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Genetic Instability and Tumorigenesis

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With 12 Figures

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Cover illustration: Diagram of some of the steps involved in modulating genetic changes and cancer development in eukaryotic cells. Cellular stresses can damage cellular targets and contribute to the development of genetic alterations. The DNA can be directly damaged by base-damaging agents or by oxidative stresses breaking the DNA backbone and damage to cellular organelles, such as mitotic spindles, could lead to abnormal chromosome segregation. A wide variety of gene products influence cellular responses to such lesions by controlling signal transduction, DNA repair and/or cell cycle progression. Damaged DNA can then either: 1) lead to cell death; or 2) be repaired; or 3) result in heritable genetic alterations. The appropriate set of genetic alterations in a given cell type can result in a transformed phenotype (i.e. cancer).

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Genetic Instability and Tumorigenesis: Introduction

M.B. KASTAN

Cancer is a disease resulting from alterations of cellular genes which cause phenotypic changes in somatic cells. Usually, when we think about genetic diseases, we think about inheriting one or two abnormal genes from our parents and these gene abnormalities confer the disease phenotype. In contrast, in the majority of cancers, no such inherited gene abnormalities can be identified (which does not mean that they do not exist) and there is no obvious family history suggesting an inherited disease. The vast majority of genes which are altered in the cancer cells are not transmitted through the germ line, but rather become abnormal in somatic cells sometime during the lifetime of the individual. Thus, the critical question which arises is "how do these genetic changes occur in somatic cells?".

Epidemiologic data suggest that exposure to environmental carcinogens contributes to the genesis of at least 80% of all human cancers (DOLL and PETO 1981). Thus, it is natural to suspect that the genetic changes in somatic cells which contribute to the transformed phenotype arise from DNA damage caused by such exposures. Therefore, understanding how cells respond to DNA-damaging agents is likely to be an important component of our understanding of the genesis of human tumors. There are three possible outcomes for a cell following exposure to a DNA-damaging agent: (a) the cell could repair the damage in a timely fashion so that no physiologically significant mutations are passed on to daughter cells; (b) the cell could die (dead cells cannot go on to form tumors) or never replicate again; or (c) a permanent mutation could result by replication of a damaged DNA template or segregation of damaged chromosomes and such mutations would be passed on to daughter cells. Once the appropriate combination of mutations accumulates in a given cell type, a transformed phenotype could result.

Based on these assumptions, the physiologic factors and the gene products which determine which of these outcomes occur following DNA damage would be predicted to be modulators of the tendencies for malignant transformation. Inherited variability in either the metabolism of environmental carcinogens or in these responses to DNA-damaging agents would thus be expected to alter cancer susceptibility. In fact, there are notable exceptions to the statement above that cancer gene abnormalities occur in somatic cells rather than being inherited. There are a handful of inherited syndromes which predispose individuals to develop cancer. The unique feature of cancer as a heritable genetic disease, however, is that these

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inherited gene abnormalities do not give the person the disease of cancer, but rather predispose them to develop the disease. Thus, the inherited gene abnormalities do not generate the transformed phenotype, but somehow set the stage for the genetic changes to occur in the somatic cells which lead to the cellular transformation.

There are two ways to envision how this might happen: (a) the inherited gene abnormality is a rate-limiting step in tumor progression and loss of its function creates a situation in which accumulation of other genetic changes could more easily transform cells; or (b) the inherited genetic change affects the rate at which the somatic genetic changes occur. Interestingly, many of the inherited cancer susceptibility syndromes appear to be defects in the cellular responses to DNA damage (Table 1). There are examples of both homozygous recessive disorders (where the patients have multiple clinical problems in addition to the cancer predisposition) and heterozygous germ line defects (where there is no other discernible phenotypic abnormality other than cancer susceptibility) predisposing to cancer.

Examples of recessive disorders with defects in DNA damage responses include xeroderma pigmentosum (defect in excision repair and susceptibility to UV-induced skin cancers), ataxia telangiectasia (defects in a variety of cellular responses to ionizing radiation and a high incidence of lymphoid malignancies), Fanconi's anemia (defect in repair of DNA cross-links and increased susceptibility to myeloid leukemias), and Bloom's syndrome (increased sister chromatid exchanges after DNA damage and susceptibility to leukemias). Examples of dominantly inherited, heterozygous germ line defects which alter cellular responses to DNA damage and increase cancer risk include hereditary non-polyposis colon cancer (with defects in DNA mismatch repair) and Li-Fraumeni syndrome (with defects in p53). Interestingly, for most of these syndromes, the increased cancer susceptibility tends to be for only one tumor type, suggesting some type of specificity for the particular defective process for the given tissue or in the carcinogenic stimulus for that tissue. An exception to this generalization is that Li-Fraumeni patients are at risk for several different tumor types, suggesting that p53 dysfunction is an important rate-limiting step for carcinogenesis in several (but not all) tissue types. The physiologic function of several other cancer susceptibility genes, such as the *BRCA-1* breast cancer susceptibility gene, are not yet clarified. In addition, there are clearly familial risks for certain other tumor types, such as prostate cancer, where the inherited gene defect remains to be identified. It will not be surprising if other cancer sus-

Table 1. Cancer susceptibility syndromes attributable to abnormalities in DNA damage responses

Cancer susceptibility syndrome/inheritance pattern	Defect/disease(s)
Fanconi's anemia/recessive	Cross-link repair/acute myeloid leukemia
Ataxia telangiectasia/recessive	Ionizing radiation responses/lymphomas
Xeroderma pigmentosum/recessive	Excision repair/skin cancers
Bloom's syndrome/recessive	Increased sister chromatid exchanges/leukemias
Li-Fraumeni syndrome/dominant	Germ line p53 mutations/multiple tumors
Hereditary nonpolyposis colorectal cancer/dominant	Mismatch repair defects/colon, ovary, endometrial carcinomas

ceptibility genes similarly play a role in modulating cellular responses to DNA damage.

It is worth repeating that inheritance of these gene defects is not sufficient for generating a transformed phenotype. These inherited physiologic abnormalities primarily appear to increase the likelihood that the cell will accumulate the other genetic changes which generate the transformed phenotype, i.e., they increase genetic instability. This observation further supports the notion discussed above of the critical role of cellular DNA damage responses in human tumorigenesis. The genes which get altered in this transformation process in the somatic cells are likely to be those involved in cell proliferation (oncogenes and tumor suppressor genes), cell death (apoptosis and anti-apoptosis genes), angiogenesis, basement membrane invasion, and metastasis. This is likely to be true whether the tumor arises in an individual with a known cancer susceptibility syndrome or arises sporadically; the concept is that, with many of these susceptibility syndromes, the chance of generating these somatic genetic errors is increased. The tissue specificity for the various genetic susceptibility syndromes is likely to depend on the role that a particular gene product plays in responding to the particular carcinogens that tissue is exposed to (e.g., xeroderma pigmentosum and UV-induced skin cancers) and on which of the various genes which need to be altered in the somatic cells are rate limiting for transformation in that tissue.

We have learned much about the molecular controls of DNA repair processes, cell cycle perturbations after DNA damage, and cell death decisions in recent years. This book tries to capture many of these insights and focuses on the role of genetic instability in tumor development. Included are basic topics, such as the insights gained by studying cell cycle control and genetic instability in genetically manipulable yeast (discussed by FOREST SPENCER) and the molecular mechanisms involved in gene amplification, a common type of chromosomal alteration seen in human tumor cells (discussed by THEA TLSTY). A general discussion of the role of increased mutation rates in tumors is presented by KEITH CHENG and LARRY LOEB. The impact of specific defects in DNA repair on carcinogenesis, with a focus on xeroderma pigmentosum, is covered by JAMES FORD and PHILIP HANAWALT and a more general treatise on chromosomal instability syndromes and tumor development, with more emphasis on ataxia telangiectasia and the recently identified *ATM* gene, is presented by STEVEN MEYN. Finally, a comprehensive consideration of the various genes which are actually found altered in human tumors, including some emphasis on the role of mismatch repair genes on cancer susceptibility, is covered in the chapter by KATHLEEN CHO and LORA HEDRICK. Though it is hard to completely cover all aspects of this subject, these chapters should be sufficient to provide the reader with a good conceptual base in the field and hopefully a good sense of the current state of the art in our concepts of the molecular controls of human tumorigenesis.

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Genomic Stability and Instability: A Working Paradigm

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1 Introduction

Nearly a century of scientific inquiry into the nature of mutation has offered an exciting glimpse of the forces that cause mutation and drive evolution. New concepts make us realize the magnitude of the challenge of duplicating and distributing an accurate copy of the genome each time a cell divides. For each of the 10^{16} somatic cell divisions that occur in a human lifetime, each copy of the genome must contain an intact sequence of some 3 billion nucleotide base pairs, and each daughter cell must receive the correct number of the 46 chromosomes into which the instruction book of life is parceled. The challenging task of accurate DNA replication and distribution must be accomplished while the cell is under constant attack by forces that damage DNA.

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Genomic instability refers to normal and abnormal tendencies of cells to undergo mutation. Here we attempt to paint a cohesive picture of our understanding of genomic instability in the context of normal and abnormal biological function. To illustrate the importance of this area of research, we open with a brief overview of the events that led to the exciting finding in 1993 that genomic instability mutations are associated with some inherited human diseases and with many human cancers. We consider cellular mechanisms that provide potential sources for genomic instability, pathways involved in maintaining genomic integrity, and the consequences of mutations in these functions. Methods for measuring mutations are briefly compared, first, in the studies of microsatellite instability and then with respect to genomic instability in human cancer. Finally, we briefly consider some potential directions in the research on genomic instability and their possible benefits to our understanding of cancer and genetic diseases.

2 Historical Perspective

Shortly after the rediscovery of Mendel's work on the nature of inheritance near the turn of the century, Theodor Boveri used sea urchin and *Ascaris* embryos to study the importance of chromosomal stability in maintaining cellular function (BOVERI 1902). Work in bacteria and their viruses over the ensuing decades led to the discovery of DNA as the repository of genetic information. Basic genetic and biochemical work led to molecular concepts about mechanisms of mutagenesis. Recent interest in genomic instability has been heightened by the remarkable discovery that gross expansion in the number of CGG triplet repeats is diagnostic for the presence of the Fragile X syndrome (FU et al. 1992). This finding presaged the discovery of multiple repetitive sequence expansion in other human diseases and suggested the possibility that simple repeat mutations might be a marker for genetic instability in human tumors (CHENG and LOEB 1993).

While early work on rare hereditary cancers suggested that loss of function in a single gene could cause cancer (KNUDSON 1971), work in the 1980s on the more common epithelial neoplasms, particularly colon cancer (FEARON and VOGELSTEIN 1990) and malignant melanoma (BALABAN et al. 1986), indicated that multiple mutations are frequently observed in the most common cancers. One mechanism to account for the number of mutations observed in common cancers is for mutations in genomic stability genes to occur early in the course of tumorigenesis. Mutations in genomic stability genes could engender mutations throughout the genome. The high error rates of DNA polymerases first suggested that errors in DNA replication might be a key mechanism to generate the multiple mutations found in human cancers (LOEB et al. 1974). This, together with Nowell's hypothesis that mutations causing genomic instability would lead to accelerated tumor progression (NOWELL 1976), led to predictions that humans carrying genomic instability mutations are cancer prone. Individuals with one mutant allele may lack an initial phenotype, but

develop cancer at an early age due to loss of the wild-type allele during growth of somatic tissues (CHENG and LOEB 1993; KNUDSON 1985). Until recently, experimental support for the hypothesis that cancer cells may have mutations in genomic stability genes and exhibit a mutator phenotype (LOEB 1991) has been equivocal. The recent demonstration that mutations in genes responsible for mismatch correction cause forms of familial colon cancer and are associated with microsatellite instability (BRONNER et al. 1994; FISHEL et al. 1993; LEACH et al. 1993; PAPADO-POULOS et al. 1994) provides strong evidence for the importance of a mutator phenotype in cancer. It is important to note that decades of groundwork by basic scientists determining the genetic and biochemical mechanisms of mutagenesis in unicellular organisms made it possible to establish the importance of mutator phenotypes in human cancer.

3 Genomic Stability

In the 1950s and 1960s, interest in the question of how rates of mutation are controlled led to the genetic and biochemical dissection of bacteriophage and bacterial mutants that exhibit elevated rates of mutation (COX 1976; DRAKE et al. 1969). The study of these mutator strains and related DNA repair mutants led to a series of important concepts. First, the accuracy of replication by DNA polymerases is inadequate to account for the observed net level of mutation, even after taking into account proofreading mechanisms exhibited by these enzymes. Second, as a corollary it follows that cells must have special mechanisms for the correction of replication errors. Third, cells are exposed to a plethora of environmental chemicals that damage genomic DNA (AMES and GOLD 1991). Moreover, normal metabolic processes such as oxidative metabolism and inflammation also potentially may produce large numbers of alterations in DNA (AMES and GOLD 1991; KLEBANOFF 1988). Fourth, cells have evolved multiple mechanisms for DNA repair (HANAWALT et al. 1979). Thus, multiple cellular biochemical processes can, if altered, increase genomic instability. A comprehensive review of these functions is given elsewhere (CHENG and LOEB 1993). We present here an updated overview, summarized in Fig. 1, which is biased toward newer aspects of genomic instability.

3.1 Endogenous Sources of Genomic Instability

Genomic stability can be viewed as two opposing sets of reactions that are maintained at equilibrium: one set of reactions alters DNA and the other restores the original sequence. When DNA damage exceeds repair, mutations are manifested. This basic paradigm is embellished by more complex phenomena, such as the induction of DNA damage pathways and the consequences of error-prone DNA repair processes. It provides a useful framework for evaluating the potential of

Consequences of Failure of Genomic Maintenance

Gene amplification
Single base substitutions
Insertions (including viruses, transposons)
Deletions
Recombination
Chromosomal abnormalities
Numerical loss or gain
Translocations
Inversions
Deletions
Epigenetic change
Inappropriate gene expression
Loss of function

Cancer-Related Targets

Oncogenes
Tumor suppressor genes
Genomic stability genes
Migration (invasion) genes
Metastasis and metastasis suppressor genes
Senescence genes
Epigenetic regulation

Genomic Maintenance Functions

Maintenance of primary DNA sequence
DNA Replication
Base-Selection
Proofreading
Mismatch Correction
Prevention of DNA damage
DNA repair
Cell cycle checkpoints
Recombination
Suppression of transposon movement
Suppression of recombination of repeat sequences
Maintenance of chromosomal ploidy and structure
Genome organization
Topological management of DNA
Chromosome segregation

Factors that Foster Genomic Instability

Error prone DNA repair
Variations in nucleotide precursor pools
Environmental agents
Oxygen free radicals
Depurination
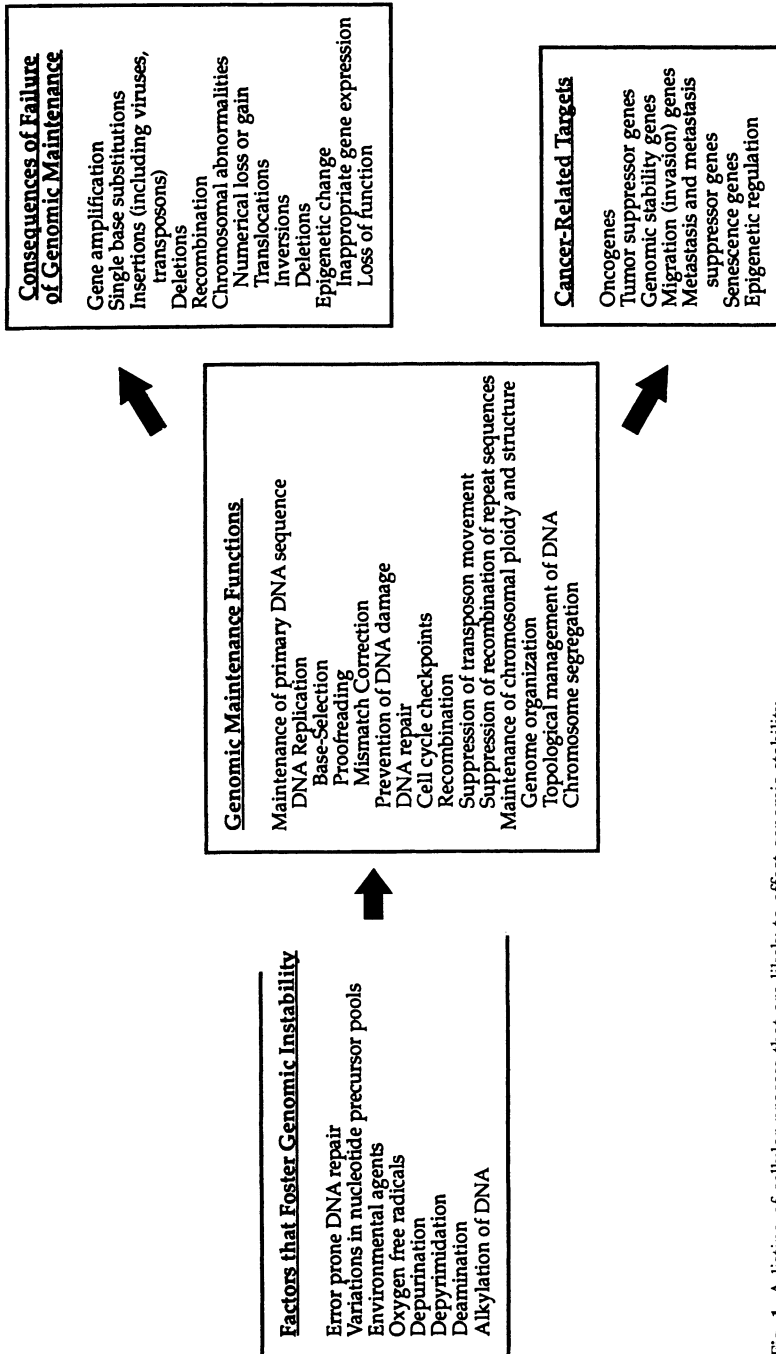Depyrimidation
Deamination
Alkylation of DNA

Fig. 1. A listing of cellular process that are likely to affect genomic stability

DNA-damaging events to cause mutations. It has long been recognized that many environmental agents, including irradiation, chemicals, and heat, have the potential to damage DNA in vivo. In somatic cells, these mutations are associated with cancer. Recently, there has been increasing evidence that normal biological processes can also damage DNA or alter the nucleotide sequence in DNA (LOEB and CHENG 1990). If the rates of DNA damage by these exogenous and endogenous sources of DNA damage exceed the corresponding rates of repair, they could contribute to the large number of mutations observed in human cancers. Three normal cellular processes with the potential to cause DNA damage and/or mutations at exceptionally high frequencies will be considered.

3.1.1 Chemical Instability of DNA

In cells, the nucleotide bases in DNA are subject to attack by water. Depurination, the cleavage of the carbon bond between the purine base and its sugar, is the most frequent hydrolytic modification of DNA. It results in the formation of an abasic site which, if not repaired, codes for the incorporation of adenine opposite the abasic site (LOEB and PRESTON 1986). Other hydrolytic reactions, such as depyrimidation and deamination, are less frequent. Based on the rates of depurination of DNA in vitro, it has been estimated that each human cell undergoes 10 000 depurinations per day (LINDAHL and NYBERG 1972). Despite the cell's multiple enzymatic activities for the repair of abasic sites, it seems reasonable that some of these abasic sites escape repair and are mutagenic at the time of DNA replication. In spite of the high frequency of depurination, a survey of nucleotide changes that have been reported in genes associated with cancer does not indicate that transversions are the most frequent mutations, suggesting other sources of mutations are more important.

3.1.2 Oxygen Free Radicals

Respiration and a variety of metabolic processes generate as intermediates a series of reactive oxygen species (FLOYD 1990; KLEBANOFF 1988). Based on the excretion of oxygen free radical-modified nucleotides in human urine, it has been estimated that each cell removes from its DNA approximately 20 000 oxygen radical-induced modifications per day (CATHCART et al. 1984). The most frequent mutations resulting from oxygen free radical damage to DNA in both prokaryotes and eukaryotes are C to T transitions (FEIG and LOEB 1993). However, the same substitutions are produced by other processes and thus are not pathognomonic of mutagenesis by oxygen free radicals. A much less frequent mutation, a double tandem CC to TT substitution, has so far been reported to result only from damage to DNA by UV irradiation (BRASH et al. 1991) and oxygen free radicals (REID and LOEB 1993). The presence of CC to TT mutations in internal human tumors that are not exposed to UV irradiation would constitute strong evidence for the involvement of oxygen free radicals in pathological processes. So far, this evidence is lacking.

3.1.3 Misincorporation by DNA Polymerases

The frequency of incorporation of non-complementary nucleotides by DNA polymerases is much greater than the observed frequency of mutations found in DNA after replication in cells. Other mechanisms in cells can increase the fidelity of DNA polymerases. These include proteins that interact with DNA polymerases and increase base selection and proofreading (FRY and LOEB 1986) as well as mismatch correction, a mechanism to excise misincorporations left after DNA synthesis (MODRICH 1987). A comparison of the error rates of replicating DNA polymerases and the spontaneous mutation rate in normal cells suggests that misincorporation by DNA polymerases in the absence of mismatch correction could account for as many as 6000 mutations per cell division cycle (LOEB 1989). Among the eukaryotic DNA polymerases that have so far been investigated, DNA polymerase-β is by far the most error prone (KUNKEL 1985, 1992). Since DNA polymerase-β is involved in DNA repair synthesis, these results suggest that DNA repair itself may be a further source of mutations. Studies on the spectrum of mutations produced by DNA polymerases have not as yet revealed specific mutations that are diagnostic of errors in DNA replication, with the possible exception of slippage during the copying of repetitive nucleotide sequences (SCHACHMAN et al. 1960) (see below).

3.2 Deficits in DNA Repair

Considering the plasticity of the DNA molecule and the frequency of DNA damage by both environmental chemicals and endogenous processes, it is not surprising that cells have evolved multiple and redundant pathways for DNA repair. It has been recognized for more than 50 years that there are rare inherited human diseases that manifest both deficits in DNA repair and an increase in the incidence of specific human tumors (FRIEDBERG 1985). Diseases such as xeroderma pigmentosum, Fanconi's anemia, and ataxia telangiectasia are characterized by repair deficiencies in DNA damage induced by specific agents. As a result, exposure of affected individuals to these same DNA-damaging agents is associated with an increased incidence of cancer. What was not adequately appreciated is that deficits in DNA repair might also be associated with the more common human cancers.

3.3 Cell Cycle Checkpoints

An important component in the maintenance of the genome is the coordination and control of DNA replication, repair, and the distribution of DNA to daughter cells during each division cycle. For example, replication of altered DNA prior to removal of modified bases by DNA repair enzymes would lead to miscoding during DNA replication. As in the study of mismatch correction, much of our knowledge about checkpoints in the cell cycle has been gained from an analysis of mutants. Mutants in these processes abolish the normal arrest in the cell cycle that occurs in

response to DNA damage. At least two stages of the cell cycle are regulated in response to DNA damage: the G_1-S and G_2-M boundaries (HARTWELL and KASTAN 1994; KASTAN et al. 1992). Cells delay cell cycle progression in order to facilitate the repair of DNA damage and to ensure that previous steps in the cell cycle are complete before proceeding. The relevance of these initial observations in yeast is underscored by the high frequency of abnormalities in p53-related checkpoint functions in human cancer (HARTWELL and KASTAN 1994; KASTAN et al. 1992). Individuals carrying germ line mutations in p53 have a proclivity toward the development of certain tumors (HARTWELL and KASTAN 1994; KASTAN et al. 1992; MALKIN et al. 1990; SRIVASTAVA et al. 1990) and the majority of human cancers have mutations in p53. In addition, mice without p53 have an increased incidence of multiple cancers (DONEHOWER et al. 1992). Perhaps the most incriminating association between p53 mutations and cell cycle delay is that tumor cells lacking p53 do not exhibit the G_1-S delay after exposure to ionizing irradiation, and that the introduction of the wild-type allele of p53 restores the G_1-S delay (KASTAN et al. 1992). The biochemical mechanisms underlying the cell cycle checkpoints for DNA repair are not known, and the coordination of these processes with DNA repair and transcription remains to be determined. Conceivably, there is a complex machinery at the G_1-S phase transition that coordinates cell cycle delays, transcription-coupled DNA repair, and DNA replication.

3.4 Recombination

While recombination has been studied in great detail, primarily in unicellular systems (LOW 1988), there is little known about the enzymological mechanisms for recombination in human cells. Recombinatorial mechanisms have been postulated to play a role in DNA repair, DNA replication, chromosome segregation, evolution, and disease (RADMAN et al. 1993). A major concern with respect to genetic stability is how the cell prevents recombination amidst the enormous repertoire of repetitive DNA elements. For example, there are hundreds of thousands of *alu* repeats in the human genome. If recombination were to occur frequently between these repeats, a myriad of chromosomal transversions, deletions, and inversions would occur. This suggests the hypothesis that specific control mechanisms limit recombination between repeats and that deficiencies in these mechanisms would cause genetic instability.

4 Multiple Mutations and Cancer

Human cancers exhibit multiple chromosomal abnormalities (MITELMAN 1991). Since these chromosomal alterations have been categorized cytologically, they involve large regions of chromosomes that contain millions of nucleotides. Chro-

mosomal aberrations in tumors could be the tip of an iceberg, with a much greater number of smaller mutations, such as single-base substitutions, lying undetected in the sea of DNA sequences (CHENG and LOEB 1993; LOEB 1991); if so, these will be identified increasingly as more sensitive molecular techniques become available. Unfortunately, this hypothesis cannot be immediately tested since we still lack practical methods to measure random single-base substitutions in DNA. A variety of studies suggest that the rate of spontaneous mutagenesis in normal human cells is about 1.4×10^{-10} mutations/nucleotide/cell generation. We calculated that this rate is only sufficient to generate three mutations per cell during a normal lifespan (LOEB 1991). A much higher mutation rate is necessary to account for the six to nine mutations that have been reported in human cancers (SUGIMURA 1992; VOGELSTEIN et al. 1988) or the much larger numbers of smaller mutations that may be present.

Different mechanisms could account for the multiple mutations that have been found in human tumors. One such mechanism is that an early event in the carcinogenic process causes a mutation in a gene that normally functions in maintaining the stability of the human genome. This mutation then engenders additional mutations throughout the genome. The subsequent random mutations might include some in other genes also involved in maintaining genetic stability. Examples of these early mutations include genes involved in DNA repair, DNA replication, and chromosomal segregation. Mutations involved in oncogenes that are involved in the cancer phenotype might occur later during the course of tumor progression.

Until recently, there has been little evidence to support the concept of a mutator phenotype in cancer. Early efforts to detect mutation relied upon single gene targets, such as *HPRT*. These lack the sensitivity of assays using microsatellites that examine multiple loci and are hypersensitive to mutation. The demonstrations that repetitive nucleotide sequences (microsatellites) are mutated in many different types of human tumors and not in normal cells now provides the strongest evidence for a mutator phenotype (LOEB 1994). Expansion or contraction of repetitive sequences provides an exceptionally sensitive indicator of mutagenesis. If the finding that cancer cells exhibiting microsatellite variation can be generalized to high mutation rates in expressed genes, then microsatellite instability provides a diagnostic marker for a general mutator phenotype in cancer. It should be noted that the mutation rate at the *HPRT* locus has been reported to be 200–600-fold higher in three colon cancer cell lines exhibiting microsatellite instability (BHATTACHARYYA et al. 1994). The mutation spectra differed, one cell line exhibited 25% frame-shift mutations at a mutational hotspot in the *HPRT* gene, and the other two lacked this hotspot, suggesting that there are at least two mutational pathways leading to microsatellite instability. Thus, microsatellite instability may indeed serve as an excellent diagnostic marker for general mutator phenotypes in cancer.

