sgs1* mutant cells were undergoing accelerated aging (49).

Given the marked nucleolar enlargement and nucleolar fragmentation that has been observed in *sgs1* mutants, the state of yeast rDNA was examined. The yeast nucleolus is a subnuclear structure that is the site of rDNA transcription and rRNA processing and contains approximately 150 copies of the 9.1-kb rDNA unit that are located on chromosome 12 as head-to-tail tandem repeats (50,51). The nontranscribed spacer of each repeat has three adjacent autonomously replicating sequences (ARSs) (51) that can support the replication of plasmids (52). However, less than one-third of the ARSs are normally used as origins of replication during each S phase (53). As in most organisms, rRNAs are coordinately synthesized, and the steady-state levels vary with growth rate and carbon, amino acid, and nitrogen sources in the medium (54–57).

Although most of the rDNA exists as an array on chromosome 12, occasional intrachromosomal recombination occurs, releasing an rDNA repeat as a 3-μm, circular episome (58,59). These ERCs have been found to accumulate in high levels in yeast cells as they age—estimated to be as many as 500 copies/cell in old yeast cells (60). This level of ERCs corresponds to between one-third to one-half of the total cellular DNA in old cells.

Because of the presence of ARS sequences within the rDNA repeat, the ERC is free to replicate once it has been excised from the chromosomal rDNA locus. ERCs accumulate in the aging yeast mother cells, indicating that segregation of ERCs is asymmetrical, with a propensity for both products of replication to remain within the mother cell. In this way, the "aging" phenotype is in most cases not passed on to the daughter cell. It has been proposed that the amplification and accumulation of rDNA sequences in old cells is responsible for the nucleolar enlargement and fragmentation that occur with aging in yeast (60). At high levels, ERCs are thought to titrate essential cellular components causing a slowing of the cell cycle and eventual cessation of replication.

That excision of an ERC can shorten yeast life span has been demonstrated using a Cre-*lox* system to introduce a single ERC into a young cell. Such artificial

introduction of an ERC shortened the life span by 40% and accelerated the onset of the age-associated phenotypes of cellular enlargement, nucleolar fragmentation, and sterility (60). It had been shown previously that daughter cells of old mother cells sometimes demonstrated a reduced life span (11). When the segregation of an artificial ERC tagged with an *ADE2* marker was examined, a strong mother cell bias in segregation was observed, which tends to break down as the mother cell ages (60). As aging mother cells accumulate high levels of ERCs, an occasional failure in asymmetrical segregation increasingly produces prematurely senescent daughter cells.

The predicted behavior of ERCs during yeast aging agrees well with that predicted for the hypothetical senescence factor proposed by Egilmez and Jazwinski (61). Those investigators initially proposed that yeast aging is best described by an initial, stochastic triggering event that leads to the exponential accumulation of a senescence factor. The majority of virgin daughter cells is produced free of ERCs due to the asymmetrical segregation of ERCs to mother cells (Fig. 3). At a stochastically determined rate, a single ERC is produced by intra-chromosomal recombination from the rDNA array. Within this cell, ERCs then begin exponentially to amplify, because ERCs have replicative potential (although given the inefficient activity of the ARS within the rDNA repeat, this may be at a rate that is substantially less than once per cell division). As ERCs accumulate to higher levels, they will tend to leak into daughter cells at increasing frequency causing the daughter cells to age prematurely. The stochastic nature of the initiating event—the generation of an ERC in a young mother cell—explains the individual cell-to-cell variation in life span in genetically identical cells.

VIII. FURTHER TESTS OF THE ROLE OF ERCS IN YEAST AGING

An rDNA circle model of yeast aging makes simple predictions of how mutations that influence the rate of rDNA circle production should affect the life span: mutations that increase the rate of ERC production should decrease the life span, and those that decrease the rate of ERC production should increase the life span. This prediction has been tested by analyzing the ERC formation and life span in *fob1* mutants (in which recombination within the rDNA locus is greatly reduced).

Each repeat of the rDNA contains sequences that encode the 35S rRNA and 5S rRNA. In addition, the spacer between adjacent 35S coding sequences encodes an initiator and enhancer for transcription of 35S by RNA pol I and *ARS* sequences for the initiation of DNA replication. Replication in the rDNA is halted in one direction at a replication fork block (RFB), preventing replication from proceeding into a 35S rDNA transcription unit (62,63). The site of the RFB is just 3′ to the termination of the 35S rRNA and overlaps the pol I enhancer (64). The *FOB1*

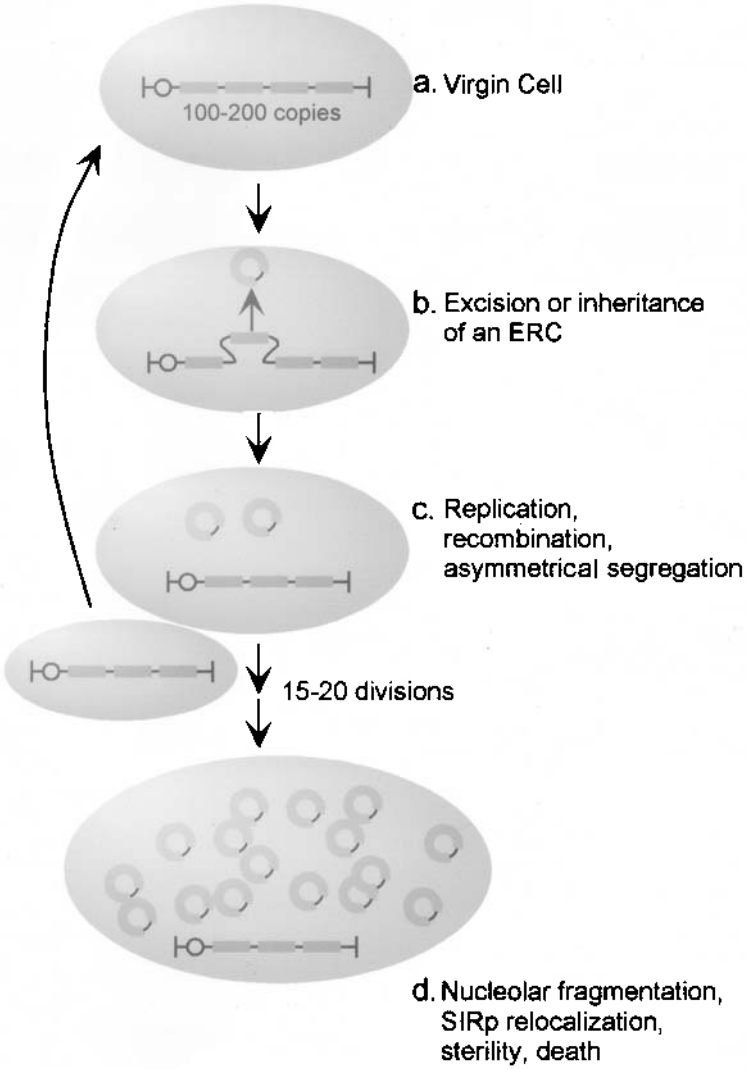


Figure 3 rDNA circle model of yeast aging. (a) Young mother cells initially produced contain no ERCs. (b) At some frequency, the initiating event of the aging process is the generation of an ERC by intrachromosomal homologous recombination between rDNA units on chromosome 12. (c) Most daughter cells do not inherit an ERC, and the aging phenotype is therefore not transmitted. The ERCs, which contain an origin of replication within the rDNA repeat, replicate during S phase and are maintained within the mother cell through asymmetrical segregation. (d) ERCs accumulate exponentially in mother cells resulting in nucleolar enlargement, nucleolar fragmentation, and cessation of cell division. In very old mother cells with high levels of ERCs, the asymmetrical segregation of ERCs may break down. This results in the accelerated senescence of some daughter cells of old mother cells that has been observed. (Adapted from Ref. 102.)

gene is required for function of the RFB; in a *fob1* mutant rDNA replication is bidirectional (65). The rDNA also contains an activity termed *HOT1*, which is contained within an rDNA spacer fragment that increases recombination when placed near markers outside the rDNA (66). *FOB1* is also required for this activity, as *fob1* mutants show reduced recombination within the rDNA as assayed by rate of marker gene loss (65).

A *fob1* deletion nearly doubles the life span, and ERC accumulation is found to be greatly reduced (67). The artificial induction of an ERC in a *fob1* mutant cell reduces the median life span to the same level as the introduction of an ERC in a *FOB1* wild-type cell, supporting the hypothesis that the extended life span of *fob1* mutants is due to decreased ERC accumulation and not a relative resistance to high levels of ERCs.

IX. CALORIC RESTRICTION AND ERC FORMATION

Caloric restriction has been shown to extend the life span in a wide number of organisms (68–71). Growth on low glucose has been used as a model for caloric restriction in yeast and was found to extend the life span (72,73). Yeast are normally grown in medium containing 2% glucose. Growth on 0.1% glucose results in a 55% extension of the median life span (72); growth on 0.5% glucose results in a more modest 24% extension in the life span (73). In yeast, glucose activates the cAMP-dependent protein kinase pathway. Components of this pathway include the GTP/GDP–binding proteins (Ras1 and Ras2 proteins), and a GTP–GDP exchange factor (Cdc25 protein) that interacts with the Ras proteins (74,75). Mutations in *CDC25* and several other genes within the protein kinase A (PKA)–signalling pathway that decreased PKA activity have been found to extend the life span (73). Growth on low glucose does not further extend the life span of the long-lived *cdc25* mutant, suggesting that low glucose and low PKA activity are functioning through the same pathway. In the long-lived *cdc25* mutant, rDNA recombination rates were decreased and ERC levels were decreased in the old *cdc25* mutant cells. These results support that the life span extension produced by caloric restriction/PKA pathway is mediated through rates of ERC accumulation.

Mutations in other genes in the pathways governing carbon source utilization also show similar effects on ERC accumulation and the life span. Snf1p is a protein kinase that plays a central role in the response to glucose starvation (76). Sip2p binds to Snf1p (77,78), and is believed to function as an adaptor molecule between Snf1p and some of its substrates (79). Deletion of *SIP2* causes accelerated aging in yeast, as evidenced by a decreased life span and early onset of the age associated phenotypes of sterility, nucleolar fragmentation, and Sir3p relocalization to the nucleolus (80). Markedly increased levels of ERCs have been observed in the *SIP2* deletion when the cells were sorted out to 12 generations.

X. SILENCING, RDNA CIRCLES, AND YEAST AGING

Among the initial observations that lead to the identification of the nucleolus as a site of genomic instability was the relocalization of the SIR proteins to the nucleolus during yeast aging (31,81). Deletions of *SIR3* or *SIR4* cause a modest decrease in the life span (31,81–83). This decrease in the life span in *SIR3* or *SIR4* deletions is caused by the simultaneous expression of both **a** and α mating-type information, as the deletions have no effect on the life span in diploid yeast, and the decrease in the life span caused by deletion of *SIR3* or *SIR4* requires the presence of the normally silenced mating-type locus. The *sir3hml*α or *sir4hml*α doubly mutant strains have normal life spans (82). *SIR2* deletion has a more pronounced effect on the life span, causing a decrease in the median life span of 50% (82,83). This effect was not mediated through the silenced mating-type locus, as the decrease in the life span was still present in diploid strains and was unaffected by the deletion of the silenced mating-type locus (82). *SIR2* overexpression results in a 30% extension of the median life span.

That the effects of SIR mutations on the life span were mediated through ERC accumulation is supported by the following three observations: (a) *SIR4* deletion increases the rate of recombination at the rDNA. This increase in recombination requires the presence of the silenced mating-type locus. (b) Increased levels of ERCs are seen young and old SIR2 deletion strains. (c) Mutation of *fob1*, which markedly decreases rDNA recombination and ERC formation, suppresses the life span defect of a *SIR2* deletion (82).

The analysis of the role of additional proteins involved in silencing in yeast also supports the hypothesis that increased silencing in the rDNA is associated with life span extension. Deletion in the yeast gene *ZDS1*, which interacts with Sir2, 3, and 4 proteins in a yeast two-hybrid assay (84), causes an increase in rDNA silencing and an increase in the life span (85). Deletion of *ZDS2*, which shares 34% sequence identity to *ZDS1* (86,87), decreases rDNA silencing and decreases the life span (85).

Transcriptional silencing in yeast is mediated, in part, by covalent modification of core histones by acetylation. The acetylation of core histones is reversibly catalyzed by histone acetylases and deacetylases (reviewed in ref. 87). Yeast *RPD3* and *HDA1* encode histone deacetylases, mutations that lead to histone hyperacetylation. In yeast, *rpd3* or *hda1sir3* deletions were found to extend the life span (83).

At first this might seem to conflict with preceding observations that an increase in silencing in the rDNA is associated with life span extension—acetylation of histones is associated with transcriptionally active chromatin, and a deletion of a histone deacetylase should therefore result in an increase in transcription. An increase in rDNA silencing has been seen in a *rpd3* deletion (83,88) and in a *hda1sir3* deletion (83). Why deletion of a histone deacetylase should increase

rDNA silencing is unclear, but it may be mediated through indirect effects on transcription at other sites.

XI. IS S*GS1* AN AGING GENE?

Although increased levels of ERCs were first noticed in a yeast deleted for *SGS1,* and it then was proposed that the accelerated aging phenotype seen in *sgs1* deletions was due to increased recombination at the rDNA (60), it has been difficult to demonstrate that ERCs accumulate at a greater rate in the presence of an *sgs1* deletion. The state of the rDNA has been examined in nonsorted (or sorted to seven generations) of wild-type and *sgs1* deletion cells, no obvious increase in the intensity of extrachromosomal rDNA bands has been seen (89,90).

Failure to detect increased levels of ERCs in *sgs1* mutants is in part explained by the finding that the effects of the *sgs1* deletion on the life span are complex (91). The terminal arrest morphology of cells undergoing life span determination has been examined in wild-type and *sgs1* mutant cells. In wild-type cells, 60% of cells arrest with large, unbudded morphology (91). In an *sgs1* deletion, only 20% of the cells arrest with large, unbudded morphology.

The effect of age on arrest state also has been examined. Growth arrest morphology is not influenced by age in wild-type cells: 60–70% arrest with the large, unbudded morphology regardless of whether they had arrested at less than 70% of maximal life span or greater than 70% maximal life span. This was not true when the effect of age on the growth-arrest phenotype of an *sgs1* mutant was examined: 65% of cells that had lived less than 70% of maximal life span arrested as large cells with buds, whereas of those cells that had survived to greater than 70% of the maximal life span, only 15% arrested as large cells with buds. Old *sgs1* mutant cells tend to arrest as large unbudded cells; that is, they arrest with the same terminal growth-arrest phenotype of as wild-type cells.

Distribution of terminal phenotypes in the *sgs1* mutant is most consistent with there being two components. The first component is a G2 arrest (cells with buds) that is stochastic and age independent, whereas the second component is a G1 arrest (unbudded cells) that is age dependent and likely related to wild-type yeast aging (91). The age-associated death rate in young cells is low, so the fixed, age-independent component predominates, and most cells arrest with budded morphology. As cells age, the age-associated component increases and eventually predominates, so cells increasingly arrest with unbudded morphology.

That old *sgs1* mutants age similarly to wild-type cells is supported by the analysis of the effect of combining mutations that increase the life span with the *sgs1* deletion. *SIR2* overexpression (82) or *fob1* deletion (67) extend the life span in wild-type *SGS1* strains. When the life span of *sgs1fob1* strains or *sgs1* dele-

tion–SIR2 overexpression strains was determined, a prolongation of the maximum life span was observed with little affect on survival within the early life span (91). This is consistent with these mutations remaining capable of modulating the age-associated death rate and not affecting age-independent death rate in *sgs1* mutant strains.

Does *sgs1* mutation accelerate yeast aging? Criteria that can be analyzed on an individual cell basis during the manual determination of the life span microscopically—sterility, cell enlargement, nucleolar fragmentation—are accelerated by *sgs1* deletion (49). These analyses can be performed on *sgs1* mutant cells nearing the end of their life span at a time when the age-dependent mortality rate predominates (91). Quantitation of ERC levels can only be performed on bulk populations of yeast cells, not on individual cells, and is biased towards cells earlier in their life spans (discussed in detail below). At earlier times, the age-independent (non-ERC) mortality rate predominates.

XII. "QUANTITATIVE" ERC DETERMINATION

If the increased rates of rDNA recombination observed in *sgs1* mutants cause an increase in the rate of ERC formation, what is the expected change in the level of ERCs compared with an isogenic, wild-type *SGS1* strain? This question is not simple to answer given the complex effects of the *sgs1* mutation discussed above. Using a computer model to describe the accumulation of ERCs in response to changing rates of production, it is possible crudely to estimate what the expected change in ERC level in an *sgs1* mutation.

In addition, the experimental quantitation of ERC levels is an intricate task. ERCs can be seen in Southern blots of total chromosomal DNA prepared from logarithmically growing yeast cultures (80,83,90), but extrachromosomal species represent only a small fraction of the total ribosomal DNA. This is believed to be due to the preferential accumulation of ERCs in old mother cells—old mother cells are rare in a growing culture. The fraction of cells that have survived long enough to accumulate significant numbers of ERCs is small—hence the levels of ERCs in a logarithmically growing culture are low.

It is also possible to analyze ERC accumulation as a function of age by studying age-sorted populations of yeast cells (10,60,67,80). Producing a sorted population of old cells is a four-step process, each step of which must be performed with great care (Fig. 4). This procedure takes advantage of the fact that the cell wall of the bud is synthesized de novo. The biotin that is attached to the surface of the mother cell will stay with the mother cell throughout its life span and is not transferred to any daughter cells. By sorting cells in this manner, it is possible to examine phenotypes—such as level of ERCs or gene expression—in populations of yeast cells of a crudely defined age.

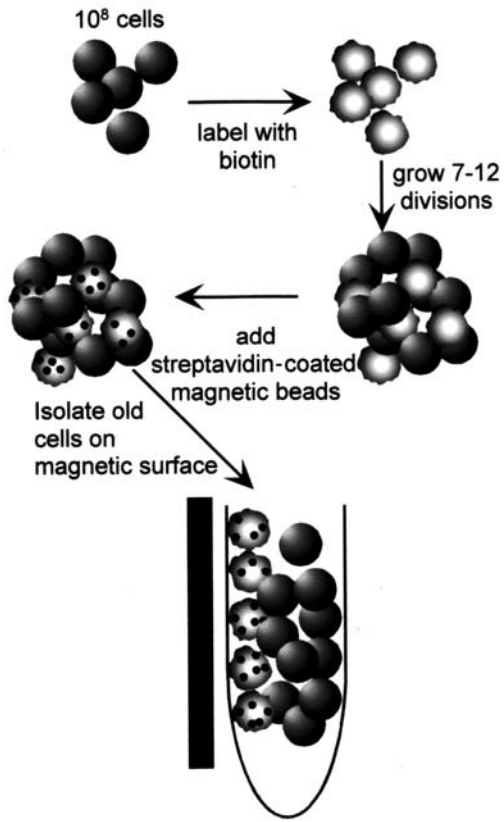


Figure 4 Purification of old yeast cells. Logarithmically growing yeast cells are chemically labeled on their surface with biotin and allowed to grow 7–12 divisions. Cells are harvested by first incubating them with paramagnetic beads that have streptavidin covalently attached, and then by separating the beads with the "old" yeast cells attached using a magnet. The vast excess of "young" yeast cells are discarded. (Adapted from Ref. 101.)

ERC levels as a function of age are determined by performing a Southern blot, probed with an rDNA probe, on total genomic DNA prepared from the age-sorted cells. Although detecting twofold differences is within the level of quantitation of single bands on Southern blots, ERCs exist as multiple species of supercoiled episomes containing one or more copies of the 9.1-kb rDNA repeat (60). To quantitate ERC levels on a Southern blot, multiple bands need to be analyzed, decreasing the accuracy of the analysis. In addition, quantitation of ERCs is more sensitive than a standard genomic Southern blot to nicking or shearing of the DNA during preparation, as these will cause the supercoiled forms of ERCs to decrease and additional bands representing relaxed or linear forms to appear.

XIII. COMPUTER MODEL OF RDNA CIRCLE ACCUMULATION

Monte Carlo computational methods can be used to estimate the magnitude of changes in experimentally measured ERC levels that would be produced by modifications in processes that influence ERC generation in cells. Computationally, the behavior of ERCs within a population of yeast cells can be modeled using a minimal set of the following six parameters:

- P^{cir}: Probability that an ERC will be formed from the rDNA array in any generation.
- E^{max}: Maximum number of ERCs that a cell will tolerate. When ERCs accumulate in a cell beyond this level, the cell stops dividing.
- P^{rep}: Probability that an ERC will replicate.
- P^{seg}: Probability that an ERC will segregate to a daughter cell.
- P^{aim}: Probability that a yeast cell dies due to age-independent factors.
- S^{post}: Number of generations a postmitotic yeast cell remains intact and can be recovered for analysis.