4.1 Microsatellite Instability and Mismatch Repair

Microsatellite instability was initially observed in tumors from patients with hereditary nonpolyposis colorectal cancer (HNPCC) (AALTONEN et al. 1993). Inheritance in 60% of these families maps to chromosome 2p (AALTONEN et al. 1993; PELTOMAKI et al. 1993), and the protein encoded by this locus is a homolog of the bacterial mutS protein, a key component of the mismatch repair pathway. Based on this homology, it was named hMSH2 (human mutS homolog) (FISHEL et al. 1993). In addition, 30% of patients with hereditary nonpolyposis families segregate with a locus on chromosome 3p (LINDBLOM et al. 1993), encoding a different mismatch repair protein, hMLH1, homologous to the bacterial mutL protein (PAPADOPOULOS et al. 1994). For the basic scientist, the finding that mutations in human homologs of these *Escherichia coli* and yeast mismatch repair genes are associated with human cancer is particularly gratifying. It vividly demonstrates that extensive studies in prokaryotes on fundamental problems can be instrumental in understanding human cancer. Since mismatch repair corrects errors in DNA synthesis in prokaryotes, it has been generally assumed that microsatellite instability is the result of errors by DNA polymerase that are too numerous to be corrected by mutated mismatch repair proteins. It is assumed that the localization of these mutations to repeats could be a manifestation of slippage of DNA during copying of the repetitive nucleotide sequences.

4.2 Microsatellite Instability and Human Cancer

In addition to hereditary nonpolyposis coli, microsatellite instability has been reported with varying frequencies in a number of sporadic tumors. In most of these tumors, it remains to be determined if this instability is also associated with mutations in mismatch repair genes. Human tumors exhibiting microsatellite instability include cancers of the colon (THIBODEAU et al. 1993), stomach (RHYU et al. 1994), and endometrium (RISINGER et al. 1993), and there is evidence for the presence of microsatellite instability in lung cancer (SHRIDHAR et al. 1994). A lack of microsatellite instability has been reported in breast cancers and in male germinal cell cancers (LOTHE et al. 1993). Interestingly, we are unaware of many reports on microsatellite instability in experimentally induced tumors in animals, but we see no reason why these will not be soon forthcoming. Studies of microsatellite instability are reported as being either positive or negative. False positives can be the result of slippage or recombination of truncated products during the polymerase chain reaction (PCR). Also, false positives can be the result of PCR-associated laddering of bands differing by two nucleotide repeat unit steps during amplification of (CA)n microsatellites. As a result, the experiments with high validity are only those that directly and simultaneously compare tumor DNA and normal DNA from the same individual. False negatives, i.e., the lack of expansion, could simply reflect the limited number of microsatellite sequences examined. Microsatellite instability

might be evident in most cancers if a sufficiently large number of the 50 000 repetitive sequences in the human genome were probed.

Tumors that exhibit microsatellite instability are frequently designated as RER$^+$ (LIU et al. 1995), indicating that the mutations are generated by replication errors; however, the source of mutations has not been unequivocally established. For example, heteroduplex DNA yielding nonparental microsatellite alleles could arise not only from errors in DNA synthesis, but also from recombination intermediates. Microsatellite instability may therefore be imagined to be caused by mutations in DNA polymerases or recombination proteins. Moreover, microsatellite instability could be a sensitive indicator for an elevation in mutation frequency in tumors caused by mutations in a variety of other DNA synthetic processes including DNA repair. Complementation assays have already indicated the presence of at least four different mutations that generate microsatellite instability in vitro utilizing extracts from endometrial and colorectal cell lines (UMAR et al. 1994). LIU et al. (1995), reported that the mutation in nine of ten cases of sporadic colon cancers which exhibited microsatellite instability was not one of those reported to be associated with mismatch repair. Thus, there are mutations other than the four which have so far been identified in mismatch repair genes that cause microsatellite instability.

The role of genomic instability in cancer may be illustrated by plotting somatic mutation rates over time in spontaneous and hereditary cancers. In Fig. 2, the gray area designates the normal range of mutation. The lower non-zero baseline level is due to the balance between mutational influences (Fig. 1) and repair mechanisms. The range of mutation rate is due to normal allelic and environmental variations. In spontaneous cancer, mutations causing genomic instability may be dominant or recessive. For dominant mutations, the time at which the mutation occurs would coincide with a stepwise increase in mutation rate. For the more common recessive
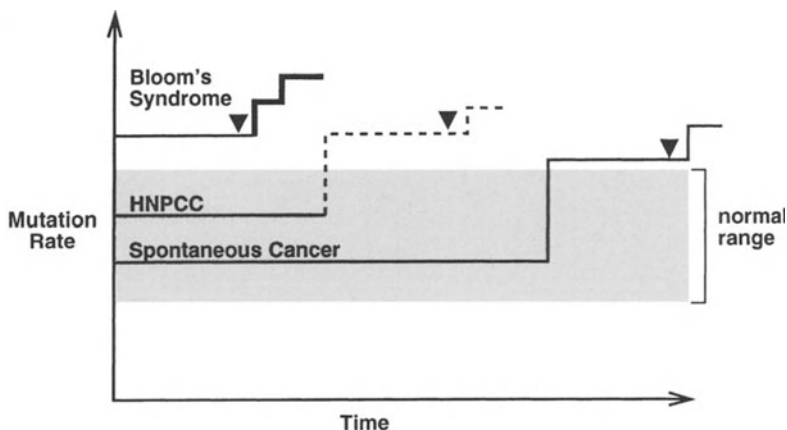

Fig. 2. Postulate increase in mutation rate over time in spontaneous and hereditary human cancers. The *triangle* indicates the clinical manifestation of a malignancy

mutations, two hits are required to elevate the mutation rate. The first mutation arises at an undesignated earlier time, and loss of the remaining wild-type allele is coincident with an increased mutation rate. Loss of expression of the remaining wild-type allele may occur by chromosome loss, recombination, or mutation. Subsequent increases in mutation rate are due to mutations in other genomic stability genes. In inherited genomic instability syndromes, such as Bloom's syndrome (WARREN et al. 1981), the baseline mutation rate is elevated from birth. Secondary mutations in genomic stability genes may or may not be necessary for the development of cancer. In the case of HNPCC, a recessive genomic instability mutation is inherited, and the baseline mutation rate at birth may be normal. Since one copy of a genomic stability gene is already mutated, loss of the wild-type allele occurs earlier, leading to an elevated mutation rate. Alternatively, the extra burden of repair associated with abnormal cellular proliferation (e.g., benign tumor) may cause otherwise recessive genomic instability mutations to become dominant (LIU et al. 1995). The mismatch repair pathway defective in HNPCC might be expected to be a general repair system, and yet there is tissue specificity for tumor susceptibility. The existence of different repair pathways for different tissues may explain this tissue specificity. Relative rates of mutation and types of mutation caused by genomic instability mutations are expected to be determined by the pathway affected.

5 Experimental Systems and Future Directions

The genetic and biochemical analysis of unicellular organisms laid the foundation for the discovery that mutations in mismatch repair genes are associated with human cancer. Further studies in genetically tractable prokaryotes and simple eukaryotes may provide insights into other mechanisms of genomic instability. For example, the pathogenic yeast *Candida albicans* spontaneously switches between alternative phenotypes (SOLL et al. 1953) and African trypanosomes undergo recombination-based antigenic variation (VAN DE PLOEG et al. 1992). Established eukaryotic systems including biochemical approaches with cell extracts and knockout mutations in mice to test homologs for genomic instability phenotypes are expected to continue to yield further insights. Newer vertebrate systems for generating mutants, such as the zebrafish, also may yield more candidate genes to test in human cancers.

New assays which detect other types of mutations or increase sensitivity of mutation detection might reveal that genomic instability is more common than we might expect, and they may also open windows of opportunity to better understand and perhaps interfere with genomic instability in the hope of arresting tumor progression. Thus, the study of genomic instability is likely to provide not only the basic insights into mechanisms of mutation and evolution, but also knowledge relevant to cancer prognosis, drug design, and differential drug sensitivity.

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Surveillance and Genome Stability in Budding Yeast: Implications for Mammalian Carcinogenesis

F. Spencer

1 Introduction

The recognition of cancer as a genetic disease has brought with it the observation that tumor development is the outcome of multiple independent mutational events occurring during somatic tissue growth (Peto et al. 1975; Fearon and Vogelstein 1990). These mutations in combination lead to abnormalities in growth control and genome instability in cancer cells. Advances in model organism studies have shown that these two attributes are frequently linked; that abnormalities in the control of cell cycle progression are often associated with increased errors in replicating and transmitting the parental genome to daughter cells. Because fundamental aspects of cell cycle control and chromosome distribution are clearly conserved in eukaryotic organisms, studies in model experimental systems are highly relevant to elucidation of the roles of these processes in tumor development in humans (Hartwell and Kastan 1994). This review will focus on recent advances in cell cycle control and genetic instability in the budding yeast *Saccharomyces cerevisiae*, with emphasis on experimental topics in which loss of cell cycle progression control decreases the fidelity of chromosome transmission to daughter cells. Viable single gene mutations that perturb both cell cycle control and genome stability in model organisms represent promising candidate tumor suppressor homologues. At this time, the connections between these functions in yeast and mammalian carcinogenesis are largely speculative, although the parallels are strong and warrant consideration.

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The area of chromosome transmission fidelity has made substantial recent progress in budding yeast. This is due in large part to the fact that chromosomal elements required for chromosome replication and segregation occupy short segments of DNA, and are easily manipulated in bacterial shuttle vector systems (reviewed in NEWLON 1988). This feature of yeast chromosome biology has allowed the manipulation of DNA elements in vitro, which can be subsequently reintroduced into a yeast host by DNA-mediated transformation for functional assay in vivo. In addition, the highly active homologous recombination system of budding yeast facilitates the incorporation of engineered structures into defined positions on natural yeast chromosomes for functional characterization in chromosomal context (reviewed in ROTHSTEIN 1991). These pragmatic experimental advantages have supported a rapidly advancing field of study focused on identifying determinants of chromosome transmission fidelity and characterizing their cellular roles. The current molecular genetic and biochemical analysis of proteins that function at budding yeast centromeres, origins of replication, and telomeres (reviewed in NEWLON 1988; ZAKIAN et al. 1990; HEGEMANN and FLEIG 1993; SUGINO 1995; TOYN et al. 1995) has already provided a firm foundation for the study of structural and regulatory aspects of the chromosome cycle in this organism.

The fidelity of chromosome transmission depends on the interaction of these structural elements with controlling proteins that ensure temporal order in the execution of cell division processes. The subject of cell cycle control embodies a major focus of productive research in a large number of organisms, and many molecular components of cell cycle progression control through the activity of cyclin-dependent kinases are clearly conserved among widely divergent species (reviewed in COLEMAN and DUNPHY 1994). As in all eukaryotes, activity of the yeast $p34^{CDC28}$ protein kinase catalytic subunit (the $p34^{CDC2}$ homologue) is a central element in the machinery that controls progression through mitosis in budding yeast (PIGGOTT et al. 1982; REED and WITTENBERG 1990; FITCH et al. 1992; RICHARDSON et al. 1992). $p34^{CDC28}$ activity in the G_2/M portion of the yeast cell cycle requires association with B-type cyclins, six of which are described in the literature (EPSTEIN and CROSS 1992; FITCH et al. 1992; RICHARDSON et al. 1992; SCHWOB and NASMYTH 1993). General themes in regulation of cyclin-dependent kinase activities include changes in phosphorylation status (reviewed in SOLOMON 1993) and association with inhibitory subunits (reviewed by PETER and HERSKO-WITZ 1994). Studies aimed at the elucidation of the specific roles of the multiple forms of p34 kinases comprise an active area of research. The target in vivo substrates and regulation of kinase activity of these complexes are not fully described in any organism, although there is no shortage of candidates (NIGG 1993). Though much further work needs to be done in multiple organisms to distinguish the fundamentally shared from species-specific mechanisms, many observations made in model systems will be of value in probing similar functions in mammalian cells.

The cell morphology of budding yeast also provides a useful experimental tool for studies in cell cycle progression. This feature was recognized 3 decades ago by HARTWELL who used it to generate and study a large collection of cell division cycle (*cdc*) mutants (reviewed in HARTWELL 1974; PRINGLE and HARTWELL 1981). Ele-

gant analyses of these mutants have pioneered much of what we now understand about the organization of the cell cycle. The relationship between bud morphology and cell cycle phase in *S. cerevisiae* is shown in the top portion of Fig. 1, which depicts easily scored changes in cell nuclear morphology. These morphological changes reflect progress through the four classically defined cell cycle phases G_1, S, G_2, and M. While the simplicity of the morphological assay makes it a powerful experimental tool, it is not without limitation. In particular, the transitions from S to G_2 and G_2 to M phases are not well represented by landmark morphological events. Many *cdc* mutants and many experimental treatments will cause arrest in a large budded uninucleate state, which may represent late S phase, G_2, or early mitosis, and thus other criteria must be applied to differentiate among these possibilities.

The lack of clarity in defining S to G_2 and G_2 to M phase transitions is not limited to budding yeast (nearly all experimental systems rely on methods such as flow cytometry to detect the completion of bulk DNA synthesis, and hence the S to G_2 transition), but the problem of identifying a G_2/M transition is especially acute. Hallmarks of the initiation of mitotic prophase in yeast are either not visible at this level of analysis (e.g., chromosomal condensation requires in situ hybridization for detection; GUACCI et al. 1994), or do not occur (e.g., nuclear envelope dissolution is not observed in budding yeast; BYERS 1981), or occur at a much earlier time in the cell cycle than in other eukaryotes (e.g., spindle pole separation and spindle microtubule formation are nearly concurrent with the initiation of DNA synthesis; BYERS 1981). The majority of cell cycle studies in budding yeast have therefore relied on anaphase to indicate mitosis, or genetic arguments to differentiate pre-anaphase mitotic arrest from late S or G_2 arrest.

Mitosis, as a component of cell division, is an irreversible process. It has been argued that cells have evolved mechanisms that ensure readiness prior to the initiation of mitosis (e.g., MURRAY and KIRSCHNER 1989; NURSE 1990; MURRAY 1992). However, mitosis is not a single event, but a complex series of steps (reviewed in AULT and RIEDER 1994; KOSHLAND 1994) within which there may be controls to ensure that the segregation of cellular contents will result in two viable daughters. The box associated with M phase in Fig. 1 lists events that are classically associated with mitosis in eukaryotes whether or not they have been visualized in budding yeast. These events are arbitrarily organized into three groups that can be thought of as representing entry into, execution of, and exit from mitosis. All of these events are essential for a successful division and clearly must occur in a coordinated fashion.

The number of operational control points represented within this series of events and their relationships remain to be determined. This is an important problem to be considered in the interpretation of studies addressing the nature of controlled progression through the G_2/M portion of the cycle, especially when considering results from different organisms in presumed conserved processes. The criteria used to identify cell cycle position are dictated by the landmarks available in the relevant experimental systems. Budding yeast landmarks are somewhat different from those available in mammalian cells and therefore perhaps worth brief

Bud Emergence
DNA Synthesis Initiation
Spindle Pole Duplication

S

Nuclear Migration
to Mother/Bud Neck

G1

G2

Cell Separation

M

Chromosome Separation

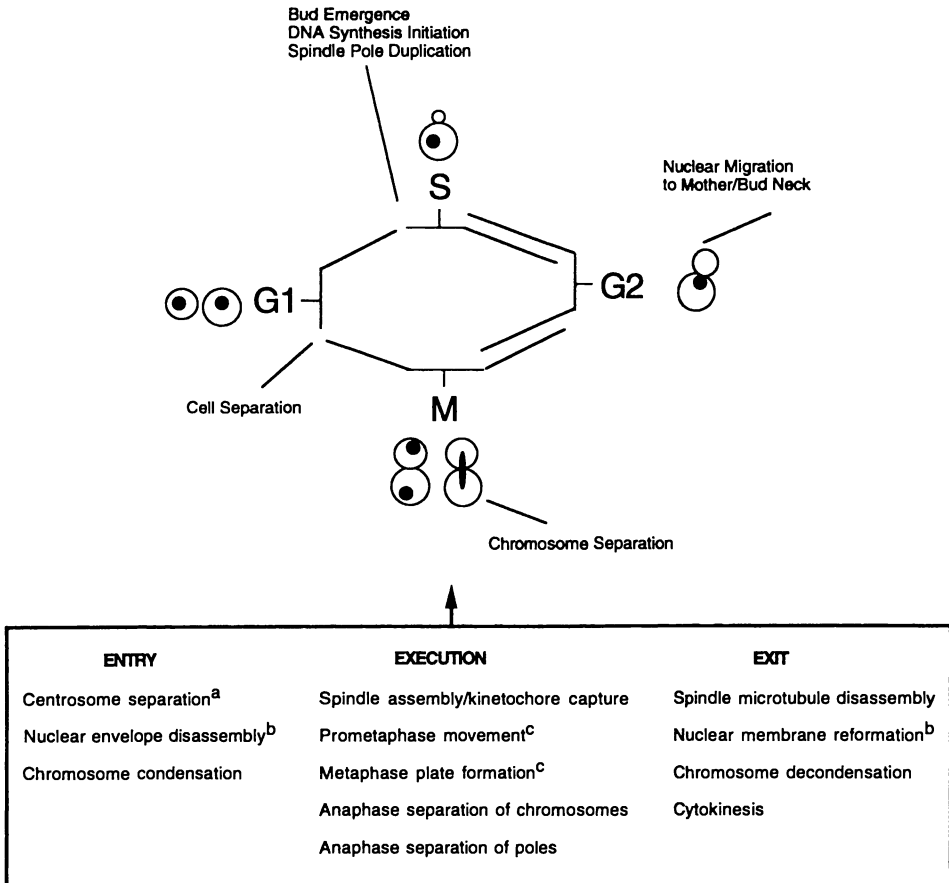| ENTRY	EXECUTION	EXIT
Centrosome separation[a]	Spindle assembly/kinetochore capture	Spindle microtubule disassembly
Nuclear envelope disassembly[b]	Prometaphase movement[c]	Nuclear membrane reformation[b]
Chromosome condensation	Metaphase plate formation[c]	Chromosome decondensation
	Anaphase separation of chromosomes	Cytokinesis
	Anaphase separation of poles	

Fig. 1. Simple morphological criteria used in determining the cell cycle stage of a yeast cell. The M to G1 and G1 to S transitions can be scored using cell separation and bud formation as criteria. The S to G2 and G2 to M transitions are inferred, and the overlap between these phases as determined by cytology is indicated. The anaphase separation of chromosomes and poles is observed as simultaneous elongation of the DNA mass and spindle. It is not known if budding yeast has distinct anaphase A (chromosomal separation) and anaphase B (pole separation) movement. The *box* contains a canonical list of mitotic events whether or not they are observed in budding yeast. The large budded uninucleate cell morphology (often referred to as being in G2/M arrest or delay) encompasses late S phase, G2, and preanaphase mitotic stages. *a*, occurs early in S phase in budding yeast; *b* does not occur in budding yeast; *c*, is not cytologically distinct in budding yeast

description here (reviewed in BYERS 1981). The spindle pole bodies (centrosomes of yeast) undergo separation very early in S phase, nuclear envelope disassembly is not observed, and chromosome condensation can be visualized using in situ hybridization techniques. Because yeast chromosomes contain centromeres that direct segregation of sister chromatids on a microtubule-based spindle (PETERSON and RIS 1976; BYERS 1981), a process in which kinetochores associate with spindle microtubules must occur. Indeed, yeast centromere DNA/protein complexes are ob-

served to interact with microtubules in vitro (KINGSBURY and KOSHLAND 1993; SORGER et al. 1994), but when this association is first established in vivo is not known. Because individual yeast chromosomes are not delimited by light- or electron-microscopic techniques (PETERSON and RIS 1976), prometaphase and metaphase states have not been distinguished. Prior to anaphase in yeast, the entire chromosome mass is seen to undergo rapid oscillatory movement (PALMER et al. 1989; YEH et al. 1995) which may represent these phases. Anaphase itself is observed as simultaneous separation of both chromosomal masses and spindle poles in wild-type cells. In exiting mitosis, spindle microtubule disassembly occurs (BYERS 1981), and chromosome decondensation is inferred (GUACCI et al. 1994). Prior to or within the processes of cytokinesis, the yeast nuclear membrane must be resolved into two distinct organelles. Cytokinesis occurs at the bud neck and requires activities associated with a 10-nm filament ring (HAARER and PRINGLE 1987). Cell separation after cytokinesis requires chitin synthesis (reviewed in BALLOU 1982).

2 Checkpoints and Surveillance: The Budding Yeast *RAD9* Example

The correct temporal order of critical events in the cell cycle is controlled by mechanisms that ensure the dependency of later processes on the completion of necessary earlier processes. This observation has been formalized and explored by WEINERT and HARTWELL (WEINERT and HARTWELL 1988; HARTWELL and WEINERT 1989), and termed "checkpoint control" which is mediated by "surveillance", and by MURRAY and KIRSCHNER (1989), who used the term "feedback control." Experimental evidence from many systems strongly suggests widespread existence of such dependencies (HARTWELL and WEINERT 1989; MURRAY and KIRSCHNER 1989). In defining the checkpoint concept, HARTWELL and WEINERT have formally defined two distinct types of dependent relationships: substrate–product limited dependency versus dependency governed by checkpoint control. In a substrate–product limited process, the rate of change is governed by the intrinsic properties of a structure, with no external regulation imposed. At a cell cycle checkpoint, however, extrinsic control governs progression via the activity of a surveillance pathway which provides a mechanism for preventing progress in a dependent process until prerequisite events have been completed. This hypothesis of checkpoint control by surveillance provides an intellectually satisfying mechanism for the temporal coordination of spatially separate events.

   In practice, how can surveillance be experimentally detected? Genetic analysis of the function of the yeast *RAD9* gene provided the first compelling experimental paradigm (WEINERT and HARTWELL 1988). DNA damage-induced delay of mitosis required wild-type *RAD9* protein function: lack of this delay in a *rad9* deletion mutant was associated with increased chromosome loss and cell death. Restoration of delay by slowing the cell cycle with a microtubule inhibitor (provided at non-

arresting concentrations) rescued viability in irradiated cells. The simplest inter-
pretation of this result was that *rad9* deletion cells were capable of repairing ra-
diation damage, but had lost the ability to delay cell cycle progression while the
repair functions did their work. Thus, it was proposed that the wild-type *RAD9*
gene operated within a surveillance system controlling the timing of cell division in
response to the state of the chromosomal DNA.

This analysis established three criteria for the recognition of surveillance at a
checkpoint. First, a cell cycle delay or arrest was induced by an experimental
treatment causing damage (in this case, irradiation). Second, the delay or arrest was
found to depend on a gene product whose removal also resulted in loss of fidelity of
the relevant process (in this case, observed as chromosome loss and cell death).
Third, reintroduction of a delay in cell cycle progress by an independent method (in
this case, low concentration of a microtubule inhibitor) restored fidelity of the
process affected in the delay-deficient mutant. In principle, these criteria can be
applied to the analysis of any mutant lacking a delay or arrest response. However,
the third and most convincing criterion depends on the identification of a delay-
inducing treatment that is both unrelated to the surveillance pathway under study,
and appropriately positioned in the cell cycle to allow the results of consequences of
repair processes to be revealed. In the *RAD9* study, partial inhibition of micro-
tubule polymerization was informative, but in other studies a similar situation
could prove to be difficult to achieve.

This is not a series deterrent as limitations encountered within classical genetic
approaches will be complemented by molecular genetics and biochemistry. The
notion of "extrinsic control" is best modeled as an inducible reaction cascade with
a diffusible or transported component. In this hypothesis, at a minimum there must
be an inducer, a signal (including the diffusible or transported component), and one
or more effectors, the timing of whose substrate–product conversion is altered by
input from the signal. Considering cumulative experience in the analysis of signal
transduction pathways, it is highly likely that molecular and biochemical ap-
proaches will link legitimate components identified by genetic studies.

3 Budding Yeast Genes That Monitor Integrity
of the Chromosomal DNA

Further screening of yeast *rad* genes identified two additional genetic loci, *RAD17*
and *RAD24*, with properties similar to *RAD9* (WEINERT and HARTWELL 1993;
WEINERT et al. 1994). In a study designed to characterize the specificity of the
RAD9 checkpoint, *cdc* mutants encoding known replication enzymes were found to
depend on *RAD9* for their G_2/M arrests and for their recovery on return to per-
missive temperature (WEINERT and HARTWELL 1993). The "rapid death" phenotype
observed in replication mutants that lacked *RAD9* was exploited in the design of a
primary screen for G_2/M surveillance genes. This screen identified three *MEC*

(mitosis entry checkpoint) loci: *MEC1/ESR1, MEC2/SAD1/RAD53/SPK1*, and *MEC3* (WEINERT et al 1994; ALLEN et al. 1994; ZHENG et al. 1993), bringing the total number of published genes with roles in DNA damage surveillance to six.

The primary amino acid sequence of three of these genes have been reported. *RAD9* exhibits no obvious homology to known proteins (WEINERT and HARTWELL 1990). *MEC2* encodes an essential dual-specificity protein kinase which is also required for a DNA damage-induced transcriptional response (ZHENG et al. 1993; ALLEN et al. 1994). The *MEC1* predicted protein sequence exhibits significant similarity to the budding yeast *TEL1* gene product as well as the recently cloned human gene for ataxia telangiectasia, *ATM* (GREENWELL et al. 1995; MORROW et al. 1995; SAVITSKY et al. 1995). These protein sequences provide additional members to a group with homology to phosphatidylinositol-3 kinases, although biochemical activities of *MEC1, TEL1,* and *ATM* have not been characterized. Abnormalities in human ataxia telangiectasia are consistent with a DNA damage checkpoint defect, and affected individuals are at increased risk of tumor development (reviewed by SHILOH 1995). It has been hypothesized that human *ATM* functions are shared by activities of *TEL1* and *MEC1* proteins in yeast (GREENWELL et al. 1995; MORROW et al. 1995). Protein and functional homology are also evident in analysis of a fruit fly homolog (*mei-41*, HARI et al. 1995) and a fission yeast homolog (*rad3*; SEATON et al. 1992). Interestingly, AT cells show defects in p53 induction by DNA-damaging agents (KASTAN et al. 1992). This observation suggests that the mammalian p53 damage response includes surveillance elements homologous to the budding yeast DNA damage response. Further work on these related genes holds promise for determining what aspects are conserved within the cell physiology of DNA damage surveillance pathways in eukaryotes.

In the pioneering study that defined the *RAD9*-dependent DNA damage checkpoint in G_2/M, logarithmically growing cell populations were subjected to DNA-damaging treatments and found to accumulate late in the cell cycle, prior to anaphase of mitosis. Subsequent studies showed that synchronous yeast cell populations irradiated in G_1 exhibit a pronounced delay prior to entry into S phase, and that this S initiation delay depends on *RAD9, RAD24,* and *MEC2/RAD53* (ALLEN et al. 1994; SIEDE et al. 1993, 1994). The fact that all three genes tested (of the six known to operate at G_2/M) are required for S phase initiation delay function suggests that a single surveillance pathway can operate to arrest cells in response to DNA damage at these two cell cycle stages.

However, the pathway appears to be branched: a subset of DNA damage response loci are required for the maintenance of cell cycle arrest in the presence of a DNA replication block at initiation of DNA synthesis (WEINERT et al. 1994). All six *MEC* and *RAD* surveillance loci were identified by their requirement for response to DNA insults that directly alter DNA structure or prevent the completion of DNA replication. When these mutants were tested for arrest in the presence of hydroxyurea, two different responses were observed: *mec3, rad9, rad17,* and *rad24* mutants arrested their cell cycles, whereas *mec1* and *mec2/rad53* mutants failed to arrest and died rapidly. The effect of hydroxyurea treatment is to block nucleotide synthesis by inhibiting ribonucleotide reductase, and thus prevent DNA replication

once the internal cellular pools are drained. This experiment suggests that the physiological response to unreplicated DNA is qualitatively different from that to damaged DNA and utilizes an overlapping but not identical control pathway.

Interestingly, surveillance pathway function is also implicated in the control of the rate of passage through S phase. In a recent study, it was observed that alkylation damage to DNA (by exposure to methylmethane sulfonate) slows progress through S phase, as measured by flow cytometric analysis, and that this slowed pace requires the *MEC1* and *RAD53* gene functions (PAULOVICH and HARTWELL 1995). This observation strongly suggests that at least some surveillance pathway functions can direct modification of the DNA replication machinery or chromosomal proteins that modulate the rate of DNA replication. The relationship between this type of progression control and those which appear to delay cell cycle phase transitions (G_1 to S and G_2 to M) is not yet apparent.

It seems likely that related surveillance pathways that monitor DNA integrity are capable of responding to a variety of abnormal structures within chromosomes. Interestingly, NAVAS et al. (1995) have recently demonstrated that the C-terminal tail of budding yeast DNA polymerase epsilon is required for the DNA damage transcriptional response in S phase and for S phase arrest in response to hydroxyurea. These results suggest that the activities of DNA polymerase epsilon are intimately associated with a DNA damage-sensing mechanism.

The relationship between DNA damage pathway control and cell cycle progression control via p34^{CDC28} kinase activity is open to speculation. Although cyclin-dependent kinases are universal cell cycle regulators (NURSE 1990; COLEMAN and DUNPHY 1994), their molecular physiology is not simple. Experiments in fission yeast and frogs have shown that an inhibitory phosphorylation on tyrosine 15 of the cyclin-dependent kinase catalytic subunit was required for cell cycle arrest in the presence of incomplete DNA replication or DNA damage (ENOCH and NURSE 1990; SMYTHE and NEWPORT 1992). Though an analogous modification clearly occurs on tyrosine 19 of p34^{CDC28} in budding yeast, the functional connection with surveillance of DNA integrity appears not to be conserved in *S. cerevisiae* (AMON et al. 1992; SORGER and MURRAY 1992; STUELAND et al. 1993). As these experiments would have been most sensitive to the G_2/M block, it is clear that G_2/M activity of the *RAD9* pathway does not act via Tyr 19 phosphorylation. Clearly, however, this result does not preclude a role for p34^{CDC28} interaction.

4 A Surveillance Pathway Monitors Integrity of the Mitotic Spindle in Budding Yeast

There is very strong evidence for a widely conserved checkpoint that responds to the state of the mitotic spindle. Drugs that inhibit the formation of spindle microtubules, such as benzidimazoles, induce an arrest in mitosis in a large number of diverse organisms (reviewed in RIEDER and PALAZZO 1992). Under conditions that

completely disrupt spindle assembly, this arrest occurs in prometaphase (as indeed there are no microtubules to interact with the chromosomes in the formation of a metaphase plate). Under conditions that interfere with but do not destroy microtubule formation, cells are observed to arrest in metaphase. Whether these are physiologically distinct arrest pathways remains an open question. The duration of drug-induced arrest varies with drug concentration, organism, and cell type (KUNG et al. 1990; RIEDER and PALAZZO 1992; SCHIMKE et al. 1994), but the presence of a pause is essentially universal, suggesting a fundamental and conserved mechanism.

Preanaphase arrest is also associated with abnormal kinetochore configuration in diverse experimental paradigms and in many distantly related eukaryotes. Methods providing experimental interference that lead to preanaphase arrest or delay include breakage of spindle fibers with a glass needle in grasshopper cells (NICKLAS 1967), breakage of spindle fibers using a UV microbeam in newt cells (ZIRKLE 1970), and microinjection of antibodies that bind chromosomal centromeres or kinetochores in cultured human (BERNAT et al. 1990; YEN et al. 1991; TOMKIEL et al. 1994; CAMPBELL and GORBSKY 1995) and mouse (SIMERLY et al. 1990) cells. Although these experiments point to a preanaphase signal that blocks chromosome segregation, they themselves do not differentiate between an arrest resulting from a steric mechanism (i.e., a substrate–product relationship as discussed above) versus one requiring extrinsic control by a surveillance pathway. The strongest evidence for the activity of a surveillance pathway is provided by studies in human PtK cells (RIEDER et al. 1994) and budding yeast (SPENCER and HIETER 1992). In human PtK cells, the timing of coordinate anaphase separation of all chromosomes is markedly delayed in cells in which a single chromosome spontaneously fails to achieve spindle attachment by the time the other chromosomes are aligned at the metaphase plate. Moreover, the time interval between bipolar attachment of all chromosomes and anaphase initiation is the same in delayed or nondelayed mitoses. This is consistent with the hypothesis that anaphase chromatid separation is extrinsically controlled by a signal that communicates the unready state of any unattacked kinetochore and that, when metaphase spindle assembly is complete, a stereotypical set of biochemical events is initiated that releases the sister chromatids and segregates them to their respective poles. Recent data from micromanipulation experiments in praying mantis spermatocytes (LI and NICKLAS 1995) strongly suggest that tension on the chromosomal kinetochores, rather than physical association between kinetochore and pole, is the important feature of bipolar attachment. The description of the differential expression of a kinetochore phosphoepitope may provide a clue to structural differences between attached and unattached kinetochores at the molecular level. This epitope is apparent in prophase on all kinetochores and absent in metaphase except on the kinetochores of unattached chromosomes (GORBSKY and RICKETTS 1993).

Strong evidence of a similar preanaphase delay in budding yeast benefits from recent work identifying the centromere DNA elements and many associated proteins essential for chromosome segregation (reviewed in HEGEMANN and FLEIG 1993; KILMARTIN 1994). The cloning and characterization of yeast centromere DNA sequences have revealed a 125 nucleotide conserved region composed of

three elements, referred to as centromere DNA elements (CDEs). CDEI and CDEIII have palindromic character, and proteins that bind these sequences have been identified by both biochemical and genetic criteria. CDEIII is clearly essential for centromere function as point mutations central to the palindrome decrease chromosome segregation function by three orders of magnitude (HEGEMANN et al. 1988; JEHN et al. 1991). A 240-kilodalton multisubunit complex of proteins that binds CDEIII in vitro has been described (LECHNER and CARBON 1991). The three major protein species present in the complex are encoded by the kinetochore protein genes *NDC10, CTF13*, and *CEP3* (DOHENEY et al. 1993; GOH and KIL-MARTIN 1993; JIANG et al. 1993; STRUNNIKOV et al. 1995). Three groups have described a helix-loop-helix protein that binds the CDEI consensus as a dimer (BAKER and MASSION 1990; CAI and DAVIS 1990; MELLOR et al. 1990). This protein is encoded by a nonessential gene *(CPF1/CBF1/CEP1)* whose deletion results in a ten fold decrease in chromosome transmission fidelity. CDEII is an A+T rich region (exhibiting little other sequence conservation among centromere DNA sequences) whose function is unknown. CDEII provides an essential activity as CDEIII alone does not confer stable chromosome transmission (PANZERI et al. 1985).

The terms "kinetochore" and "centromere" have evolved highly specific, but different, meanings among groups of scientists working in different systems. A mammalian kinetochore can be visualized in electron micrographs and is defined as a multilayered structure associated with the primary constriction of mitotic chromosome (RIEDER 1982; EARNSHAW 1991). In budding yeast, the chromosomes are not cytologically distinct structures, and thus there is no visible constriction to which this term could refer. For the purposes of this review, the term yeast "kinetochore" will refer to the entire DNA/protein complex responsible for chromosome attachment to the mitotic spindle, and chromosome movement to the poles in mitosis.

Preanaphase delay induced by budding yeast kinetochore alteration has been detected in temperature-sensitive mutants of *NDC10, CTF13*, and *CEP3* (DOHE-NEY et al. 1993; GOH and KILMARTIN 1993; STRUNNIKOV et al. 1995). The delay is observed as accumulation of large budded uninucleate cells in culture at semipermissive or nonpermissive temperatures. To date, the period that is elongated prior to anaphase remains largely uncharacterized, but is generally assumed to occur in G_2 or early M. Preanaphase delay is also observed in yeast cells containing a single test chromosome with a centromere DNA mutation (SPENCER and HIETER 1992). Interestingly, in these experiments only some centromere DNA mutations caused delay, and the extent of delay did not correlate with the degree of chromosome missegregation. This observation indicates that kinetochore abnormalities that compromise chromosome segregation differ in kind. The ability of a single abnormal kinetochore to induce preanaphase delay strongly supports the existence of a preanaphase surveillance pathway in budding yeast and suggests that mammalian and budding yeast are likely to share conserved elements in progression control through mitosis.

Recently, two groups have identified mutant strains that bypass arrest or delay in the presence of the microtubule-inhibiting drug benomyl. The *bub* (budding uninhibited by benzidimazole) mutants (HOYT et al. 1991) were isolated for two diagnostic phenotypes: they are unable to recover from brief exposure to arresting concentrations of drug (sufficient to completely prevent the formation of a spindle), and they undergo unscheduled bud formation in the absence of nuclear division. The *mad* (mitotic arrest defective) mutants (LI and MURRAY 1991) were identified by decreased viability in the presence of low (nonarresting) concentrations of drug and an inability to slow cell division time under these conditions. The *bub* mutant screen yielded three mutants defining two genetic loci, *BUB1* and *BUB2*. During the cloning of the *BUB1* gene, *BUB3* was discovered as a low copy suppressor of *bub1-1*. The *mad* screen generated three mutants defining three loci: *MAD1*, *MAD2*, and *MAD3*. Neither screen was performed to saturation, and there are therefore likely to be many more genetic loci that could be identified using this approach.

Recent data (PANGILINAN and SPENCER, 1996) indicate that the components of the spindle surveillance pathway active in the presence of antimicrotubulte drugs are also required for the delay induced by mutant kinetochores in yeast. In *bub1* and *mad2* mutants, single chromosomes containing a delaying centromere DNA mutation are specifically and profoundly destabilized. This result indicates not only that *BUB1* and *MAD2* are required for the cell cycle delay, but that the delay is important for mitotic segregation of the mutant kinetochore. Further, the delay induced by the temperature-sensitive kinetochore protein mutant *ctf13-30* is abolished in *bub1* and *mad2* mutants. These data are consistent with the hypothesis that the kinetochores are inducers of a spindle surveillance pathway, although there is no evidence to suggest that kinetochores are the only inducers. It is also not clear what abnormal kinetochore property is important for delay induction although, by analogy with the observations in mammalian cells, it is tempting to speculate on the existence of a conserved response to microtubule attachment defect.

The analysis of spindle surveillance at the molecular level is just beginning. The predicted amino acid sequences for *BUB1, BUB2, BUB3, MAD1,* and *MAD2* have been determined (HOYT et al. 1991; LI and MURRAY 1991; and the public databases). Unfortunately, the primary sequences of four of these (*BUB2, BUB3, MAD1* and *MAD2*) do not predict known molecular activities. *MAD2* was originally reported to encode a prenyl transferase; it has since been discovered that the cell cycle control function isolated in the original mutant is encoded by the neighboring open reading frame (LI et al. 1994). Interestingly, *BUB1* encodes a dual specificity protein kinase (ROBERTS et al. 1994). It has been speculated that *BUB3* may encode a regulator of the *BUB1* kinase: the *BUB3* gene was originally isolated as a low copy suppressor of *bub1-1*, *BUB3* coimmunoprecipitates with *BUB1*, and *BUB3* is phosphorylated by *BUB1* in vitro (ROBERTS et al. 1994).

DNA damage surveillance and spindle surveillance impose arrests in the G_2/M portion of the cell cycle. Do these surveillance pathways intersect? It is clear that *rad9* mutants delay in the presence of benomyl (WEINERT and HARTWELL 1988) and do not play a role in the yeast kinetochore-induced delay (PANGILINAN and SPENCER 1996). Moreover, the three *mad* mutants regain benomyl resistance in the

presence of hydroxyurea, suggesting that a DNA damage surveillance pathway is operative (LI and MURRAY 1991). These observations argue that the surveillance pathways are distinct. However, a large body of evidence indicates that in normal cells the execution of mitosis depends on prior DNA replication, and in fission yeast and frogs this linkage involves control of cell cycle progression through p34^{CDC2} (reviewed in NURSE 1990; MURRAY 1992). The relationship between cyclin-dependent kinase cell cycle control and the surveillance pathways monitoring DNA integrity and spindle structure has not been determined.

An intriguing recent observation from the study of mammalian p53 suggests a relationship between DNA damage and spindle structure surveillance pathways. Although the S phase-related functions of p53 are best characterized, the behaviour of cells from p53-deficient mice are consistent with an additional functional role in responding to simple damage at G_2/M (CROSS et al. 1995). In these experiments, mouse embryonic fibroblasts lacking p53 (homozygous nulls) treated with nocodazole (an antimicrotubule drug) fail to accumulate in metaphase and rapidly generate 8N and 16N subpopulations. Polyploidy was also seen to develop spontaneously in vivo. These observations on p53 null mutants suggest that searches for common elements between DNA damage and spindle structure surveillance pathways may prove to be fruitful.

5 A Surveillance Pathway May Monitor Budding Yeast Cell Morphogenesis

A recent study in budding yeast suggests that temporal control of mitosis may be sensitive to timely cytoskeletal rearrangement (LEW and REED 1995). Bud initiation is associated with a polarization of the actin cytoskeleton and delivery of cytoplasmic materials to the growing bud site. These events do not occur in cdc24 and cdc42 mutants at nonpermissive temperature, even though DNA synthesis proceeds and microtubules form a spindle. Anaphase timing under these conditions occurs ≥2h later than in control cells, timing that is essentially a full generation behind schedule.

Evidence that this delay may be due to the activity of an extrinsic control is found in the role played by p34^{CDC28} (LEW and REED 1995). In cdc24 and cdc42 cells at nonpermissive temperature, the delay observed is associated with increased phosphorylation of tyrosine 19 of p34^{CDC28}. Moreover, a phenylalanine substitution mutation (which cannot be phosphorylated) in p34^{CDC28} substantially decreases the preanaphase delay in cdc24 and cdc42 mutants. Consistent with this observation, overexpression of the tyrosine phosphatase M1H1 (the S. cerevisiae homolog of the S. pombe CDC25 phosphatase that functions in controlling p34^{CDC2} activity) partially abrogates the delay.

It is worth pointing out that this study presents experimental evidence for surveillance by a fundamentally different approach than the identification of a

control pathway typified by the *RAD9* mutational analysis. In the LEW and REED study, an alteration of p34^{CDC28} activity implies the presence of a surveillance pathway which acts directly on the controlling M phase kinase and represents the first such regulation through p34^{CDC28} demonstrated in budding yeast. Although cytoskeletal defects are the most obvious morphological consequences of loss of *CDC42* and *CDC24* function, further work is needed to identify the molecular nature of the "damage" that is sensed by the cell: *CDC42* and *CDC24* encode a rho-like GTPase and interacting nucleotide exchange factor, respectively. Might there be a related surveillance activity in mammalian cells? It is a tempting speculation: in mammals, the rho-related proteins direct actin reorganization and associate with protooncogene products (reviewed in RIDLEY 1995).

Experimental evidence demonstrating the involvement of p34^{CDC28} in extrinsic control of anaphase timing in yeast is particularly intriguing in light of the several lines of evidence pointing toward a role for altered p34 control in tumor formation in mammals. GALAKTIONOV et al. (1995b) have recently shown that human *CDC25* overexpression in rodent cells (predicted to promote tyrosine 15 dephosphorylation of cyclin-dependent kinases) cooperated with Ha-RASG$_1$2V or Rb loss in oncogenic focus formation, and that human *CDC25B* was overexpressed in 32% of human breast cancers tested (GALAKTIONOV et al. 1995b). Furthermore, human *CDC25* has been observed to associate with Raf1, a Ras-associated guanine nucleotide binding protein (GALAKTIONOV et al. 1995a). It will be interesting to learn whether one or more species of cyclin-dependent kinase is important for tumor development in humans, and which if any of the candidate cellular processes appear to be governed by altered control.

6 Concluding Remarks

Proteins that function to ensure high-fidelity chromosome replication and transmission present promising candidates for proteins with roles in tumor suppression in mammalian systems. Those that are not essential for cell viability, but are important for high-fidelity execution of chromosomal events, provide especially attractive candidates. In budding yeast, the pathways that provide surveillance of DNA and spindle integrity are clearly important for genome stability. Chromosome loss rates are elevated in the range of 10- to 50-fold in *rad9*, *mad1*, *mad2*, *mad3*, *bub1*, and *bub3* mutants (WEINERT and HARTWELL 1988; LI and MURRAY 1991; PANGILINAN and SPENCER 1996), indicating that surveillance activities are utilized in unperturbed cell populations. The current repertoire of surveillance genes provides a rich starting point, with the promise of additional genes to emerge from related work in the near future.

Although parallels in cell physiology indicate conservation of fundamental processes, the protein sequences of homologous genes provide the molecular links between related studies in yeast cells and mammals. The first such link between

surveillance pathways under study in budding yeast and mammalian cells has recently been found in the structural and functional ATM/MEC1/TEL1 homologies. This observation provides material for the design of direct experiments to evaluate the degree of conservation of protein and pathway function in two very distantly related organisms.

The recent formulation of the concept of surveillance has affected the way in which many experiments in cell cycle control and genome stability are designed and interpreted. At this stage, there are clearly more questions about mechanism than unifying hypotheses. Happily, the armament of experimental tools available in model organisms and mammals is undergoing rapid expansion. While the cytological description of the behavior of chromosomes throughout the cell cycle is vastly superior in animal systems (especially for the study of events in mitosis), the study of homologous phenomena in yeast opens avenues to efficient molecular genetic manipulations. The utilization of the strengths of both yeast and mammalian experimental systems will greatly facilitate progress in understanding the underlying molecular mechanisms underlying surveillance.

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Genomic Instability and Its Role in Neoplasia

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1 Genomic Instability and Tumor Progression

The study of how genomic integrity is regulated is important not only in the formation and progression of a neoplasia, but also in how a tumor responds to therapy. Genomic instability has been hypothesized to be a driving force behind multistep carcinogenesis (NOWELL 1976). A number of genetic changes are required for a normal cell to become tumorigenic (FOULDS 1959; FEARON and VOGELSTEIN 1990). If genomic instability increases the rate at which these alterations occur, then the accumulation of changes and subsequent selection for growth and motility advantage may lead to the formation of a neoplasia. Thus, the new variants generated during tumor progression may be fueled by an underlying genomic instability. Once a cell becomes neoplastic, its evolution may continue to malignancy. Further genetic changes are required to confer metastatic properties on the tumor cell. These properties include the ability to invade surrounding tissues, enter the vasculature, extravasate, and colonize a secondary site. Proficiency at each step is necessary for a tumor cell to become fully metastatic. Genetic alterations are the basis for this acquired variation (RUBIN 1987; LIOTTA et al. 1987). The emergence of drug-resistant or radiation-resistant variants is one of the most disappointing aspects of treating a neoplasia. These variants are generated by the same forces that allow the tumor to become established and progress.

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Neoplastic growth may result from activation of stimulatory mechanisms (proto-oncogenes) and/or from inactivation of inhibitory mechanisms (tumor suppressor genes). A disturbance of homeostasis in a number of cellular systems is neccessary to progress to malignancy. The most commonly studied cellular system is that of growth control. Improper expression of growth factors and growth factor receptors has long been known to contribute to the development of a neoplasm. More recently, it has been found that overexpression of positive regulators of the cell cycle may contribute to tumor progression. For example, experimental evidence has shown that cyclin D1 overexpression leads to hyperplasia and carcinogenesis in a mouse model system (WANG et al. 1994). Amplification of cyclin D1 has been found in primary tumors (MOTOKURA et al. 1991) and tumor cell lines (LUKAS et al. 1994). Mutations in the negative controls on this system are also found. While some cyclin-dependent kinases (CDKs) are overexpressed in tumor lines (TAM et al. 1994), the loss of the cyclin-dependent inhibitors (CDIs), negative regulators which act as tumor suppressors, is also observed in tumor cells (SERRANO et al. 1993). These data suggest that abnormalities in cell cycle control elements can underlie tumorigenesis. Likewise, disturbance in a cell's ability to move from one place to another (used to invade surrounding tissues, enter the vasculature, and extravasate) is a key factor in malignancy.

The processes of generating neoplastic variants (carcinogenesis) and generating drug-resistant variants shares several similarities:

1. Both of these events are known to occur at a rare frequency. Given the multitude of cells that comprise the body and the number of divisions that each cell goes through, it is significant that the frequency of neoplasia is as low as it is. Likewise, out of a population of tumor cells that is exposed to adverse selection pressures, it is only one out of every 10 000 or 100 000 that can emerge to survive. The generation of drug-resistant variants often occurs at frequencies that range between 10^{-3} and 10^{-6} in tissue culture models.

2. Both processes are known to be multistep in nature. It is well established that the accumulation of mutations in both oncogenes and tumor suppressor genes leads to the formation of a neoplasia. In the generation of drug-resistant variants, such as those due to gene amplification, simple duplications of genetic material is followed by the formation of more gene copies and the rearrangement of DNA sequences. Deletions, translocations, and further amplifications are known to occur as the tumor cell increases its resistance to physical and chemical therapies.

3. Each process seems to depend on the occurrence of an S phase transition. The mutations that occur and contribute to neoplasia have no effect if the cell does not proliferate (i.e., traverse the S phase of the cell cycle). It is in this phase that the mutations are fixed and passed to the daughter material. Experiments analyzing gene amplification and the generation of drug-resistant variants also point to the involvement of the S phase of the cell cycle. If cells are held at confluence during a pretreatment period that is documented to enhance amplification frequency, no increase occurs. Only if cells are cycling does the enhancement of gene amplification frequency occur in mammalian cells.

4. Both tumor progression and the generation of drug-resistant variants are typified by the emergence of a monoclonal population from a heterogeneous population. In tumor cells it is believed that selective pressure provides the screen that allows for the dominance of a monoclonal population. Those cells having the greatest advantage eventually take over or dominate the proliferative fraction of a neoplastic population. Likewise, in drug-resistant variants, one variant emerges from the heterogeneous parental population that is responding to the selection pressure.

5. Both events are known to be accompanied by widespread chromosomal rearrangements. The rearrangements that take place in tumor progression have been documented and have been reported to increase as the population becomes more malignant. Increased chromosomal rearrangements are also seen as the drug-resistant variants emerge. Deletions, translocations, aneuploidy, and inversions have accompanied the amplification events that provide resistance to certain drugs.

6. Finally, amplification of target genes is known to further each process. Amplification of oncogenes is well documented in the progression of neuroblastomas and other neoplastic samples. It is believed that amplification of these oncogenes provides a selective growth advantage to the cells that contain them. Likewise, amplification of the genes that are targeted by various chemotherapies also provides a selective growth advantage and allows for the emergence of the drug-resistant variants.

2 Systems Which Modulate Genomic Instability

In thinking of cellular processes that would affect the endpoint of genomic instability, several candidates come to mind. It has long been appreciated that the repair capacity of a cell has an important effect on the level of genetic instability found in tumor cells. The early description of human cancer syndromes, such as xeroderma pigmentosum, Bloom's syndrome, etc., and the realization that cells from these patients have problems in DNA metabolism and increased chromosomal abnormalities supports the involvement of DNA repair processes in governing the amount of genomic instability a cell experiences.

Systems which provide for the activation or inactivation of chemical and physical carcinogens could also be envisioned to influence the mutation rate in mammalian cells. Enhancement of enzyme systems which detoxify chemicals should decrease the mutation rate while inactivation of these systems should increase the mutation rate. The field of genetic toxicology is examining the contribution of these systems to human disease.

Perhaps less expected to contribute to genomic instability would be mutations in the cellular systems that govern the rearrangements of the immunoglobulin genes. These recombination and mutation events are part of a carefully controlled

developmental system that produces heterogeneity in a lymphocytic cell. If this system is improperly regulated, it could conceivably generate rough rearrangements that could contribute to the activation of oncogenes and the inactivation of tumor suppressor genes. Recent work has documented a contribution of this system to the generation of lymphomas (Kirsh et al. 1993).

Logically, one could also postulate that improprieties in chromatin structure could also contribute to genomic abnormalities. If the genetic material is not properly packaged or cannot navigate the structural changes that are repeatedly necessary as a cell replicates and divides, chromosomal breakage and abnormalities would result. At the present time, the documentation of these types of mutations to the generation of genomic instability has not occurred. A subgroup of chromatin alterations that can lead to changes in gene expression has its basis as an epigenetic event. Preferential methylation of specific genes can contribute to the neoplastic process. Recent studies have identified abnormal methylation as the trigger for turning off the expression of a specific kinase inhibitor in bladder cancer and prostate cancer. The small molecular-weight cyclin-dependent kinase inhibitor p16 has been shown to be silenced by methylation in a good proportion of these tumors. This is the first example of methylation changes that are known to contribute to tumor progression and are found in end-stage carcinomas.

In procaryotes and lower eucaryotes there are cellular systems in place which respond to environmental stress and generate a plethora of genetic changes. These inducible systems, which include the SOS response of *Escherichia coli*, generate mutations as a response to DNA damage and are believed to allow for the generation of genetic diversity that is necessary to overcome adverse growth conditions. The activation or misregulation of this type of system could be a key force in the generation of mutational changes which fuel neoplasia and malignancy.

Finally, alterations in cell cycle control can contribute to genomic instability in a variety of different ways. Recent data in this area have documented several of these contributions, and the ways in which cell cycle control is altered is an active area of study. Cell proliferation in the normal cell is regulated by both positive and negative regulatory mechanisms that control growth state transitions and progression through the cell cycle. Early in G_1, the gap phase just prior to DNA replication, the cell is responsive to exogenous signals which reflect environmental conditions. These signals may convey the presence or absence of nutrients as well as contact inhibition. At the restriction (R) point (in mammalian cells) just prior to DNA replication (S phase), the cell commits to cell division, no longer requiring mitogens for cell cycle progression, and becomes unresponsive to external growth signals (Pardee 1974).

From the R point, cell cycle progression is regulated by endogenous signals primarily from a family of CDKs. The temporal activity of CDKs is regulated by cyclin partners whose levels and activity are phase specific (Sherr 1993). As a result, the activity of a given cyclin *cdk* complex varies throughout the cell cycle. Cyclins D, E, and C are active in G_1, with cyclin E being required for traversing the G_1/S boundary. Cyclin A is required for progression through S phase and for transition to mitosis (M phase). Entry into M is signaled by activation of cyclin B,

while degradation of cyclin B allows the cell to prepare for S phase (for a review see PINES 1994). Thus, cell growth is regulated by the strictly regimented progression of cell cycle.