All time-dependent parameters are expressed per generation. Only the first five parameters affect the life span in an ERC model of yeast aging. Old yeast cells that have stopped dividing do not live indefinitely; they eventually lyse and are lost to analysis. Although it does not affect the life span as defined as number of cell divisions, the post-mitotic survival of cells does affect the number of ERCs that are recovered from age-sorted populations.

A minimal ERC model of yeast aging can be described:

1. Daughter cells may be produced with ERCs. The probability that any ERC in a dividing mother cell will segregate to the newly produced daughter cells is P^{rep}, and is assumed to be independent of segregation of any other ERCs in the mother cell, and is assumed to be unaffected by the age of the mother or total number of ERCs. The asymmetrical segregation of ERCs to daughter cells is not 100% (60).
2. An ERC is generated by recombination from the rDNA array with fixed probability P^{cir}.
3. ERCs accumulate exponentially in the mother cell at a rate determined by P^{rep}. The ARS present in the rDNA may not be sufficient to replicate an ERC once per cell division (92).
4. When ERCs reach some fixed level within the cell, cell replication ceases (60) at the ERC level given by E^{max}.

Yeast cells can die owing to non–ERC related and non–age dependent factors at a fixed rate given by P^{aim}. This parameter is necessary as the age-independent mortality rate is likely increased in *sgs1* mutants (91).

Many assumptions are necessary to create even a simple model of the behavior of ERCs in yeast aging. Factors such as the recombination of ERCs back into the chromosomal rDNA array, ERC–ERC recombination producing multimers with greater number of ARSs, and the possible effects of growth or other conditions on generating ERCs are ignored. The purpose of this analysis is not to try and fit the data to obtain accurate estimates of what the values of parameters may be but rather to estimate what magnitude of change in parameters may produce what magnitude of change in the life span and levels of ERC accumulation and in what direction those changes will be.

Table 1 and Figure 5 give the output of the Monte Carlo simulation of yeast aging. For a given set of values for the input parameters, the output of the model is the predicted life span of the yeast strain, the predicted steady-state level of circles in a logarithmically growing population, and the predicted level of circles in age-sorted populations.

The most obvious result of the computer analysis of ERC accumulation is that the increase in the expected number of ERCs—either in logarithmically growing or age-sorted populations—is not great. A three-fold increase in the rate of circle formation is predicted to cause only a three-fold increase in the expected number of ERCs in an unsorted or sorted to seven-generation population (compare Table 1, row b to row a; Fig. 5b to 5a). Initially, one might believe that if ERCs are being produced and are accumulating at a faster rate, then the difference in ERC levels should continue to increase with successive generations. Although the absolute difference in the ERC level will increase with successive generations, the relative levels (the measure that can be experimentally determined) remain constant or decrease with successive generations.

Some factors that clearly affect the life span, even within an ERC model of yeast aging, will not be reflected in ERC levels that are measured. How ERCs might cause mitotic arrest is not known; it is believed that when they reach a sufficient level they may titrate essential cellular factors (60,93). It is likely that the sensitivity of cells to ERC accumulation (E^{max}) may vary in different strains. Given the exponential kinetics of ERC accumulation, changes in E^{max} have a minor effect on life span but no measurable effect on ERC accumulation (see Table 1, row c; Fig. 5c).

With several parameters, changes that cause an increase in the life span cause a corresponding decrease in predicted ERC levels, and changes that cause a decrease in the life span cause an increase in predicted ERC levels. This is not true for all parameters. Like *SGS1* (91), other single-gene mutations are likely to cause alterations in more than one parameter of the model. As discussed above, increasing the ability of cells to tolerate ERCs (E^{max}) can extend the life span and leave unchanged or minimally increase the measured levels of ERCs in cells (see Fig. 5c). Increasing the rate that ERCs segregate to daughter cells (P^{seg}) can greatly extend the life span and also increase the ERC level in an unsorted population (see

Table 1 Predicted Life Span and ERC Accumulation

	Input parameters					Life span		ERCs/Cell						
									Sorted to 7 generations			Sorted to 15 generations		
									Postmitotic Survival					
	P^{cir}	E^{max}	P^{rep}	P^{seg}	P^{aim}	Median	Max	Log phase	0	4	∞	0	4	∞
a. "WT"	0.2	500	0.4	0.05	0.0	23	40	0.48	8.6	8.6	8.6	80.9	87.2	87.6
b. $3 \times P^{cir}$	0.6	–	–	–	–	18	24	1.4	25.5	25.6	25.6	214.5	245.5	247.3
c. $3 \times E^{max}$	–	1500	–	–	–	27	43	0.5	8.6	8.6	8.6	88.4	89.8	89.9
d. $2 \times P^{rep}$	–	–	0.8	–	–	13	28	1.3	46.2	57.6	58.3	144.7	444.7	497.6
e. $4 \times P^{seg}$	–	–	–	0.2	–	51	86	0.52	3.4	3.4	3.4	11.2	11.2	11.2
f. $P^{aim} = 0.8$	–	–	–	–	0.08	8	28	0.35	7.4	6.2	4.7	73.3	66.7	27.2
SGS1	0.6	–	–	–	0.08	8	21	1.1	23.4	19.8	14.8	172.5	148.2	64.9

Parameters input to the Monte Carlo computer model of ERC accumulation during yeast aging are shown. "WT" parameters were calculated using the predicted level of circles in old clels (Ref. 60) for E^{max}, assuming a low probability of segregation of ERCs to daughter cells and adjusting P^{cir} (probability of ERC formation) and P^{rep} (replication efficiency of ERCs) to fit a median life span of 23 and a maximum life span of 40. A (–) for an input parameters signifies that this parameter was the same as that used in "WT." *SGS1* value for P^{aim} is that estimated in McVey et al. (91); a three-fold increase in P^{cir} was estimated from the aproximate three-fold increase in the rate of marker loss in the rDNA in *sgs1* mutants (Ref. 89).

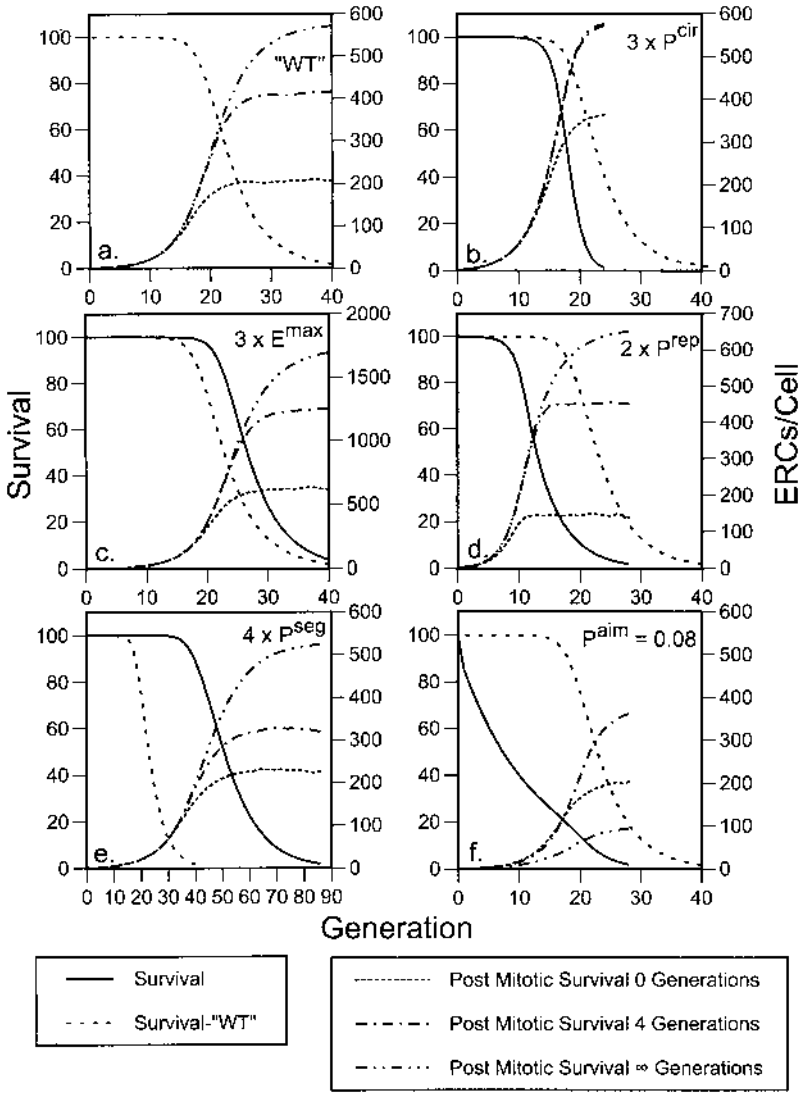


Figure 5 Monte Carlo model of ERC accumulation during yeast aging. The graph shows the predicted survival curve, as would be calculated by a yeast life span analysis, and the predicted number of ERCs/cell, as a function of generation. This corresponds to the level of ERCs that would be detected on a Southern blot of yeast cells sorted to the indicated generation. Values of the parameters of the model used are given in Table 1. ERCs/cell are given for postmitotic survivals of 0, 4 and infinite generations, as the levels of ERCs that are measured are dependent on this parameter. The dashed "wild-type" survival curve is reproduced in all panels for comparison. (a) Wild-type yeast life span; (b–f) parameters were kept the same as those used for wild-type life span except a single parameter that was altered as indicated in Table 1.

Table 1, row e; Fig. 5e). The level of ERCs in an unsorted population of yeast is most reflective of the levels in young cells, and decreasing the asymmetry of ERC segregation provides a means of transferring ERCs into young cells. Increasing the age-independent mortality rate (discussed regarding *sgs1* mutations above) will cause a decreased life span and modestly decreased levels of ERCs (see Table 1, row f; Fig. 5f). The simple interpretation of an ERC model of yeast aging that higher levels of ERCs observed in an unsorted population imply a shorter life span and lower levels imply a shorter life span is not correct.

This issue may be pertinent to the case of petite yeast (83). Petite yeast, which are deficient for aerobic respiration due to deletions within the mitochondrial genomes, are long lived in some strain backgrounds (94). It has also been reported that petite yeast also have increased levels of ERCs in non–age sorted populations (95). Whether there is an increase in ERCs specific petite strains in which the life span has been analyzed has not been reported. It will be informative to analyze those factors in petite yeast that do not show an inverse correlation between the life span and the ERC level. Segregation of marked episomes can be analyzed (96), changes in the terminal arrest phenotype can be quantitated as a function of age as a potential measure of changes in age-independent mortality rates (91), and E^{max} can be crudely tested through the experimental introduction of an ERC using the Cre-*lox* system (60).

No increase in ERC levels in *sgs1* mutants has been found (89,90). The predicted increase in ERC levels in the *sgs1* mutant compared with isogenic *SGS1* wild type, taking into account changes in the rates of ERC accumulation and the increase in age-independent mortality (91) can be calculated (see Table 1). A 2.3-fold increase in ERCs is predicted in an unsorted population and between 1.7- and 2.7-fold increase in levels is predicted in comparing seven-generation sorted populations. Published reports at the time of his writing have not demonstrated sufficient sensitivity—given the complexity in quantitation of ERC levels—to rule out differences this small.

XIV. FUTURE DIRECTIONS

Many genes have been shown to affect the life span in yeast. *LAG1* encodes a 47-kD protein that was identified in the earliest genetic analysis of aging in yeast using a differential hybridization screen to identify genes whose expression changes over the course of the yeast life span (14). Moderate levels of *LAG1* overexpression, controlled by the glucocorticoid receptor response element, increased the life span of the parent strain by 60% (97). Two members of the *RAS* signal transduction protein family exist in yeast. Overexpression of *RAS2* (98,99) or deletion of *RAS1* (99) increases the yeast life span. Mutations in nuclear genes required for mitochondrial function increase the life span by compromising mitochondrial

function. This requires the retrograde response, a signaling pathway coordinating gene expression between the nucleus and mitochondria (72,94). How, if at all, these genes alter rDNA processing or ERC accumulation has not yet been investigated.

The precise quantitation of extrachromosomal rDNA levels is difficult. Predicting the expected change in the ERC level sufficient explain a change in the life span is not possible without analyzing how a genetic alteration affects all the parameters responsible for determining ERC levels in populations. It will be productive to test further predictions of an ERC model of yeast aging. The prediction that genetic manipulations that decrease the rate of formation of ERCs extend the yeast life span has been tested and confirmed (67,82). The ERC model of yeast aging also suggests that genetic manipulations that decrease the fidelity of segregation of episomes to mother cells should extend replicative life span; this prediction has not yet been tested.

XV. CONCLUSION

Several lines of evidence implicate a causative role for the accumulation of ERCs in aging in yeast. Evidence in support of this role includes (a) the fact that high levels of ERCs are found in old yeast cells, (b) artificially generating an ERC markedly decreases the life span, and (c) the correlation between the life span and genetic mutations that are predicted to alter the formation of ERCs (*FOB1*, *SIR2*).

In yeast strains that were proposed to be short lived owing to accelerated ERC accumulation, it has been difficult to demonstrate conclusively that ERCs accumulate at greater rates. This failure does not strongly argue against the importance of ERCs in normal yeast aging, as the relationship between the experimentally measured level of ERCs in a population and ERC-induced cellular senescence is complex. In a simple model of ERC-induced senescence, some alterations (e.g., increased rate of ERC formation) that are predicted to cause an increase in the measured level of ERCs cause a *decrease* in the life span, whereas other alterations (e.g., decrease in asymmetry of ERC segregation) that are predicted under some conditions to cause an increase in the measured level of ERCs cause an *increase* in the life span. Single-gene mutations can have multiple effects within cells. Predicting the effects of such mutations on measured levels of ERCs and the life span has proved more complex than initially thought.

Mutants in *fob1* that do not accumulate ERCs, although long lived, still die with normal-appearing life span curves (67). Yeast passaged through stationary phase have a shortened life span without any detectable increase in ERCs (100). Thirty percent of wild-type yeast cells arrest in G2/M with a budded morphology, a terminal arrest morphology not associated with aging (91). It is probable that during normal aging, yeast mother cells die from more than a single cause. Other

factors, in addition to ERCs, likely contribute to the age-associated increase in mortality in yeast mother cells.

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Genetics of Aging in the Nematode *Caenorhabditis elegans*

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I. *CAENORHABDITIS ELEGANS* AS A MODEL SYSTEM FOR AGING RESEARCH

Caenorhabditis elegans is a small nematode worm containing somewhat less than 1000 postmitotic, somatic cells at adulthood. Its invariant, mosaic pattern of development and self-fertilizing hermaphroditic life style have made it favorite of developmental biologists second only to *Drosophila.* Details on numerous aspects of its development and other aspects of current studies have been compiled in two books (1,2), and detailed methodologies can be found in the journal *Methods in Cell Biology*, Vol. 48 (3). Several online sources of information are available, including an informal newsletter, published by the *C. elegans* Stock Center (http://elegans.swmed.edu/), which also includes access to all nematode publications (4654 at the time of this writing, including 146 cross referenced to aging). Numerous bioinformatics resources are available and can be accessed through the same URL or at http://www.wormbase.org/ and other sites.

Biologists identified *C. elegans* as a good model for studying aging in the 1970s (reviewed in refs. 4–6). However, studies on aging were galvanized with the recognition that a lack of inbreeding depression and significant heritabilities makes it possible to identify genetic variants affecting the processes of

aging (7). Soon the first single-gene mutation (*age-1*) leading to longer-than-normal life span was identified (8) and subsequently mapped and shown to behave as a single gene (9). Mutations in *age-1* are recessive to the wild type and dramatically lengthen life expectancy by an average of about 40% and maximum life span by about 70% (10). These mutants result in a decreased mortality almost 10-fold lower by 13 days of age (11), but have no effect on fertility or development (9).

Soon it was recognized that numerous other genes (notably *spe-26* [12], *daf-2* [13], *clk-1* [14], and *old-1* [formerly *tkr-1,* (15)]) could also be found to lengthen the life span and slow the rate of mortality (15a). Currently, more than 50 distinct genes have been identified that are directly associated with the aging process (in that altering these genes results in increased longevity [see http://ibgwww.colorado.edu/tj-lab/ for an up-to-date list of such mutants]). A number of mutants with shorter life spans also have proven informative.

The recognition that several of these genes interact with each other in regulating dauer development (reviewed in ref. 16) and their subsequent cloning has resulted in numerous publications in major journals, making this area of research one of the "hottest" in current molecular genetics. The subsequent completion of the DNA sequence of the *C. elegans* genome (17) and the discovery that genes can be selectively silenced using RNA interference (18–19) have provided tools that are being increasingly applied to the analysis of the aging process in *C. elegans.*

There is a wide recognition that the genetic approach to aging offers an abundance of analytical tools hitherto unavailable to the researcher in aging, and this chapter will focus on recent studies in which such tools as long- and short-lived genetic variants are analyzed. Numerous other reviews on aging in *C. elegans* have been published during the last few years (15,20–26). Here we will focus on one aspect of aging not covered elsewhere; that is, the role of genomic maintenance in worm aging, although other areas will also be reviewed.

II. LONG LIFE IS ASSOCIATED WITH INCREASED RESISTANCE TO STRESS

A number of early studies on the long-lived (referred to as *age*) mutants focused on stress resistance. Long-lived mutants show increased resistance to oxidative stress, heat, and ultraviolet (UV) light (summarized in refs. 20 and 27). A summary of data showing correlations between mean longevity in a variety of mutant strains and subsequent life spans is shown in Figure 1. This information can be supplemented with studies from the Johnson laboratory that are available online (see http://ibgwww.colorado.edu/tj-lab/) and will be continually updated. Among the various classes of mutants that have been studied for increased stress

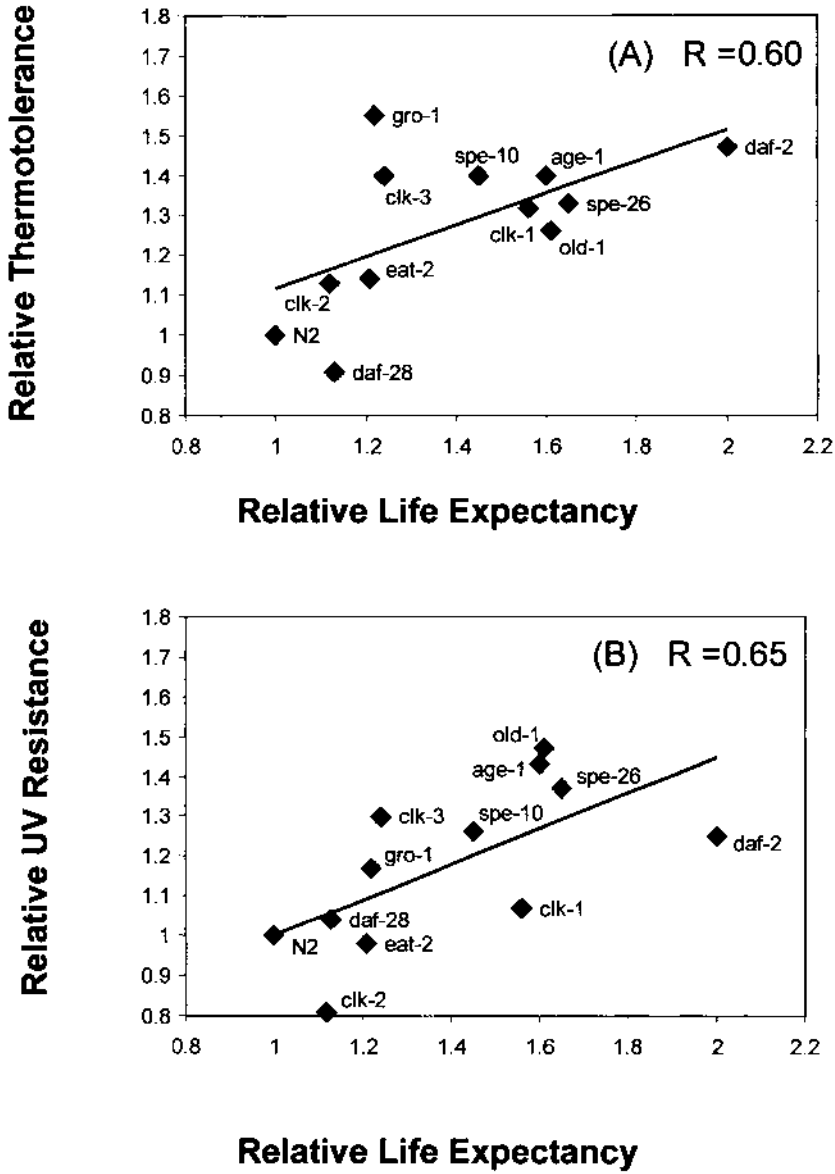

Figure 1 Correlation between life expectancy and stress resistance. (A) Correlation between relative thermotolerance and life expectancy. (B) Correlation between relative UV resistance and life expectancy. All data are normalized to control for environmental variation by dividing by the corresponding wild-type (N2) life expectancy. Mean life span on agar (life expectancy at birth) are all derived from published literature (except for *eat-2[ad456]* and *clk-1*[e2519]) as follows: *age-1(hx546)* in (9); *spe-26(hc138)* in (12); *daf-2(e1370)* (13); *clk-1(e2519), clk-2(qm37), clk-3(qm38),* and *gro-1(e2400)* in (14) with supplemental survival from our laboratory for *clk-1*); *daf-28(sa191*); *old-1(Is104)* in (29); and *spe-10(hc104)* in (28).

resistance, all have shown increases. Those in the dauer pathway (*age-1, daf-2*) have been well described, as have the clock class (e.g., *clk-1*), and mutants affecting fertility (*spe-26*). Among the most complete studies on stress resistance is that of *spe-10* (28), which was resistant to heat and UV light but not to the oxidative stressor paraquat. The overexpression mutant *old-1* (29) was originally identified by its increased resistance to stress (15), and also shows increased longevity. Resistance to stress also has been effectively used in screens for new mutations that increase the life span (29–32) (E. deCastro and T.E.J., unpublished data). In all of these studies, no evidence for increased resistance to ionizing radiation has been observed, which is consistent with the notion that DNA repair and chromosomal stability are not important for an organism that lives for only 3 weeks under laboratory conditions and probably less than a day, on the average, in the wild.

The stability of the mitochondrial genome has been studied in some detail in a variety of different species, and the reader is referred to Chapter 23 on this topic. In particular, however, mitochondrial DNA rearrangements have been found to occur with increasing chronological age in *C. elegans* (33). In studies in which entire worms or parts of individual worms were analyzed for mitochondrial DNA deletions, numerous rearrangements were found. These rearrangements deleted from 3231 to 3899 bp and were found to occur at 4- to 8-bp direct repeats in the mitochondrial genome, which is consistent with a mechanism involving slipped mispairing or imprecise recombination. Notably, the frequency of these events seems to be dramatically reduced in the *age-1* mutants (34), but precise quantitation of the frequency at which mitochondrial deletions occur has not been achieved.

III. SHORT LIFE IS ASSOCIATED WITH INCREASED SENSITIVITY TO STRESS

It has been argued that more interesting insights into aging are revealed by mutations that confer longer life spans, because mutations that shorten the life span can do so in many ways that are indirect and presumably uninteresting (7, 35, 36). However, by careful analysis and judicious choices, short-lived mutants in *C. elegans* have been shown to affect biological processes known to be important in aging.

Most notable among these are mutations in two genes, *gas-1* and *mev-1*, that encode subunits of complex I and complex II of the mitochondrial respiratory chain. The mutants were isolated based upon their abilities to confer hypersensitivity to two seemingly unrelated agents. Specifically, *mev-1*(*kn-1*) was isolated by virtue of its hypersensitivity to oxidative damage, presented in the form of either methyl viologen or hyperoxia (37). Conversely, the *gas-1*(*fc21*) mutant was

isolated on the basis of its hypersensitivity to a number of volatile anesthetics (38). As indicated above, both genes have been cloned and the specific mutations identified (39,40). The mutations confer a considerable overlap in phenotypes (P.S.H., N.I., and P.G. Morgan, unpublished data). Most notably, hypersensitivity to free radicals was increased in both as measured during development. In addition, whereas hyperoxia had minimal effects on the life span of wild-type animals, the same treatment dramatically shortened the life spans of both *gas-1* and *mev-1* mutants. However, the *gas-1* and *mev-1* mutations differentially affected mutability and anesthetic sensitivity. Specifically, *gas-1* but not *mev-1* animals were hypersensitive to volatile anesthetics. In addition, whereas the *gas-1* mutation had little, if any, effect on spontaneous mutation frequency, *mev-1* animals had significantly elevated mutation frequencies at all oxygen concentrations tested, including at atmospheric.

The most salient question is that of how these specific molecular defects in complexes I and II mediate precocious aging. At least in the case of *mev-1*, preliminary data suggest several answers to this question (N.I. and P.S.H., unpublished data). First, superoxide anion levels are elevated in *mev-1* animals, which undoubtedly leads to a wide variety of cellular damage. Second, the concentration of the free radical scavenger glutathione is reduced in *mev*-1 animals. These two pathologies render *mev*-1 animals more susceptible to oxidative stress. Third, *mev-1* mutants suffer a number of metabolic imbalances, which in turn may lead to a reduced ability to deal with oxidative stress. For example, the ability to repair UV radiation–induced DNA damage in the nucleus is compromised in *mev-1* animals, which also may explain its oxygen hypermutability. The importance of reactive oxygen species in contributing to the premature aging of *mev-1* is indicated by the ability of superoxide dismutase/catalase mimetics to restore the life span of mutant animals to that of wild type (41).

IV. MAINTENANCE OF THE GERM LINE

Research into mechanisms of metazoan aging typically focuses on events that occur in either somatic cells or the entire organism. However, important lessons have been gained through examination of the *C. elegans* germ line. Inappropriate "aging" of the germ line can render it mortal such that its afflicted genome cannot be perpetuated from generation to generation. Telomeric maintenance, DNA repair capacity, cell cycle checkpoints, and apoptosis are each extensively studied biological processes that have been implicated in the prevention of aging of the *C. elegans* germ line.

Ahmed and Hodgkin (42) have isolated mutants with mortal germ lines. The screen was to score for strains that can reproduce for several generations but eventually became sterile. For the purposes of long-term maintenance, the offending

mutations must be maintained in the heterozygous condition. One of these, *mrt-2,* results in a progressive telomeric shortening from generation to generation. Homozygotes of *mrt-2* also accumulate chromosomal fusions in their last several generations. The phenotypes of telomeric shortening and late-onset chromosomal fusion have been observed in certain mutants of mouse and yeast, indicating that telomeric loss can age the germ line (43–45). Interestingly, *mrt-2* encodes a homolog of the *Schizosaccharomyces pombe rad1* gene, a cell cycle checkpoint gene. The gene functions to delay cell cycle progression in response to either DNA damage or a block in replication. The delays afforded by this and other cell cycle checkpoints are thought to be an important complement to DNA-repair mechanisms, because they provide additional time to ameliorate damage that might otherwise be fixed into lethal events or mutations.

Related research indicates that ionizing radiation transiently blocks 0cell proliferation and induces programmed cell death (i.e., apoptosis) in the germ line (46). In some organisms, apoptosis serves to eliminate cells that have been extensively damaged. The genetic control of apoptosis in the somatic cells of *C. elegans* has been extensively studied (47,48) and is meditated by the same core apoptotic machinery (e.g., *ced-9, ced-4, ced-3*) as employed in the germ line. The dual responses of cell cycle arrest and apoptosis upon irradiation suggest that an active checkpoint is operative in the *C. elegans* germ line. Indeed, these phenotypes are reversed by mutations in three genes, *mrt*-2, *rad*-5, and *him*-7; that is, irradiation of these mutants does not inhibit germ line proliferation and induces apoptosis as it does in wild type. Interestingly, these mutants were isolated using three different types of mutant hunts; namely, for a mortal germ line (*mrt-2* [42]), for somatic cell radiation hypersensitivity (*rad-5* [49]), and for elevated meiotic nondisjunction (*him*-7 [50]). In addition to these historical differences, *rad-5* and *him-7* mutants do not display telomeric erosion and germ line mortality. This indicates that checkpoint-mediated apoptosis and telomeric maintenance require overlapping but nonidentical machinery, although it is also possible that this genetic heterogeneity reflects allele-specific differences.

A spindle assembly checkpoint also has been identified in *C. elegans* that is responsible for ensuring that the mitotic apparatus is intact before anaphase proceeds (51). In the absence of the *mdf-1* and *mdf-2* gene products, which bear sequence homology to *mad-1* and *mad-2* of *Saccharomyces*, errors accumulate in chromosomal segregation and ultimately lead to inviability. Finally, Chin and Villeneuve (52) have recently isolated and characterized a *mre-11* mutant of *C. elegans* whose yeast counterpart encodes a multifunctional protein important for mitotic recombination, DNA repair, and maintenance of telomeric length. The germ line of *mre-11* mutants of *C. elegans* is hypersensitive to ionizing radiation, suggesting a DNA repair deficiency. Genetic recombination also is abolished in these mutants. As with the *mrt* mutants, *mre-11* is essential to germ

line maintenance, as homozygotes cannot be propagated for more than a few generations.

In summary, an examination of the genetic factors responsible for maintenance of the germ line has revealed the essential nature of a number of biological processes thought to be important in somatic cell and organismal aging. These include apoptosis, DNA repair, cell cycle checkpoints, and telomeric maintenance.

V. POLYGENIC CONTRIBUTIONS TO AGING

Although most efforts have focused on investigating single-gene mutations that influence life span, the polygenic nature of aging also has received some attention. This approach usually begins by crossing various wild-type strains to generate recombinant inbred (RI) strains. Since C. *elegans* normally reproduces as a self-fertilizing hermaphrodite, virtually complete homozygosity is achieved after only a few generations. Owing to independent assortment and genetic recombination, and in the absence of any selective pressure, each RI contains a more or less random mixture of the two parental genomes. Johnson and Wood (7) first employed this approach in *C. elegans* to generate a series of RIs that had mean life spans ranging from 10 to 31 days. This was as compared with the 18-day mean life spans of the two parental strains. It has been possible to employ RIs to map quantitative trait loci (QTLs) that affect a variety of life history traits. To do so, polymerase chain reaction (PCR) amplification is employed to detect polymorphisms that exist between the two parental wild-type strains. The polymorphisms serve as genetic markers, enabling the investigator to determine from which parent a given linked region was inherited. For example, Ebert and colleagues (53) identified five regions in which the allele from one parent was significantly enriched in long-lived strains. Shock and Johnson (54, 55) employed a similar strategy to identify QTLs for survival, early fertility, onset of sexual maturity, and population growth rate.

The analysis of RIs also has illuminated the roles played by DNA-repair capacity and oxidative stress in determining the life span in wild-type *C. elegans*. With respect to the former (56), analyses have demonstrated that there is no significant correlation between the mean life span and sensitivity to a number of DNA-damaging agents in four RIs with mean life spans ranging from 13 to 31 days. In addition, excision repair of two UV radiation–induced DNA photoproducts was experimentally identical in a short-lived and a long-lived strain. These data suggest that, at least in the wild-type strains employed to create these RIs, DNA repair plays a minor role in the aging process. Conversely, there is a demonstrated overlap between the polygenes that influence the life span and those that control resistance to reactive oxygen species (ROS). Hartman and associates (57)

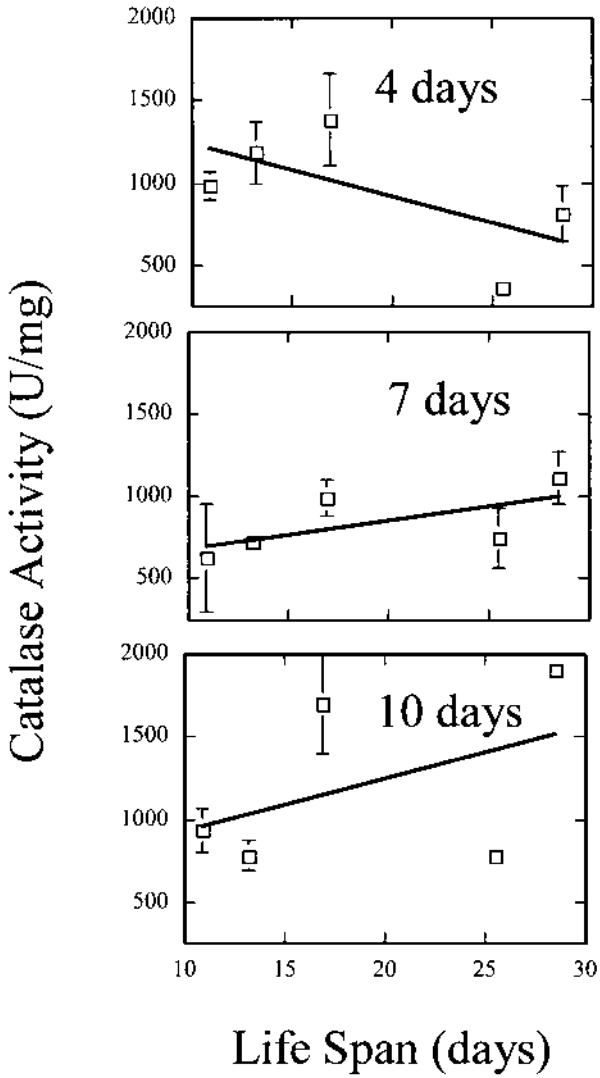

Figure 2 Relationship between catalase levels and life span in different RI strains of *C. elegans.* As reflected in the three panels, catalase levels were determined in staged populations of animals that were 4, 7, and 10 days of age.

have determined that the RI life span is inversely proportional to the growth rate inhibition imposed either by hyperoxia or methyl viologen. More recently, a positive correlation has been observed between the life spans of these same RI strains and catalase levels (P.S.H., unpublished data). Specifically, mRNA and protein levels were quantitated (using Northern blots and enzyme assays, respectively) in preparations of 10-day-old synchronous populations. The long-lived strains tended to possess higher levels of both *ctl-1* mRNA (the cytosolic catalase) and total enzyme levels (Fig. 2) than did the short-lived strains. Ebert and coworkers (58) employed a slightly different approach to address the polygenic nature of ROS resistance. They exposed a mixed RI population of animals to a toxic concentration of hydrogen peroxide and identified among the survivors a specific chromosomal region from one parent that was overrepresented, suggesting it conferred resistance to ROS.

A limitation that characterizes all such polygenic approaches, including those just described, is the inherent difficulty in identifying the specific gene or genes that endow a given QTL with its phenotype. Nonetheless, these data serve to remind us that a considerable determinant of the life span is polygenic in nature. Finally, these data also indicate a substantial overlap exists between those genes that influence the life span and those responsible for regulating expression of various defense components against oxidative stress.

VI. HORMESIS

Hormesis can be defined as an increase in functional ability resulting from a pretreatment with nontoxic levels of a toxic agent (reviewed in Ref. 59). In addition to the increased resistance to environmental stressors that is seen in long-lived mutants, as described above, there is an increase in the life span readily discernible in *C. elegans* after exposure to a variety of stressors. The most extensively studied example of these hormetic responses shows that heat treatment can increase longevity 15–25% (60–62 and J. Cypser and T.E.J., unpublished data). Other agents such as high oxygen and reactive oxidants also can induce subsequent increases in longevity (62a).

There is an intimate association between increased ability to resist subsequent challenges by the hormesis inducing agent and subsequent increases in longevity, but these are not the same response (62a). Using a variety of mutant strains, we have shown that some ability to affect longevity hormesis can be genetically separated from the effects on stress resistance alone. Studies on radiation-sensitive mutants showed that hormetic effects on longevity could be observed even in these mutant backgrounds (56), suggesting that hormesis is not dependent on DNA-repair processes altered in these mutants.

VII. FUTURE PROSPECTS

Although *C. elegans* already has proven itself as a paradigm in gerontology, its future holds even more promise. Complementing the traditional strengths of this model system, articulated in the first section of this chapter, are a number of emerging and powerful methodologies that will facilitate even more incisive exploration into the molecular and cellular mechanisms that govern aging. Several notable examples of selected methodologies and their application are given below.

First, DNA microarrays can be used to quantitate gene expression on a genomewide basis (63–66). Microarrays consist of many different DNA sequences, each mobilized to a specific location in a matrix. The intensity of mRNA hybridization to each immobilized sequence can be employed to give a measure of steady-state mRNA levels on a gene-by-gene basis. Such microarrays exist for *C. elegans* and have been used by one of us to survey systematically the effects of aging and mutations affecting aging on gene expression of the entire *C. elegans* genome (J. Lund, E. Tedesco, S.K. Kim, and T.E.J., unpublished results).

Second, database searches for sequence homology are becoming increasingly powerful, as the genomes of more and more organisms have been sequenced and annotated. Such an identification of orthologues can serve as a point of departure to explore systematically the genetic basis of essentially any biological process. For example, Boulton and colleagues (67) have identified 75 *C. elegans* proteins that are homologous to DNA-repair proteins in other organisms. The same group has cloned the majority of these. Similarly, Rikke and associates (68) have searched for homologs to *old-1*, finding no homologs of this subclass of tyrosine kinases in mammals. These clones currently are being used to determine protein–protein interactions using a third sophisticated technique—the yeast two-hybrid system (69,70).

The two-hybrid system uses the protein product of a given cloned gene as "bait" to identify and capture proteins with which it interacts. This enables cloning of genes whose protein products interact, allowing determination of the structural and functional relationships between the gene products controlling a particular biological process. In the example given above, such an approach should not only reveal interactions between DNA-repair proteins, but also illuminate mechanisms by which checkpoint responses communicate and coordinate with DNA-repair systems. The use of these sophisticated methodologies stands in stark contrast to the traditional approach of studying the genetics of DNA repair, which was to conduct laborious mutant hunts for radiation-sensitive mutants with the hope that some would prove to be defective in DNA repair (49).

A fourth emerging methodology is that of RNA interference (RNAi). This consists of exposing animals to double-stranded RNA either by microinjection, soaking, or simple ingestion. An organismal response is triggered that mimics the phenotype of a null mutation in the gene corresponding to the double-stranded

RNA. Given that the entire genome has been cloned and sequenced, RNAi lets the investigator determine the phenotype resulting from inactivation of literally every *C. elegans* gene without the laborious process of mutant isolation. In fact, RNAi already has been employed to ascertain systematically the phenotypes derived by inhibiting expression of most genes on two of the six *C. elegans* chromosomes (18,19). The potential applications of RNAi are manifold. For example, there are approximately 75 putative DNA-repair genes in *C. elegans*. Using RNAi would enable the corresponding phenotypes associated with defects in each of these genes to be systematically determined. In addition, systematic inactivation of mitochondrial proteins through RNAi should provide insight into the relationships among mitochondrial function, oxidative stress, apoptosis, and aging.

Two additional approaches have and should continue to provide insights into how individual cells contribute to organismal aging. First, it is possible to ablate specific cells with a laser microbeam. Given the invariant nature of cellular development in *C. elegans*, this technique can be employed to determine how individual cells contribute to the organismal phenotype of aging. Hsin and Kenyon (71) have demonstrated that elimination of the germ line by laser ablation significantly increased the life span of *C. elegans*. This result implicates germ line signals as modulators of the life span. Functional *daf-16* and *daf-12* genes are required for this extension, which suggests that the *C. elegans* insulin/IGF-1 system mediates this response.

Second, genetic mosaics can be created in which a single animal is composed of cells of two different genotypes. With respect to aging, Apfeld and Kenyon (72) have employed a strain that is homozygous for a *daf-2* mutation but also carries a semistable free duplication that includes the wild-type *daf-2* gene. An unrelated genetic marker was included, which enabled detection of mosaics that had lost the duplication during somatic development. Such mosaics consisted of a mixture of mutant and wild-type cells. In these experiments, *daf-2* proved to function nonautonomously; that is, the presence of some *daf-2* mutants cells influenced the life span of the entire animal. Multiple lineages also had an effect on longevity. An even more powerful methodology demonstrating nonautonomous regulation was used by Wolkow and colleagues (73) to demonstrate that expression of DAF-2 in only neurons only resulted in rescue of the normal longevity phenotype.

Collectively, these specific examples provide evidence that the application of emerging methodologies will synergize aging research in *C. elegans*.

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21

Aging, Somatic Maintenance, and Genomic Stability in *Drosophila melanogaster*

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I. INTRODUCTION

Powerful genetic and molecular techniques have allowed manipulation of the *Drosophila* life span by genetic selection of populations, single-gene mutations, and using transgenes. The data have made *Drosophila* a leading model for aging studies and implicate oxidative damage and metabolic capacity/reserves as major factors. Numerous genes involved in DNA repair and genomic stability have been identified in *Drosophila* by mutations, and many more are suggested by sequence homology and analysis of the *Drosophila* genome sequence. As a consequence, *Drosophila* is also emerging as a leading model system for the study of DNA repair, cell cycle control, and genomic stability. Relatively few studies have bridged these areas to investigate the potential role of DNA repair and genomic stability in aging and life span regulation. The wealth of research tools available suggests that the interface of these areas may be a particularly rewarding area for research in the next decade.