3 Genomic Integrity and Cell Cycle Progression

During the proliferation and differentiation of a normal cell there are four stages where the integrity of the genome may be compromised resulting in spontaneous mutations and chromosome alterations. First, errors in DNA sequence are most likely to occur during S phase when the total genome is replicated prior to cell division. Second, during cell division, replicated DNA must be partitioned evenly into two daughter cells. This may provide an opportunity for improper chromosome segregation resulting in abnormal chromosome numbers. Third, repair of damaged DNA can provide an opportunity for errors in DNA sequence to occur. Finally, genomic rearrangements, such as the VDJ rejoining of immunoglobulin chains, can go awry and result in abnormal recombination events.

   During the processes of proliferation and differentiation, one might expect to observe many more spontaneous mutations than are experimentally determined (10^{-7}/cell per generation; ELMORE et al. 1983) given the amount of DNA to be duplicated and the number of cell divisions occurring during the life span of a cell. Relatively few errors are detected, however, because the cell is equipped with mechanisms designed to prevent the propagation of errors. Normal cells are able to maintain the fidelity of the genomic sequence through proofreading mechanisms and repair processes.

   Progression of the cell cycle relies on a delicate balance of timing. Replication should precede division and should be completed before division begins. Therefore, not only is the order of events important, but an early event must be completed before a later event begins. This order is ensured by the imposition of safeguard mechanisms which evaluate where the cell is in its cycle, mechanisms termed "checkpoints" by HARTWELL and WEINERT (1989). Genes controlling these points serve to generate an inhibitory signal when upstream events are delayed, thus preventing onset of the next downstream event. Yeast and somatic cells have evolved checkpoint controls that ensure that a later event does not occur if the previous event has not yet been completed. Checkpoint controls delay cell cycle progression, presumably to permit repair of errors incurred during DNA replication or chromosome segregation.

   Three of the best-defined checkpoints in the cell cycle, the G_1/S, S phase, and the G_2/M transitions, are negatively regulated in response to DNA damage. Cell cycle progress may be delayed to allow repair of damage before entering either S or M phase. At these growth state transitions, DNA damage would be replicated or DNA would be lost due to chromosome breaks. HARTWELL (1992) suggests that checkpoints may possess three types of activities including: "1) surveillance mechanisms that can detect DNA damage, 2) signal transduction pathways that

transmit and amplify the signal to the replication or segregation machinery, and 3) possibly repair activities." Mutants that abolish the arrest or delay its occurrence in wild-type cells in response to DNA damage have been isolated (WEINERT and HARTWELL 1988; MORENO and NURSE 1994), indicating that the two checkpoints are under genetic control. These checkpoints are instrumental in facilitating cell viability after damage and in determining whether cells will survive with heritable genetic alterations. All somatic cells display a variety of such checkpoints that monitor cell growth, DNA replication, and DNA repair (HUNTER and PINES 1994).

4 Consequences of Loss of Cell Cycle Control

Cell proliferation is controlled by regulatory mechanisms, both positive and negative, that control growth state transitions and cell cycle progression. Relaxation of these control mechanisms may result in an accumulation of genetic alterations and/ or uncontrolled growth. Chromosomal alterations, such as deletions, translocations, amplifications, aneuploidy, and polyploidy, are often observed in tumor cells. These rearrangements may result from defective checkpoints controlling cellular surveillance mechanisms and lead to errors in replication, nondisjunction, and improper chromosome segregation (HARTWELL and KASTAN 1994). In fact, experimental evidence has proven that chromosomal aberrations are evident in cells lacking proper checkpoint control (NISHIMOTO et al. 1978; WEINERT and HARTWELL 1988; WHITE et al. 1994). In G_2, many yeast strains are most resistant to ultraviolet and ionizing radiation due to efficient repair mechanisms (CHANET et al. 1973; BRUNBORG and WILLIAMSON 1978). It is likely that an arrest in G_2 facilitates this repair. In mammalian cells, failure to arrest in G_2 following nitrogen mustard treatment is associated with nuclear fragmentation and enhanced lethality (LAU and PARDEE 1982). Temperature-sensitive *BN-2* mutants in BHK cells and *rad9* mutants in *Saccharomyces cerevisiae* are deficient in G_2 checkpoint control when shifted to nonpermissive temperature or irradiated, respectively, and enter mitosis with unrepaired DNA, resulting in chromosome breaks and cell death (NISHIMOTO et al. 1978; WEINERT and HARTWELL 1988). These checkpoint mutants show elevated rates of mutation and reduced viability (WEINERT and HARTWELL 1990).

Even in human cells, one may observe chromosomal instability in the form of chromosomal rearrangements and gene amplification when cell cycle regulation is compromised (LIVINGSTONE et al. 1992; SCHAEFER et al. 1993; WHITE et al. 1994), suggesting that loss of cell cycle control may be an underlying cause of genomic instability. Recent work (WHITE et al. 1994) demonstrates that compromise of checkpoint function results in the loss of genomic integrity and that integrity can be regained upon restoration of checkpoint function (YIN et al. 1992).

5 Genomic Instability and Gene Amplification

Genomic instability, the increased rate of chromosomal alterations, can take many forms: point mutation, deletion, inversion, translocation, gene amplification, and aneuploidy. Measurements of the rates of spontaneous point mutations have failed to show consistent correlations with tumorigenicity. Some studies support a correlation between spontaneous point mutation rates and tumorigenicity (SESHADRI et al. 1987), and some studies do not (ELMORE et al. 1983). Another marker for instability, gene amplification, has shown a more consistent correlation with tumorigenicity (reviewed in TLSTY et al. 1993) with normal and tumorigenic cells showing vastly different propensities for this phenotype.

Gene amplification was first postulated by BIEDLER and SPENGLER (1976) in methotrexate (MTX)-resistant Chinese hamster cells containing "strikingly long marker chromosomes." Later the mechanism of MTX resistance was determined to be increased copy number (amplification) of *DHFR* genes on these marker chromosomes, resulting in increased expression of DHFR protein (NUNBERG et al. 1978; SCHIMKE et al. 1978; DOLNICK et al. 1979). Subsequently, gene amplification has been determined to be a common mechanism of resistance to a number of agents (reviewed in STARK and WAHL 1984; SCHIMKE 1984).

Amplification of the *CAD* gene is the primary mechanism of resistance to the drug *N*-(phosphonoacetyl)-L-aspartate (PALA). PALA inhibits the aspartate transcarbamylase activity of the trifunctional CAD protein which contains carbamyl phosphate synthetase, aspartate transcarbamylase, and dihydroorotase activities. These enzymes catalyze the first three steps of UMP biosynthesis (WAHL et al. 1979). PALA specifically blocks de novo pyrimidine nucleotide biosynthesis (SWYRYD et al. 1974). Inhibition of aspartate transcarbamylase by PALA therefore results in decreased pyrimidine pools for DNA synthesis. Although amplification of CAD is the major mechanism of resistance, aneuploidy has been detected in virally transformed lines (SCHAEFER et al. 1993; WHITE et al. 1994). Amplification is also seen in tumors where the selection pressure is not apparent (McGILL et al. 1993; BAND et al. 1989; Cox et al. 1965). In many instances, the amplified DNA contains multiple copies of identified oncogenes and correlates with poor prognosis (SEEGER et al. 1985; SLAMON et al. 1987; HENRY et al. 1993). Since amplification is the mechanism of resistance to many agents, including those used in chemotherapy, and is often an indicator of more aggressive tumors, it is important to understand the regulation of gene amplification. For these studies, genomic instability is defined as the ability to generate colonies in PALA due to increased CAD gene copy number. Copy number may be increased by gene amplification or aneuploidy.

A number of models have been proposed as mechanisms of gene amplification (for review see STARK and WAHL 1984; HAMLIN et al. 1991): (a) unequal sister chromatid exchange; (b) rereplication at a locus in a single-cell cycle; (c) episome formation of a locus deletion; and (d) rolling replication. Analysis of early products of *DHFR* gene amplification in CHO cells demonstrates that a double strand break may serve as an initiating event in gene amplification (MA et al. 1993). Chromo-

somes containing these breaks may fuse at the ends forming dicentrics. These dicentrics are then subject to further cycles of breakage and fusion during chromosome segregation in mitosis.

It appears that chromosome breaks are needed for amplification and other chromosome rearrangements such as inversions and translocations. DNA double-strand breaks initiate amplification and also serve as a signal for cellular DNA damage response (NELSON and KASTAN 1994). The p53 protein induction, a key feature of this response, occurs after double-strand breaks are introduced into DNA by endonucleases (NELSON and KASTAN 1994). Any number of mutations could alter the efficiency with which these breaks are detected by the cell and/or repaired. Questions concerning the relationship between genomic instability, tumor progression, regulation of amplification potential, cell cycle, and cellular response to DNA damage can have important ramifications for therapy.

6 Cellular Response to DNA Damage

The correlation between loss of cell cycle control and amplification potential has led to an analysis of effectors of cell cycle regulation. Inactivation of wild-type p53 function leads to an increased rate of genomic instability (LIVINGSTONE et al. 1992; WHITE et al. 1994; YIN et al. 1992) and tumorigenesis (LI et al. 1988; DONEHOWER et al. 1992). It is thought that the p53 protein acts as a checkpoint control in the cell cycle to permit the repair of damaged DNA by executing cell cycle arrest in G_1 (KASTAN et al. 1991). Loss of the G_1 arrest function in p53 mutant cells results in the accumulation of mutations and genomic instability with the potential to contribute to malignant progression. In cells where p53 is mutated and no G_1 arrest occurs following damage, a G_1 arrest can be restored by the introduction of wild-type p53 (YIN et al. 1992).

In addition to being implicated in amplification and G_1 checkpoint function (WHITE et al. 1994; YIN et al. 1992), p53 has been shown to be instrumental in a cascade which mediates cellular growth arrest in response to DNA-damaging agents (KUERBITZ et al. 1992) including PALA. The transcriptional transactivation activity of p53 suggests that p53 can act indirectly to arrest cell growth. A gene transcriptionally responsive to p53 has been identified, $p21^{WAF1/CIP1}$, and has been demonstrated to inhibit the activity of cyclin cdk complexes which regulate cell cycle progression (XIONG et al. 1993; HARPER et al. 1993). Overexpression of p21 protein delays cell cycle progression (GYURIS et al. 1993), and thus p21 appears to be an effector of p53-mediated growth-suppressive activity. The evaluation of the function of the p53/p21 response to DNA damage in normal cells and tumor lines is ongoing and is expected to yield molecular targets for more effective chemotherapy. It is the detection and processing of DNA lesions along with their choreographed sequelae that will provide the variables that determine which cells are vulnerable to probability events that generate genomic rearrangements.

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Role of DNA Excision Repair Gene Defects in the Etiology of Cancer

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1 Introduction

Experimental and clinical evidence strongly suggests that exposure to environmental carcinogens is a critical event in the development of the majority of human cancers. Mutations in genes important for normal cellular functions and growth properties, including many proto-oncogenes and tumor suppressor genes, may result from spontaneous or environmentally induced alterations in DNA and contribute directly to the multistage process leading to malignancy. In addition, alterations in the specific genes required for processing and responding to DNA damage may result in an enhanced rate of accumulation of additional mutations, recombinational events, chromosomal abnormalities, and gene amplification (LOEB 1991). Therefore, the removal of lesions from DNA is essential not only for the basic processes of transcription and replication necessary for cellular survival,

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but also for maintaining genomic stability and avoiding the development of malignancies.

Many recently converging lines of experimental evidence reveal the complexity of the cellular responses to DNA damage and their role in malignant transformation. A myriad of interrelated biochemical pathways exist which influence: (a) the metabolism of potentially mutagenic or carcinogenic agents; (b) the efficiency and manner by which damaged DNA is recognized and repaired; (c) cell cycle progression and the coordination of DNA replication and cell division relative to the repair of lesions; and (d) the decision point determining survival or the active induction of programmed death of cells carrying different types and amounts of DNA damage. The discovery of several classes of DNA repair genes, which when defective result in a predisposition to the development of certain malignancies, highlights the central role of DNA damage responses in neoplastic transformation. For example, inherited mutations in genes involved in post-replication mismatch repair predispose individuals to the development of colon cancer and several other malignancies associated with the hereditary non-polyposis colon cancer phenotype (FISHEL et al. 1993; LEACH et al. 1993; BRONNER et al. 1994; PAPADOPOULOS et al. 1994). Somatic mutations or allelic loss of the DNA damage-inducible cell cycle checkpoint control protein encoded for by the *p53* tumor suppressor gene is the most commonly described genetic abnormality in the majority of all human cancers (HOLLSTEIN et al. 1991), and germ line mutations in the *p53* gene result in the inherited cancer susceptibility disease Li-Fraumeni syndrome (MALKIN et al. 1990). These two important models of hereditary cancer susceptibility are discussed in detail in several other chapters in this volume.

Because of the importance of DNA repair mechanisms in the cellular response to DNA damage, one would predict that deficiencies in repair enzymes, if compatible with viability, would predispose individuals to malignancies. In fact, a number of rare, inherited disorders have been described which appear to be caused by defects in the repair of DNA lesions (Table 1), and several of these are associated with an increased risk of developing certain cancers (HANAWALT and SARASIN 1986). In this chapter, we will focus on the clinical, biochemical, and molecular characteristics of two autosomal recessive hereditary diseases associated with defects in nucleotide excision repair (NER): xeroderma pigmentosum (XP) and Cockayne's syndrome (CS). Since only XP, but not CS, predisposes individuals to an increased incidence of malignancy, an analysis of the molecular defects underlying these disorders and their effect on mutagenesis and carcinogenesis should help to define the role of enzymes involved in NER for malignant transformation, as well as other cellular processes.

Table 1. Major human hereditary disorders that involve defects in processing DNA damage (adapted from HANAWALT and SARASIN 1986)

Syndrome	Clinical features	Increased incidence of cancers	Hypersensitivities

Xeroderma pigmentosum (classical and variant)	Sunlight hypersensitivity
	Exposed skin epithelioma
	Frequent skin cancers
	Often neurologic abnormalities
	  (none in XP variant)
	Occular defects
		+ + +	UV and chemical
			  carcinogens

Cockayne's syndrome	Cachectic dwarfism Mental retardation (microcephaly) Premature aging Sunlight hypersensitivity	–	UV and chemical carcinogens

Ataxia telangiectasia	Cerebellar ataxia
	Telangiectasia
	Neurologic deterioration
	Partial immunodeficiency
	High incidence of lymphomas
	Increased risk of breast cancer
	  in heterozygotes
		+ + +	Ionizing radiation
			  bleomycin

Fanconi's anemia	Growth retardation Pancytopenia Bone marrow deficiency Anatomical defects Increased incidence of leukemia	+	Bifunctional alkylators
Bloom's syndrome	Sunlight hypersensitivity Growth retardation High incidence of malignancies	+ + +	UV and bifunctional alkylators

2 Nucleotide Excision Repair

DNA repair may be defined as those cellular responses associated with the restoration of the normal nucleotide sequence following events which damage or alter the genome (FRIEDBERG 1985). A wide variety of endogenous and exogenous agents cause various types of damage to DNA, and multiple enzymatic processes exist to repair these different lesions. The most versatile and ubiquitous mechanism for DNA repair is NER, which functions to remove many types of lesions, including bulky base adducts of chemical carcinogens, interstrand and intrastrand cross-links, and UV-induced cyclobutane pyrimidine dimers (CPD) and 6-4 photo-products. Such lesions may serve as structural blocks to transcription and replication due to distortion of the helical conformation of DNA, and they may also result in mutations if translesional replication occurs or if they are not repaired correctly. Thus, mechanisms for NER are vital for both genomic stability and cellular survival in humans. While most of the proteins involved in eukaryotic NER have now been identified, the basic biochemical model for NER has been based mainly on studies with bacteria and is assumed to be at least functionally analogous

in higher organisms. Studies of NER in mammalian cells have benefitted greatly from analysis of cell lines deficient in particular steps in the repair process.

2.1 Biochemical Mechanism of NER

The general pathway for NER has been understood for 30 years since the discovery of pyrimidine dimer excision (SETLOW and CARRIER 1964; BOYCE and HOWARD-FLANDERS 1964) and repair replication (PETTIJOHN and HANAWALT 1964). The sequential steps are illustrated in Fig. 1 and include: (a) recognition of the damaged site; (b) incision of the damaged DNA strand at or near the site of the defect; (c) removal of a stretch of the affected strand containing the lesion; (d) repair replication to replace the excised region with a corresponding stretch of normal nucleotides using the complementary strand as a template; and (e) ligation to join the
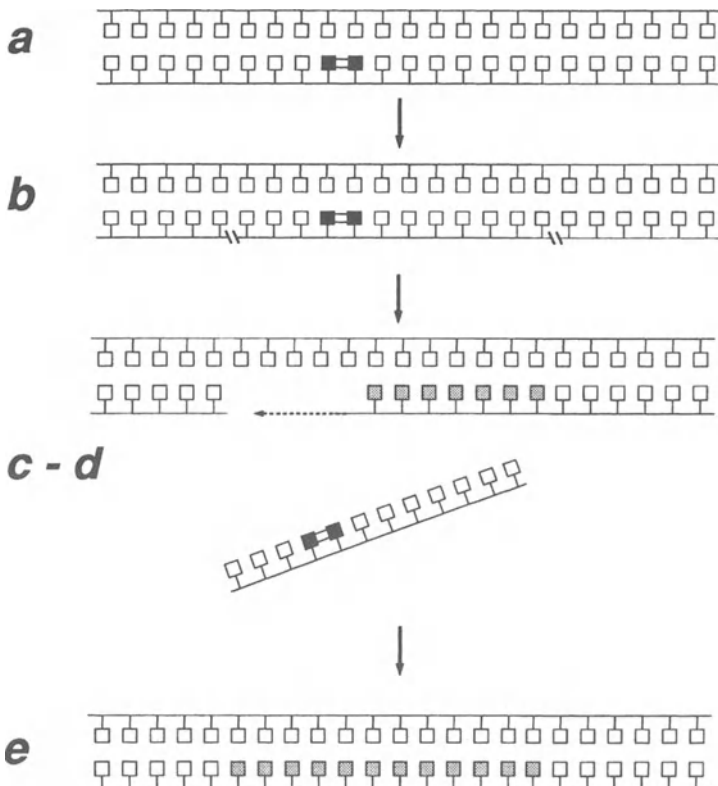
Fig.1 a-e. Nucleotide excision repair. Following the formation of a DNA lesion, the following steps occur sequentially: **a** recognition of the damaged site; **b** incision of the damaged DNA strand near the site of the defect; **c** removal of a stretch of the affected strand containing the lesion; **d** repair replication to replace the excised region with a corresponding stretch of normal nucleotides using the complementary strand as a template; and **e** ligation to join the repair patch to the contiguous parental DNA strand

repair patch at its 3' end to the contiguous parental DNA strand (HOEIJMAKERS 1993a,b).

Molecular details of the mechanism of NER are known primarily from work with the prokaryote *Escherichia coli* and have been recently reviewed elsewhere (HOEIJMAKERS 1993a; SANCAR and TANG 1993). Briefly, the basic components of the bacterial system include three proteins, termed UvrA, UvrB, and UvrC, which participate in the initial steps of lesion recognition and incision on both sides of the lesion. Excision of the 12-13-mer damage-containing oligonucleotide requires the concerted action of UvrD (helicase II) and DNA polymerase I. Polymerase I then fills the resultant gap and ligase seals the newly synthesized DNA to the preexisting strand. The cloning and expression of bacterial genes involved in NER has enabled the reconstitution of repair in vitro (SELBY et al. 1991; SELBY and SANCAR 1991, 1993) and has facilitated the identification of additional factors that modulate the activity of NER. As will be discussed more extensively below, investigators in our laboratory first demonstrated in mammalian cells that UV-induced CPD are more efficiently removed from actively expressed genes than from unexpressed regions of the genome (BOHR et al. 1985; MELLON et al. 1986), and that this preferential repair is due to more efficient repair in the transcribed than the non-transcribed DNA strand (MELLON et al. 1987) suggesting a direct association between transcription and repair. This phenomenon was subsequently shown to occur in *E. coli*, in which a pronounced preferential repair of the transcribed strand of the induced *lac* operon was observed compared to that in the non-transcribed strand (MELLON and HA-NAWALT 1989). Recently, the product of the bacterial *mfd* gene was found to be required for transcription-coupled repair in *E. coli* (SELBY et al. 1991), suggesting that it may function as a transcription-repair coupling factor to target NER to lesions within active genes (SELBY and SANCAR 1993; HANAWALT and MELLON 1993).

To what extent the detailed enzymatic features of prokaryotic NER are preserved in eukaryotes is largely unknown, though the basic steps of lesion recognition, dual incision of the damaged strand and repair synthesis have been clearly documented (HOEIJMAKERS 1993b). The eukaryotic yeast *Saccharomyces cerevisiae* has been studied as a paradigm for mammalian NER, facilitated by the large number of excision repair-deficient yeast mutants available (SWEDER 1994). To date, more than 30 genetic loci have been identified which affect the sensitivity of yeast to UV irradiation, and at least 12 genes have been identified which are specifically involved in NER. While even less is known regarding the specific molecular components of NER in humans, it is certainly likely to be at least as complex as that in yeast. Most of the genes implicated in human excision repair processes have been cloned and demonstrate a remarkable conservation of certain nucleotide sequence motifs with their yeast counterparts.

2.2 Heterogeneity of NER

The preferential repair of expressed genes was first demonstrated when it was discovered in our laboratory that UV-irradiated Chinese hamster ovary cells repair CPD much more efficiently in the expressed dihydrofolate reductase (*DHFR*) gene than in an unexpressed DNA sequence downstream or in the genome overall (BOHR et al. 1985). Similarly, a preferential rate of repair of the *DHFR* gene was observed in human cells (MELLON et al. 1986), though overall genomic repair was significantly more efficient than in murine or rodent cells. We suggested that the blocked RNA polymerase might serve as a signal to recruit repair enzymes to these sites in expressed genes (MELLON et al. 1986). It was subsequently shown that the preferential repair of the expressed *DHFR* gene in hamster and human cells was primarily due to preferential repair of the transcribed DNA strand (MELLON et al. 1987). This so-called transcription-coupled repair has since been demonstrated in a number of other expressed mammalian genes (cf. LEADON and LAWRENCE 1991; VENEMA et al. 1992; FORD et al. 1994), in bacteria (MELLON and HANAWALT 1989), and in yeast (SMERDON and THOMA 1990; SWEDER and HANAWALT 1992; LEADON and LAWRENCE 1992) implicating it as a highly conserved and ubiquitous molecular repair pathway.

A number of experimental approaches suggest that the phenomenon of transcription-coupled DNA repair is strictly dependent upon RNA polymerase II transcriptional elongation activity. For example, strand-selective repair is not observed in human and rodent ribosomal RNA genes which are transcribed by polymerase I (VOS and WAUTHIER 1991; CHRISTIANS and HANAWALT 1993), and it is abolished by the RNA polymerase II inhibitor, α-amanatin (CHRISTIANS and HANAWALT 1992). Furthermore, in yeast containing a temperature-sensitive mutation in the *rpb1-1* subunit of RNA polymerase II, the more rapid and preferential repair of the transcribed strand of active genes was lost at the non-permissive temperature, whereas repair on the non-transcribed strand and genome overall was not significantly affected (SWEDER and HANAWALT 1992; LEADON and LAWRENCE 1992). Therefore, it appears that some NER is directly associated with transcription, and a great deal has been recently learned regarding this association (HANAWALT et al. 1994). We have proposed a model for transcription-coupled repair in which a transcription-repair coupling factor specifically binds to the blocked RNA polymerase at a site of transcription blockage and provides both a high-affinity binding site for the repair complex involved in initiating NER and also facilitates the removal of RNA polymerase and truncation of the nascent RNA transcript to provide access for repair enzymes to the lesion site (HANAWALT 1993). In fact, recent studies have shown that transcript shortening occurs at the site of a CPD and is dependent on the transcription factor SII (TFIIS), which may also be essential for the resumption of transcription after the template has been repaired (DONAHUE et al. 1994).

The existence of a mechanism to facilitate the preferential repair of the transcribed strand of active genes in both eukaryotes and prokaryotes raises a number of questions as to its evolutionary role. Certainly, one could presume that strand-

specific repair of active genes would be important for maintaining genomic stability in multicellular organisms by helping to avoid transforming mutations in expressed proto-oncogenes and tumor suppressor genes in humans. However, the lack of an increased incidence of malignancy in individuals with CS, a disease in which transcription-coupled repair has been selectively lost but overall genomic NER retained, argues against this NER pathway as being critical in the process of transformation. This issue will be further explored in a later section with regard to actual mutational spectra in cancers from normal compared to NER-deficient patients. Furthermore, the existence of transcription-coupled repair in unicellular and prokaryotic organisms suggests that its function is more important to the basic processes of transcription and replication required for cellular survival.

2.3 NER Genes

Considerable progress has been made recently in identifying and characterizing human NER genes (WEEDA et al. 1993). The most successful strategy has involved identifying human genomic sequences that correct the UV sensitivity and DNA repair deficiency in mutant rodent cell lines. Corresponding human cDNA clones have been isolated and termed excision repair cross-complementing (ERCC) genes. Many of the identified genes have been shown to be mutated in human NER deficiencies, such as XP and CS (Table 2). For example, the ERCC6 gene was first cloned from a human sequence that corrected the UV-sensitive Chinese hamster UV61 cell mutant (TROELSTRA et al. 1990) and was subsequently found to correct the molecular defect in human CS-B cells, which lack transcription-coupled repair (TROELSTRA et al. 1992), but not to correct the repair defect in CS-A cells. A human CS-A cDNA was subsequently cloned by functional complementation of CS-A cells, and lacked the ability to restore UV-resistance to CS-B cells (HENNING et al. 1995). Thus, the CS-A and CS-B proteins, perhaps as a heterodimer, appear to be directly involved in linking DNA repair and transcription, and may function in an analogous manner to the prokaryotic *mfd* transcription-repair coupling factor.

Table 2. Cloned human nucleotide excision repair genes

Human gene	Mutant rodent cell line(s)	Human disease	UV sensitivity
ERCC1	UV20, 43-3B	?	+ + +
ERCC2	UV5, V-HI	XP-D	+ + +
ERCC3	UV24	XP-B/CS-C	+ + +
ERCC4	UV41, UV47	XP-F	+ + +
ERCC5	UV135	XP-G	+ +
ERCC6	UV61	CS-B	+
XPA		XP-A	+ + +
XPC		XP-C	+
CSA		*CSA*	+

Another recently identified human repair gene, *ERCC3*, has been implicated in patients with XP-B/CS-C, a rare group of XP patients who also display clinical features of CS and are completely deficient in the repair of overall genomic DNA, including transcribed sequences. *ERCC3* may thus constitute another functional element involved in transcription-repair coupling (WEEDA et al. 1990). However, this gene is also essential for overall genomic excision repair (SWEDER and HANA-WALT 1993, 1994; HANAWALT et al. 1994; MA et al. 1994). Similarly, the human repair gene ERCC2 has been cloned and found mutant in patients with XP-D/CS (TAKAYAMA et al. 1995). Remarkably, both the ERCC2 and ERCC3 gene products have now been shown to also be components of the basal transcription initiation factor TFIIH (SCHAEFFER et al. 1993; VAN VUUREN et al. 1993; DRAPKIN et al. 1994; SCHAEFFER et al. 1994), and to possess helicase activities which may help in open complex formation and promoter clearance. Indeed, the convergence at the molecular level of NER and transcription is supported by a growing number of reports in which essential protein components of the eukaryotic transcription factor TFIIH are required for NER (reviewed in HANAWALT et al. 1994; FRIEDBERG et al. 1994). The presumed dual role for many of these proteins in both NER and gene transcription therefore provides new and provocative hypotheses regarding the cellular and clinical consequences of mutations in these genes. For example, it has been suggested that some of the developmental abnormalities in CS may in fact represent a class of "transcription disease," rather than consequences of a repair deficiency (BOOTSMA and HOEIJMAKERS 1993).