II. *DROSOPHILA* AS A MODEL FOR AGING STUDIES

The fruit fly, *Drosophila melanogaster,* has been a leading model for aging research for more than 80 years (1,2). The benefits of *Drosophila* include its short

life span (1–2 months), ease of culture, and the availability of powerful genetic and molecular biological tools. The latter includes the P element: a class II DNA transposable element that was engineered to carry modified or foreign genes into the *Drosophila* genome and allow germ line transformation, as well as insertional mutagenesis and other genetic manipulations (3,4). One disadvantage of *Drosophila* is that its small size precludes a detailed study of pathology and cause of death. For this reason, life span is still the most reliable measure of *Drosophila* aging rate.

The use of *Drosophila* as a model is supported by the numerous similarities between aging in *Drosophila* and mammals (2,5). These include a decline in performance of functions such as reproduction, learning, behavior, and locomotion. At the ultrastructural level, similarities include deterioration of muscle and nervous tissue and accumulation of intracellular inclusions such as pigments (lipofuscin), abnormal mitochondria, and viruslike particles. At the molecular level, similarities include the accumulation of oxidatively damaged DNA and proteins and mitochondrial genomic deletions (6,7). Finally, both *Drosophila* and mammals exhibit a tight link between stress responses and aging, as discussed below.

An exponentially increasing rate of mortality with age previously was thought to be a defining characteristic of the aging process in all organisms. However, experiments with *Drosophila* and medflies and examination of human survival data have demonstrated that when large numbers of organisms are analyzed, mortality rates level off at the most advanced ages (8,9). The mechanism of this mortality rate plateau is currently unknown, and its occurrence has significant implications for models and definitions of aging.

An important difference between *Drosophila* and mammals is the fact that *Drosophila* is cold-blooded (poikilothermic). Raising environmental temperature increases the rate of *Drosophila* metabolism and aging and decreases the life span (2). The ability to manipulate the *Drosophila* life span in this way has proven to be useful in many studies, and has provided some of the first evidence of the link between metabolic activity and life span.

"Metabolic potential" has been defined as the total lifetime metabolic activity of an organism (6,10,11). In *Drosophila*, not only will reduced temperature increase life span but so will other environmental and genetic manipulations that decrease metabolic activity (11). For this reason, it is important to determine whether any life span increases associated with mutations or transgenes are associated with altered metabolic activity. A manipulation that increases the life span without lowering metabolic activity would increase metabolic potential, and it appears certain that such an intervention is of interest. However, it remains an open question whether life span increases associated with reduced metabolic activity in poikilotherms will be informative.

III. SELECTION EXPERIMENTS AND QUANTITATIVE GENETICS

Current theory suggests that aging exists because of the decreasing force of natural selection as a function of age, and that it has an underlying genetic basis (12–15). Much of the experimental support for this theory has come from study of *Drosophila*. When a genetically heterogeneous population of *Drosophila* is cultured in the laboratory using only the oldest individuals to reproduce, the force of selection now acts on the older individuals. Over many generations, this selection results in populations with a significantly increased fertility at older ages and with a significantly increased life span relative to control populations (16–19). The long-lived strains exhibit a number of correlated phenotypes, including increased stress-resistance and increased expression of stress-response genes (20–22). The increased life span and correlated phenotypes are expected to result from the selection for increases and decreases in the frequency of particular gene alleles preexisting in the population. Genes with altered allele frequency include *phosphoglucomutase* (involved in glycogen metabolism) and *Cu/Zn superoxide dismutase* (involved in oxidative stress resistance) (23). Life span is a quantitative trait, and the chromosomal loci that affect the life span are examples of "quantitative trait loci" (QTLs). QTLs affecting the life span have been identified and genetically mapped using appropriate chromosomal markers and crosses between strains of different life span (24,25).

IV. DIFFERENTIAL GENE EXPRESSION DURING AGING

Aging in *Drosophila* is associated with characteristic changes in gene expression. During aging, the expression of a subset of stress-response genes, particularly *hsp70* and *hsp22*, is upregulated at the transcriptional and posttranscriptional levels, in specific temporal and spatial patterns (20,26–28). Induction appears to be in part a response to oxidative stress. The response of heat shock genes to heat stress is also altered during aging (29,30). Recent data suggest that a similar pattern of stress-response gene expression occurs in aging mammals: a tissue-specific upregulation of a subset of hsp genes, and decreased induction in response to acute stresses (31–33). Several other *Drosophila* genes exhibit characteristic dynamic expression patterns in the antenna during aging (34–37). These include genes with important functions during development; however, the significance of their altered expression during aging is currently unknown. DNA microarrays have been used to survey approximately half of the *Drosophila* genome for changes in RNA levels during aging (38). Numerous genes exhibited changes of two- to fourfold, but no readily interpretable pattern of changes has been identified.

V. TRANSGENICS

One way to identify genes that directly regulate aging is to increase or decrease their expression experimentally and assay for effects on the life span. A decreased life span is problematic, as it is likely to result from novel pathologies that do not normally limit the life span. In contrast, an increased life span should result from alterations in limiting processes, and is therefore more likely to identify genes directly related to aging. A strength of the *Drosophila* model system is that there are a variety of transgenic methods for upregulating or downregulating the expression of specific genes under well-controlled conditions (39).

Extensive correlative evidence suggests that for most organisms, oxidative damage may be a primary cause of aging and functional decline (40–42). Reactive oxygen species (ROS) are generated as a by-product of normal metabolism, and oxidatively damaged molecules and organelles have been found to accumulate in all aging organisms examined, including *Drosophila*. Not surprisingly, the transgenes tested for effects on the life span in *Drosophila* have been ones linked to oxidative stress resistance or response. The *hsp70* gene was originally identified as one induced in response to heat and oxidative stress (43). The *hsp70* family proteins can prevent protein aggregation, facilitate protein refolding, and facilitate entry of damaged proteins into proteolytic pathways (44). The enzymes superoxide dismutase (SOD) and catalase are primary defenses against ROS in all cells. SOD exists in two forms: a primarily cytoplasmic form (Cu/ZnSOD) and a mitochondrial form (MnSOD). SOD converts superoxide to H_2O_2, and catalase converts H_2O_2 to H_20 and O_2. Another important defense against ROS involves the enzyme glutathione reductase. This enzyme generates reduced glutathione, which is an abundant low molecular weight antioxidant.

If overexpression of a gene increases the life span, that gene is by definition a positive regulator of the life span. Transgenic *Drosophila* containing an extra copy of the native *catalase*, *CuZnSOD*, *MnSOD*, *hsp70*, or *glutathione reductase* genes generally exhibited increased gene expression, but were not found to exhibit any consistent increase in the life span under normal culture conditions (30,45–49,54). However, extra copies of *hsp70* produced small increases in the life span following mild heat stress, and glutathione reductase overexpression increased survival in hyperoxic atmosphere, a condition known to increase oxidative stress.

Relatively large increases in the life span have recently been achieved by driving transgenic overexpression with more complex binary transgenic systems. The GAL4/UAS system (55) was used to produce overexpression of human Cu/ZnSOD in a tissue-specific pattern during *Drosophila* development and aging, with expression in the adult occurring primarily in motor neurons (56). This expression pattern caused increases in the mean life span of up to 40%. Importantly, O_2 consumption was not altered, indicating that metabolic potential was in-

creased. In other studies, a conditional gene expression system called "FLP-*out*" (57) was used to drive overexpression of transgenes specifically in the adult (58). Using the FLP-*out* system, catalase enzyme was overexpressed up to 2.5-fold. Catalase overexpression significantly increased resistance to H_2O_2 toxicity, but had neutral or slight negative effects on the mean life span. In contrast, overexpression of Cu/ZnSOD using FLP-*out* extended the mean life span up to 48%. Simultaneous overexpression of catalase with Cu/ZnSOD had no added benefit, apparently due to a preexisting excess of catalase. FLP-*out* recently has been used to demonstrate that overexpression of the mitochondrial MnSOD also can increase the life span (59). Moreover, the increased mean and maximum life span caused by either Cu/ZnSOD or MnSOD overexpression was not correlated with decreased metabolic activity, suggesting that metabolic potential is increased in each case.

VI. SINGLE-GENE MUTATIONS THAT INCREASE LIFE SPAN

Several negative regulators of the *Drosophila* life span also have been identified. A P element insertion mutation in the *methuselah* gene was found to increase the life span by up to 35% (60). The *methuselah* mutation also was found to increase body size and stress resistance. P element insertion mutations of the *INDY* gene (which is related to human and rat renal sodium carboxylate cotransporters) also increased the life span (61). Finally, mutations of either the insulin-like receptor (*InR*) or its substrate *chico* can extend the *Drosophila* life span (62,63). The data demonstrate that an insulin-like growth factor pathway negatively regulates the life span in *Drosophila*, as had previously been shown for *Caenorhabditis elegans* (64).

VII. DNA REPAIR AND GENOMIC STABILITY IN *DROSOPHILA*

Several dozen genes implicated in DNA repair, replication, recombination, and/or cell cycle control have been identified in *Drosophila* by mutations that render larvae hypersensitive to mutagens (65,66). Many more genes of this class are suggested by sequence homology and analysis of the *Drosophila* genomic sequence. A comprehensive list of *Drosophila* DNA repair genes has been made available by several investigators, at http://www.dmrepair.ucdavis.edu (67). With the interesting exception of "transcription-coupled" repair, most of the major pathways of DNA repair and genomic stability maintenance in humans are now represented by one or more genes and an active research effort in *Drosophila*.

When eukaryotic cells incur DNA damage, checkpoint pathways are activated to delay the cell cycle until the damage is repaired. The sensors of the DNA damage, the transducers of the signal, and the cell cycle regulatory proteins involved in mammals are each represented in *Drosophila* (68–73). Sufficient DNA damage also can induce programmed cell death, or apoptosis. The apoptotic pathway and its central transcriptional regulator, *p53* (74), are also conserved in *Drosophila* (75,76).

Single-base mismatches and small insertion/deletion loops can be generated by errors in DNA replication and other pathways. In *Escherichia coli* and metazoan cells, the correction of these errors is initiated by the MutS and MutL classes of mismatch repair proteins, and *Drosophila* contains members of each class (67,77,78). Mutations of the *Drosophila* MutS homolog *spellchecker1* reveal a function in maintaining genomic stability (78). The *Drosophila mei-9* gene product appears to play a role in mismatch repair as well as in other repair pathways (79–81). Mei-9 is homologous to the RAD1 subunit of the RAD1/RAD10 yeast nucleotide excision repair (NER) endonuclease. In yeast, RAD1/RAD10 functions as the 3′ endonuclease required for excision of damaged DNA during NER. Mutations of *Drosophila mei-9* reveal broader roles in meiotic recombination, NER, mismatch repair, and double-strand break repair. *Drosophila* also contains other evolutionarily conserved and unique genes involved in double-strand break repair (82,83).

During DNA repair, new DNA is synthesized by DNA polymerases. The number of unique DNA-dependent DNA polymerases identified in metazoans has grown rapidly in recent years (84). *Drosophila* contains homologs for each class with the notable exception of Polβ. Additional key components of recombination and repair pathways in eukaryotes are the "Rad51 family" of strand-exchange proteins; named for the first of four members identified in *Saccharomyces cerevisiae*. These proteins are related to *E. coli* RecA, and there appear to be at least four family members in *Drosophila* with functions in meiotic and mitotic cells (67,85).

Base excision repair (BER) and NER are distinct pathways through which various types of lesions are removed from the DNA. In mammals and yeast, such lesions are more rapidly removed from the template strand of actively transcribed genes in a process called transcription coupled repair (TCR). Although NER and BER pathways and genes are found in *Drosophila*, the more rapid repair of the template strand characteristic of TCR was not observed in *Drosophila*, at least in studies of ultraviolet-induced damage (86,87). *Drosophila* also lacks homologs for two critical TCR pathway genes characterized in humans, *CS-A* and *CS-B*. Taken together, the data suggest the possibility that *Drosophila* lacks the TCR pathway (67).

In humans, mutation of the *WRN* gene causes a syndrome marked by genomic instability, increased rates of certain cancers, and several other features re-

sembling premature aging (88). *WRN* encodes a DNA helicase related to *E. coli recQ*. Mutation of human *recQ* family members *BLM* and *RECQ4* also causes syndromes marked by increased genomic instability and cancer (89). *Drosophila* contains at least three *recQ* family members; however, their mutant phenotypes are currently unknown (67).

VIII. *DROSOPHILA* AGING AND GENOMIC STABILITY

DNA damage and somatic mutation have long been hypothesized to be possible contributors to aging (90). However, there have been relatively few studies directly linking aging and genomic stability in *Drosophila*. Aging *Drosophila* have been shown to accumulate the oxidatively damaged DNA base 8-oxo-guanine (6). In addition, mitochondrial DNA mutations have been found to be more abundant in older flies (7). Therefore, at least some types of DNA damage are accumulating in the aging *Drosophila* adult. A more detailed analysis of the types and extent of DNA damage, mutation, and genomic instability occurring during adult *Drosophila* aging would be particularly informative. Several mutagen-sensitive mutations in *Drosophila* reduce the life span, as would be expected if DNA repair and genomic stability were required for a normal life span (91,92). However, these data are problematic for two reasons: First, the reduction in the life span does not mean that the particular repair pathway is normally limiting for the life span. A convincing demonstration of this would likely require demonstration of an increased life span by upregulation of the pathway. Second, the DNA repair mutations analyzed have phenotypic effects during development. Therefore, the data do not distinguish between a reduced life span resulting from damage occurring during development and damage accumulating during adult aging.

Mismatch repair usually has been thought to occur primarily after DNA replication in dividing cells. However, a biochemical study found evidence for abundant expression of mismatch repair activities in the entirely postmitotic cells of young and old (senescent) adult *Drosophila* (81). Extracts of *Drosophila* embryos and adults were found to catalyze highly efficient DNA mismatch repair, as well as repair of 1- and 5-bp loops. The activity was reduced in extracts of animals mutant for the *mei-9* gene. The specific activity of embryonic extracts was found to be similar to that of extracts derived from the entirely postmitotic cells of young and senescent adults. Thus, DNA mismatch repair activity is expressed in *Drosophila* cells during both development and aging, suggesting that there may be a function or requirement for mismatch repair throughout the *Drosophila* life span. Genetic experiments are required to determine if, in fact, mismatch repair is occurring in the postmitotic cells of young and old adults as predicted, and whether such activity is required for maintaining a normal life span.

Recent technological and methodological advances in *Drosophila* should allow for a more detailed analysis of the role of DNA repair and genomic stability in aging. The transgenic systems used to assay SOD and other stress-response genes for effects on aging reviewed above should be equally applicable to testing of DNA-repair genes (39). Conditional overexpression systems such as FLP-*out* and tetracycline-inducible promoters ("tet-on") allow assay of gene function specifically in the aging adult by induced overexpression (58,59,93,94). Transcription of inverted repeats recently has been shown to allow sequence-specific gene inactivation in *Drosophila,* called either "RNA interference" (RNAi) or "posttranscriptional gene silencing" (PTGS) (95,96). Driving expression of an inverted repeat with an inducible system allows a gene to be specifically inactivated in the aging adult (M. Allikian and J. T., unpublished data). The potential ability to overexpress or inactivate any gene specifically in the aging *Drosophila* adult should allow for detailed analyses of the roles of DNA repair and genomic maintenance in future studies.

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22
Telomerase-Deficient Mouse Model

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I. INTRODUCTION

A. Structure and Function of Telomeres and Telomerase

Telomeres are the ends of eukaryotic chromosomes and consist of tandem repeats of a G-rich DNA sequence, which in all vertebrates is TTAGGG (1,2). There are proteins specifically bound to mammalian telomeres (3). Some of these proteins bind to double-stranded telomeric DNA, such as TRF1 and TRF2 (4–5), which in turn recruit to the telomeric proteins important in the DNA-damage response such as tankyrase (PARP-5), Ku, DNA-PKcs, and the Mre11/Rad50/Nsb1 complex (6–11). Recently, a novel telomeric protein, POT-1, has been shown specifically to recognize the 3′ single-stranded telomeric DNA or G-strand overhang (12). Telomeres protect chromosomal ends from degradation, recombination, and DNA-repair activities. Loss of telomeric function, either due to loss of telomeric sequences or to mutation of telomeric proteins, results in end-to-end fusions and loss of cell viability (9,11,13–15). The current model is that telomeres can adopt two different conformations: One is an "open" telomere that is accessible to telomerase and other cellular activities and the other is a "protected" telomere in which the single-stranded telomeric G-strand overhang invades the double-stranded telomeric DNA, forming a loop structure named the t-loop (16) (see Sec. II.D) (Fig. 1).

What regulates the length of TTAGGG repeats in mammalian cells is not fully understood; however, the activity of telomerase—a cellular reverse transcriptase that synthesizes telomeric repeats de novo—is crucial in this process. Telomerase is composed of a catalytic subunit known as Tert (telomerase reverse transcriptase) (17–23), an RNA molecule (Terc, telomerase RNA component) that

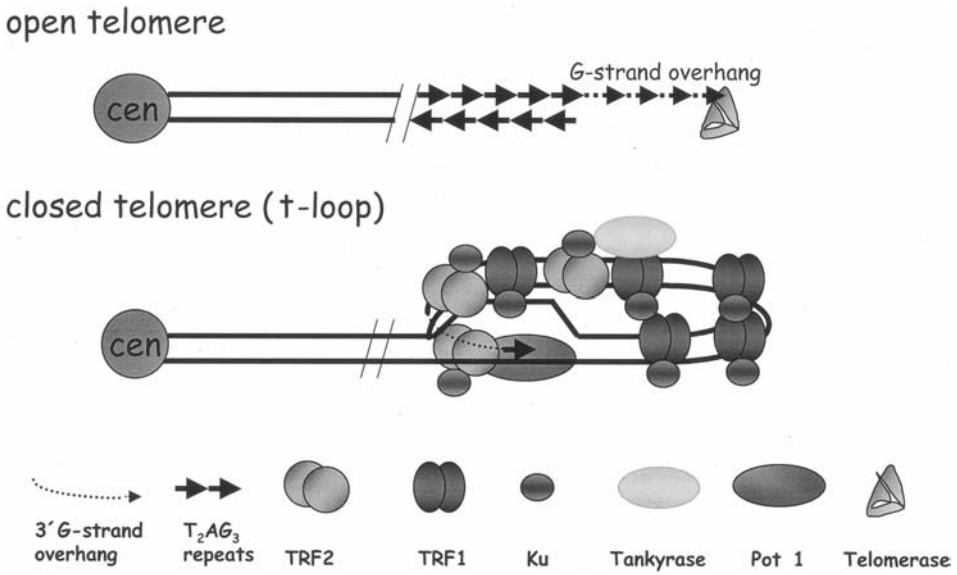

Figure 1 Mammalian telomeric composition. Scheme of mammalian telomeres. The upper part shows a naked telomere in an "open" conformation with telomerase at the 3′overhang (G-strand overhang). The bottom depicts a closed telomeric conformation or t-loop with telomere-binding proteins.

is used as a template for addition of new telomeric repeats (24–27), and associated proteins (28–32). The C-terminus of the telomerase Tert component shows all the characteristic amino acid motifs of the reverse transcriptase family, including the aspartic acid residue that forms the catalytic triad (17), suggesting that they may share a similar structure. The N-terminus is conserved only in telomerases, and it is likely to constitute a different structural domain from the reverse transcriptase active site. In the case of the vertebrate telomerase RNA component, a model of secondary structure has been recently proposed (33), which shows that vertebrate Terc shares a structural domain with the snoRNAs. This domain is characterized by the Box H/ACA motifs and binds a family of proteins that includes dyskerin (32).

B. Telomeres, Telomerase, and Cancer

Telomerase activity is upregulated in the vast majority of human tumors as compared with normal somatic tissues, where it is undetectable or present at very low levels (34). It has been shown that expression of the Tert catalytic subunit of telomerase in cultured human primary cells reconstitutes telomerase activity and

allows immortal growth (35–39). In addition, Tert-mediated telomerase reconstitution is able to cooperate with oncogenes in transforming cultured primary human cells into neoplastic cells (40). These findings have opened up the possibility that telomerase upregulation, which occurs in more than 90% of all human tumors (34), may contribute actively to tumor growth (41). Hence, telomerase inhibition may be an effective way to interrupt tumor growth.

C. Telomeres, Telomerase, and Aging

In contrast to what is thought to occur in tumors, telomeric sequences are lost with each cell division in mortal cells and in most somatic tissues as a consequence of the so-called "end replication problem." This telomeric shortening with increasing age coincides with an absence of detectable telomerase activity in adult somatic tissues and has been proposed to limit the proliferative capacity of cells and contribute to the aging process. Telomerase activity reconstitution in adult somatic cells or tissues is thus envisioned as a potential approach for gene therapy in age-related diseases (39).

II. CHARACTERIZATION OF MICE WITHOUT TELOMERASE

To address directly the potential role of telomerase in cancer and aging, four different telomerase-deficient mouse models have been generated to date. In two of the models, the gene encoding the telomerase RNA component Terc was eliminated from the mouse germ line (14,42); these mice lack telomerase activity, although they still express the Tert subunit (23). In two different knockout models, the gene coding for Tert was eliminated (43,44). The phenotypes of the four telomerase knockout mice appear to be identical, suggesting that the catalytic subunit and the telomerase RNA are essential for telomerase activity, and that they do not seem to have differential roles in the cell. This chapter will focus mainly on the extensively studied mouse telomerase knockout model, Terc$^{-/-}$ mice, originally described by Blasco and coworkers. (14).

A. Telomeric Shortening in the Absence of Telomerase

Since mouse telomeres are very long (~40 kb of average length in some mouse strains), it has been possible to derive several Terc$^{-/-}$ mouse generations before loss of viability is observed in the colony. As expected from the absence of telomerase activity, Terc$^{-/-}$ mice show progressive telomeric shortening with increasing mouse generations in different adult cell types. Figure 2 shows telomeric quantitative fluorescence in situ hybridization (FISH) on metaphases from early (p1) and late passage (p50) wild-type and G6 Terc$^{-/-}$ mouse embryonic

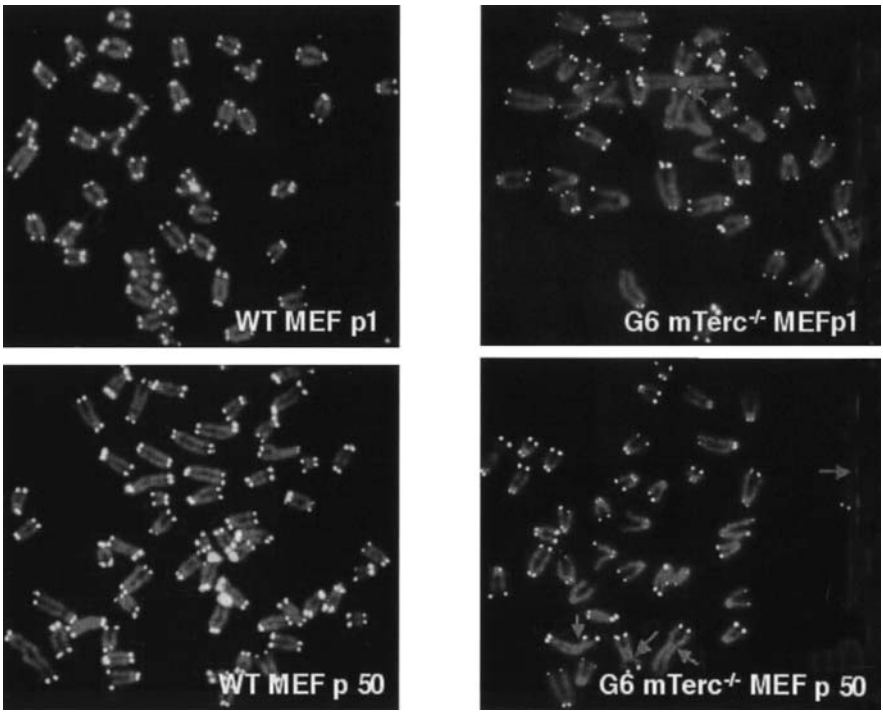

Figure 2 Telomeric shortening and chromosomal instability in late-generation Terc$^{-/-}$ primary cells. Representative telomeric FISH images of metaphases from early-passage (p1) and late-passage (p50) wild-type and G6 Terc$^{-/-}$ mouse embryonic fibroblasts (MEF). A clear telomeric shortening can be seen in the late-generation Terc$^{-/-}$ cells, which is more dramatic at later passages. Arrows point to end-to-end fusions. Wt, wild-type; G6, sixth mouse generation; p, passage.

fibroblasts (MEFs); absence of detectable TTAGGG signals from some chromosomal ends can be seen in late-generation Terc$^{-/-}$ cells, which is more dramatic at later passages (see Fig. 2). Telomeric shortening in Terc$^{-/-}$ mice is 3–5 kb per mouse generation, which corresponds to 100–150 bp of telomeric loss per cell division, similar to that previously described for human cells. The number of mouse generations that can be obtained in the absence of telomerase is directly proportional to the initial telomeric length of the strain (45). Whereas six generations were derived on the original mouse background, which showed an average telomeric length of 40 kb, only three to four generations were derived on the pure C57BL/6 background, with an average telomeric length of 25 kb (45). These results indicate that telomerase activity is necessary and sufficient for long-term maintenance of the species, and furthermore show that telomerase

activity is the only efficient pathway for maintaining telomeres in a mammalian organism (see below).

B. Critical Telomeric Shortening Results in Chromosomal Instability

The study of the telomerase knockout mouse model has established that critical loss of TTAGGG repeats from telomeres results in increased end-to-end chromosomal fusions and loss of viability (see Fig. 2 for end-to-end fusions, [*arrow*]) (14,46,47). Hence, a minimal length of TTAGGG repeats at the telomeres is required for proper telomeric function. However, telomeric loss is not the only way to impair telomeric function. As mentioned above (Sec. I.A), loss of telomeric binding proteins such as TRF2, Ku, and DNA–PKcs, also renders an end-to-end fusion phenotype in the absence of telomeric shortening (9,15). In the case of TRF2 loss of function, the G-strand overhang at the telomere is disrupted, which may affect the formation of t-loops (15,16).

C. Cell Growth and Neoplastic Transformation of Cells Derived from the Telomerase Knockout Mouse: Fibroblasts versus Embryonic Stem Cells

As described above, late-generation telomerase-deficient mice show telomeric shortening and increased numbers of end-to-end fusions. Several groups have addressed the effects of these alterations in viability, immortalization, and neoplastic transformation ability of cells derived from these mice. In particular, mouse embryonic fibroblasts (MEF) and embryonic stem cells (ESCs) have been studied (14,42,47).

1. Mouse Embryonic Fibroblasts

Telomerase-deficient MEFs never showed a decreased growth rate with increasing population doublings (PD) in culture—even when reaching more than 600 PD in the case of first-generation MEFs and up to 300 PD in late-generation Terc$^{-/-}$ MEFs (47). Furthermore, MEFs derived from different-generation telomerase knockout mice immortalize spontaneously in culture and can be neoplastically transformed by oncogenes (14). These results suggest that, at least in cultured MEFs, telomerase deficiency may be compensated for by activation of alternative mechanisms to lengthen telomeres (alternative lengthening of telomeres, ALT) (48). Indeed, a study using different passage number MEFs from all telomerase knockout generations showed that once telomeres shorten to a critical length (which differed for the distinct chromosomes studied), they could be maintained at this length or elongated in the absence of telomerase activity (47). These ALT

cells generally show high chromosomal instability. It is estimated that there is a 40-fold increase in chromosomal aberrations in cells growing without telomerase compared with wild-type cells (47). Figure 2 shows chromosomal aberrations detected in wild-type and Terc$^{-/-}$ cells that have been passaged in culture for increasing PD. All together, these results illustrate the critical role of telomeres and telomerase in maintaining chromosomal stability.

2. ES Cells

In contrast to cultured Terc$^{-/-}$ MEFs, Terc$^{-/-}$ ESCs showed a decrease in viability after 450 PD in culture, suggesting that ESCs are more susceptible to telomeric dysfunction than MEFs (42). ESC clones were selected, however, that were able to survive beyond 450 PD at a high growth rate. These Terc$^{-/-}$ ESC survivors showed high chromosomal instability as well as telomeric maintenance without telomerase, as described for late passage number MEFs (49).

All together, these results suggest that, at least in cultured cells, telomerase deficiency and telomeric shortening to a critical length can be rescued by the activation of telomerase-independent telomeric lengthening mechanisms which, based on the work carried out in yeast, are likely to involve illegitimate recombination at the telomeres (see below).

D. Telomerase-Independent Telomeric Rescue in Telomerase-Deficient Mice

In the absence of telomerase, yeast cells can overcome a culture crisis through the appearance of rapidly growing survivors that have activated alternative mechanisms of telomeric maintenance (ALT) (50). These telomere-rescue mechanisms are dependent on the Rad52 family of recombination proteins and on helicases of the RecQ family, such as SGS1 (51–53). In yeast, there are two types of survivors according to their telomeres: type I survivors have short and stable telomeres generated by amplification of Y′ subtelomeric element and type II survivors have unstable, long, and heterogeneous telomeres generated by intertelomere recombination mediated by the Rad52 and SGS1 family of proteins (52–54) (Fig. 3). This illegitimate recombination at the telomere is inhibited by the Rif proteins (54). More recently, it has been shown that defects in mismatch repair genes (MSH2, MSH3, MSH6, MLH1, and PMS1) also promote telomerase-independent proliferation in yeast (55).

In the case of human cells, ALT activation is associated with long and very heterogeneous telomeres (56). Human ALT cells also use intertelomere recombination to elongate their telomeres (57) similar to that shown for type II yeast survivors. Furthermore, human ALT has been associated with the presence of doughnut-shaped nuclear structures called ALT-associated promyelocytic leukemia

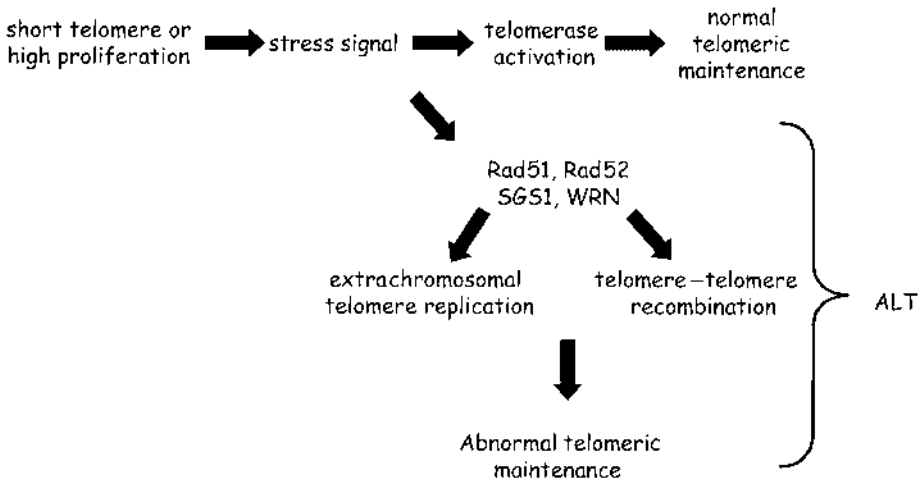

Figure 3 Rescue of short telomeres by telomerase-dependent or independent mechanisms. A high proliferation in the absence of telomerase activity results in critically short telomeres, which in turn are regarded as a stress signal and can result in cell arrest or apoptosis unless telomere-rescue mechanisms are activated. Telomerase reactivation is the preferred mechanism to elongate telomeres without compromising genomic stability. However, in the absence of telomerase, proteins important in homologous recombination (Rad51, Rad52) or helicases (SGS1, WRN) can promote illegitimate telomerase-independent telomeric rescue. This telomerase-independent telomeric rescue is known as ALT (alternative lengthening of telomeres), and it is found in cells that actively grow in the absence of telomerase activity.

bodies (APBs) (56,58). These APBs contain PML proteins, RAD51 and RAD52, TRF1 and TRF2, replication factor A, and telomeric DNA. The APBs are present in 5% of cells in those cell lines and tumors that maintain their telomeres in the absence of telomerase. Additionally, DNA-repair proteins NBS 1 and Mre11 seem to interact with telomeres and with PML bodies (59). Interestingly, the SGS1 homologous WRN helicase also interacts with telomeres, and patients with Werner syndrome show accelerated telomeric shortening and high chromosomal instability and suffer immunodeficiency, cancer, and accelerated aging.

As mentioned above, primary cells from the Terc$^{-/-}$ mouse erode their telomeres at about 3–5 kb per generation, but when spontaneously immortalized cell lines were studied, it was found that telomeres were maintained in the absence of telomerase (47). Moreover, when the telomeres of individual chromosomes were measured, it was found that they could increase in size (47). In the case of the Terc$^{-/-}$ ESC lines, at about 450 PD, survivors emerged and took over two independent Terc$^{-/-}$ ESC cultures (49). One of the clones activated ALT and showed telomeric elongation after a prolonged period in culture. In this case, the

amplified DNA at the telomeres was found to consist only of TTAGGG repeats. The other clone amplified >100-fold a fragment of 1.6 kb containing 700 bp of TTAGGG repeats flanked by nontelomeric sequences of unknown origin. The amplified fragment in the second clone also localized to the telomeres (49).

In both cases (MEFs and ESCs), it was shown that although ALT mechanisms were taking place and partially rescuing short telomeres, they did not prevent chromosomal instability, which in turn could be favoring immortal growth (47,49). Therefore, there is increasing evidence that ALT mechanisms of telomeric maintenance take place in mammalian cells and permit telomeric rescue and rapid proliferation (see Fig. 3). Nevertheless, ALT seems to function in a manner that favors genetic instability, and this probably explains why ALT is not able to rescue organismal viability in the telomerase-deficient mice (45), although it can efficiently rescue immortal cell cultures (14,47).

E. G-Strand Overhangs in Telomerase-Deficient Mice

Telomeres are characterized by the ability to form a special structure termed the t-loop, which is proposed to protect telomeres from DNA-repair and recombination activities (see Fig. 1). Telomeric loops are maintained by the existence of a G-strand overhang at the telomere that invades the double-stranded DNA telomeric region. Telomeric proteins TRF1 and TRF2 are present at t-loops and have been proposed to stabilize them (16) (see Fig. 1). Indeed, loss of TRF2 function due to a dominant negative mutation results in telomeric fusions and loss of the G-strand overhang, which in turn can compromise the formation of t-loops (15). It has been postulated that the G-strand overhang may originate from telomerase activity, since telomerase elongates the 3′ G-strand at the telomeres. The telomerase knockout models rule out this possibility, however, since both Terc$^{-/-}$ and Tert$^{-/-}$ mice have normal G-strands at the telomeres independent of generation number (43,60).

III. LIFE WITHOUT TELOMERASE

A. Embryonic Development

In humans and mice, telomerase activity is abundantly expressed during embryonic development and is rapidly downregulated after birth, concurring with the fact that it is absent in most normal adult somatic tissues (27,61). High telomerase levels are postulated to be required during embryonic development to sustain the elevated proliferation that occurs in this process. In agreement with this, Terc$^{-/-}$ mice show increased embryonic mortality with increasing Terc$^{-/-}$ mouse generations (62). In particular, Terc$^{-/-}$ mice of later generations show a larger percentage of neural tube defects (NTDs), consisting of an open neural tube, indicat-

ing that the process most sensitive to telomeric loss during embryonic development is neural tube formation and closure. This phenotype concurs with the fact that TERC is most abundantly expressed in the central nervous system during the human embryonic development (63). Neural tube defects–including spina bifida, anencephaly, and congenital hydrocephalus–are among the major causes of infant mortality. It is possible that telomeric shortening–mediated chromosomal instability could be one of the multiple factors determining the occurrence of NTDs in humans.

B. Fertility

The genetic information of a species must be transmitted intact from generation to generation. In humans and mice, telomerase activity is abundantly expressed in the germ line in contrast to its absence or low levels of expression in most adult somatic tissues (64). Terc$^{-/-}$ mice from increasing generations show a progressive decrease in litter size until no progeny are produced in late-generation Terc$^{-/-}$ mouse intercrosses. This decrease in litter size is due to embryonic death as a consequence of a neural tube defect in these mice (see above), as well as to infertility. Fertility decreases with increasing generations in Terc$^{-/-}$ mice, eventually resulting in completely sterile Terc$^{-/-}$ males and females. The Terc$^{-/-}$ male infertility phenotype is well characterized in two different genetic backgrounds (45,46). The Terc$^{-/-}$ female infertility phenotype has been only partially studied (46).

Late-generation Terc$^{-/-}$ males show testicular atrophy (a reduction of 60% in normal testicular weight after correction by total body weight), as well as a depletion of male germ cells. Histological analysis of late-generation Terc$^{-/-}$ testes shows various abnormalities, including depletion of germ cells in the epithelium (which shows only vacuolated Sertoli cells) and marked hyperplasia of Leydig cells (45). These lesions are similar to those found in patients affected by the Sertoli cell only syndrome (65), and are also found in 40% of wild-type mice of 15–17 months of age (66), suggesting premature aging in late-generation Terc$^{-/-}$ males. These lesions are the result of increased apoptosis and a decreased mitogenic index in the germ cells with increasing generations of Terc$^{-/-}$ males (46).

C. Decreased Viability with Increasing Generations of Mice Deficient for Telomerase Activity

Late-generation Terc$^{-/-}$ mice show increased mortality with age compared with wild-type littermates. Age at death depends on the genetic background. On the original mixed genetic background (C57Bl6/129Sv 50%/50%), which shows long, heterogeneous telomeres, there is decreased viability in Terc$^{-/-}$ mice older than 18 months compared with wild-type mice (67). Nonetheless, on a pure

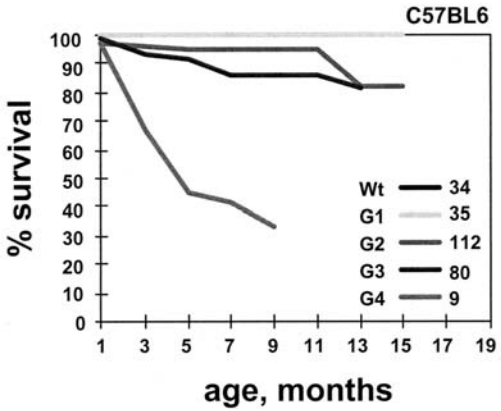

Figure 4 Decreased viability of late-generation Terc$^{-/-}$ mice. Late-generation Terc$^{-/-}$ mice in a pure C57BL6 background show decreased viability with age. Fifty percent of the G4 Terc$^{-/-}$ mice died at 5 months of age.

C57BL/6 background, which shows shorter, more homogeneous telomeres, 50% of late-generation Terc$^{-/-}$ mice died at 5 months of age (45) (Fig. 4). Death of these mice occurred within 48 hr of the detection of symptoms of poor health, such as reduced activity, lowered responsiveness to stimuli, bristly hair, dehydrated skin, a hunched position, and small size (45). Affected mice showed severe defects in some highly proliferative organs (see below). It is important to note, however, that 40% of the Terc$^{-/-}$ mice died suddenly with no apparent signs of disease. These sudden death cases are currently under investigation and may be related to cardiovascular failure in these mice.

D. The Digestive System

Late-generation Terc$^{-/-}$ mice are significantly smaller than age-matched wild-type controls (Fig. 5). This is particularly evident in late-generation Terc$^{-/-}$ mice on a pure C57BL/6 background. A 20% reduction in body weight has been observed in both C57BL/6 Terc$^{-/-}$ males and females older than 4 months (45); this loss in body weight was not apparent on the mixed background until Terc$^{-/-}$ mice were 15–18 months old (67). Reduced body size was coincidental with an increased number of deaths in the colony. The fact that this weight reduction is more apparent in adult mice suggests a relationship to a digestive system malfunction. Indeed, in all C57BL/6 Terc$^{-/-}$ mice showing signs of poor health, but not in age-matched wild-type mice, histological analysis of the small intestine reveals various abnormalities, such as atrophy of the villi and mucosal flattening in some areas and epithelial and glandular hyperplasia in other areas (45). These histological

alterations have been described as typical age-related lesions in C57BL/6/C3H hybrids more than 2 years old (68). The abnormal small intestine mucosa may result in reduced nutrient absorption, which could account for decreased body weight, dehydrated skin, and hunched position. In accordance with this, lymphangiectasia and increased lymphocyte numbers, which are typical signs of a malabsorption syndrome, are also observed in the affected intestine. Ulcerations and initial stages of peritonitis are also detected in some areas of the intestine, which may result in rapid death of affected mice (45).

The small intestine epithelium is completely regenerated every 4–5 days (69); hence, telomeric shortening associated with cell division and aging may result in growth impairment of epithelial cells, triggering the alterations described (45).