3 Human NER-Deficient Syndromes and Cancer

A direct correlation between unrepaired DNA damage and carcinogenesis in humans was first established when CLEAVER (1968) found that the cancer-prone hereditary disease XP involved a defect in the repair of DNA lesions produced by UV light. Since that discovery, a great deal has been learned regarding the clinical characteristics and molecular defects of this and the related UV hypersensitivity disorder CS. In this section, we will review these findings and discuss the potential role of specific defects in NER in oncogenesis.

3.1 Clinical Characteristics

Xeroderma pigmentosum is a rare, autosomal recessive disease in which homozygous individuals display several characteristics: (a) extreme sensitivity of the skin to sun (UV) exposure evident by 1 year of age; (b) pigmentation abnormalities and premalignant lesions in sun-exposed skin; (c) up to 4000-fold increases in incidence of skin cancers (predominantly squamous and basal cell carcinomas, but also melanomas) and ocular neoplasms, occurring 3–5 decades earlier than in the gen-

eral population; and (d) a 10- to 20-fold increased incidence of internal cancers in non-sun-exposed sites (KRAEMER et al. 1984; CLEAVER and KRAEMER 1989). Overall, there is an approximately 30-year reduction in life span among XP patients, and many die due to malignancies (KRAEMER and SLOR 1985). Approximately 20% of XP patients also display progressive neurologic degeneration, characterized by peripheral neuropathy, sensorineural deafness, progressive mental retardation, and cerebellar and pyramidal tract involvement (ROBBINS 1988). The incidence of XP is approximately one in 250 000 individuals in the United States and in Europe, but significantly higher (one in 40 000) in Japan and Egypt.

Cockayne's syndrome is also an autosomal recessive disease with defective repair of UV-damaged DNA (SCHMICKEL et al. 1977) and is characterized by cutaneous photosensitivity, cachectic dwarfism, skeletal abnormalities, retinal degeneration, cataracts, severe mental retardation, and neurologic degeneration characterized by primary demyelination, calcific deposits in the brain, and normal pressure hydrocephalus (ROBBINS 1988; TIMME and MOSES 1988). However, in contrast to patients with XP, those with CS are not at an increased risk for developing skin cancers. The average life span of individuals with CS is only 12 years, with most patients succumbing to infectious or renal complications, rather than cancer (NANCE and BERRY 1992).

3.2 Complementation Groups

Cultured fibroblasts from XP patients are hypersensitive to UV irradiation and to carcinogens that form bulky adducts in DNA. The biochemical defect in cells from most XP individuals is in NER (CLEAVER 1968), though in a small group of cases (termed XP variants), excision repair appears normal, and a defect apparently exists in bypass replication at unrepaired lesions (WANG et al. 1993). Complementation analysis via fusion of cells or cell nuclei from different patients has demonstrated genetic heterogeneity within XP and provided evidence for the existence of at least seven excision-deficient complementation groups, termed XP-A through XP-G, in addition to the above-mentioned XP variant (Table 3). Similarly, CS is characterized by the existence of at least three complementation groups, termed CS-A through CS-C. Presumably, each XP and CS complementation group represents a defect in a distinct DNA repair gene. Furthermore, three patients have been described in XP group B (ROBBINS et al. 1974) and several in XP groups D and G who share the DNA repair defects and clinical features of CS together with the cutaneous manifestations of XP (XP-B/CS-C, XP-D/CS, and XP-G/CS, respectively). The two most recently identified patients (siblings) with XP-B are hypersensitive to UV, severely defective in NER, display some cutaneous and neurologic abnormalities common to XP and CS, but have not developed skin cancers even beyond age 40 (VERMEULEN et al. 1994). Molecular analysis of the XP-B/CS-C-correcting *ERCC3* gene has revealed a single-base missense mutation in a conserved region for both these patients. Thus, the relationship between defects in repair proteins and UV-induced skin carcinogenesis may, at least for cer-

Table 3. Properties of XP and CS complementation groups

Complementation group	Associated CS	Frequency	UDS (%)	Overall repair	Transcription-coupled repair	Skin cancer	Neurologic abnormalities
XP-A	–	High	< 5	–	–	+	+
XP-B	+	Only 3	< 8	–	–	+/–	+
XP-C	–	High	15–30	–	+	+	–
XP-D	+/–	Intermediate	20–50	–	–	+	+
XP-E	–	Rare	< 50	–	?	–	–
XP-F	–	Rare	15–30	+/–	–	–	--
XP-G	+/?	Rare	5–15	–	?	+/–	+
XP variant	–	High	100	+	+	+	–
CS-A	+	Rare	100	+	–	–	+
CS-B	+	High	100	+	–	–	+

tain genes, be more complex than can be simply accounted for by abnormalities in DNA repair.

Cells from XP complementation groups A through G all appear to be defective in an early step in NER, prior to and including incision. However, they display variable degrees of UV sensitivity and corresponding deficiencies in their capacity to carry out the incision step of NER (Table 3). For example, XP-C cells are only moderately UV sensitive and have been shown to repair limited domains of their genome (MANSBRIDGE and HANAWALT 1983), while the repair deficiency and UV sensitivity is more pronounced for XP groups A, B, and G.

3.3 Biochemical and Molecular Aspects of NER-Deficient Diseases

The development of methods to measure the efficiency and extent of NER within specific regions of the genome (BOHR et al. 1985) has greatly advanced our understanding of the underlying defects within many of the XP and CS complementation groups (Table 3). For example, XP-A cells demonstrate severe loss of both overall genomic and transcription-coupled DNA repair (EVANS et al. 1993a) and display the greatest UV sensitivity. XP-C cells have been shown to selectively retain the ability to repair CPD in the transcribed strands of expressed genes (VENEMA et al. 1990b, 1991), though they are deficient in overall genomic NER (MANSBRIDGE and HANAWALT 1983; KANTOR et al. 1990). Therefore, the relative resistance of XP-C cells to UV irradiation, compared to XP-A, may be due to persistent transcription-coupled repair of active genes. This view is supported by studies of a partial revertant of an XP-A cell line called XP129 (LOMMEL and HANAWALT 1993). The revertant has regained the full UV resistance of normal human fibroblasts and it also has regained transcription-coupled repair. The features of repair in the XP129 cells interestingly correspond to those of most rodent cells. That is, repair of 6–4 photoproducts is efficient throughout the genome, while CPD repair is restricted to transcribed DNA strands. It is concluded that tran-

scription-coupled repair is more important to cell survival than is overall genomic repair, at least for CPD.

In contrast, while CS cells appear normal in their ability to repair overall genomic DNA, MAYNE and LEHMANN (1982) first observed that these cells were defective in the recovery of RNA synthesis following UV irradiation, suggesting a deficiency in the removal of CPD from active genes. Indeed, we (HANAWALT 1993), and others (VENEMA et al. 1990a), have directly demonstrated that CS cells are selectively defective in the preferential repair of transcribed strands of active genes, which likely accounts for the UV sensitivity of these cells. In fact, our studies demonstrated a reduction of repair within the expressed *c-abl* gene to levels even below that of overall genomic repair (HANAWALT and MELLON 1993), suggesting that in the absence of a mechanism for transcription-coupled repair, a stalled RNA polymerase blocks access of recognition and repair proteins to lesions within transcribed DNA strands. However, it has also been reported that CS cells repair the transcribed strand of the active *DHFR* and *ADA* gene at similar rates as the non-transcribed strand (VAN HOFFEN et al. 1993), yet more slowly than either strand in normal cells. The defect in CS may also affect more than simply the preferential repair of polymerase II-transcribed genes, since CHRISTIANS and HANAWALT (1994), recently showed markedly deficient repair of CPD in polymerase I-transcribed ribosomal RNA gene sequences in cell lines from both CS-A and CS-B patients, genes that do not exhibit transcription-coupled repair in normal cells.

Therefore, analysis of cell lines and individuals from several of the XP and CS complementation groups provides a useful model system by which to determine the individual contribution and role of overall and transcription-coupled NER pathways to UV-induced carcinogenesis.

3.4 Relationship Between XP and CS Repair Defects and Clinical Phenotype

Analysis of the specific abnormalities in NER displayed by the various genetic complementation groups of XP and CS allows correlations to be drawn with their heterogeneous clinical features. Specifically, only those subgroups of patients who display a defect in overall repair of genomic DNA are at significantly increased risk for developing UV-induced malignancies. In contrast, the neurologic symptoms associated with certain other complementation groups of XP and CS are found only in those groups which are defective in transcription-coupled repair (Table 4). In fact, recent data implicating particular human NER enzymes as also being components of a transcription factor complex suggest that the clinical phenotype of patients with defects in transcription-coupled repair may actually be due to abnormalities in transcription rather than in repair itself, as noted above.

For example, patients with XP-A and XP-D generally possess both severe neurologic disorders and a high incidence of skin cancers, and their cells lack the ability to perform both overall and transcription-coupled repair (EVANS et al. 1993a). Patients with XP-C develop skin disorders, but remain neurologically intact

and retain the selective repair of the transcribed strand of active genes. Patients in the XP variant group have mild to severe skin symptoms and are neurologically normal (CLEAVER and KRAEMER 1989). XP variant cells have intact transcription-coupled repair and overall genomic repair, but are defective in a post-replication repair process. Patients from all CS complementation groups are distinguished by a complex phenotype, including developmental and neurologic abnormalities, but they are not at increased risk for malignancies. As noted above, cells from CS patients are defective in transcription-coupled repair, but are generally intact for overall repair functions.

While potentially explaining the molecular basis for many of the clinical characteristics of XP and CS, these observations present an apparent paradox with regard to these patients' cancer risk. The current theory of multistage carcinogenesis suggests that activating mutations in proto-oncogenes and inactivating mutations in tumor suppressor genes drive the progression of cells from normal to transformed to frankly malignant. Many currently recognized oncogenes and tumor suppressor genes are known to possess important cellular functions, and to be actively expressed in normal cells. Since CS cells are defective in the repair of actively expressed genes and have also been shown to be hypermutable (MAYNE et al. 1982), a reasonable prediction would be that these patients would acquire mutations in genes leading to transformation more readily than normal patients. However, this is not supported by the clinical picture.

In contrast, XP patients all share a defect in overall NER and a dramatic propensity to develop UV-induced skin cancers, even though some XP patients retain efficient repair of expressed genes. It is not readily apparent why a defect in overall NER, but not transcription-coupled repair, predisposes individuals to cancer. However, it appears that the more rapid and preferential repair of transcriptionally active genomic regions is mainly important to facilitate the formation of full-length mRNA transcripts and directly affects cellular viability, while repair of transcriptionally silent DNA is much more critical for reducing the mutational load of cells and protecting against neoplastic transformation.

Recent analyses of the DNA damage-inducible, p53-dependent apoptotic pathway in XP and CS cells provides an interesting alternative explanation for this paradox. YAMAIZUMI and SUGANO (1994) first demonstrated that DNA damage-induced activation of the wild-type p53 gene product occurred following lower doses of UV irradiation in human fibroblasts deficient in transcription-coupled repair (XP-A and CS-B) than in those proficient in transcription-coupled repair (XP-C and normal fibroblasts), regardless of the efficiency of global nucleotide excision repair. LJUNGMAN and ZHANG (1996) have confirmed and extended these findings to demonstrate that apoptosis also occurs following lower doses of UV irradiation in XP-A and CS-B cells than in normal or XP-C human skin fibroblasts, and that these results correlated with the induction of p53.

These results suggest that UV-induced activation of p53 is evoked specifically by lesions in the transcribed strand of expressed genes, and that perhaps the blockage of RNA polymerase II by UV photoproducts may serve as a signal for the induction of p53 and subsequent apoptosis. Furthermore, these results provide

support for a provocative additional model to explain the differences in clinical phenotypes and cancer predisposition among individuals with different nucleotide excision repair deficiency syndromes. In this model, cells from patients defective in transcription-coupled repair are more prone to undergo apoptosis following exogenous or endogenous DNA damage, perhaps leading to the development of the neurological abnormalities seen in these groups. Conversely, enhanced damage-induced apoptotic cell death may result in the elimination of DNA damaged, pre-mutagenic cells, and lower the risk of carcinogenesis (LJUNGMAN and ZHANG 1996).

Another puzzling aspect of the clinical phenotype of XP is why these patients do not appear to be at a greater risk for developing neoplasms other than skin cancers. While a disproportionate number of relatively rare tumors such as brain sarcomas and extra-glossal carcinomas of the oral cavity have been described in XP patients under 40 years of age (KRAEMER et al. 1984), individuals with XP do not appear to be at significantly increased risk for more common solid or hematologic malignancies. Owing to the presumed importance of NER pathways for removing bulky DNA adducts resulting from environmental carcinogen exposure, it is surprising that a greater number of cancers such as lung, liver, breast, colon, gastric, and other gastrointestinal malignancies are not seen in XP patients. Based on this apparent lack of an increased number of internal malignancies, CAIRNS (1981) has argued that such common cancers in the normal population are not caused by the particular DNA lesions which XP patients are unable to repair. However, alternative explanations include a potential sample bias due to the early mortality experienced by XP patients, or a decreased exposure to non-UV environmental carcinogens during early life for XP patients.

3.5 Disease Due to Defects in Transcription

As discussed previously, many recently identified human excision repair proteins have been found to be integral components of the basal transcription factor complex TFIIH. These observations raise the provocative possibility that many of the heterogeneous and systemic clinical symptoms manifested by patients with XP and CS may in fact be due to subtle alterations in gene transcription and regulation, rather than caused directly by the corollary defects in NER. This hypothesis is particularly attractive for explaining the pathogenesis of the seemingly developmentally related abnormalities in these patients, such as neurologic and growth defects. However, it is more difficult to generalize this explanation to include CS-B, since the *ERCC6* gene, which when mutant results in this disease, has not been found to associate with or function as a transcription factor. Furthermore, while a defining feature of CS is a lack of resumption of RNA synthesis following UV irradiation of cells in vitro (MAYNE and LEHMANN 1982), evidence for specific transcriptional abnormalities in the absence of DNA damage has not been reported for CS cells, nor for XP cells which display defects in transcription-coupled repair due to mutations in genes whose products are associated with TFIIH (XP-B/CS-C, XP-D/CS).

4 Role of NER in Mutagenesis

One approach to evaluate the influence and significance of certain DNA repair functions on carcinogenesis and transformation is to analyze patterns of mutagenesis in clinical tumor samples from repair-deficient patients and in experimental systems using cells derived from these patients. The following sections will discuss several experimental approaches employed to probe these questions, by looking at UV-induced mutational frequency and patterns in NER-deficient cells and in clinical samples from patients with deficiencies in NER.

Heterogeneity of DNA repair processing may have implications for induction of mutations since the frequency of mutations will depend in part on the extent of repair of pre-mutagenic lesions prior to replication, as well as the consequences on cellular survival of the damage event or its mutagenic result. Furthermore, preferential repair of transcribed DNA strands should be expected to have measurable consequences for the resulting pattern of mutations. Specifically, transcription-coupled repair should result in an accumulation of DNA adducts in non-active regions of the chromosome and in the non-transcribed strands of active genes, leading to a relative abundance of mutations at these sites. Therefore, the mutational spectrum resulting from UV irradiation will be influenced by the more efficient repair of the transcribed strand and predicts a bias toward mutations resulting from lesions in the non-transcribed strand.

4.1 UV-Induced Mutational Spectra in Shuttle Vectors

The development of a host cell reactivation assay using a replicating plasmid has allowed for the analysis of UV-induced base substitution mutagenesis in various human cells. A "shuttle vector," derived from viral genomes such as SV40 to ensure replication in mammalian cells, and, containing a marker gene as a mutational target, is UV irradiated and transfected into mammalian cells. Following DNA repair, plasmids which have replicated are recovered, screened for mutations, and sequenced (BRASH 1988). In this manner, the effect of specific host cell DNA repair enzymes on UV-induced base mutations may be determined for a defined DNA sequence.

Several groups have now used the pZ189 shuttle vector to perform such experiments in various normal, XP, and CS cell lines. This plasmid contains a 150-bp bacterial suppressor tRNA gene, *supF*, that allows for selection of mutants in an indicator strain of bacteria (BREDBERG et al. 1986). The mutational spectrum of UV-irradiated pZ189 following replication in cells from normal, repair-proficient individuals demonstrates predominantly C to T transition mutations at dipyrimidine sequences, similar to results previously obtained in bacteria (BRASH 1988). This observation has been explained by TESSMAN (1976) as the "A rule," whereby polymerase preferentially inserts an adenine opposite the site of uninformative damaged bases. Similar experiments utilizing pZ189 in fibroblasts or lymphoblastoid cells from XP-A, XP-C, and XP-D patients consistently demonstrated a

higher frequency of mutated plasmids compared to experiments using normal cells, but an altered spectrum of mutations characterized by a relative increase in the frequency of C to T transitions (90% compared to 70%) and a decrease in other mutations, such as transversions and multiple-base substitutions (BREDBERG et al. 1986; SEETHARAM et al. 1987, 1991; YAGI et al. 1991, 1992). These results suggest that UV-induced C to T transition mutations may be particularly important for UV-related skin cancers, especially in patients with XP. In fact, this prediction has been borne out, as discussed below.

The use of this shuttle vector system to probe the effect of transcription-coupled repair on UV-induced mutagenesis is problematic, as it is unlikely that the bacterial *supF* gene is transcribed by RNA polymerase II in human cells. Nevertheless, several investigators have determined the UV-induced mutational spectrum of pZ189 following replication in CS cells and found a pattern similar to that seen with fibroblasts from normal humans (MURIEL et al. 1991; PARRIS and KRAEMER 1993), as would be expected for a non-transcribed DNA sequence. The ability to determine the effect of transcription-coupled repair on UV-induced mutations in a shuttle vector system awaits the construction of an appropriate plasmid containing an inducible, polymerase II-transcribed gene selectable for mutations. Such a vector will prove very interesting for use in XP-C and CS cell lines to determine the effect of the presence and absence, respectively, of transcription-coupled repair on mutational frequency and potential strand bias.

4.2 Experimental UV-Induced Mutations in Endogenous Genes

The use of the polymerase chain reaction has facilitated the study of the effect of NER on mutations in endogenous genes in mammalian cells and tissues. VRIELING et al. (1991) first demonstrated that strand-specific repair of an expressed mammalian gene was associated with a bias toward the non-transcribed strand for the dipyrimidine sites at which the majority of UV-induced mutations occurred by mapping mutations in the *HPRT* gene in several repair-proficient hamster cell lines (which display a CPD-repair phenotype similar to that of human XP-C cells). These results were extended to human cells by Maher and colleagues (McGREGOR et al. (1991), who demonstrated that when synchronized, repair-proficient primary human fibroblasts were UV irradiated in early G_1 of the cell cycle (6 h prior to S phase), 80% of the resulting *HPRT* mutations arose at dipyrimidine sites situated in the non-transcribed strand, suggesting that the bias is due to preferential repair of the transcribed strand prior to replication. In contrast, when the same cells were irradiated in S phase, thus eliminating time for DNA repair prior to replication, or when human XP-A cells were used to eliminate NER entirely, an average of 75% of *HPRT* mutations were due to lesions in the transcribed strand. Similarly, VRIELING et al. (1989) found that 90% of mutations in repair-deficient hamster cells were due to photoproducts in the transcribed strand of *HPRT*. These authors argue that the transcribed strand bias seen for mutations in this gene in the absence of transcription-coupled repair which cannot be explained by the distribution of

dipyrimidine sites may be due to differences in polymerase error rate between synthesis of the leading and lagging DNA strand on damaged templates (MUL-LENDERS et al. 1991). Alternatively, in the absence of transcription-coupled repair, the blocked RNA polymerase may preclude repair at these sites, thus giving the reverse bias.

Mutations at dipyrimidine sites in the p53 tumor suppressor gene characteristic of UV-induced DNA damage have been identified in a high frequency of human skin cancers, providing genetic evidence for a direct relationship between sunlight and the development of skin cancer (BRASH et al. 1991). Murine skin cancers induced by repeated UV-exposure provide an excellent model for investigating the mutagenic effects of UV, by determining the resulting sequences of the *p53* gene. Furthermore, unlike the *HPRT* mutation assay, which relies on phenotypic selection of mutants, this cancer-related gene mutation assay is based on genotype analysis and reflects the carcinogenic as well as mutagenic effects of UV-induced DNA damage. Since the *p53* gene can be functionally mutated at more than 100 sites (in contrast, for example, to the *ras* proto-oncogene), it is a particularly good target for determining in vivo mutational spectra, though most analyses only look at restricted regions of the gene. KRESS et al. (1992) reported that 32% of 22 squamous cell skin carcinomas from several strains of mice chronically exposed to UV-B irradiation contained base substitutions in the p53 gene, and all were located at dipyrimidine sites in the non-transcribed strand of the gene. Similarly, analysis of 11 tumor cell lines derived from UV-induced murine skin cancers revealed all contained one or more p53 mutations at dipyrimidine sites, 76% of which arose from lesions in the non-transcribed strand (KANJILAL et al. 1993). Since rodent cells are very poor at repairing CPD in non-transcribed DNA sequences but repair the transcribed strands of active genes quite well (MELLON et al. 1987), these results strongly implicate transcription-coupled repair in causing the resulting mutational strand bias in the p53 gene.

The mutational spectrum of the p53 gene has also been analyzed in UV-irradiated cultured human keratinocytes (NAKAZAWA et al. 1994). CC to TT transition mutations at two specific codons were measured and found to increase in a UV dose-dependent manner, while no mutations occurred in non-irradiated controls. Similar experiments using cells from XP and CS individuals would be revealing regarding the relative frequency of mutation induction and potential strand bias.

Thus, these data from a variety of experimental systems allow one to make predictions of the mutational spectra and strand bias for UV-associated mutations in genes from tumors arising spontaneously in normal patients compared to those from patients with various NER deficiencies.

4.3 *p53* Mutational Spectra in Human Tumors

Until recently, the role of sunlight exposure in the pathogenesis of skin cancers was inferred based upon strong epidemiological evidence since the molecular basis for these tumors was unclear. However, recent observations show that mutations of the *p53* tumor suppressor gene, the most commonly altered gene in the majority of all human cancers (HOLLSTEIN et al. 1991), occur in over half of human squamous and basal cell carcinomas of the skin, and that the spectrum of these mutations directly implicates UV-induced DNA damage as the primary carcinogenic event (BRASH et al. 1991; ZIEGLER et al. 1993). For example, BRASH et al. and ZIEGLER et al. found that nearly all p53 mutations in human squamous and basal cell carcinomas occurred at dipyrimidine sites, and that a quarter of these resulted from CC to TT double-base transitions, virtually pathogneumonic for UV-induced mutations. This observation has been extended to demonstrate that UV-specific p53 gene mutations occur in normal skin and may be useful as a biologically relevant measure of UV exposure and a possible predictor for skin cancer risk (NAKAZAWA et al. 1994). In this study, 17 of 23 (74%) normal skin samples from sun-exposed sites contained CC to TT transition mutations in the p53 gene, while only one of 20 (5%) samples from non-sun-exposed sites displayed such an alteration in this gene.

An analysis of the strand distribution of p53 transition mutations at dipyrimidine sites in skin cancers provides the first opportunity to assess directly the role of overall and transcription-coupled NER in carcinogenesis of a specific human tumor. In fact, for basal cell carcinomas, a trend has been observed for a higher frequency of mutations at dipyrimidine sites in the non-transcribed strand of p53 in several small series (ZIEGLER et al. 1993; RADY et al. 1992; MOLES et al. 1993), though p53 mutations were evenly distributed between DNA strands in reported squamous cell skin carcinomas (BRASH et al. 1991; PIERCEALL et al. 1991). Of note, a pronounced predominance of guanine transversion mutations arose from lesions in the non-transcribed strand of p53 in human lung cancers (HOLL-STEIN et al. 1991; PUISIEUX et al. 1991; MILLER et al. 1992). The preferential repair of tobacco carcinogen benzo[*a*]pyrene diol epoxide or similar adducts in the transcribed strand of active genes (CHEN et al. 1992) may account for this bias.

Several recent reports have also described the *p53* mutational spectrum in skin cancers from patients with XP. While still insufficient to correlate particular mutational patterns to specific XP complementation groups, the findings as a whole are remarkably similar to those predicted from the in vitro and in vivo model systems previously described. SATO et al. (1993) reported that five of eight non-melanoma skin cancers from six Arabic XP patients contained mutations in the p53 gene. All base changes were located at dipyrimidine sites containing cytosine residues. Of the four patients for whom complementation groups were known, mutations likely arose at dipyrimidines in the non-transcribed strand of *p53* in an individual with XP-A and one with XP-C, and from the transcribed strand in an individual with XP-F and one XP variant.

DUMAZ et al. (1993) analyzed *p53* mutations in a larger series of XP skin cancers: 48% of 23 squamous cell carcinomas and 27% of 11 basal cell carcinomas of the skin

contained *p53* mutations, all at dipyrimidine sites and 61% CC to TT tandem base transitions. Furthermore, 15 of the 17 documented mutations arose from lesions in the non-transcribed strand. Because complemention group information could only be inferred from clinical features and UDS measurements for most patients, a specific correlation between the non-transcribed strand bias for mutations and NER defects cannot be made. However, the authors claimed that at least 50% of the tumors were likely from patients with XP-C, 25% from patients with XP-A, and 25% from XP-variant patients. Of note, one of the two samples in which a mutation arose from a lesion in the transcribed strand was from a patient documented to have XP variant with normal overall NER.

These investigators have gone on to review all reported *p53* mutations in XP and non-XP human skin cancers and compared them to the *p53* mutational spectrum of internal tumors, utilizing a database of nearly 2000 p53 sequences (DUMAZ et al. 1994). This analysis is significant for demonstrating that the mutational spectra of *p53* from skin tumors is statistically different from all other reported human cancers, but very similar to those observed in other UV-treated genes in model systems, and that these characteristics were exacerbated in a predictable manner in XP skin tumors, consistent with the absence of efficient NER. For example, 74% of all skin tumor mutations were C to T transitions, whereas only 47% of internal tumors displayed this base substitution pattern, and most of those were located at CpG sequences implicating deamination of 5-methyl cytosine as the mutagenic event. In XP skin tumors, 100% of mutations were located at dipyrimidine sites and 55% of these were tandem CC to TT transitions, compared to only 14% CC to TT double-base mutations in skin cancers from normal individuals. Internal tumors only rarely displayed these double cytosine base mutations (0.1%). Finally, 95% of mutations from XP skin cancers arose from lesions presumed to be on the non-transcribed strand, while internal tumors or non-XP skin cancers did not show this strand bias. This last observation is suggestive of a role for transcription-coupled repair, in the absence of overall NER, for causing a non-transcribed strand bias for *p53* mutations by selectively repairing lesions in the transcribed strand, as would be predicted for patients with XP-C. However, this mechanistic explanation should not hold true for patients from other XP complementation groups in which both overall and transcription-coupled repair are defective (e.g., XP-A). Since the actual number of patients from various complementation groups in these studies remains unknown, it is difficult to assess the significance of the *p53* mutational strand bias and its potential association with transcription-coupled repair.

Taken together, the data from UV-induced mutations in human skin cancers is consistent with, though not proof of, the notion that in the normal population, under conditions leading to skin carcinogenesis, transcription-coupled repair after UV irradiation does not play a fundamental role in avoidance of malignancies. This is in agreement with the previously discussed observations that CS patients who lack transcription-coupled repair are not at an increased risk for UV-induced skin tumors. The mutational frequency and spectra of the *p53* gene in sun-exposed or UV-irradiated normal skin samples from CS individuals would therefore be of interest and further advance this hypothesis.

We (FORD et al. 1994), and others (EVANS et al. 1993b), have now provided direct experimental evidence for preferential repair of the human *p53* gene by measuring the rate of removal of CPD from each strand of this gene in several human fibroblast cell lines. For example, we found more rapid repair of UV-induced DNA damage in the transcribed than in the non-transcribed strand of the *p53* gene in a normal, diploid fibroblast cell line, with repair efficiency of the non-transcribed strand similar to that of other regions of unexpressed DNA (88% repair of the transcribed strand and 60% repair of the non-transcribed strand 24 h following irradiation; FORD et al. 1994). EVANS et al. (1993b) reported similar results for another normal fibroblast cell line and further demonstrated that, in a human XP-C cell line, the transcribed strand of *p53* was repaired with an efficiency similar to that of normal cells, but that the non-transcribed strand displayed no repair at all 24 h after UV irradiation, as expected. Therefore, these results suggest that the repair characteristics of the non-transcribed strand, and by implication those DNA sequences targeted by overall genomic NER mechanisms in general, are most relevant for UV-induced carcinogenesis.

5 Conclusions

Cellular responses to DNA damage are highly complex, with many factors actively influencing the outcome of damage events, whether resulting in complete repair, survival with mutations, or cell death. The study of UV-induced DNA damage and its repair provides a useful model to examine the role of NER in mutagenesis and carcinogenesis, due to our relatively complete understanding of the lesions and the repair mechanisms involved. The clinical, biochemical, and molecular evidence presented regarding diseases caused by deficiencies in NER, such as XP, clearly indicate that UV irradiation directly contributes to skin cancers in humans. However, a close analysis of the specific NER pathways which result in heterogenous repair of DNA, and the specific clinical phenotype resulting from mutations in the various genes involved, reveal a more complex relationship between repair and carcinogenesis. In fact, it appears from the study of patients with CS that defects in mechanisms for the preferential repair of transcribed DNA sequences do not result in a propensity to develop carcinogen-induced malignancies, but rather display clinical features that may be due to resulting abnormalities in transcription. In contrast, molecular defects in the repair of UV-induced DNA damage in the overall genome correlate closely with the dramatically increased incidence of skin cancers in XP patients. Thus, the recent discoveries at a molecular level of the various gene products and pathways involved in NER, and their interactions with other cellular processes, such as transcription, has greatly expanded our understanding of the pathogenesis of the clinically heterogeneous set of human diseases characterized by deficiencies in NER. Furthermore, analysis of the molecular defects underlying the different phenotypic presentations of these diseases allows

insight into the repair processes important for affecting mutagenesis and the development of cancers.

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Chromosome Instability Syndromes: Lessons for Carcinogenesis

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1 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, e.g., the SOS system in *Escherichia coli* (Battista et al. 1990). The means by which cells achieve this goal are complex and involve DNA repair, genetic recombination, alterations in the cell cycle, and programmed cell death. This article discusses the chromosome instability syndromes, human diseases in which these homeostatic processes have broken down, resulting in cancer, immunodeficiency, growth failure, neurologic abnormalities, and mutagen sensitivity. Genetic instability in these syndromes may result from changes in DNA topology, loss of cell cycle checkpoint control, or dysregulation of lymphokine-mediated signal-transduction pathways, illustrating the diverse nature of the cellular processes that can affect the integrity of the genome.

The chromosome instability syndromes are a group of autosomal recessive conditions that include ataxia telangiectasia (A-T), Fanconi anemia, Bloom syndrome, Werner syndrome (MONNAT 1992), Nijmegen breakage syndrome (WEEMAES et al. 1994), and ataxia pancytopenia (LI et al. 1981). Focusing on the first three conditions, this article summarizes their homozygote and heterozygote phenotypes with an emphasis on genetic instability and cancer. Previous models for these diseases are discussed and new hypotheses proposed to explain how the underlying molecular defects in these syndromes give rise to their phenotypic abnormalities. The involvement of these genes in inherited and sporadic cancers is reviewed in depth, followed by discussion of the implications that these diseases have for both understanding the basic biology of cancer and improving the treatment of cancer patients.

2 Chromosome Instability Syndromes

2.1 A-T Phenotype

The first published report of individuals with A-T was probably that of SULLABA and HENNER (1926), who described three adolescent siblings with progressive choreoathetosis and ocular telangiectasias, whom they thought represented a variant of Hunt's familial athetosis. Fifteen years later, LOUIS-BAR (1941) independently described a 9-year-old boy with cerebellar ataxia and telangiectasias, whom he proposed represented a new syndrome. The condition became known as Louis-Bar syndrome until the name ataxia telangiectasia was proposed by BODER and SEDGWICK (1957). The pleiotrophic phenotype of A-T homozygotes is described below.

2.1.1 Neurologic Abnormalities

Ataxia telangiectasia homozygotes have multiple neurologic problems (reviewed in SEDGWICK and BODER 1991; WOODS and TAYLOR 1992). Although they typically have no functional abnormalities in the 1st year of life, they gradually lose cerebellar function, resulting in progressive ataxia, dysarthric speech, ocular apraxia, drooling, and choreoathetoid movements. Most A-T patients are wheelchair bound by the end of the 1st decade of life due to cerebellar dysfunction, and older A-T patients may develop intellectual arrest. Their functional neurologic abnormalities are accompanied by a continual loss of neurons from the central nervous system. The cerebellum is particularly affected due to the cumulative effects of ongoing Purkinjie cell death.

2.1.2 Immunodeficiencies

Ataxia telangiectasia homozygotes express both humeral and cellular immune defects (PETERSON and GOOD 1968; STROBER et al. 1968). They have absent or hypoplastic thymuses, low numbers of circulating T cells and functional impairment of T cell mediated immunity (e.g., delayed hypersensitivity and proliferative responses). They also have a distinctive pattern of immunoglobulin (Ig) deficiencies, including low levels of IgA, IgE, IgG_2, and IgG_4. The combination of immunodeficiency and progressive loss of cerebellar function seen in A-T homozygotes makes aspiration pneumonia the leading cause of death in these children, whose median life expectancy was estimated in a 1986 survey to be ~17 years (MORRELL et al. 1986).

2.1.3 Malignancy

In a retrospective review of the first 101 known cases, BODER and SEDGWICK (1963) noted that lymphoreticular malignancy is the second most common cause of death in children with A-T. Since then there have been three separate large-scale surveys that illustrate the range of tumors in A-T homozygotes: (a) the Immunodeficiency Cancer Registry periodically has reported on those A-T patients with cancer that have been entered into this international registry (SPECTOR et al. 1982; HECHT and HECHT 1990); (b) MORRELL et al. (1986) described the incidence of cancer in 263 A-T homozygotes as part of a survey of A-T in the United States; and (c) SEDGWICK and BODER (1991) recently reviewed published autopsy reports that included 30 A-T homozygotes who had developed cancer. The following comments are based on these studies plus additional published case reports.

   Cancer is a frequent complication of ataxia-telangiectasia. For example, in the 1986 MORRELL; et al. survey, ~20% of 263 A-T patients had developed cancer at the time of ascertainment. Malignancies were a contributing cause of death in almost half of published autopsies surveyed by SEDGWICK and BODER (1991). Recent estimates place the lifetime risk of developing cancer at 30%–40% (e.g., PETERSON et al. 1992). MORRELL et al. (1986) calculated that Caucasian A-T homozygotes had a 61-fold excess risk of developing cancer. Their risk for lymphoma was even higher: a 252-fold increase. In the same study, the relative risks for African-American A-T homozygotes were calculated to be 184-fold higher than controls for all cancers and 750-fold higher for lymphomas.

   Published surveys and case reports have consistently shown an age effect on the tumor type seen in A-T homozygotes. Figure 1 plots malignancies in A-T patients by age at onset and type, using data from the Immunodeficiency Cancer Registry (SPECTOR et al. 1982). As Fig. 1 illustrates, there is a bias towards lymphomas and acute lymphocytic leukemias prior to age 10, while epithelial tumors tend to predominate beginning in adolescence. The slight fall off in epithelial tumors after age 20 seen in this and other surveys may be a reflection of the declining population of A-T homozygotes who survive past age 20 rather than a true drop in
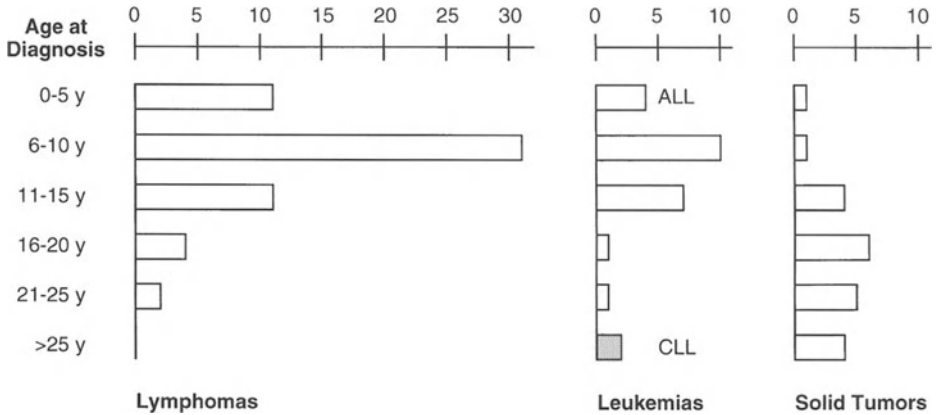
Fig. 1. Malignancies in A-T homozygotes as a function of age of onset (based on data from the Immunodeficiency Cancer Registry, SPECTOR et al. 1982)

the incidence of solid tumors in this age group. As noted by TAYLOR (1992), those few A-T homozygotes who live into their 20s and 30s are at risk to develop chronic T cell leukemias in addition to solid tumors (HECHT and HECHT 1990; SEDGWICK and BODER 1991; TAYLOR 1992).

The most common malignancies in A-T homozygotes are tumors of the immune system occurring in the first 15 years of life. For example, lymphomas and leukemias together account for 67% of the 52 tumors found in the 1986 MORRELL et al. survey and 74% of tumors seen in the 108 A-T patients listed in the Immunodeficiency Cancer Registry report of 1990 (HECHT and HECHT 1990). More than 40% of all tumors in A-T homozygotes are non-Hodgkin's lymphomas, another ~20% of tumors are acute lymphocytic leukemias (ALL) and ~5% are Hodgkin's lymphomas (MORRELL et al. 1986; HECHT and HECHT 1990; SEDGWICK and BODER 1991). Lymphomas in A-T homozygotes are commonly of B cell origin, while the majority of cases of ALL in A-T homozygotes express T cell markers in contrast to the low proportion of T cell-derived ALLs seen in other children (SPECTOR et al. 1982).

Solid tumors account for only about one quarter of tumors seen in A-T homozygotes. Nonetheless, they still are unusually frequent for such a young population. A wide range of cancers have been reported (SPECTOR et al. 1982; MORRELL et al. 1986; SEDGWICK and BODER 1991), the most frequent being gastric carcinoma, breast carcinoma, medulloblastoma, basal cell carcinoma, ovarian dysgerminoma, hepatoma, and uterine leiomyoma (in approximate order of frequency). Less common solid tumors include pyloric carcinoma, laryngeal carcinoma, gonadoblastoma, parotid carcinoma, thyroid carcinoma, renal cell carcinoma, pancreatic carcinoma, bladder carcinoma, epithelioma and leiomyosarcoma.

2.1.4 Other Clinical Abnormalities

Most A-T homozygotes develop telangiectasias of the sclera, face and ears, beginning at 3–6 years of age. Adolescent A-T homozygotes frequently have other cutaneous signs of premature aging, including greying of the hair, senile keratoses, skin atrophy, and areas of hyper- and hypopigmentation (SEDGWICK and BODER 1991). Gonadal abnormalities are another feature of the syndrome. Many female homozygotes have congenital absence or hypoplasia of the ovaries and menarche may be delayed or absent (SEDGWICK and BODER 1991; WOODS and TAYLOR 1992). Male homozygotes have been shown to have histological abnormalities of their testes, and incomplete spermatogenesis has been reported (STRICH 1963; AGUILAR et al. 1968), although hypogonadism is less frequent and milder than in female homozygotes. Some A-T homozygotes develop diabetes in adolescence, although this is not the norm (SCHALCH et al. 1970). Although older A-T patients develop growth retardation, congenital malformations are not a feature of A-T.

2.1.5 Genetic Instability in A-T Homozygotes

Genetic instability is an intrinsic feature of the A-T phenotype. The first direct evidence came from cytogenetic studies by HECHT et al. (1966), who found an increased frequency of chromatid and isochromatid breaks in PHA-stimulated lymphocytes from children with A-T. Since then, a variety of spontaneous in vivo chromosomal aberrations have been documented in multiple tissues from A-T homozygotes (reviewed in COHEN and LEVY 1989; KOJIS et al. 1991). The range of spontaneous aberrations includes chromatid and chromosome breaks, chromosome gaps, acentric fragments, and dicentric chromosomes, as well as increased frequencies of structural rearrangements and aneuploidy. Multiple cell lineages have been shown to express chromosomal instability, including T lymphocytes, keratinocytes, fibroblasts, hepatocytes, and neurons (e.g, HECHT et al. 1966; AGUILAR et al. 1968; KOJIS et al. 1991). However, most studies of bone marrow stem cells in A-T homozygotes have not documented increased chromosome aberrations (e.g., COHEN et al. 1975; KOHN et al. 1982b), and in vitro Epstein-Barr virus (EBV)-transformed lymphoblastoid lines do not express spontaneous karyotypic abnormalities (KOJIS et al. 1991). In addition, spontaneous sister chromatid exchange frequencies appear to be normal (GALLOWAY and EVANS 1975; BARTRAM et al. 1976; KOHN et al. 1982b). Interestingly, breakpoints have never been reported near the 11q23 location of the *ATM* gene itself. Telomere fusions, resulting in dicentric, multicentric, and ring chromosomes, are distinctive features of A-T homozygotes that are not seen in other chromosome instability syndromes. They occur in multiple cell types (PFEIFFER 1970; HAYASHI and SCHMID 1975; TAYLOR et al. 1981) and may be the result of shortened or abnormal telomeres (PANDITA et al. 1995).

In addition to the generalized increase in chromosome aberrations seen in other tissues from A-T homozygotes, their peripheral T lymphocytes have a specific predilection for rearrangements involving four sites: 7p14, 7q35, 14q11.2, and

14q32. Although translocations and inversions of chromosomes 7 and 14 also are common in normal individuals (AURIAS et al. 1985b; AURIAS and DUTRILLAUX 1986), they occur 30- to 50-fold more often in A-T homozygotes (AURIAS et al. 1980). These breakpoint regions contain the α, β, and γ T cell antigen receptor genes as well as the Ig heavy chain genes, immune genes that normally undergo obligatory rearrangement during the maturation of the immune system. When these breakpoints have been sequenced in A-T patients' T cells, they have interrupted their respective TCR and Ig heavy chain genes (reviewed in KOJIS et al. 1991). These T cell-specific rearrangements frequently exhibit clonality, and, with time, T cell clones carrying these chromosome 14 rearrangements can become the majority of peripheral T cells in A-T homozygotes (HECHT et al. 1973; OXFORD et al. 1975; SAADI et al. 1980). Although these rearrangements do not appear to be sufficient for malignant transformation, T cell leukemias in A-T homozygotes frequently arise from these clones (TAYLOR 1992). Inversions and 14 : 14 translocations involving the α-TCR and Ig heavy chain genes are common in chronic T cell leukemia in normal individuals as well (RABBITTS 1991), suggesting that these rearrangements may facilitate the development of T cell leukemia.

Spontaneous genetic instability also has been documented by molecular and immunologic means. A study of individuals heterozygous for the M and N alleles of the glycophorin locus found 7- to 14-fold elevations in frequencies of conversion to hemizygosity and homozygosity in erythrocytes from A-T homozygotes (BIGBEE et al. 1989). A-T homozygotes have approximately 70-fold elevation in their frequency of circulating T lymphocytes expressing γ/β, δ/β, α/δ, or γ/δ heavy chain TCRs as a result of aberrant interlocus gene rearrangements (LIPKOWITZ et al. 1990, 1992; KOBAYASHI et al. 1991). Their T lymphocytes also have significant elevation in the spontaneous frequency of *HPRT* mutations (COLE and ARLETT 1994). Finally, A-T fibroblast lines grown in culture have 30- to 200-fold increases in spontaneous intrachromosomal recombination rates (MEYN 1993).

2.1.6 Abnormal Responses to DNA Damage

In 1967, GOTOFF et al. described a 10-year-old A-T patient who developed lymphosarcoma and subsequently died from a severe reaction to radiation therapy directed at the tumor. This case report was the first of several that documented the in vivo sensitivity of A-T patients to the killing effects of ionizing radiation (see also MORGAN et al. 1968; FEIGIN et al. 1970; EYRE et al. 1988). In 1975 TAYLOR et al. confirmed this sensitivity in A-T fibroblasts grown in vitro. Since then, radiosensitivity has been documented for a variety of cell types, including primary fibroblasts, lymphocytes and keratinocytes as well as transformed lymphoblasts and fibroblasts (e.g., ARLETT et al. 1988; COLE et al. 1988; STACEY et al. 1989). Cells from A-T homozygotes are typically three to five times more sensitive to ionizing radiation than control cells, based on D_0 and D_{37} values (ARLETT et al. 1988; COLE et al. 1988). A-T cells are easily killed by other agents that induce double-strand breaks, particularly those that induce broken ends with 3′ phosphoglycolates (SHILOH et al. 1982a; BURGER et al. 1994; NELSON and KASTAN 1994). Most studies

have found that A-T cells have normal resistance to UV irradiation (e.g., HEDDLE et al. 1983), suggesting that their sensitivity may be specific for double-strand breaks and gaps.

Another part of A-T radiosensitivity is an increase in the number of chromosomal aberrations induced by ionizing radiation or radiomimetic agents. Following treatment with agents that induce double-strand breaks, cells from A-T homozygotes express significant increases in the frequencies of induced chromatid breaks, gaps, and rearrangements (reviewed in COHEN and LEVY 1989). This has been documented in peripheral T cells, lymphoblastoid lines, primary fibroblasts, and fibroblast lines (COHEN and LEVY 1989). These increases in induced aberrations are not due to an increased susceptibility to the induction of DNA breaks by ionizing radiation (CORNFORTH and BEDFORD 1985; PANDITA and HITTELMAN 1992b).

2.1.7 Repair Abnormalities in A-T Homozygotes

Although cells from A-T homozygotes are exquisitely sensitive to the cytotoxic effects of ionizing radiation, their ability to repair DNA damage appears to be largely intact. Multiple biochemical studies have failed to detect gross abnormalities in the kinetics of single-strand and double-strand break repair in A-T cells (e.g., TAYLOR et al. 1975; LEHMAN and STEVENS 1977). Other reports have found no evidence that A-T cells are functionally defective in DNA repair (MURIEL et al. 1991; EADY et al. 1992). On the other hand, A-T homozygotes may have subtle defects in their ability to repair DNA breaks. Several studies found slight increases in the fraction of DNA breaks left unrepaired in irradiated A-T cells (CORNFORTH and BEDFORD 1985; BLOCHER et al. 1991), as well as abnormalities in the rejoining of restriction enzyme breaks in plasmids transfected into A-T fibroblasts (Cox et al. 1984; DEBENHAM et al. 1988) This may be the result of impaired accuracy in strand rejoining (Cox et al. 1984; DEBENHAM et al. 1988) or inability to repair a small but critical fraction of double-strand breaks (TAYLOR 1978; CORNFORTH and BEDFORD 1985; TAYLOR et al. 1989; PANDITA and HITTELMAN 1992a).

The subtle repair defects observed in A-T homozygotes may be the result of their inability to activate DNA repair following DNA damage. Exposing normal human cells to low doses of radiation prior to infection of irradiated virus improves viral survival and increases the number of mutant viruses recovered (SUMMERS et al. 1985). These effects, termed enhanced survival and enhanced mutagenesis, have been demonstrated using both single-stranded and double-stranded DNA viruses (SUMMERS et al. 1985; JEEVES and RAINBOW 1986; HILGERS et al. 1989) and is presumed to be the result of activation of one or more DNA repair processes by DNA damage. Although not extensively studied, damage-activated DNA repair appears to be impaired in A-T homozygotes, since A-T fibroblasts have been shown to lack the enhanced survival and enhanced mutagenesis expressed by control human cells for irradiated H-1 parvovirus and adenovirus 2 (JEEVES and RAINBOW 1986; HILGERS et al. 1987; BENNETT and RAINBOW 1988).

2.1.8 A-T Homozygotes Lack Cell Cycle Checkpoints

There are at least three damage-sensitive cell cycle checkpoints in mammalian cells: one at the G_1/S border, another in S phase, and one at the G_2/M boundary. In normal cells, these checkpoints are triggered following the induction of strand breaks in cellular DNA by a variety of agents (reviewed in MURRAY 1992; HARTWELL and KASTAN 1994). These checkpoints also may restrain the cell cycle temporarily in response to the generation of strand breaks, shortened telomeres, and other 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).

The tumor suppressor protein p53 plays a key role in activating the G_1/S checkpoint following certain types of DNA damage (reviewed in HARTWELL and KASTAN 1994). G_1/S cell cycle arrest occurs via p53-mediated transcriptional activation of the *p21(WAF-1/CIP1/SDI1)* gene, which codes for a protein that binds to Cdk-cyclin complexes and inhibits their kinase activities (KASTAN et al. 1992; DULIC et al. 1994; EL-DEIRY et al. 1994). The S phase and G_2/M damage-activated checkpoints may be p53 independent (KOMATSU et al. 1989; RUSSELL et al. 1995); however, relatively little is known about their genetics and biochemistry.

Ataxia telangiectasia homozygotes lack the p53-mediated G_1/S damage-sensitive checkpoint, and the kinetics of p53, p21, and GADD45 induction by ionizing radiation are abnormal in the cells of A-T homozygotes (KASTAN et al. 1992; KHANNA and LAVIN 1993; CANMAN et al. 1994). A-T cells do not have the S phase checkpoint, since they fail to arrest DNA synthesis when irradiated in S phase, resulting in the phenomenon of radioresistant DNA synthesis (PAINTER and YOUNG 1980). The G_2/M checkpoint also appears to be defective in A-T cells, in that both A-T fibroblasts and lymphocytes irradiated in G_2 fail to undergo the initial radiation-induced G_2/M delay seen in normal cells (ZAMBETI-BOSSELER and SCOTT 1980; RUDOLPH and LATT 1989; BEAMISH and LAVIN 1994).

2.1.9 Dysfunctional Apoptosis in A-T Homozygotes

Although researchers have begun only recently to focus on the possible role of programmed cell death in the phenotype of A-T homozygotes, several in vivo and in vitro findings suggest that inappropriate apoptosis is responsible for certain aspects of the disease. Histologic analyses of cerebella taken from A-T homozygotes at autopsy document a high frequency of abnormal Purkinje and granule cells that exhibit the highly condensed, pyknotic nuclei expected from programmed cell death in neurons (BODER and SEDGWICK 1958; AGAMANOLIS and GREENSTEIN 1979; AMROMIN et al. 1979). We recently demonstrated that fibroblasts and lymphoblasts undergo apoptotic death in culture following exposure to low radiation and streptonigrin doses that do not induce appreciable apoptosis in control cells (MEYN et al. 1994, submitted). This inappropriate apoptosis appears to be p53 mediated, in that it is suppressed in A-T fibroblasts whose p53 protein has been functionally inactivated by transfection with either a dominant-negative *p53* gene

or a human papilloma virus E6 gene. In these experiments, transfection-induced loss of p53 function did not affect survival of control fibroblasts, but transfected A-T cells acquired near-normal resistance to ionizing radiation, suggesting that p53-mediated apoptosis is the major cause of radiosensitivity in A-T cells in culture (MEYN et al. 1994, submitted).

2.2 *ATM* Gene

In the summer of 1995, an intensive search for the genes responsible for A-T culminated with the positional cloning of the *ATM* (Ataxia Telangiectasia Mutated) gene by SAVITSKY et al. (1995). 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 putative ATM protein is homologous to that of at least four checkpoint proteins from other organisms: *Drosophila melanogaster* MEI-41, *Schizosaccharomyces pombe* Rad3, *Saccharomyces cerevesiae* MEC1p, and *S. cerevesiae* TEL1p (GREENWELL et al. 1995; HARI et al. 1995; SAVITSKY et al. 1995). The region of strongest homology between these five proteins contains a phosphatidylinositol 3-kinase (PI3-kinase) domain, suggesting that proteins are involved in signal transduction.

The *ATM* gene also shares phenotypic similarities with these genes. Like A-T homozygotes, *mei-41, rad3*, and *mec1* mutants are X-ray sensitive and lack damage-induced cell cycle checkpoints (JIMENEZ et al. 1992; WEINERT et al. 1994; HARI et al. 1995). In addition, A-T, *mei-41, rad3*, and *tel1* homozygotes express increased chromosomal instability and have high spontaneous rates of mitotic recombination (PHIPPS et al. 1985; SEDGWICK and BODER 1991; MEYN 1993; GREENWELL et al. 1995; HARI et al. 1995). Taken together, the physical and phenotypic similarities between these checkpoint genes and *ATM* suggest that the normal function of ATM protein is to activate multiple cellular functions in response to spontaneous and induced DNA damage.

2.3 Damage Surveillance Network Model
as a Working Hypothesis for *ATM* Function

In the past, abnormalities of DNA repair, genetic recombination, chromatin structure, and cell cycle checkpoint control all have been proposed as the underlying defect in A-T (for a review see MEYN 1995). However, with the cloning of *ATM* gene and the recognition of the putative ATM protein's similarity to other cell cycle checkpoint proteins, it now is assumed that A-T is caused by the failure of mutant *ATM* genes to play their proper role in activating cellular responses to DNA damage (ENOCH and NORBURY 1995; KASTAN 1995; MEYN 1995; SAVITSKY et al. 1995).

Several years ago, we proposed a model for A-T in which the A-T defect results in inability to activate a group of diverse cellular functions in response to DNA damage (MEYN et al. 1994; MEYN 1995). Figure 2a depicts such a damage response

network in which the ATM gene product plays a critical role. In this model, the detection of certain types of spontaneous or induced DNA damage triggers a signal transduction network, resulting in the activation of a group of cellular functions that promote genetic stability by temporarily arresting the cell cycle and enhancing DNA repair. At the same time, the ATM-dependent network promotes cellular survival by inhibiting execution of damage-induced programmed cell death. In addition to the five responses illustrated in Fig. 2a, there also may be other, as yet undefined, ATM-dependent functions.

The primary abnormality in A-T homozygotes presumably creates a defect in this network that prevents the activation of these cellular functions in response to strand breaks, shortened telomeres, and other DNA lesions (Fig. 2b). This inability to respond to spontaneous and induced DNA damage results in increased genomic instability as well as in an unusually low threshold for triggering of p53-mediated apoptosis by otherwise non-lethal DNA damage. These abnormalities lead, in turn, to the multiple in vivo and in vitro abnormalities seen in A-T homozygotes (Fig. 2b).