E. Skin and Hair

An increased incidence of hair graying and alopecia has been detected in late-generation Terc$^{-/-}$ mice as compared with wild-type littermates (67). This phenotype was particularly evident on the C57BL/6 background, which normally has black hair (Fig. 5 shows representative images of a wild-type and a late-generation

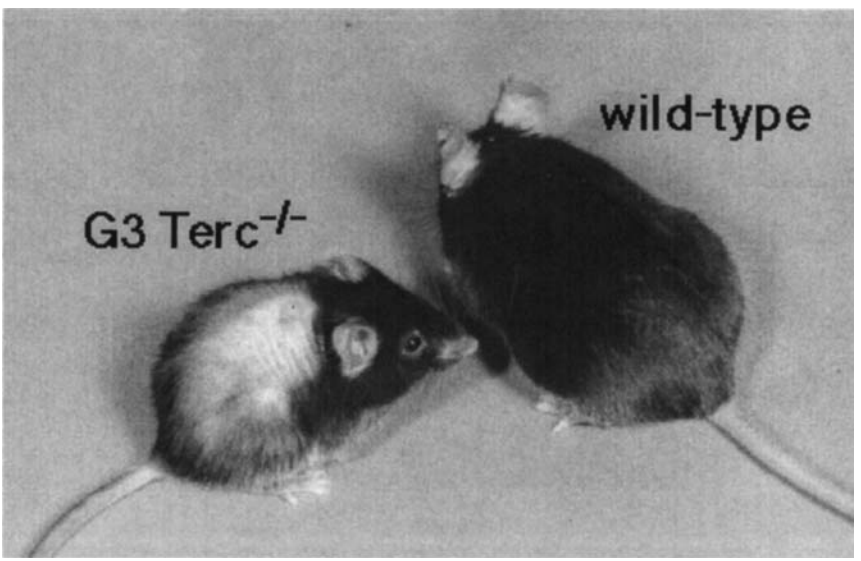

Figure 5 A diseased late-generation Terc$^{-/-}$ mouse. Representative images of a wild-type mouse and a late-generation C57Bl6 Terc$^{-/-}$ mouse showing signs of disease. Notice alopecia, hair graying, hunched position, and small size of the Terc$^{-/-}$ mouse as compared with an age-matched wild-type.

Terc$^{-/-}$ mouse on the C57BL/6 background; notice the hair graying, alopecia, and smaller size of the Terc$^{-/-}$ mouse) (45). Skin ulcerations also were detected in late-generation Terc$^{-/-}$ mice at a higher frequency than in wild-type mice. These lesions were located predominantly at anatomical sites exposed to chronic mechanical stress, such as the distal limbs, perineum, snout, and throat area. Histologically, the lesions appeared as ulcerations with epidermal hyperplasia, hyperkeratosis, and underlying dermal fibrosis (67), and are similar to those seen following chronic superficial trauma, particularly in debilitated elderly humans. These skin defects suggest decreased proliferative capacity of the skin in late-generation mice. In this regard, old (>18 months) sixth generation mice are reported to show delayed wound healing compared with wild-type animals (67).

F. Liver

Histological analysis of the liver in Terc$^{-/-}$ mice on two different genetic backgrounds reveals no pathologies compared with wild-type mice (45,67). When late-generation Terc$^{-/-}$ mouse livers were challenged either by repetitive exposure to hepatotoxic substances (CCl_4) or by partial hepatectomy (surgical removal of two-thirds of the liver) (70), a marked difference was apparent between wild-type and Terc$^{-/-}$ mice. In wild-type mice, normal liver histology was reestablished within 1 week of surgery, whereas late-generation Terc$^{-/-}$ mice showed decreased organ weight compared with wild types, and some of these mice died of postoperative complications (70). The late-generation Terc$^{-/-}$ mice that died were those with shorter telomeres, suggesting that the shorter telomeres of late-generation Terc$^{-/-}$ mice impair the regenerative capacity of the liver (70). From a clinical point of view, these results suggest that end-stage liver cirrhosis patients might benefit from telomerase-based gene therapy.

G. Immune System

A reduction in immune system reactivity with age is commonly known as immunosenescence, and is characterized by impairment of B- and T-lymphocyte function (diminished response to mitogens) coincident with decreased germinal center (GC) reaction (71). Germinal centers are characterized by extensive clonal expansion and selection of B lymphocytes to generate the B memory cell pool. The decline in immune system reactivity in humans may be due to exhaustion of the proliferative potential of naive B cells as a consequence of telomeric shortening with increasing age (72,73). Telomerase is activated when B cells enter the GC, and is subsequently downregulated when B cells differentiate to memory B cells (74–78). Diminished lymphocytic reactivity and impaired GC reaction have been described in late-generation Terc$^{-/-}$ mice (45,46,79). In particular, late-generation Terc$^{-/-}$ mice, which are telomerase deficient and have short telomeres, show a dra-

matic reduction in the number of GCs formed following in vivo antigen immunization (79). Furthermore, telomeric length analysis of wild-type and Terc$^{-/-}$ mice showed that B-cell telomeres were 5 kb shorter in immunized first-generation Terc$^{-/-}$ mice than in unimmunized Terc$^{-/-}$ controls in agreement with the absence of telomerase activity in these mice (79). In contrast, B-cell telomeres were 5 kb longer in immunized wild-type than in unimmunized wild-type control mice (79). These results indicate that the telomerase elongation detected in wild-type spleens following in vivo immunization is mediated by telomerase activity. Telomeres in B cells of immunized late-generation Terc$^{-/-}$ mice were similar in length to wild-type telomeres (79). These late-generation Terc$^{-/-}$ cells with long telomeres may derive from a surviving subpopulation of late-generation Terc$^{-/-}$ splenocytes that preserved long telomeres or from activation of telomerase-independent telomere-rescue mechanisms in late-generation Terc$^{-/-}$ splenocytes. Either way, telomeres are crucial to sustaining cell proliferation during the GC reaction.

In these mice, the diminished proliferative capacity of late-generation Terc$^{-/-}$ B and T lymphocytes to mitogen treatment is also a landmark of immunosenescence (45,46,79). Late-generation Terc$^{-/-}$ mice on the C57BL/6 background show severe atrophy of the spleen with reduced follicle numbers (45,79). This severe spleen phenotype has never been observed in Terc$^{-/-}$ mice on the original mixed genetic background; this is in accord with the milder phenotype of these mice (46).

H. Hematopoietic System

Initial peripheral blood counts in different-generation Terc$^{-/-}$ mice on the mixed background revealed no significant differences with wild-type mice (46). Nevertheless, study of late-generation Terc$^{-/-}$ mice on a C57BL6 background showed significant differences in lymphocyte and neutrophil numbers; lymphocyte numbers decreased significantly in late-generation Terc$^{-/-}$ mice, whereas neutrophils were significantly increased (45). The greater neutrophil numbers may be a compensatory mechanism of the immune system triggered by the decreased lymphocyte numbers. Similarly increased neutrophil numbers have been described in 27-month-old wild-type animals (80). No large differences have been detected in total leukocyte numbers or in hematocrit between late-generation Terc$^{-/-}$ mice and wild-type controls.

Colony-forming unit (CFU) assays have been performed in Terc$^{-/-}$ mice to determine whether the early progenitor cell compartment is compromised. These studies show statistically significant decreases in the number of CFU–granulocyte, monocyte (CFU-GM), CFU–granulocyte, erythrocyte, monocyte, megakaryocyte (CFU-GEMM), and highly proliferative potential colony-forming cell (HPP-CFC) colonies (46). These results indicate that the long-term renewal of hematopoietic stem cells is decreased upon telomeric loss.

I. Other Organs

Initial histopathological studies of lung, brain, kidney, heart, and blood vessels reveal no major differences between late-generation Terc$^{-/-}$ mice and wild-type controls. Nonetheless, more detailed studies are currently underway that may reveal defects in these organs.

IV. CANCER AND TELOMERASE

A. Spontaneous Tumors in Telomerase-Deficient Mice

Late-generation Terc$^{-/-}$ mice show telomeric shortening, increased chromosomal instability, and severe proliferative defects in highly proliferative organs. One might thus predict decreased tumor incidence in these mice. In contrast, Rudolph et al. have reported a moderately increased (fivefold) incidence of spontaneous tumors in late-generation Terc$^{-/-}$ mice compared with age-matched wild-type controls (67). According to these authors, 18-month-old late-generation Terc$^{-/-}$ mice on the original mixed background developed a low incidence of lymphomas and an even lower incidence of teratocarcinomas, hepatomas, squamous cell carcinomas, and sarcomas compared to wild-type controls. This modest increase in tumor incidence was not detected, however, in a different colony of Terc$^{-/-}$ mice on the same genetic background nor in late-generation Terc$^{-/-}$ mice on a pure C57BL/6 background (45), suggesting that the difference in tumor incidence reported by Rudolph et al. may be dependant on the genetic background (67).

B. Cancer in Mice Deficient for Telomerase and the INK4a/ARF Tumor Suppressor Locus

To analyze the impact of short telomeres on tumorigenesis in telomerase-deficient mice, Terc$^{-/-}$ mice were crossed with mouse models that develop spontaneous tumors at a high incidence—these mice carry mutations in tumor suppressor genes, such as mice deficient for the INK4a/ARF locus or for p53 (see below).

Telomeric shortening in double (INK4a/ARF)$^{-/-}$/Terc$^{-/-}$ mice decreased tumor incidence by 50% and increased survival of these animals. Cells derived from these mice also showed impaired colony formation, transformation, and plating efficiencies. Reintroduction of the *Terc* gene in these cells restored their normal transformation potential (81). These results are also in contrast with the increased incidence of spontaneous tumors described in late-generation Terc$^{-/-}$ mice (67), and suggest that short telomeres result in decreased tumor cell growth.

C. Cancer in Mice Deficient for Telomerase and the APC Tumor Suppressor

The role of Terc in the appearance of intestinal carcinomas in mice with the Apc^{min} mutation has been studied recently (82). In humans, mutation of the APC gene causes an inheritable disease called familial adenomatous polyposis, in which the patient shows a predisposition toward colorectal cancer (83). In mice, a nonsense mutation in the APC gene (Apc^{min} mutation) causes multiple intestinal neoplasias (84). When successive generations of $Apc^{min}Terc^{-/-}$ mice are derived, progressive telomeric shortening leads to a modest increase in initiating lesions but a significant decline in the progression of the lesions to macroscopic adenomas (82). The results also suggest that telomere shortening may in fact protect the organism from tumor progression.

D. Cancer in Mice Deficient for Telomerase and the p53 Tumor Suppressor

To examine the impact of short telomeres in tumorigenesis in the absence of the p53 tumor suppressor protein, $Terc^{-/-}/p53^{-/-}$ double knockout mice were generated and characterized (85). Deletion of p53 significantly attenuated the adverse cellular and organismal effects of telomeric dysfunction in the $mTerc^{-/-}$ mice, and these mice could be maintained for more generations than in the case of the single $Terc^{-/-}$ mutation, suggesting that p53 is one of the key regulators in the cellular

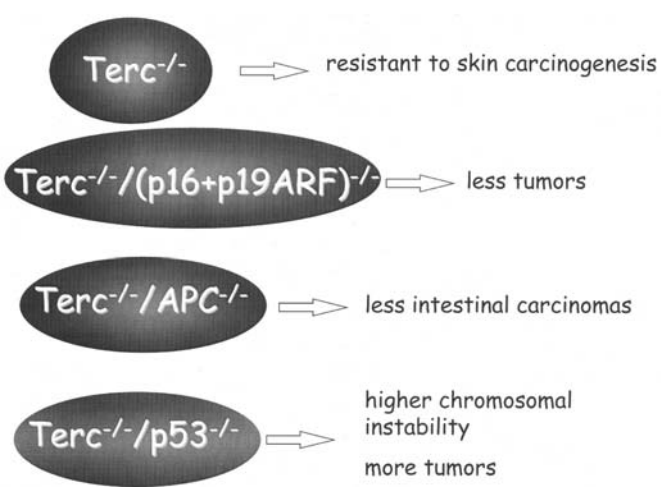

Figure 6 Cancer in $Terc^{-/-}$ mice that possess or lack several tumor suppressor proteins.

response to short telomeres (85). Additionally, telomeric attrition in aging telomerase-deficient p53$^{+/-}$ mutant mice promotes development of epithelial cancers in the digestive tract (86). It has been established that the occurrence of these epithelial cancers is mediated by a process of fusion-bridge breakage, leading to the formation of nonreciprocal translocations (86), a classic cytogenetic feature of human carcinomas. These results suggest that p53 loss in cell with short and dysfunctional telomeres may promote tumorigenesis, at least in the context of a mouse.

E. Carcinogen-Induced Tumors in Telomerase-Deficient Mice

The study of Terc$^{-/-}$ mice in (p16/p19ARF)$^{-/-}$ and APC^{min} backgrounds supports the notion that short telomeres have a negative effect on tumor growth. This was directly tested using chemical carcinogenesis to induce skin papillomas in wild-type and different-generation Terc$^{-/-}$ mice (87). The results of this study indicate that late-generation Terc$^{-/-}$ mice are resistant to carcinogens. In particular, the number of skin papillomas in mTerc$^{-/-}$ mice was dramatically reduced compared with wild-type mice, and all late-generation mTerc$^{-/-}$ mouse papillomas disappeared after termination of the treatment. Importantly, those small papillomas that appeared in late-generation Terc$^{-/-}$ mice showed increased levels of the p53 protein (87).

These results suggest that short telomeres act as a tumor suppressor mechanism when in a wild-type p53 genetic background, preventing the appearance of tumors in the skin and coinciding with an upregulation of the p53 tumor suppressor. Indeed, several studies suggest that p53 plays a significant role in signaling dysfunctional telomeres as DNA damage (88). González-Suárez et al. also have reported a lower incidence of papillomas in first-generation telomerase-deficient mice, which have sufficiently long telomeres (87). This suggests that telomerase activation during tumor progression may have an active role in favoring tumor growth.

F. Cancer in Tissue-Specific Telomerase Transgenics

Recently, construction and characterization of the first transgenic mouse model that overexpresses telomerase in adult tissues have been reported (89). In particular, these mice have constitutive telomerase activity in all stratified epithelia. This was achieved by expressing the *Tert* gene under the bovine keratin 5 promoter (K5), which is specific to all stratified epithelia. The K5–Tert transgenics show normal stratified epithelia in the absence of any treatment. Upon skin carcinogenesis, however, K5–Tert mice show a higher incidence of skin papillomas and carcinomas than corresponding wild-type controls (89). The K5–Tert epithelia also show a faster rate of wound healing and a higher reactivity to mitogens (89).

Taken together, these results suggest that high telomerase activity favors tumor growth even in the presence of long telomeres. These studies also have important implications for the use of telomerase in gene therapy of age-related disease states.

V. DNA REPAIR AND TELOMERIC FUNCTION

A. Telomerase-Deficient Mice with Short Telomeres Are Hypersensitive to Ionizing Radiation

Double-strand breaks (DSBs) are generated by reactive by-products of oxygen metabolism, by exposure to ionizing radiation, and in V(D)J recombination in lymphocytes. Efficient DSB DNA repair machinery eliminates these breaks, which might otherwise cause increased death or tumorigenesis. There is increasing evidence that short telomeres result in increased organismal sensitivity to ionizing radiation. Short telomeres render yeast and *Caenorhabditis elegans* more radiosensitive. Based on work in yeast, it has been proposed that short telomeres may directly impair the efficiency of DSBs generated by ionizing radiation, since they may be the storage sites for DNA-repair proteins (90,91), although this has not been formally addressed to date. Terc$^{-/-}$ mice with progressively shorter telomeres provide a unique system with which to address whether there is a causal relationship between short telomeres and radiation sensitivity in mammals; whether telomerase, per se, influences radiation sensitivity, even in the presence of long telomeres; and whether short telomeres result in defective DSB DNA repair in mammals.

Two independent studies using Terc$^{-/-}$ mice have revealed that telomeres affect organismal sensitivity to ionizing radiation (92, 93). Wong et al. (93) have shown that late-generation Terc$^{-/-}$ mice are less resistant to single radiation doses of gamma rays (7 Gy), and Goytisolo et al. (92), using fractionated gamma ray doses (1.75 Gy per dose), have demonstrated that late-generation mice are similarly radiosensitive. This increased radiation sensitivity of Terc$^{-/-}$ mice with short telomeres has been associated with increased apoptosis as well as with increased chromosomal instability as a consequence of irradiation (92). Intriguingly, no defect in the nonhomologous end-joining DNA-repair machinery was found in either study, suggesting that the major DNA-repair pathway in mammals is not affected in late-generation Terc$^{-/-}$ mice. Similarly, the frequency of homologous recombination, measured as a sister chromatid exchange frequency, was normal or slightly elevated in late-generation Terc$^{-/-}$ mice (92). These results indicate that short telomeres are one of the biological determinants of radiation sensitivity, and that telomeric dysfunction nonetheless does not affect the efficiency of the major NHEJ and HR recombination DNA-repair pathways, invoking the existence of a defect in a different and still to be determined repair pathway. Similarly, in humans, McIlrath et al. have also described a reverse correlation between the telomeric length of a radiosensitivity (94).

Transformed fibroblasts expressing Myc/Ras^{v12} from late-generation Terc$^{-/-}$/Ink4a$^{-/-}$ mice with short telomeres and no telomerase activity are also hypersensitive to chemotherapeutic drugs of the doxorubicin family, which cause double-stranded breaks (95). In fact, a strong correlation was found between the chemosensitivity of these cells and the number of end-to-end fusions. When Terc was reintroduced into these fibroblasts, chemoresistance was regained. Importantly, this study described that the chemosensitivity of the G6 Terc$^{-/-}$/Ink4a$^{-/-}$ was dependent on the status of the p53 protein. In particular, if the transformed cell lines were p53 null, chemosensitivity appeared much later than in similar p53-positive cells and was not rescued by reintroduction of telomerase (95). These results are in accordance with the fact that p53 appears to be the key mediator of telomeric dysfunction in mammalian cells (85)

B. Essential Role of DNA-Repair Proteins at the Telomere

Recent studies in mammals have shown that DNA repair proteins such as Ku and the Mre11/Rad50/Nbs1 complex are components of the mammalian telomere. In addition, Ku and DNA–PKcs prevent end-to-end fusions independent of the length of TTAGGG repeats and of the integrity of the G-strand overhang, suggesting that they contribute to the functionality of telomeres by still to be defined mechanisms (9,11,96,97).

VI. FUTURE DIRECTIONS

Knocking out telomerase activity from mice has provided a wealth of information on the role of telomerase and telomeres in such important aspects of human health as aging and cancer. There are a number of aspects of human aging, such as neurodegenerative disorders and cardiovascular disease, which have not been addressed in telomerase knockout mice as yet. Future, more detailed studies of mice with short telomeres should try to address these issues carefully.

New mouse models overexpressing telomerase activity in specific tissues also should be developed to address the role of telomerase activity in tumorigenesis. Only after a clear understanding of the role of telomerase in aging and cancer has been established will it be possible to design telomerase-based therapies for these processes.

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Animal Models of Oxidative Stress and Aging

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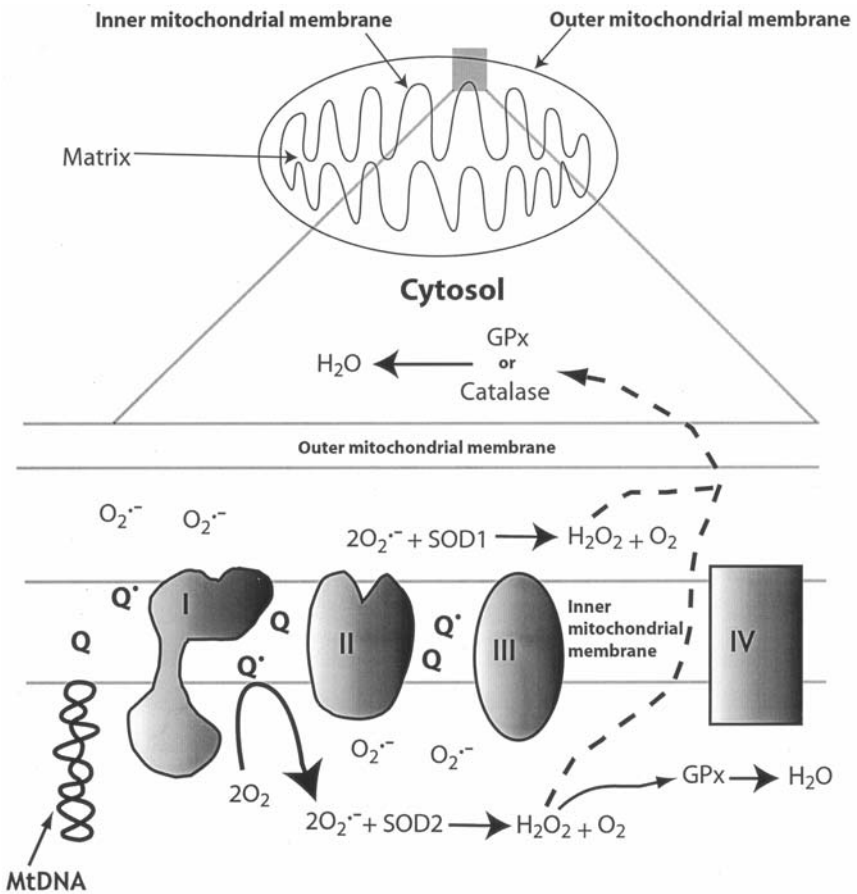
I. INTRODUCTION

The free radical theory of aging, originally proposed in 1956, hypothesizes that aging is a result of an accumulation of oxidative damage to macromolecules by free radicals produced during normal metabolism (1). Mitochondria are the major source of reactive oxygen species (ROS) within the cell and are essential for maintaining cellular homeostasis. Therefore, it has been proposed that a loss of mitochondrial function with age potentially contributes to the degenerative processes of aging. Mitochondria contain their own genome that typically encodes 13 proteins of the respiratory chain, as well as their own ribosomal RNAs and tRNAs. The exact number of proteins encoded by the mitochondrial DNA (mtDNA) varies among species. mtDNA is in close proximity to the site of production of ROS, and therefore, it has been hypothesized that mtDNA unusually will be prone to an accumulation of oxidative damage, and consequently might accumulate potentially deleterious mutations during aging. It is speculated that these mutations in turn may lead to many of the degenerative changes associated with aging. This theory was originally proposed by Miguel in 1980 and further elaborated on by others (2–6).

Each mitochondrion within the cell contains multiple copies of the mitochondrial genome. MtDNA encodes genes for proteins that are required for the production of adenosine triphosphate (ATP) through oxidative phosphorylation (Fig. 1). There are four holoenzyme complexes that facilitate the transfer of electrons from reducing equivalents to water during oxidative phosphorylation. Complex I (NADH-UQ oxidoreductase) is the largest of these complexes, containing



Nuclear encoded	36	4	10	10
MtDNA encoded	7	0	1	3

at least 43 subunits (7,8), although the final structure has yet to be determined. Complex II (succinate dehydrogenase) is entirely nuclear encoded and is composed of 4 subunits (9), complex III (ubiquinone–cytochrome *c* oxidoreductase) is composed of 11 subunits, only 1 of which is mitochondrial encoded (10), and complex IV (cytochrome *c* oxidase) has 3 mitochondrial encoded subunits and 10 nuclear encoded subunits (11). Oxygen radicals produced as a by-product of normal respiration can theoretically damage mtDNA, perhaps resulting in a reduction of necessary components of the oxidative phosphorylation machinery and thus a reduction in the amount of net ATP produced. This theory, espoused over the last decade by numerous investigators, states that this creates a "vicious cycle," whereby increased oxidative stress arising from mtDNA mutations engenders more mtDNA mutations, which further impairs mitochondrial function and eventually results in many of the degenerative changes of aging (6,12,13). It is clear that this hypothesis is intellectually appealing, primarily because mitochondria occupy an undisputed central role in maintaining cellular homeostasis, and eukaryotes have an absolute requirement for optimal mitochondrial function in order to survive. Although this theory is intellectually attractive, careful review

Figure 1 Mitochondrial Production of the Reactive Oxygen Species. This figure has been simplified for clarity. A Roman numeral represents each respiratory chain complex, and mtDNA is depicted in its supercoiled form attached to the inner membrane. O_2 = superoxide, Q = ubiquinone, Q = ubisemiquinone, GpX = glutathione peroxidase. For a full description of the process of oxidative phosphorylation, see Ref. 10. Briefly, reducing equivalents such as NADH or succinate donate electrons at either complex I or II. Sequential electron transfers take place using redox components of each of the complexes of the respiratory chain (complexes I–IV), which ultimately lead to the reduction of oxygen to water concomitant with the production of ATP at the ATP synthase (not shown) through utilization a chemiosmotic proton circuit. Each of these complexes is composed of nuclear and mitochondrially encoded subunits with the exception of complex II. Complex I (NADH–UQ oxidoreductase), the largest respiratory complex (at least 43 subunits) is also believed to be a major site of production of superoxide (Ref. 93). Complex II (succinate dehydrogenase) contains four subunits as well as a cytochrome *b* of unknown function. Mutations in this cytochrome in *C. elegans* cause increased oxidative stress and a shortened life span (Refs. 60, 61, 65). Complex III (ubiquinone–cytochrome *c* oxidoreductase) is composed of 11 subunits and also is believed to be a site of ROS production within the mitochondria (Ref. 94). Complex IV (cytochrome *c* oxidase) is composed of 13 subunits. Ubiquinone (Q) is a major electron carrier within the respiratory chain and is present at high levels within the inner mitochondrial membrane (Ref. 95). When reduced, it forms ubiquinol (Q), which can donate electrons to oxygen present within the mitochondria. This forms superoxide, which can be detoxified by SOD2 in the matrix to yield hydrogen peroxide, which is free to diffuse out of the mitochondria owing to its uncharged nature. Recent data indicate that SOD1 is present in the intermembrane space (V. Culotta, personal communication), indicating that superoxide must exist in this location as well.

demonstrates that although there are some intriguing correlations with aging for the incidence of mitochondrial DNA mutations, there is little evidence for a central role in the aging process (14). Perhaps the best test for evaluating this hypothesis is an animal model with high levels of mtDNA mutations and an examination of tissues from this animal for evidence of oxidative stress. Such a model has recently been reported (15,16), and we will summarize their findings later in this chapter.

Mitochondrial proteins, lipids, and DNA are all targets of oxidative stress, and damage to any one of these components could result in an increase in damage to other constituents of the mitochondria. Research into the role of oxidative modifications to these three components of the mitochondria in aging has been the focus of research in many laboratories (for a comprehensive review of this topic, see Ref. 17). This chapter will review current data concerning the potential targets of oxidative damage within mitochondria in animal models, endogenous and exogenous defenses against endogenously produced reactive oxygen species, and the role that this oxidative damage may play in aging.

II. OXIDATIVE STRESS AND AGING

The free radical theory of aging has consistently lacked direct, definitive support. This primarily has been due to the failures of many intervention studies. The theory lost considerable standing among researchers during the early 1990s, particularly in the light of the then growing evidence that specific genetic loci can influence the rate of aging in model organisms such as *Caenorhabditis elegans.* In fact, by the mid-1990s, prominent researchers in the molecular gerontological community voiced the opinion that specific genetic regulatory mechanisms, and *not* oxidative mechanisms, were the likely place to look for the control of aging, and that antioxidant interventions would never forestall aging. The model that then gained adherents postulated specific regulatory mechanisms rather than entropic processes. More recently still, a synthesis of the two views has emerged, as some of the *C. elegans* genes implicated in the aging process (sometimes called "gerontogenes") have revealed themselves as being components of regulatory networks for antioxidant enzymes (18,19). Aging research may be at a point of synthesizing these two disparate perspectives (entropic decay versus specific genetic control).

Ongoing genetic and genomic approaches undoubtedly will continue to define genetic mechanisms involved in the control of oxidative stress. Recent studies have emphasized the utility of model organisms for studying the consequences of oxidative stress, as well as identifying the most relevant sources of oxidative stress within the cell. Specifically, animal models of oxidative stress have been used extensively to test the oxidative stress theory of aging.

III. CORRELATIONS OF OXIDATIVE STRESS AND AGING

The hypothesis that oxidative stress is a major factor in the aging process has existed for more than 40 years (1). During this period, much correlative data were reported that lent support to the idea that cumulative damage inflicted by endogenous pro-oxidants is deleterious to a cell or to an organism's ability to maintain homeostasis (17). For example, a number of interesting findings have recently been reported in relation to specific protein and carbonyl modifications with age (20,21). In *Drosophila,* carbonyl modifications have been found to accumulate with aging (22,23). In addition, specific carbonyl modification of mitochondrial aconitase and the adenine nucleotide translocator (ANT) have been reported with age (20,21). In addition to model systems such as *Drosophila* and the mouse, protein oxidation has been described immunohistochemically in tissue sections of aged human brain in association with lipid peroxidation and proteins indicative of stress (24–27).

Much work has been done correlating the differential production of ROS from mitochondria with life spans of different species (28–36). Although there are broad correlations in the level of ROS produced from isolated mitochondria relative to the maximum and mean life span, there are a number of notable exceptions to this generalization (37,38). Perhaps the best-known example is the comparison of nonpasserine birds to rats (39). Pigeons and rats have high metabolic rates that are approximately equivalent, yet pigeons live about three to five times longer than the rats. Mitochondria isolated from each species and compared for ROS production do not show equivalence, as might be expected based on the metabolic rate (38). Instead, isolated mitochondria from a variety of tissues of the pigeon have approximately two- to four-fold lower levels of ROS production than the rat (38). Hence, ROS production and its effects must be taken into account in addition to the metabolic rate in testing the free radical theory.

IV. GENETIC MODELS OF OXIDATIVE STRESS AND AGING

Recently, genetic studies using invertebrates and the mouse have provided some compelling arguments that oxidative stresses are a major factor limiting the life span (40–47). The mean and maximum life spans of many organisms have been extended through genetic manipulation. Both classic and reverse genetic (transgenic) approaches have been used to identify genes involved in influencing the life span. Many of these experiments have increased our understanding of the role of oxidative stress in aging, and we have focused on these models in this chapter. In terms of oxidative stress and aging, there are two obvious pathways through which genetic manipulation of an organism might increase its life span—decreas-

ing the level of ROS and/or increasing the level of defense against ROS. Alternatively, the issue of whether increasing the levels of ROS and/or decreasing defenses against ROS of an organism result in some form of accelerated aging is relevant to understanding the role that oxidative stress may play in aging.

Overexpression of genes that are responsible for neutralizing the superoxide free radical (O_2) have shown that the mean and maximum life span of *Drosophila melanogaster* can be increased, indicating that endogenous superoxide production limits the life span in the fly (44,45). In addition, recent work using *C. elegans* demonstrates that one of the major mechanisms that limit life span in *C. elegans* occurs through signal transduction modulating oxidative stress responses (18,19). A recently published study using synthetic catalytic antioxidant compounds to extend the life span in *C. elegans* also provides support to the notion that oxidative stress is a major limiter of the life span in multicellular organisms, and that appropriate antioxidant intervention can forestall and extend the life span (48). Overall, these results demonstrate the utility of *C. elegans* for the study of oxidative stress and aging.

This utility is well recognized, with "the worm" being much used for more than a decade to study the genetics of aging (49–52). Its small size, rapid life cycle, well-characterized genetics, and defined cell lineage make it an excellent choice for testing aging hypotheses. In addition, it is the first multicellular organism to have its genome completely sequenced (53). A number of well-characterized mutants that lengthen life have been identified in *C. elegans,* which by definition implicate those genes in senescence (49,52,54,55). Most of these genes fall into the dauer pathway, which is an alternate developmental pathway induced by hardship, food scarcity, or overpopulation (56). Mutations in some genes within this pathway increase the adult life span of *C. elegans* by as much as 100%. However, until recently, the emphasis has been on characterizing the genetics of these mutants rather than on the unique physiology that may arise as a result of the mutation.

The role that oxidative stress may play in life span extension was first tested in *age-1,* which was the first identified single-gene mutation that resulted in an increased life span of multicellular organism. The *age-1(hx546)* mutation in *C. elegans* was isolated using a classic genetic approach and results in a 65% increase in the life span (49,57). Several studies have been published that link oxidative stress resistance with the AGE-1 life-extension phenotype. The mutant strain has increased levels of the ROS scavenging enzymes superoxide dismutase (SOD) and catalase (42) and has increased thermotolerance and resistance to hydrogen peroxide and superoxide when compared with the wild type (42,43,58). In addition, the *age-1* mutant sustains a reduced amount of mtDNA deletions with age compared with the wild type, which suggests either improved mitochondrial DNA repair, or alternately, slower aging (59). Together, these results suggest that the life span extension of the *age-1* mutant is a result of an increased tolerance for oxidative stress.

Another mutant of *C. elegans, mev-1,* is mutant for the cytochrome *b* subunit of the respiratory chain, which presumably prevents the efficient passage of electrons from complex II to complex III via ubiquinone (60,61). Phenotypically, this has the effect of reducing the mean life span of these worms by about 25% and impairing their ability to survive at increased partial pressures of oxygen (60). Carbonyl modifications to proteins, a product of metal catalyzed oxidation, have been shown to accumulate with age in different organisms (20,21,62–64). Adachi and coworkers have demonstrated that isogenic strains of *C. elegans* with different life spans accumulate carbonyl modifications to proteins at a rate that correlates with the life span (65). Total carbonyl content of wild-type N2, long-lived *age-1* and short-lived *mev-1* was assessed. As previously described, the *age-1* mutant has an increased life span and elevated levels of catalase and SOD. In addition to the shortened life span, the *mev-1* mutant has a 50% reduction in SOD (60). The fact that the life span and free radical scavenging enzymes inversely correlate with the level of carbonyl content again strengthens the argument that altering the capacity of an organism to defend against ROS is critical in the determination of the life span.

A more direct test of the link between oxidative stress and aging comes from transgenic studies in *Drosophila melanogaster.* In an initial study by Orr and Sohal, the life span in *Drosophila* was apparently increased by overexpressing SOD1 and catalase together (66). However, upon careful examination of the survival data, this study was found to be statistically lacking when compared with survival studies on the transgenic lines, and its conclusions were called into doubt (67). More recent studies have taken a rigorous statistical and genetic approach to test the idea that innate oxidative stresses that arise through normal metabolism are a major limiter of life span. Sun and Tower have demonstrated that an increase in the level of SOD1 (the cytosolic form of SOD) results in a significant increase in the life span in *Drosophila* (45). They exploited a transgenic technique that allowed for inducible expression of the SOD1 transgene. This was important, because expression of the transgene could be induced at a specific time to determine its effect on aging but not on development. Therefore, the fact that overexpression of SOD1 increases the life span, independent of developmental effects, provides compelling evidence that the oxidative damage by superoxide is an important determinant of the life span. Moreover, the researchers evaluated levels of metabolism in terms of oxygen consumption and found no decrease in the metabolic activity of transgenic flies compared with controls, indicating that the extension of life was achieved without metabolic cost (J. Tower, personal communication). Similar experiments by Parkes and colleagues have demonstrated that adult motor neuron–specific overexpression of SOD1 extends the life span of flies by 40% (44). Even more recently, the same group demonstrated that the life span of wild-type flies can be extended by 30% by overexpression of SOD2 (the mitochondrial form of SOD), and expression of SOD2 in an SOD1-null fly par-

tially rescues the reduced life span of the SOD1 mutant (68). Because the transgenic SOD2 resides almost completely in the mitochondria, these results suggest that the function of SOD1 must be, at least in part, to detoxify mitochondria-generated superoxide. Tower's group also has shown that not only can the life span be increased through overexpression of the cytosolic SOD1 but also by overproduction of the mitochondrial SOD (J. Tower, personal communication).

Perhaps due to the difficulties in using genetic screens to identify genes that regulate the life span in mammals, no life-extending mutations have been identified in mammals using classic genetic approaches. However, using a reverse genetic approach to delete the $p66^{shc}$ gene from mice (69), it has been determined that loss of this gene results in an increased life span and stress response with no other detectable abnormal phenotypes. In wild-type cells exposed to oxidative stress, $p66^{shc}$ is phosphorylated at serine 36, and cells lacking this gene or containing an alanine in place of the serine are more resistant to oxidative stress. Therefore, the normal function of this protein somehow represses the cell's ability to resist oxidative stress, and by doing so, limits the life span. For some time, it has been known that mutation of a single gene in invertebrates can also increase the oxidative stress resistance and life span of a mammal. It will be important to know whether these $p66^{shc}$ mutant mice sustain a decreased level of oxidative stress to macromolecules throughout their life span.

An alternative to developing model organisms with increased life spans is to use genetic approaches to develop models with reduced life spans. Clearly, there are many genetic alterations that can lead to premature death, with no relevance to the aging process. However, the fact that overexpression of ROS scavenging enzymes has the potential to extend the life span suggests that mutations that increase the production of ROS or decrease defense mechanisms against ROS may accelerate the aging process, and thus result in decreased life spans.

One approach to investigating the factors that limit the mammalian life span has been the selective breeding of mice with a more rapid onset of senescence and a decreased life span. These mice are known as the senescence-accelerated mouse (SAM) strain and have a 27% reduction in their life span compared with the SAMR (senescence-resistant) mice (70,71). Evidence that the reduced life span and accelerated senescence of SAM is a result of oxidative stress has come partly from the demonstration that the free radical spin-trapping agent *N-tert*-butyl-α-phenylnitrone (PBN) significantly increases the life span of SAM (72). In addition, there is evidence of mitochondrial dysfunction in SAM compared with SAMR (73). These results suggest that an increase in oxidative stress is at least partially responsible for the decreased life span of SAM.

Using reverse genetic approaches, mutations have been engineered into mouse genes encoding ROS scavenging enzymes or important components of the mitochondrial energy production machinery. There are three isoforms of SOD: *sod1,* a cytosolic Cu/Zn SOD; *sod2,* a mitochondrial MnSOD; and *sod3,* an ex-

tracellular Cu/Zn SOD. Comparing and contrasting the inactivation of these three isoforms of superoxide dismutase in the mouse highlights the importance of scavenging mitochondrial superoxide. Inactivation of *sod1* or *sod3* via homologous recombination in mice results in mild, nonlethal phenotypes (74,75). In marked contrast, inactivation of the mitochondrial SOD results in a neonatal lethal phenotype, characterized by dilated cardiomyopathy and fibrosis, neurodegeneration, metabolic acidosis, hepatic fat accumulation, DNA oxidative damage, tissue-specific mitochondrial respiratory chain abnormalities, and abnormalities in tricarboxylic acid cycle enzymes (40,46,47).

Another mouse mutant of mitochondrial oxidative stress is the adenine nucleotide translocator mutant mouse (ANT1). This protein is responsible for translocating ADP into the mitochondria while exporting ATP out of the mitochondria after oxidative phosphorylation has occurred. Hence, ANT is responsible for ensuring the availability of substrate for the ATP synthase. As electron flow through the respiratory chain will not occur without phosphorylation of adenosine diphosphate (ADP) at the ATP synthase, ANT indirectly facilitates passage of electrons through the respiratory chain. Inactivation of the heart/skeletal muscle isoform (ANT1) in mice through homologous recombination results in a mild myopathy associated with mitochondrial proliferation in the skeletal muscle (76). Owing to the inability of the mitochondria to obtain substrate (ADP), electrons stall throughout the respiratory chain, resulting in the reduction of the electron transport chain. This increases the propensity for the mitochondria to make superoxide. This is observed through an induction at the mRNA level of *sod2* in skeletal muscle and a parallel four- to eightfold increase in the production of hydrogen peroxide from isolated mitochondria of the mutant mice compared with controls (77).

To test whether mtDNA mutations result in oxidative stress and disease, Zhang and coworkers engineered transgenic mice that overexpress a proofreading–deficient mitochondrial DNA polymerase from a heart-specific promoter (16). This resulted in detectable levels of mtDNA mutations by 7 days of age and profound cardiomyopathy by 4–5 weeks of age. Interestingly, there was no difference in DNA, RNA, and protein content in the mitochondria, no decrease in mitochondrial respiratory function, and no increase in DNA and protein oxidative adducts in transgenic hearts (15, 16). Especially striking in this model are the observations that even though many cells have high levels of mtDNA mutations, at the cellular level, there was no evidence of carbonyl modifications through immunohistochemical staining (15). In relation to the "vicious cycle" theory of mtDNA mutations and oxidative stress (see Sec. I), this implies that either the types of mtDNA mutations induced by overexpression of the mutant mtDNA polymerase cannot give rise to oxidative stress, or that there are synergistic factors present in old tissues that enhance the likelihood that there will be oxidative stress in conjunction with mtDNA mutations. The most conservative explanation

is that these mutations do not give rise to oxidative stress. However, it is clear from this model that such mutations can give rise to severe pathology, and so the physiological consequences of high levels of heterogeneous mutations are not in doubt. The authors suggest that the increase in mtDNA mutations induces a signaling pathway between the mitochondria and the nucleus that has pathological results but does not increase the level of oxidative stress. It is possible that this response is impaired with age (as are stress responses in general), and hence, in some contexts, mtDNA mutations may give rise to an oxidative stress.