The damage surveillance network (DSN) model offers a unifying explanation of how a single-gene defect can cause the pleiotropic phenotype seen in A-T homozygotes and explains several puzzling aspects of the disease. The model assumes that the enzymatic machinery for DNA repair and genetic recombination is essentially intact and emphasizes the contribution of defective cell cycle checkpoints to genetic instability and immune defects, two cardinal features of A-T. By ascribing the disruption of immunoglobulin switch recombination and TCR rearrangements to cell cycle checkpoint abnormalities and postulating that disruptions of immune gene rearrangements and of repair of spontaneous DNA
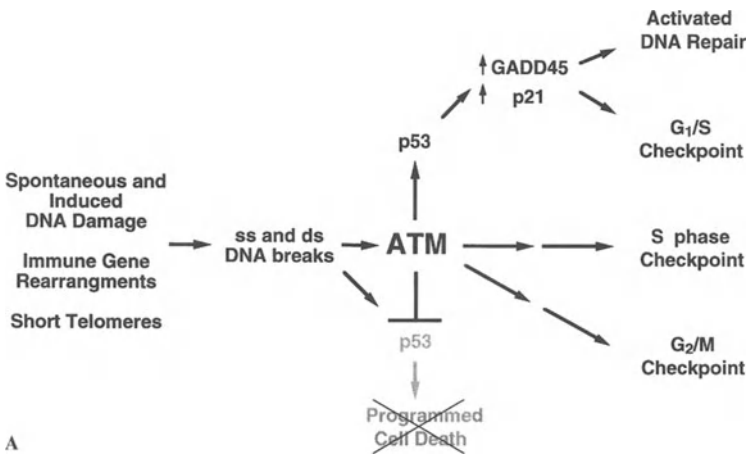
Fig. 2. A DNA damage surveillance network. As part of this signal transduction network, the ATM protein activates at least five cellular functions in response to the detection of spontaneous or induced DNA damage. B The DNA damage surveillance network is defective in A-T homozygotes. A-T homozygotes cannot activate ATM-dependent functions in response to DNA damage, resulting in the pleiotropic A-T phenotype
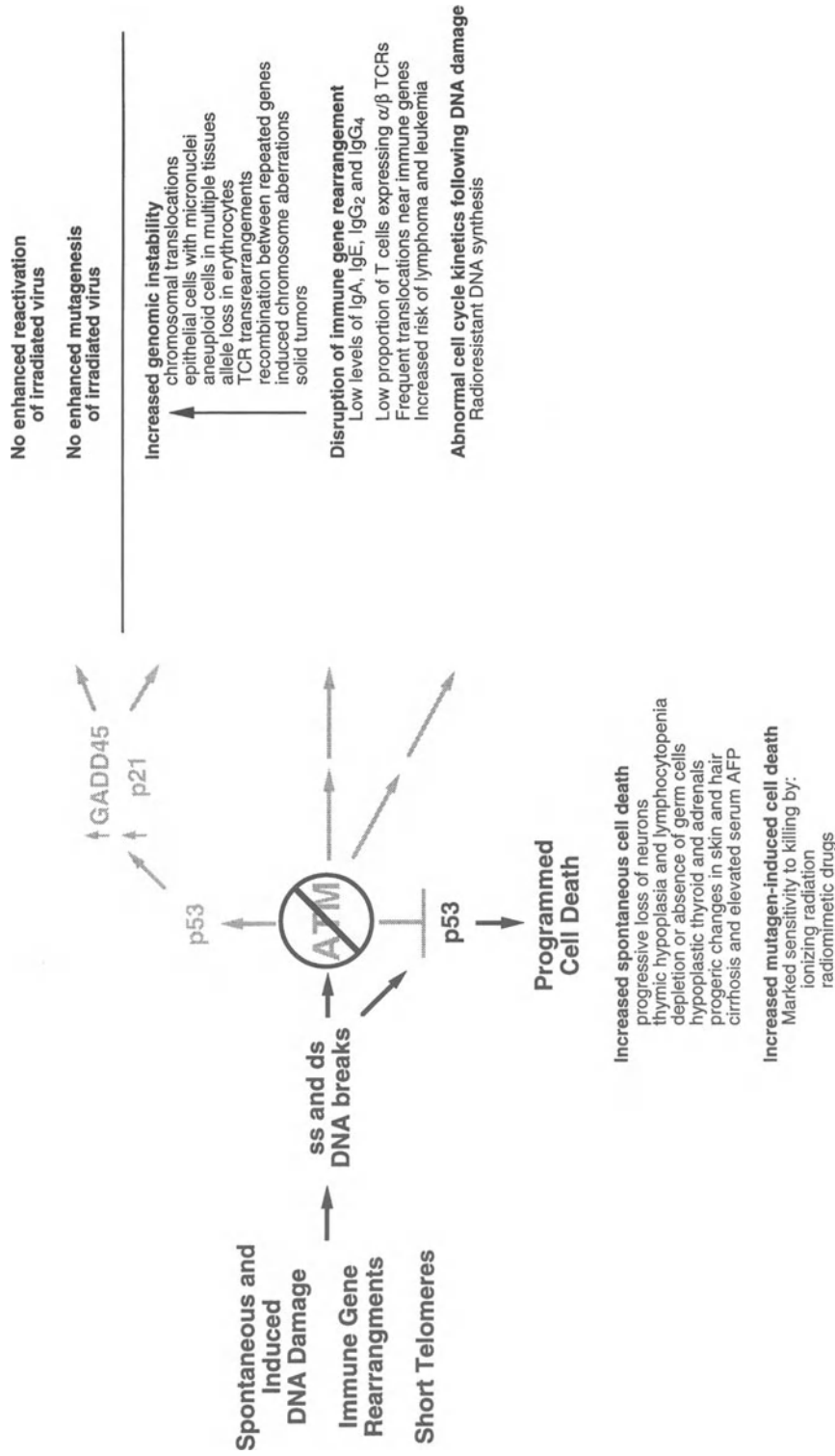
No enhanced reactivation
of irradiated virus

No enhanced mutagenesis
of irradiated virus

Increased genomic instability
chromosomal translocations
epithelial cells with micronuclei
aneuploid cells in multiple tissues
allele loss in erythrocytes
TCR transrearrangements
recombination between repeated genes
induced chromosome aberrations
solid tumors

Disruption of immune gene rearrangement
Low levels of IgA, IgE, IgG$_2$ and IgG$_4$
Low proportion of T cells expressing α/β TCRs
Frequent translocations near immune genes
Increased risk of lymphoma and leukemia

Abnormal cell cycle kinetics following DNA damage
Radioresistant DNA synthesis

GADD45

p21

p53

ATM

p53

Programmed Cell Death

ss and ds DNA breaks

Spontaneous and Induced DNA Damage

Immune Gene Rearrangments

Short Telomeres

Increased spontaneous cell death
progressive loss of neurons
thymic hypoplasia and lymphocytopenia
depletion or absence of germ cells
hypoplastic thyroid and adrenals
progeric changes in skin and hair
cirrhosis and elevated serum AFP

Increased mutagen-induced cell death
Marked sensitivity to killing by:
ionizing radiation
radiomimetic drugs

Fig. 2B

damage lead to generation of recombinogenic breaks and gaps in DNA, the DSN model explains why immunoglobulin switch recombination and TCR rearrangement appear to be defective in A-T homozygotes, while spontaneous rates of recombination between directly repeated non-immune genes in A-T fibroblasts are markedly higher than normal (MEYN 1993). The model also can account for the observation that chromosomal translocations in A-T lymphocytes cluster near immune genes, while translocations in A-T fibroblasts appear to involve random sites throughout the genome (KOJIS et al. 1989). By assuming that the ATM-dependent DSN normally monitors telomere integrity, the model explains telomeric abnormalities seen in A-T cells (PANDITA and HITTELMAN 1995).

The range of DNA lesions that might trigger the ATM network is uncertain, although strand breaks and gaps containing modified 3′ termini are likely to play a major role, given the sensitivity of A-T homozygotes to physical and chemical agents which induce strand breaks and small gaps containing 3′ phosphoglycolates (SHILOH et al. 1982a; BURGER et al. 1994; NELSON and KASTAN 1994). Short telomeres also may activate the ATM network, suggesting that the ends of abnormally short telomeres may be structurally similar to DNA breaks generated by these agents. As indicated in Fig. 2, the same lesions that activate A-T-dependent cellular functions also may trigger p53-mediated programmed cell death.

How far upstream of p53 the ATM protein functions in the signal transduction network is not certain. However, consideration of a related human protein, DNA-PK$_{CS}$, may be instructive in this regard. DNA-PK$_{CS}$ is the catalytic subunit of DNA-PK, a DNA-dependent protein kinase (ANDERSON 1993). Ku70 and Ku80, the other subunits of DNA-PK, form a heterodimer that binds without sequence specificity to double-strand DNA breaks, gaps, and short hairpins (GOTTLIEB and JACKSON 1993). Once bound to DNA ends via the Ku polypeptides, DNA-PK activates its DNA-PK$_{CS}$ subunit, which then can phosphorylate a variety of proteins in vitro, including p53 (ANDERSON 1993). This ability of the DNA-PK holoenzyme to phosphorylate proteins when bound to damaged DNA suggests that DNA-PK not only may have a direct role in promoting the repair of certain types of DNA damage, but also may serve as the front end to a signal transduction pathway that activates cellular responses to DNA damage. Further experimental support for a role in cellular damage responses for DNA-PK$_{CS}$ is provided by recent evidence that a mutation in the DNA-PK$_{CS}$ gene is responsible for the immune-deficient SCID mouse (BLUNT et al. 1995; KURCHGESSNER et al. 1995), and that mutant DNA-PK$_{CS}$, ku70, and ku80 genes are associated with radiosensitivity, defects in double-strand break repair and abnormalities of VDJ recombination (TACCIOLI et al. 1994; TROELSTRA and JASPERS 1994; BUUL et al. 1995; LEES-MILLER et al. 1995). Although ATM and DNA-PK$_{CS}$ proteins share strong sequence homology in their PI3-kinase domains (LEES-MILLER et al. 1995), their mutant phenotypes differ (e.g., Buul et al. 1995). In addition, A-T fibroblasts have normal intracellular amounts of the ku70, ku80, and DNA-PK$_{CS}$ polypeptides and the DNA-PK enzymatic activity of A-T cell extracts is normal (LEES-MILLER et al. 1995). Taken together, these observations suggest that ATM and DNA-PK$_{CS}$ act early and independently in separate damage response pathways. The similarities

between ATM and DNA-PK$_{CS}$ suggest that, like DNA-PK$_{CS}$, the ATM protein may be directly involved in the recognition of DNA damage, perhaps serving as the protein kinase subunit of a functional complex that also includes ku70- and ku80-like polypeptides.

It is generally assumed that a major function of ATM protein is signal transduction. It is not yet clear how this occurs. However, sequence analysis of the ATM protein indicates that it has a PI3-kinase domain (SAVITSKY et al. 1995). Similar PI3-kinase domains are found in the related proteins DNA-PK$_{CS}$, MEI-41, MEC1, RAD3, and TEL1 (GREENWELL et al. 1995; HARI et al. 1995; HARTLEY et al. 1995; SAVITSKY et al. 1995). The existence of a PI3-kinase domain in the ATM protein raises the possibility that a phosphoinositide might serve as a secondary messenger for the ATM signal transduction network. However, although the ATM protein has a PI3-kinase domain, its enzymatic activity is unknown, and it is far from certain that phosphoinositols are the biologically relevant targets for its putative phosphotransferase activity. The most closely related mammalian protein, DNA-PK$_{CS}$, has no detectable phosphinositol kinase activity in vitro, but can phosphorylate many proteins, including p53 (HARTLEY et al. 1995). Recently, it was demonstrated that, following irradiation, the *S. cerevisiae* ATM homologue Mec lp activates its downstream target RAD53p by phosphorylation, thereby triggering multiple cell cycle checkpoints and other cellular responses (SANCHEZ et al. 1996). The behavior of these two ATM homologues suggests that the true in vivo targets of the ATM protein's phosphotransferase activity might be one or more proteins. This conclusion is supported by the recent demonstration that ATM protein has in vitro protein kinase activity (KEEGAN et al. 1996). One target of a putative ATM kinase activity may be p53, since p53 has multiple phosphorylation sites and its DNA binding and transcriptional activities are activated by phosphorylation (reviewed in BOULIKAS 1995).

2.3.1 How Defects in Cellular Damage Responses Promote Cancer in A-T Homozygotes

The lack of multiple cell cycle checkpoints in the somatic cells of A-T homozygotes presumably increases the frequency of spontaneous and induced chromosome aberrations, mitotic recombination, and loss of heterozygosity. The result is a loss of control of genetic integrity and an unusually rapid accumulation of the multiple genetic changes in tumor suppressor genes and oncogenes necessary to give rise to tumors.

The highest cancer risk faced by A-T homozygotes is for leukemia and non-Hodgkin's lymphoma, tumors which frequently harbor chromosome rearrangements involving Ig supergene family genes. These rearrangements may be a result of loss of checkpoint control in response to a specific set of strand breaks, those that occur during the normal rearrangement and repair of immune gene DNA. The high proportion of lymphoid tumors seen in A-T homozygotes suggests that the initial production of strand breaks and other DNA damage is a rate-limiting step in oncogenesis, even in cells that are genetically unstable due to a lack of DNA damage-sensitive cell cycle checkpoints.

2.4 Fanconi Anemia Phenotype

In 1927 FANCONI published a report in which he described a new familial aplastic anemia (FANCONI 1927). Later, VAN LEEUVEN (1933) gave the name Fanconi anemia (FA) to this autosomal recessive syndrome of pancytopenia, multiple congenital malformations, malignancy, and genetic instability (for recent clinical reviews see ALTER 1994a; AUERBACH 1995). FA is the most common cause of inherited aplastic anemia, accounting for 10%–15% of all children with aplastic anemia (ALTER 1994b). FA homozygotes typically survive into their mid-20s, with death from bone marrow failure most common, followed by cancer (AUERBACH et al. 1989; ALTER 1994a).

2.4.1 Hematological Abnormalities

Fanconi anemia homozygotes have multiple hematological problems (reviewed in ALTER 1992, 1994a). The pancytopenia in FA is initially mild but relentlessly progressive, with an estimated 98% risk of bone marrow failure by age 40 (BUTTURINI et al. 1994). By the time FA patients are symptomatic, their bone marrow is hypocellular (CHU et al. 1979). Late in their pancytopenia, bone marrow cells from FA homozygotes can exhibit clonal chromosomal abnormalities (reviewed in BERGER and CONIAT 1989). The anemia responds to androgen therapy, but only bone marrow transplantation is curative (GLUCKMAN et al. 1995).

2.4.2 Malignancy

More than 40 years ago COWDELL et al. (1955) reported a 27-year-old man with FA who developed acute leukemia. Since then case reports and periodic surveys of the International Fanconi Anemia Registry (IFAR) (AUERBACH and ALLEN 1991; AUERBACH 1995) have documented that cancer is a common complication of FA. For example, in a recent survey of 836 published cases of FA, ALTER (1994a) found that 17% had developed cancer. The same survey found a shift towards early ages of onset and the occurrence of multiple tumors in 10% of FA homozygotes with cancer.

The most common malignancy in FA homozygotes is myeloid leukemia, which accounts for nearly half the malignancies seen in FA (ALTER 1994a). It has been estimated that the relative risk for developing myeloid leukemia in individuals with FA is 15 000-fold higher than normal (AUERBACH and ALLEN 1991), with 52% risk of developing myelodysplastic syndrome and/or acute myeloid leukemia by age 40 (BUTTURINI et al. 1994). Unlike A-T and Bloom syndrome (BS) lymphoma is uncommon in FA homozygotes, with only one case in 144 malignancies reported in the literature (ALTER 1994a).

Solid tumors account for half of the tumors seen in FA homozygotes and are unusually frequent for such a young population. A wide range of cancers is found (ALTER 1994a), the most frequent being hepatomas and hepatocellular carcinomas

and oropharyngeal, gastrointestinal, and gynecological tumors (in approximate order of frequency).

Like A-T and BS, there is an age effect on the tumor type in FA homozygotes. Figure 3 plots malignancies (excluding liver tumors) in FA patients by age at onset and type, using data from the largest survey of the literature (ALTER 1994a). There is a bias towards leukemia prior to age 20, while solid tumors tend to predominate after age 25. The fall off in solid tumors after age 30 may be a reflection of the declining population of surviving FA homozygotes rather than a true drop in the incidence of solid tumors in older individuals.

Liver tumors account for 18% of malignancies in FA homozygotes. GERMAN (1983) suggested a possible link between cancer in FA and exposure to the anabolic steroids commonly used to treat anemia in these individuals. His original hypothesis that androgens play a major role in the development of *all* cancers in FA has not been supported by subsequent data. For example, among published cases, 45% of leukemias and 40% of solid tumors occurred in FA homozygotes who had never been exposed to androgens (ALTER 1994a). However, 94% of published cases of liver cancer in FA homozygotes occurred in individuals treated with androgens (ALTER 1994a), suggesting a specific link between liver cancer and androgens in these individuals.

2.4.3 Other Clinical Abnormalities

Fanconi anemia homozygotes "classically" have a variety of congenital malformations. However, 30% of patients in the 1989 IFAR survey had no physical
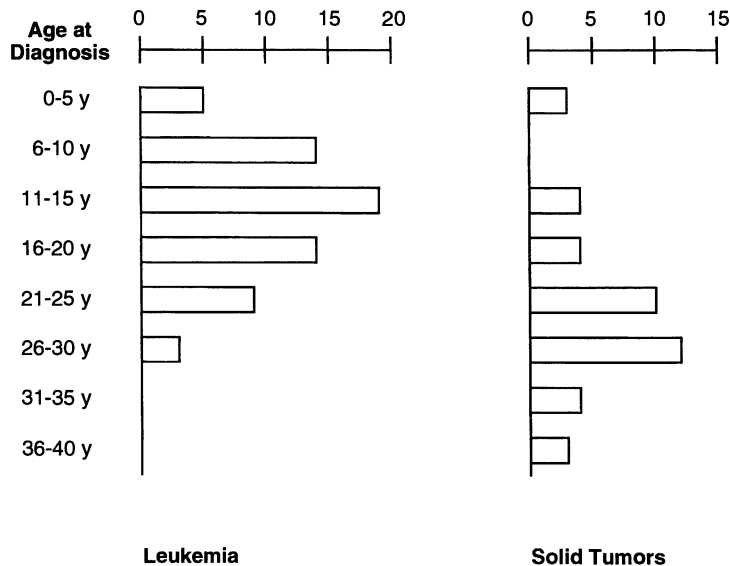
Fig. 3. Malignancies in FA homozygotes as a function of age of onset (based on the literature survey of ALTER 1994a)

malformations (AUERBACH et al. 1989), and in studies of the affected siblings of index cases, only ~25% had morphologic anomalies (RILEY et al. 1979; GLANZ and FRASER 1982). Almost half of FA homozygotes have some degree of growth retardation. Other common (i.e., >20% incidence) physical abnormalities include skin lesions (generalized hyperpigmentation, hypopigmented areas, and café-au-lait spots), facial dysmorphology (including microcephaly, frontal bossing, micrognathia), absent thumbs and/or other radial ray defects, structural abnormalities of the male genitals, and renal malformations (reviewed in ALTER 1994a; AUERBACH 1995). Less common morphologic abnormalities include vertebral anomalies, cardiac malformations, lower limb defects, deafness and malformations of the female genitourinary tract (ALTER 1994a; AUERBACH 1995). Like A-T and BS, gonadal abnormalities and reduced fertility are common problems in FA. Men with FA typically have azoospermia (BARGMAN et al. 1977), and female homozygotes undergo early menopause (ALTER 1994a). However, FA females have borne children, despite their anemia (ALTER 1994a). Unlike A-T, FA homozygotes do not appear to have intrinsic immune defects (ALTER 1992), although they occasionally have low serum levels of Ig (ALTER 1994a), and one report found decreased T cell function in vitro (JOHANSSON et al. 1982). Neurodevelopmental problems also have been reported in 25%–33% of FA homozygotes (PAVLAKIS et al. 1992; AUERBACH 1995).

2.4.4 Genetic Instability in FA

Genetic instability is a defining characteristic of FA. The first direct evidence for genetic instability in FA came from cytogenetic studies by SCHROEDER et al. (1964), who found an increased frequency of spontaneous chromosome aberrations in lymphocytes from children with FA. Since then, a variety of spontaneous in vivo chromosomal aberrations have been documented in circulating lymphocytes, fibroblasts, and bone marrow cells of FA homozygotes in vivo as well as in FA lymphocytes, fibroblasts, and lymphoblasts grown in culture (reviewed in SCHROEDER 1982; COHEN and LEVY 1989). The predominant and characteristic aberrations seen in FA homozygotes are single chromatid breaks and gaps and multiradials involving non-homologous chromosomes (SCHROEDER 1982; COHEN and LEVY 1989). Other abnormalities include endoreduplication (e.g., SCHROEDER et al. 1964) and the appearance of clonal populations of lymphocytes with marker chromosomes (HURET et al. 1988). Spontaneous sister chromatid exchange (SCE) frequencies are normal (e.g., LATT et al. 1975), but increased frequencies of spontaneous chromosome aberrations are nearly universal in FA homozygotes (SCHROEDER and KURTH 1971) and predate their development of anemia (PERKINS et al. 1969). Clones of bone marrow stem cells harboring chromosomal translocations do arise (e.g., HURET et al. 1988), but their role, if any, in bone marrow failure and the development of leukemia is not yet clear (see ALTER et al. 1993).

Several molecular studies have documented spontaneous genetic instability in FA homozygotes. SALA-TREPAT et al. (1993) found 30-fold elevations in the frequency of NO variants in erythrocytes from FA homozygotes and eight fold ele-

vations in NN variant frequencies. BIGBEE et al. (1991) found similar elevations in glycophorin variant frequencies in nine FA homozygotes. VIJAYALAXMI et al. (1985) noted high spontaneous mutation frequencies at the *HPRT* locus in circulating T lymphocytes from FA homozygotes, although this result contrasts with that of SALA-TREPAT (1993), who found no significant differences between *HPRT⁻* mutant frequencies in lymphocytes from 23 FA homozygotes, when compared to 19 age-matched controls.

An unusually high proportion of spontaneous *HPRT⁻* mutants in the cells of FA homozygotes are deletions rather than point mutations (PAPADOPOULO et al. 1990a; LAQUERBE et al. 1995). LAQUERBE et al. (1995) sequenced the breakpoint junctions of 11 *HPRT⁻* deletion mutants in FA lymphoblasts and found that most of the deletion junctions involved a putative signal sequence similar to that used in VDJ recombination. Taken together, these results suggest that one consequence of the underlying defect in FA might be an inappropriate involvement of the cell's recombination machinery in the repair of spontaneous DNA lesions. As pointed out by SALA-TREPAT et al. (1993), the preferential elevation of glycophorin but not *HPRT⁻* variants in FA homozygotes is consistent with this possibility, as glycophorin variants frequently arise as a result of genetic recombination, while recombination does not usually play a major role in the repair of DNA damage at the hemizygous *HPRT* locus. This idea that the genetic recombination is involved in the genetic instability seen in FA homozygotes is further supported by our own observations that a group A FA fibroblast line grown in culture averaged 17-fold higher spontaneous intrachromosomal recombination rates than control human fibroblasts (MEYN et al. 1993).

2.4.5 Abnormal Responses to DNA Damage

The characteristic response of cells from FA homozygotes to many DNA-damaging agents is an unusually low threshold for the induction of chromosomal aberrations (reviewed in SCHROEDER 1982; COHEN and LEVY 1989). FA cells are particularly sensitive to the induction of clastogenic damage by bifunctional DNA cross-linking agents such as mitomycin C (MMC), diepoxybutane (DEB), UV-activated 8-MOP, *cis*-diaminedichloroplatinum (II), and HN$_2$ (SASAKI and TONOMURA 1973; LATT et al. 1975; AUERBACH and WOLMAN 1976; BERGER et al. 1980; POLL et al. 1985). For example, chromosomal breaks induced by 100 ng/ml DEB average 8.96 breaks/cell in peripheral lymphocytes from FA homozygotes compared to an average of 0.06 breaks/cell for controls (AUERBACH et al. 1989). DEB-induced chromosomal instability is such a consistent characteristic of FA that it is used as the diagnostic benchmark by the IFAR (AUERBACH 1993). Both spontaneous and induced aberrations in FA consist primarily of chromatid breaks (SCHROEDER and KURTH 1971; LATT et al. 1975; PARSHAD et al. 1983), suggesting the functional defect(s) in FA are manifest in G$_2$. In contrast to BS, induction of SCEs by many DNA-damaging agents is either normal or decreased in FA cells (LATT et al. 1975; PORFIRIO et al. 1983).

The sensitivity of FA cells grown in culture to the clastogenic effects of bifunctional DNA cross-linking agents is paralleled by a marked susceptibility to

their cytotoxic effects, as demonstrated by the relative ease by which FA cells are killed by exposure to MMC, NH$_2$, DEB, and UV-activated 8-MOP (FUJIWARA and TATSUMI 1977; FORNACE et al. 1979; WEKSBERG et al. 1979; ISHIDA and BUCHWALD 1982; WUNDER and FLEISCHER-REISCHMANN 1983). Interestingly, FA cells have relatively normal resistance to clastogenic and cytotoxic effects of monofunctional DNA-alkylating agents such as 4NQO, MMS, MNNG, *trans*-diaminedi-chloroplatinum (II), and decarbamoyl-MMC (SASAKI and TONOMURA 1973; SASAKI 1978; POLL et al. 1985), suggesting a specific sensitivity to interstrand cross-links. Some, but not all studies, have reported that FA cells are sensitive to the clastogenic and cytotoxic effects of ionizing and UV radiation (HIGURASHI and CONEN 1971; SASAKI and TONOMURA 1973; FUJIWARA and TATSUMI 1977; SASAKI 1978; BIGELOW et al. 1979; ARLETT and HARCOURT 1980; DRITSCHILO et al. 1984; DUCKWORTH-RYSIECKI and TAYLOR 1985). Many FA homozygotes express an in vivo sensitivity to the toxic effects of X-irradiation and cyclophosphamide that are used to ablate bone marrow prior to transplantation (e.g., GLUCKMAN et al. 1983).

Despite their marked sensitivity for the induction of chromosome aberrations by DNA cross-linking agents, FA cells may be hypomutable at certain genomic loci. For example, exposure to UV-activated 8-MOP induced fewer mutations at the *HPRT* and Na^+/K^+-*ATPase* loci in FA(A) and FA(B) cells than in controls (PAPADOPOULO et al. 1990a,b)

2.4.6 Oxygen Sensitivity in FA

A large body of literature documents the in vitro sensitivity of FA cells to the toxic effects of oxygen and other reactive oxygen species (ROS) as well as the beneficial effects of antioxidant enzymes and low molecular weight antioxidants on the growth and chromosomal stability of FA cells (reviewed in ALTER 1994c; DEGAN et al. 1995). For example, growth in low oxygen concentrations decreases spontaneous chromosomal aberrations in FA cells (JOENJE et al. 1981) and corrects their slow growth and G$_2$ cell cycle abnormalities (SCHINDLER and HOEHN 1988). Addition of superoxide dismutase (SOD) or catalase to the media of FA lymphocytes can both reduce their spontaneous chromosome breakage (NORDENSON 1977) and increase their survival after exposure to MMC (NAGASAWA and LITTLE 1983), while the presence of low molecular weight antioxidants in the culture media partially protects FA lymphocytes against the clastogenic effects of DEB and H$_2$O$_2$ (DAL-LAPICCOLA et al. 1985).