V. SYNTHETIC ANTIOXIDANTS AND OXIDATIVE STRESS

As there is a credible and substantial body of evidence implicating oxidative stress as a major factor in aging, are there therapeutic approaches that can be used to modulate endogenous oxidative stresses and thereby further test the free radical theory as well as point toward potential treatments in humans? A variety of studies have been carried out with regard to ameliorating or attenuating aging or the progression of disease by chronic or acute antioxidant treatments (78–86). Such studies have yielded, at best, equivocal or mildly beneficial results with regard to slowing disease progression or improving the mean or maximal life span (85). Chronic dietary supplementation of antioxidants has either failed or met with very limited success in extending the life span or attenuating the rate of physiological decline in organisms from *C. elegans* to humans (80,82–86). The antioxidants used in previous studies (such as vitamin E) are not particularly effective antioxidants compared with more efficient *catalytic* antioxidants in a number of acute and chronic studies (87). Hence, this may explain the preciousness of previous results that have attempted to manipulate the life span through dietary supplementation with antioxidants (85).

Eukarion (Bedford, MA) has developed a class of small molecular weight molecules that scavenge ROS. These molecules are salen–manganese complexes, believed to act as SOD and catalase "mimics," catalytically destroying superoxide and H_2O_2 (87,88). The "EUK" (from Eukarion) class of compounds is effective in a wide variety of experimental models of oxidative stress. Compared with vitamin E, EUK-8 effectively inhibits lipid peroxidation in a brain microsome/ROS system (87). Further, EUK-8 has been shown to be effective in two mouse models of Parkinson disease, protecting nigrostriatal dopaminergic neurons from damage induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) or 6-hydroxydopamine (87). EUK-8 also has shown efficacy in a cell culture model of Alzheimer disease, protecting hippocampal slices from the cytotoxicity of β-amyloid peptide (89). Compared with EUK-8, EUK-134 (an analog of EUK-8) has increased catalase activity and has equal SOD activity (90). It has been shown to confer a high degree of protection in a rat stroke model, an MPTP mouse model,

and in kidney ischemia reperfusion–induced injury (90,91). As both EUK-8 and EUK-134 readily cross the blood–brain barrier in pharmacologically active amounts, they are attractive candidates for testing their efficacy in inhibiting mitochondrial superoxide in the brain in addition to peripheral organ systems.

Other antioxidants that may be useful in this regard have been specifically targeted to the mitochondria via a novel approach using a lipophilic triphenylphosphonium ion. This ion is widely used for measuring the membrane potential in mitochondria (10). Lipophilic cations are selectively taken up into the mitochondria through virtue of the membrane potential of mitochondria. In an ingenious strategy, Murphy and colleagues have linked a variety of antioxidants to the triphenylphosphonium ion, thereby facilitating the delivery of antioxidants to mitochondria, the source of most ROS within the cell (92). They have demonstrated the efficacy of such targeted antioxidants, as well as their presence in liver mitochondria of rats stressed with iron/ascorbate. The mitochondria treated with the targeted antioxidant had significantly less lipid peroxidation and protein damage as assessed by protein carbonyls. Moreover, the antioxidant was taken up into the mitochondria more than 80-fold over endogenous levels (92).

Illustrating a direct test of the efficacy of antioxidants against the pathologies of endogenous oxidative stress, mice that are nullizygous for *sod2* (40,46) were treated with a catalytic antioxidant in an attempt to ameliorate the pathologies that arise owing to a lack of SOD2 (47). Inactivation of the mitochondrial form of superoxide dismutase via homologous recombination in the mouse is neonatal lethal, showing the serious consequences of endogenous mitochondrial superoxide production in a mammal. SOD2 nullizygous mice treated with effective catalytic antioxidants increased their mean and maximum life span, with concomitant rescue of a number of biochemical and physiological indicators of oxidative stress (46). This included rescue of a dilated cardiomyopathy and a severe accumulation of lipid within the liver. Taken together with the results of studies that have used antioxidants to extend the life span in the nematode *C. elegans* (discussed above), these results imply that in pathological states caused by oxidative stress, appropriate intervention can have highly successful outcomes. Future studies with additional compounds in nematodes and mice may further our understanding of the targets of oxidative damage in pathology and aging.

VI. FUTURE DIRECTIONS

Although much work has been done to correlate oxidative stress with aging and a variety of pathological states, we are only just beginning to use the tremendous potential of animal models to elucidate cause and effect in oxidative stress and aging. In addition to animal models, new technologies such as microarrays and proteomics will facilitate identification of relevant and novel targets of oxidative

stress in acute (SOD2 nullizygous mice) as well as chronic states (aging). Although it has been nearly 50 years since the original hypothesis was proposed linking oxidative stress to aging, in the near future we may be able to design experiments that will either disprove the hypothesis or provide definitive evidence of its merits.

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Conclusions and Future Directions

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I. INTRODUCTION

In this book, we have taken a broad view of chromosomal instability and its relationship to aging. The study of chromosomal instability in the traditional sense refers to nucleic acid damage and the cellular systems devoted to its repair. Many of the phenotypes of aging are not simply related to nuclear DNA damage associated with replication. If this were so, one would expect the appearance of age-related phenomena first and foremost in rapidly replicating tissues. Although it can be argued that skin and hair show rapid turnover and early signs of aging, this is highly variable. At least part of the variability arises from the stochastic nature of the initiating events. The hair-graying trait is eventually universal, affecting men and women and all ethnic groups, and extends to many mammals. On average, 50% of persons by age 50 years have 50% gray hair (1). Pigment loss is caused by loss of active melanocytes in the hair bulb and incorporation of fewer melanosomes into the hair shaft cortex. It has been suggested that melanogenesis may produce large amounts of ROS, and that melanocytes in graying and white hair bulbs show cellular changes of oxidative stress (see Ref. 2 for a comprehensive review). Graying hair, however, does not proceed in a linear fashion, occurs relatively synchronously, and usually follows a particular anatomical distribution, suggesting the need for cooperative events with memory to cross a certain cellular threshold. Furthermore, the failure of rapidly replicating cell populations in the bone marrow and in the intestinal crypts is rather uncommon in the older population compared with

the prevalence of arthritis, hearing loss, diabetes, hypertension, and neurodegenerative and cardiovascular diseases. Perhaps one of the best arguments for the role of classic chromosomal instability playing an important role in human aging has been the study of premature aging syndromes with many features of normal aging, including increased cancer risk. These syndromes, including ataxia-telangiectasia and the Rothmund–Thomson, Cockayne, and Werner syndromes, are sufficient to cause many, if not all, features of premature aging, and their effects are mediated by genomic instability. In these human diseases, where damage to nucleic acid appears to be central, the mechanisms are being rapidly elucidated as presented in this volume, but the detailed connections upstream and downstream remain obscure. Nevertheless, these syndromes strongly suggest that chromosomal instability may be an underlying basis for the striking age-dependent risk of developing cancer, as well as for some aspects of the aging process itself.

Some of the most important clinical changes associated with aging occur in post-mitotic, nondividing cell populations in the nervous system and in muscle. For example, in Parkinson's disease (PD), there is a strong increase in its prevalence with age attributed to the linear decline of dopaminergic neurons. PD does not become clinically apparent until a certain threshold (50–60%) of midbrain dopaminergic neurons are lost, depleting the striatum of 80–85% of its dopamine content. The high cellular energy requirements of brain and muscle along with the production of reactive oxygen species by mitochondria suggest that these tissues may be particularly vulnerable to oxidative stress. Reactive oxygen species damage both nuclear and mitochondrial DNA. These changes accumulate with aging, and have been documented in neurodegenerative diseases including PD. Mice have been created with a targeted deletion of either manganese–superoxide dismutase (SOD2), a free radical scavenging enzyme or of an adenine nucleotide transporter 1, a mitochondrial membrane protein important in ATP/ADP exchange (see Refs. 3–6 and Chap. 23). The SOD2-deficient mice are more sensitive to a drug known to induce Parkinsonism, and they develop cardiomyopathy and lipid deposition in liver. The ANT1-deficient mice develop mitochondrial myopathy and hypertrophic cardiomyopathy. Both show significant increases in mitochondrial DNA rearrangements, providing compelling evidence that oxidative stress can induce mitochondrial DNA damage and further mitochondrial dysfunction (reviewed in Ref. 7). The roles of more recently described types of chromosomal instability, such as alterations in gene silencing and telomeric shortening, are just now being elucidated in yeast, cultured cells, and animal models. The relationship of these phenomena to human aging at the level of the whole organism or in populations remains an open question.

II. IS THERE A FINAL COMMON IF NOT UNIVERSAL PATH(S) RESULTING IN AGING?

To return to the third question (How do we age?) at the beginning of this book, it is now possible to address some long-standing questions in light of recent exper-

imental evidence. No single theory of aging to date has been able to account for all observed phenomena in invertebrates and mammals. Additional complications arise if one attempts to construct a theory of aging that encompasses phenomena such as extrachromosomal ribosomal DNA circle formation, which is sufficient to cause aging in yeast (8) but has not been observed in other species. Indeed, not all of the cellular damage accumulating with aging may be a consequence of nucleic acid damage. For example, proteins are also subject to chemical modifications with aging, and the life span may be extended in *Drosophila* by overexpression of a protein repair methyltransferase. This effect is dependent upon the ambient temperature—it is observed at 29°C but is abolished at 25°C (9).

Work by many investigators, however, has characterized an insulin/insulin like growth factor 1 (IGF-1) endocrine system that regulates the life span (reviewed in Ref. 10). Because this pathway is conserved, and mutations in some genetic elements of the pathway can increase the life span in *Caenorhabditis elegans,* yeast, *Drosophila,* and possibly mammals, this strongly supports an evolutionarily conserved mechanism that participates in the regulation of the life span. This pathway was first discovered in *C. elegans,* and is related to dauer formation (11). Under conditions of crowding or limited food, juvenile *C. elegans* enter a diapause state called dauer, characterized by developmental arrest, resistance to oxidative stress, inability to reproduce, storage and metabolism of fat, and prolonged survival. When environmental conditions become favorable, the worms exit the dauer state, become adults, and reproduce, thereby increasing the chances of survival for their progeny. A fascinating observation is that mutations that lower the level of daf-2, an insulin/IGF-1 receptor homolog, or mutation in age-1, a presumptive cytosolic phosphatidylinositol 3 kinase, cause the animal to remain youthful for an extended period and to live more than twice as long, although there is a very wide distribution of ages of death (12,13). Genetic studies to identify its mechanism of action have shown that daf-2 activates a phosphatidylinositol-3-OH kinase/3-phosphoinositide–dependent kinase-1/Akt signal transduction pathway, which in turn downregulates the activity of daf-16, a forkhead/winged–helix transcription factor family member. daf-16 subcellular localization can change in response to environmental cues, so that starvation, heat, and oxidative stress cause daf-16 to switch from diffuse cytoplasmic localization to nuclear localization (14). Old-1 was discovered as a positive regulator of the life span by overexpression in transgenic worms and encodes a putative transmembrane tyrosine kinase. Recently, evidence has been presented that daf-16 mediates transcription of old-1(15), raising the question of whether old-1 is the critical target for life span modulation by daf-16, and whether there are specific ligands and targets for old-1.

An additional consideration is which cell lineages are critical to the observed effects. The daf-2 pathway is capable of acting non-cell autonomously (signaling to cells not expressing the receptor) to regulate the life span. Studies with mosaic animals show that expression of daf-2 and age-1 restricted to neurons is as effective as ubiquitous expression in affecting the life span (16,17). The

central role of neurons in non-cell autonomous regulation of life span has also been demonstrated by studies showing that expression of the human antioxidant enzyme superoxide dismutase gene in the motor neurons of *Drosophila* can also effectively lengthen the life span (18). In the best-studied metazoan genetic systems, that is, *C. elegans* and *Drosophila,* the biochemical studies lag behind these elegant genetic studies. This limits a comprehensive view of the process of aging.

When one realizes that there are significant parallels between the insulin/IGF-1 systems in the fly and worm, including life span extension in certain *Drosophila* insulin/IGF-1 receptor mutants (19); that certain pituitary hormone–deficient strains of dwarf mice live longer than normal mice (20); and that small breeds of dogs typically have lower levels of IGF-1 and live longer than large breeds of dogs (21), we begin to approach a common pathway.

What is known about the effects of mutations in the insulin/IGF-1 signaling pathway in mice or in humans? Mice homozygous for disruption of the growth hormone receptor/binding protein (GHR/BP) gene display small size, low IGF-1 levels and increased life span compared with their wild-type or heterozygous littermates (22,23). The GHR/BP null mice are a small animal model for a human disease, Laron syndrome, reported in 1966 (24). Persons with Laron syndrome are short with truncal obesity, delayed puberty, high levels of growth hormone, and low serum IGF-1 with an underlying defect in the GHR. But do they live longer? In Laron's cohort of Israeli patients, the oldest patient was 70 years old in 1999, making it too early to answer this question with certainty (25).

An important consideration is that many organisms with a mutation of the insulin/IGF-1 pathway have additional traits such as small size, sterility, reduced metabolic rate, or resistance to oxidative stress. Although the ultimate downstream effectors of the insulin/IGF-1 pathway have not been identified, as a means to identifying them, it is useful to ask which of the commonly associated traits can be uncoupled from life span extension. Small size is not essential to longevity, since caloric restriction is effective at increasing the life span in many species of adult animals, and *Drosophila* heterozygous for a null mutation of *chico*, the insulin/IGF-1 receptor, display normal body size and live 36% longer than normal (19). Reduced fertility and metabolic rate can similarly be uncoupled from the life span by studying additional mutations affecting oocyte formation or metabolism. In *C. elegans,* the only trait that has not been uncoupled from longevity is stress resistance. Because antioxidants extend the life span of wild-type *C. elegans* (26) and overexpression of superoxide dismutase extends life span in *Drosophila* (27), it would seem that aging is caused by damage from reactive oxygen species, a theory proposed in 1957 by Harman (28). Thus, although the free radical theory of aging is alive and well, and may be a common mechanism, thus far definitive proof of the hypothesis remains elusive.

In the *chico Drosophila* mutant, longevity is observed, but is not coupled to resistance to reactive oxygen species (ROS) generated by paraquat treatment. In

the best-characterized human mitochondrial diseases, which at the cellular level affect various components of mitochondrial function leading to an increase in ROS, the phenotypic overlap with normal aging is only approximate. Although hearing loss, diabetes, peripheral neuropathy, exercise intolerance, and muscle weakness are seen in both mitochondrial diseases and aging, childhood- or adolescent-onset mitochondrial diseases due to point mutations or deletions in the mitochondrial genome are not associated with the early onset of Alzheimer's disease, Parkinson's disease, cancer, osteoporosis, atherosclerosis, or early graying. The production of ROS has traditionally been thought to act stochastically to result in DNA, lipid, and protein damage. More recently, ROS have been found to play a specific cell-signaling role, and are capable of inducing apoptosis or activating signal cascades through stimulation of growth factor receptors (reviewed in Refs. 29 and 30). How this new view of ROS will be incorporated into the "big picture" of the biological basis of aging remains an unanswered question. One intriguing clue comes from a mouse with a targeted mutation of the $p66^{shc}$ gene (31). The effects appear to be mediated by serine phosphorylation of $p66^{shc}$ in response to oxidative damage. Further, $p66^{shc}$ phosphorylation appears to be part of a signal transduction pathway that is activated by ROS, leads to apoptosis, and thereby eliminates cells injured by oxidative damage. However, phosphorylation of $p66^{shc}$ occurs in response to a variety of ligands in different cell types (32) and presumably may activate partially different signal transduction pathways. Although it has been suggested that $p66^{shc}$ regulates apoptosis in response to oxidative stress, the observations were made in mouse embryonic fibroblasts and may or may not be fundamental to the effects on the life span. Ablation of $p66^{shc}$ expression by targeted mutation has been shown to result in increased resistance to oxidative stress and a 30% increase in the life span. However, this is a puzzling observation. Most laboratory mice die of B-cell lymphomas. One would therefore expect apoptosis to protect against the survival of cells with DNA damage and, therefore, the potential to form neoplasms. More detailed reports of the progression of the aging process, pathology, and causes of death of the $p66^{shc}$ null mice would be valuable.

III. CALORIC RESTRICTION AND AGING

Caloric restriction (30–40% reduction in total caloric intake without malnutrition) has been recognized for over 60 years (33) as a means to extend the life span in many species, including rodents and probably primates. Similar to the dauer pathway in *C. elegans,* caloric restriction increases stress resistance and postpones reproduction. Certain mutations in *C. elegans* affect the ability to eat ("eat" mutants) and the rate of living, including feeding ("clk" mutants). Genetic epistasis experiments indicate that mutations in eat and clk affect the same pathway (reviewed in Ref. 34). In contrast, the daf pathway is independent, because *daf*-2 *clk*-1 double mutants live longer

than either single mutant. Which of these mutants most closely resembles caloric restriction (CR) is not clear. The mechanism of CR has not been elucidated, but one hypothesis is that is that it slows the production of toxic ROS, and thus decreases the accumulation of oxidative damage. Interestingly, some important connections have been recently described among diet, clk-1 mutants, and daf mutants. Coenzyme Q is an essential cofactor that acts as a carrier of electrons and protons across the inner mitochondrial membrane, maintaining the proton gradient driving ATP synthesis. Larsen and Clarke (35) have shown that withdrawl of coenzyme Q from the diet extends the life span of wild-type or *daf* or *clk*-1 mutant *C. elegans.*

CR also draws a connection between reproduction and aging. In the absence of reproduction, suppressed either by adverse environmental conditions, or by ablation of germ cells in *C. elegans* or *Drosophila,* the life span in prolonged. This occurs presumably in an attempt to delay reproduction until a more favorable time in order to ensure the survival of the species. Thus, we begin to see stretching the life span as another potential mechanism of evolution, and successful aging as possibly subject to evolutionary pressure rather than being beyond its effects. It is obvious from an examination of the remarkable ranges of the life span among related species, that given certain appropriate ecological niches, species can indeed evolve new life history strategies, including decreases in rates of aging and substantial enhancements of longevities. Field studies of sibling species have provided strong support for this statement (36). The genomic remodeling that accompanies such enhanced life spans can be presumed to include various "longevity assurance genes" (37). These observations do not obviate significant roles for evolutionary theories of aging that are concerned with different classes of gene action, such as Medawar's suggestion of the accumulation of constitutional mutations that escape the force of natural selection (or the concept of antagonistic pleiotrophy) (38) in which certain alleles are beneficial early in life, prior to reproduction, at the expense of deleterious effects of these same alleles late in life. A mix of these and other classes of gene action (39) will evolve to optimize reproductive fitness in given ecological niches. Given high environmental hazard functions, species will evolve with comparatively short life spans and many offspring (fish, mice). Low hazard environments will favor the emergence of species with comparatively long life spans and few offspring (elephants, humans). The existence of a latent mechanism for increasing the life span may be more prominent in short-lived species. Hypothetically, some of these mechanisms may already have been constitutively activated or maximally exploited during evolution from a short-lived ancestor to their present day long-lived descendents.

IV. FUTURE DIRECTIONS

It would be fair to say that we are in the golden era of aging research. Much has been learned. Perhaps the most surprising fact it that the life span can be greatly

extended in many organisms by mutation in a single gene, and conversely striking segmental progeroid phenotypes can be produced by single-gene mutations in mice and in humans. This suggests that there are a relatively few cellular processes that limit the rate of aging. Although a single, universal cause of aging in all species and in all tissues has not been identified, several candidate pathways with far-reaching implications for aging have emerged. In the short term, questions that may be answered quite soon are: Are the effects of caloric restriction and mutations in the insulin/IGF-1 pathway additive? What about caloric restriction and $p66^{shc}$ ablation? What are the ultimate targets of the daf-16 transcription factor and the old-1 transmembrane tyrosine kinase? Do non-cell autonomous mechanisms govern life span in mammals? Will the critical cell type turn out to be neuronal, as in flies and worms? Given the extreme regional specialization of the brains of higher mammals, will specific neuronal subpopulations have the capability of regulating life span? With the advent of genomic approaches, it is possible to ask: In long-lived animals within a species (centenarians in our own), are there particular polymorphisms associated with longevity? Some obvious candidates would be members of the insulin/IGF-1 signaling pathway, superoxide dismutases, WRN, $p66^{shc}$, apolipoprotein E. In the long term, will it be possible to develop therapies based on these new insights that mimic the actions of some of the beneficial mutations or that provide the benefits of caloric restriction without the necessity of counting calories? The answers will come first in animal models. With the promise of an increased life span comes the important question: What will the quality of the later years be like? In other words, will debilitating diseases of old age be delayed or eliminated? Observations of long-lived roundworms, fruitflies, and mice suggest that the answer may be "yes," but detailed cognitive studies are not possible in lower organisms and are time consuming and expensive in mice and primates. In our own species, there have always been a few individuals who survived to old age, and were revered for this special achievement and its accompanying wisdom. One of the most striking transformations in the last century is how commonplace old age has become, with a near doubling of life span of the average person. In this century, the challenge we face will be finding ways to ensure that the aging population remains healthy, vital, and productive. Only then will the "golden years" be truly golden.

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