The etiology of this sensitivity to reactive oxygen species is not clear. No consistent abnormalities have been identified in the major cellular pathways for detoxification of reactive oxygen species. In various reports, FA cells have been found to have either normal, decreased, or increased intracellular levels of SOD, catalase, and glutathione peroxidase (JOENJE et al. 1979; OKAHATA et al. 1980; MAVELLI et al. 1982; DALLAPICCOLA et al. 1984; YOSHIMITSU et al. 1984; SCARPA et al. 1985; GILLE et al. 1987). In the FA cells that have been examined, the specific activity and electrophoretic mobility of CuZn-SOD are normal (JOENJE et al. 1979), as are intracellular levels of glutathione (POOT et al. 1986).

Reactive oxygen species can be mutagenic as well as cause increased chromosome aberrations (IMLAY and LINN 1988). NORDENSON (1977) first postulated that ROS-induced DNA damage was responsible for spontaneous chromosomal instability in FA. Consistent with this view that FA cells are particularly vulnerable to ROS-induced DNA damage, DEGAN et al. (1995) found that DNA isolated from the circulating lymphocytes of FA homozygotes contains four- to fivefold more 8-hydroxy 2'-deoxyguanosine (a DNA-H_2O_2 reaction product) than DNA from control individuals. However, SERES and FORNACE (1982) found that high levels of oxygen induced the same number of single-strand DNA breaks in FA and control cells, suggesting that, if ROS affect chromosomal instability in FA cells, their effect may not be mediated through direct ROS damage of DNA.

In order to account for the sensitivity of FA cells to both ROS and bifunctional cross-linking agents, it has been suggested that compounds such as MMC exert their genotoxic effects on FA cells via the production of oxygen-dependent free radical intermediates (NAGASAWA and LITTLE 1983; PRITSOS and SARTORELLI 1986). However, several observations suggest that this may not be the case. Although MMC and DEB may generate intracellular free radical intermediates, FA cells also are sensitive to the UV-activated 8-MOP, a DNA cross-linking agent whose action is thought to be relatively independent of ROS species (AVERBECK and AVERBECK 1985). In addition, oxygen toxicity and exposure to DNA cross-linking agents have different effects on the cell cycle in FA cells (HOEHN et al. 1989), and SV40 transformation of FA fibroblasts eliminates most of their oxygen sensitivity without affecting their sensitivity to the cytotoxic effects of MMC (SAITO et al. 1993), demonstrating that these two aspects of the FA phenotype can be dissociated in certain circumstances.

2.4.7 Repair Abnormalities in FA Homozygotes

SASAKI (1975) first proposed that the underlying problem in FA is a defect in the repair of interstrand cross-links in DNA. Initial studies suggested problems with an early step in the removal of DNA interstrand cross-links, the endonucleolytic unhooking of the lesion from one strand (SASAKI 1975; FUJIWARA and TATSUMI 1977; FUJIWARA 1982). However, other reports found no defects in cross-link repair in FA cells (FORNACE et al. 1979; KAYE et al. 1980; POLL et al. 1984). One potential explanation for these inconsistencies is that they reflect different complementation groups. In support of this possibility, ROUSSET et al. (1990) found that FA(A) cells had impaired recognition and/or removal of 8-MOP-induced cross-links while FA(B) cells were normal. In addition, LAMBERT et al. (1992) and HANG et al. (1993) have demonstrated that extracts from FA(A) cells, but not FA(B) cells, have somewhat less endonuclease activity than controls against psoralin-induced interstrand cross-links in vitro. Multiple enzyme defects have been proposed for FA (reviewed in COHEN and LEVY 1989; ALTER 1994c). However, except for the putative cross-link endonuclease deficiency found by LAMBERT et al. and HANG et al., those enzyme activities that have been examined have been normal (e.g., TEEBOR and DUKER 1975; WILLIS and LINDAHL 1987).

Studies that use viruses to probe the repair of DNA cross-links have shown that FA cells that are sensitive to psoralin and *cis*-platinum express normal host-cell reactivation of UV plus psoralin-treated herpes simplex virus (HALL and SCHERER 1981; FENDRICK and HALLICK 1984), UV plus psoralin-treated adenovirus (DAY et al. 1975), and *cis*-platinum-treated SV40 DNA (POLL et al. 1984). These results suggest that, despite FA cells' sensitivity to the cytotoxic and clastogenic effects of DNA cross-linking agents, their repair of interstrand cross-links in episomal DNA is normal, raising doubts as to whether there is a defect in the enzymatic machinery for DNA cross-link repair.

2.4.8 Cell Cycle in FA

Primary cultures of FA cells grow poorly in culture due to delayed progression through S and G_2 (SASAKI 1975; WEKSBERG et al. 1979; DUTRILLAUX et al. 1982). However, no defect in DNA replication has been documented. α, β and γ DNA polymerases appear to be normal in FA cells (BERTAZZONI et al. 1978), and rates of DNA chain elongation and fork displacements have been found to be the same as controls (HAND and GERMAN 1975; KAPP and PAINTER 1981). The etiology of this slow growth is not clear; however, it may be linked to ROS sensitivity, since low concentrations of ambient oxygen correct both the slow growth rate and unusually prolonged G_2 phase seen in FA cells grown in culture (SCHINDLER and HOEHN 1988).

The induction of p53 and activation of cell cycle checkpoints by DNA damage have not been examined extensively in FA cells. However, ROSSELLI et al. (1995) have demonstrated that, compared to normal cells, p53 is poorly induced by X-rays, MMC, and UV-B in group C and group D FA lymphoblasts. Surprisingly, they found that these same FA lymphoblasts had an intact X-ray induced G_1/S checkpoint, suggesting that induction of p53 and activation of the G_1/S checkpoint can be uncoupled under certain circumstances. Further evidence for abnormalities in the cell cycle checkpoint responses of FA cells is provided by DEAN and FOX (1983) who found that HN_2 exposure induced transient S phase delays in normal cells but not FA fibroblasts. FA cells treated with bifunctional cross-linking agents typically arrest in the first G_2 following exposure (DUTRILLAUX et al. 1982; KUBBIES et al. 1985; SEYSCHAB et al. 1994). This arrest can be overcome by treatment with caffeine (SEYSCHAB et al. 1994), a known inhibitor of the G_2 DNA damage-sensitive checkpoint, suggesting that the G_2/M checkpoint is intact in FA cells.

2.4.9 Apoptosis in FA Homozygotes

Concentrations of bifunctional cross-linking agents needed to induce even one to three chromosome breaks per cell are typically 100- to 1000-fold higher than those needed to effectively kill either normal or FA cells (ISHIDA and BUCHWALD 1982; WUNDER and FLEISCHER-REISCHMANN 1983), suggesting that these agents do not kill cells by the induction of visible chromosome breaks. An alternative mode of death, apoptosis, has been demonstrated to be the mechanism of MMC and *cis*-

platinum cytotoxicity for several types of non-FA cells (e.g., LOWE et al. 1993; REY et al. 1994; BORSELLINO et al. 1995; MIZUTANI et al. 1995). FA cells exposed to lethal amounts of DNA cross-linking agents typically arrest irreversibly in the first G_2 following exposure (e.g., KAISER et al. 1982; DEAN and FOX 1983; SEYSCHAB et al. 1995) rather than divide and missegregate damaged chromosomes. This behaviour is similar to that of A-T cells, which arrest in G_2 and then die from apoptosis following X-irradiation (MEYN et al. 1994, submitted).

These observations raise the possibility that apoptosis plays a role in the killing of FA cells by DNA cross-linking agents. However, investigations of mutagen-induced apoptosis in FA cells have produced mixed results. ROSSELLI et al. (1995) found that FA(C) and FA(D) lymphocytes expressed increased spontaneous apoptosis, but the induction of apoptosis by X-irradiation was *diminished* in these FA cells. As to the induction of apoptosis in FA cells by DNA cross-linking agents, REY et al. (1994) reported that both control and FA cells underwent the same degree of apoptosis following exposure to highly toxic concentrations of MMC (10 µg/ml). However, we have found that exposure to low doses of either MMC (0.6 µg/ml) or DEB (4 ng/ml) induces proportionately much more apoptosis in FA(A), FA(C), and FA(D) lymphoblasts than in controls (ALLEN and MEYN 1995). A more definitive evaluation of the possible role of apoptosis in FA may come from ongoing studies of FA knockout mice as well as more extensive studies of FA cells in culture.

2.4.10 Lymphokines and FA

Several recent observations provide evidence that lymphokine abnormalities may play a role in the FA phenotype. ROSSELLI et al. (1992) found that addition of interleukin-6 (IL-6) to culture media corrected the sensitivity of FA(A) and FA(B) lymphoblasts to the clastogenic and cytotoxic effects of MMC, while addition of IL-1, IL-2, or IL-3 had no effect. The correction by IL-6 was specific for FA cells and could be inhibited by the addition of anti-IL-6 antibody. The FA lymphoblasts used in this study were found to have decreased secretion of IL-6 in culture and were insensitive to the induction of IL-6 by exposure to tumor necrosis factor alpha (TNFα). Production of IL-6 and the cytokine granulocyte–macrophage colony-stimulating factor (GM-CSF) have been found to be consistently decreased in both long-term bone marrow cultures and peripheral blood mononuclear cells from FA homozygotes (BAGNARA et al. 1993; STARK et al. 1993; WUNDER et al. 1993), although BAGBY et al. (1993) found mixed results in FA fibroblasts.

Tumor necrosis factor alpha also may be dysregulated in FA cells. Reporting the results of a survey of cytokine production in lymphoblasts, ROSSELLI et al. (1994) found up to eightfold increases over controls in the amounts of TNFα found in conditioned media from lymphoblasts representing FA complementation groups A, B, C, and D. These increases were not due to detectable differences in the genomic structure of the *TNFα* gene or TNFα mRNA expression. They also found that 36 FA homozygotes and 21 FA heterozygote parents all had serum levels of TNFα that were higher than 14 controls, confirming the previous observation of

SCHULTZ and SHAHIDI (1993). The relevance of overproduction of this cytokine to the rest of the FA phenotype was suggested by the demonstration that addition of anti-TNFα antibodies to the media of FA lymphoblasts partially corrected their sensitivity to the cytotoxic and clastogenic effects of MMC (ROSSELLI et al. 1994).

2.5 FA Genes

Unlike A-T, the multiple complementation groups of FA represent at least three separate genes: *FACC*, the gene for FA(C), at 9q22.3 (STRATHDEE et al. 1992a), the locus for FA(A) at 16q24.3 (PRONK et al. 1995), and the FA(D) locus on 3p (WHITNEY et al. 1995). Somatic cell hybrid studies indicate that there are at least five complementation groups (JOENJE et al. 1995), raising the possibility of five separate FA genes.

FACC, the only FA gene isolated to date, is expressed ubiquitously, although expression is particularly high in mesenchymal tissues and bone during embryonic development in the mouse (STRATHDEE et al. 1992b; WEVRICK et al. 1993). It codes for a 558-amino acid protein with no known homologies (STRATHDEE et al. 1992b), produces three different mRNAs due to alternate polyadenylation sites and has two alternative 5' exons which do not alter the coding sequence (CHEN et al. 1995; SAVOIA et al. 1995). The most 5' exon is used for constitutive expression, while the other appears to be preferentially expressed following DNA damage (CHEN et al. 1995). The normal function of *FACC* is not clear. Somewhat surprisingly, immunohistochemical analyses indicate that the FACC protein is part of a complex of cytosolic proteins and does not enter the nucleus, even in response to DNA damage (YAMASHITA et al. 1994; YOUSSOUFIAN 1994; YOUSSOUFIAN et al. 1995).

Mouse knockouts for *FACC* have been created using deletions of exon 8 or exon 9 (CHEN et al. 1995; GROMPE et al. 1995). Fibroblasts from mice homozygous for either of the *FACC* deletions express sensitivity to bifunctional cross-linking agents, and the female homozygotes have markedly reduced fertility (CHEN et al. 1995; GROMPE et al. 1995). However, the mice do not have widespread malformations, nor have they yet developed pancytopenia or leukemia (CHEN et al. 1995; GROMPE et al. 1995).

2.5.1 FA Genes and Phenotypic Variability

Variability of phenotypic expression is common, as indicated by a recent analysis of the IFAR data, in which 39% of 328 FA homozygotes were found to have had both hematological problems and malformations, 24% had only malformations, and the rest only hematological abnormalities (AUERBACH et al. 1989). The existence of multiple FA genes and mutations may contribute to the clinical variability of FA. For example, the *IVS4* mutation of the *FACC* gene is associated with an unusually early age at onset of pancytopenia and more than the usual frequency of congenital malformations (VERLANDER et al. 1994). However, some phenotypic variability may be independent of allele differences in FA genes, as indicated by lack of

concordance for congenital malformations between monozygotic twins and siblings (GLANZ and FRASER 1982; ADLER-BRECHER et al. 1992; GIAMPIETRO et al. 1993, 1994).

2.6 Underlying Defect(s) in FA

In 1969, COHEN and LEVY wrote that "a potentially unifying theory capable of explaining the diverse clinical, cellular and molecular manifestations of FA has yet to emerge." Twenty-seven years later, we still do not understand the molecular pathology that underlines the pleiotropic phenotype of FA. Several different hypotheses have been put forth. One general explanation assumes that an underlying deficiency in the handling of certain types of spontaneous and induced DNA damage is responsible for both the in vivo and in vitro abnormalities seen in FA. In this model, an inability to correctly repair DNA damage results in the accumulation of chromosome breaks and other forms of genetic damage. This damage to the genome can lead in turn to: (a) death during embryogenesis of cells responsible for normal limb and other organ formation; (b) postnatal death of bone marrow stem cells; and (c) the inactivation of tumor suppressor genes and activation of oncogenes in somatic tissues. This combination of increased prenatal and postnatal cell death and a genetic instability phenotype leads to the congenital malformations, bone marrow failure, and cancer risk seen in FA homozygotes. The most straightforward versions of this model postulate that FA mutations occur in genes coding for enzymes directly involved in the recognition, processing, and repair of DNA cross-links (e.g., LATT et al. 1975; SASAKI 1975; FUJIWARA and TATSUMI 1977; ALTER 1994c).

Another model emphasizes the sensitivity of FA cells to oxygen and other ROS, suggesting that the intrinsic defects in FA are in genes involved in cellular defenses against reactive oxygen radicals. In one form of this hypothesis, a pro-oxidant intracellular environment secondarily impairs the enzymatic machinery for interstrand DNA cross-link repair. This, in turn, leads to bone marrow failure, mutagen sensitivity, genetic instability, and cancer (SCHINDLER and HOEHN 1988; JOENJE and GILLE 1989; DEGAN et al. 1995). Alternatively, the bone marrow failure is thought to be a direct toxic effect of ROS on stem cells, rather than the gradual accumulation of oxidative damage to DNA (JOENJE and GILLE 1989).

ROSSINI et al. (1992, 1994) have proposed a third hypothesis for FA, one that developed out of their studies of lymphokine abnormalities in FA. They suggest that FA genes control "the expression of a network of genes involved in the development of the hematopoietic system," including *IL-6* and *TNFα*. In this model, mutations in FA genes cause perturbations in this network which, in an unspecified manner, lead to bone marrow failure, alterations in the oxidative state of cells, and an inability to properly repair DNA damage.

We recently suggested a fourth possibility, that dysfunctional apoptosis is involved in several aspects of the FA phenotype (ALLEN and MEYN 1995). The radial ray defects as well as other congenital anomalies seen in FA homozygotes could

result from inappropriate apoptosis during embryonic morphogenesis, increased spontaneous apoptosis in bone marrow stem cells could account for the pancyto-penia and a low threshold for DNA damage-induced apoptosis could be responsible for the sensitivity of FA cells to cross-linking agents. However, as discussed in Sect. 2.4, the possible involvement of apoptosis in the FA phenotype has yet to be thoroughly examined, even though several experimental observations are suggestive.

How might defects in FA genes lead to dysfunctional apoptosis? As discussed previously, FA cells do not appear to have the global defects in DNA damage responses that are seen in A-T. An alternative explanation as to how apoptosis might be involved in the FA phenotype comes from the studies of Rossini et al. (1992, 1994), who documented abnormalities of IL-6 and TNFα in FA. TNFα is a potent negative regulator of hematopoietic precursor cells in vivo that appears to suppress the growth of bone marrow stem cells through the activation of FAS-mediated apoptosis (NAGAFUJI et al. 1995; SELLERI et al. 1995; ZHANG et al. 1995). In addition to its role in life/death decisions in the bone marrow, TNFα can induce apoptosis in non-hematopoietic cells (reviewed in LARRICK and WRIGHT 1990; SCHWARZ et al. 1995). TNFα may also be involved in modulating apoptosis during embryogenesis (reviewed in WRIDE and SANDERS 1995). TNFα is expressed in early embryos (ROTHSTEIN et al. 1992; KOHCHI et al. 1994; WRIDE et al. 1994), and anti-TNFα antibodies localize at sites within the embryo that are undergoing apoptosis (WRIDE et al. 1994). As noted by WRIDE and SANDERS (1995), thalidomide is a potent inducer of limb malformations that specifically alters the stability of TNFα mRNA (MOREIRA et al. 1993), raising the possibility that TNFα normally plays a role in limb development and that the drug exerts its teratogenic effects by disrupting TNFα expression at a critical time during embryogenesis.

TNFα expression affects the oxidative state of cells. Intracellular expression of recombinant TNFα increases oxidative damage to DNA and other macromolecules (ZIMMERMAN et al. 1989), and treatment of cells in culture with exogenous TNFα causes the accumulation of free radicals within the cell (reviewed in LARRICK and WRIGHT 1990). The ability of TNFα to induce apoptosis in cells grown in culture can be prevented by growth in anaerobic conditions (MATTHEWS et al. 1987) or treatment with antioxidants (ZIMMERMAN et al. 1989; LARRICK and WRIGHT 1990), suggesting that the triggering of apoptosis by TNFα is mediated, at least in part, by ROS.

In contrast to the apoptosis-promoting effects of TNFα, IL-6 appears to act as an anti-apoptosis factor, as indicated by its ability to prevent p53-mediated apoptosis in myeloid leukemia cells (YONISH-ROUACH et al. 1991). Administration of IL-6 to mice protects their bone marrow from the lethal effects of ionizing radiation (PATCHEN et al. 1991), and treatment of renal carcinoma or prostate carcinoma cells with monoclonal antibodies against IL-6 synergistically potentiates the cytotoxic effects of cis-platinum and MMC (BORSELLINO et al. 1995; MIZUTANI et al. 1995). These results suggest that a normal function of IL-6 is to inhibit DNA damage-induced apoptosis. This is in contrast to the observation that TNFα can act synergistically to potentiate apoptosis induced by ionizing and UV radiation (e.g., HALLAHAN et al. 1990; SCHWARZ et al. 1995).

Taken together, these observations about TNFα and IL-6 suggest that much of the FA phenotype may be due to dysregulation of these, and perhaps other, lymphokines. It has been suggested that TNFα plays a role in bone marrow suppression in sporadic aplastic anemia through the induction of FAS-mediated apoptosis (MACIEJEWSKI et al. 1995), and apoptosis is thought to play a role in another hereditary anemia, Diamond-Blackfan syndrome (PERDAHL et al. 1994). Overexpression of TNFα could be responsible for the ongoing depletion of bone marrow stem cells that is thought to underlie the development of aplastic anemia in FA homozygotes. Dysregulation of TNFα could lead to inappropriate apoptosis during embryonic development, accounting for the congenital malformations seen in FA homozygotes. In addition, high levels of TNFα could be responsible for the increased spontaneous frequency of apoptosis seen in FA cells grown in culture. The "prooxidative" state of FA cells, their increased baseline oxidative DNA damage, and the poor growth of FA cells in the presence of oxygen may also be the result of inappropriately high levels of TNFα. Increases in the amount of spontaneous and induced oxidative DNA damage in FA cells could in turn be responsible for their chromosome instability.

TNFα and IL-6 exert opposing effects on the cellular decision to undergo apoptosis following DNA damage. The increased levels of TNFα and decreased levels of IL-6 seen in FA would predict that, for a given level of DNA damage, FA cells would be more likely to undergo apoptosis than controls, as we have documented for FA cells grown in culture (ALLEN and MEYN 1995). Proposing that the sensitivity of FA cells to the cytotoxic effects of DNA cross-linking agents is the result of a low threshold for triggering apoptosis resolves several issues. It explains why investigators have not found a consistent defect in DNA cross-link repair in the various FA complementation groups, why FA cells repair damaged viral DNA to the same extent as normal cells, and how FACC, an exclusively cytoplasmic protein, affects the survival of cells that have sustained DNA damage.

Based on map position, sequence information, and gene expression, the *TNFα* and *IL-6* genes are not FA genes (e.g., ROSSELLI et al. 1994). If they are playing a pivotal role in the FA phenotype, then it may be, as suggested by ROSSELLI et al. (1994), because FA genes disturb the tightly regulated expression of these critical members of a signal transduction network that regulates cell growth and death (BAKER and REDDY 1996). How multiple FA genes might affect lymphokine regulation so as to have similar phenotype is not clear. They may form a functional complex together, or they may work in the same pathway. There is much work to be done before this hypothesis can be validated and these issues resolved.

2.6.1 Genetic Instability and Cancer in FA Homozygotes

Whatever the nature of the underlying defects in FA, the spontaneous and induced genetic instability seen in this condition is presumably behind much of the cancer risk. Despite their genetic instability, FA cells are relatively hypomutable for point mutations (PAPADOPOULO et al. 1990a; LAQUERBE et al. 1995), suggesting that deletions and other rearrangements play key roles in the accumulation of genetic

damage necessary for the development of cancer in this syndrome. It has been suggested that the clonal abnormalities seen in the bone marrow of FA homozygotes predispose to the development of AML, since the leukemia risk is higher in those FA homozygotes with karyotypic abnormalities of their bone marrow stem cells (BUTTURINI et al. 1994). However, clonal abnormalities in FA bone marrow wax and wane (ALTER 1994a), and an etiologic link between the occurrence of karyotypically abnormal bone marrow stem cells and the development of leukemia has yet to be established.

2.7 BS Phenotype

Over 40 years ago, BLOOM (1954) described "congenital telangiectatic erythema resembling lupus erythematous in dwarfs." Nine years later, SZALAY (1963) reported familial clustering of this condition, which later was shown to be the result of autosomal recessive inheritance (GERMAN 1969). BS homozygotes typically survive into their early 20s (GERMAN 1995), despite the clinical and laboratory abnormalities summarized below. Additional phenotypic detail is available in recent reviews by GERMAN (1993, 1995).

2.7.1 Growth Deficiency

Proportional dwarfism is a constant finding in BS homozygotes, whose average size and weight at term birth are at the 50th percentile for a 32-week premature infant (GERMAN 1995). They remain well below the third percentile in all growth parameters throughout their lives. Slow growth is also a well-known attribute of BS cells grown in culture.

2.7.2 Immunodeficiency

LANDAU et al., (1996) first documented low levels of circulating Igs in BS homozygotes, and most individuals have defective delayed hypersensitivity as well (reviewed in WEEMAES et al. 1991). These generalized immunodeficiencies contribute to the serious bacterial sinopulmonary infections that occur in these children, making chronic respiratory failure the second leading cause of death (GERMAN 1995).

2.7.3 Malignancy

Bloom syndrome was the first chromosome instability syndrome in which a link was recognized between genetic instability and cancer risk (GERMAN et al. 1965). BS is a much less common condition than A-T and FA, and there is only one large ongoing study of BS homozygotes, the Bloom's Syndrome Registry established in the early 1960s by James German. The following discussion of malignancy in BS is

based on data contained in a recent analysis of the first 165 BS homozygotes entered into the registry (GERMAN 1993).

As expected for a cancer predisposition syndrome, there is a high risk of cancer; tumors occur early in life and multiple primary neoplasms are frequent. Of the individuals in the Bloom's Syndrome Registry report of 1993, 37% had developed malignant tumors, with a mean age at onset of 24.4 years; 28% of the BS homozygotes with cancer developed more than one primary tumor, and five of 60 had three or more malignancies (GERMAN 1993). The result is that cancer is the leading cause of death in BS homozygotes, accounting for 80% of deaths seen in the Registry (GERMAN 1995).

As shown in Fig. 4, lymphoreticular malignancies are the most common form of cancer in BS homozygotes, with leukemias and lymphomas occuring in roughly equal proportion. Together, they accounted for 45% of the malignant tumors reported in the 1993 survey (GERMAN 1993). As with A-T, lymphomas and leukemias are most common in younger patients, although the peak ages for lymphoreticular malignancies in BS are 11–25 years rather than the 6–10 years seen in A-T (compare Figs. 1 and 4). A variety of common solid tumors account for the remaining malignancies in BS homozygotes (GERMAN 1993), the most frequent carcinomas being colon, skin, breast, and cervix. Less common solid tumors include carcinomas of the tongue, larynx, lung, and esophagus as well as Wilms tumor, osteosarcoma, retinoblastoma, and medulloblastoma (GERMAN 1993; GIBBONS et al. 1995).
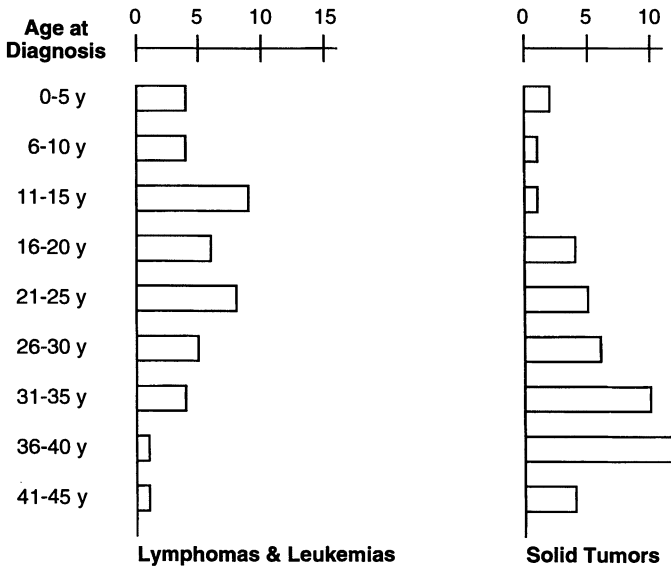
Fig. 4. Malignancies in BS homozygotes as a function of age of onset (based on data from the Bloom's Syndrome Registry report of 1993; GERMAN 1993)

Non-surgical treatment of cancer in BS is made difficult by the apparent sensitivity of some, but not all, BS homozygotes to radiation therapy and/or chemotherapy. Several case reports have documented severe and prolonged marrow suppression, esophageal strictures and other adverse effects following normal courses of radiation or chemotherapy (e.g., PASSARGE 1991; OTO et al. 1992). Unlike A-T homozygotes, however, an in vivo sensitivity to DNA-damaging agents does not appear to be universal in BS since many BS homozygotes have tolerated normal therapeutic regimens without incident (PASSARGE 1991).

2.7.4 Other Clinical Abnormalities

Bloom Syndrome homozygotes express a characteristic sun-sensitive erythematous facial rash that usually develops in the first summer of life (BLOOM 1954; GERMAN 1993). They frequently develop hyper- and hypopigmented macular skin lesions, which may represent dermatologic manifestations of increased somatic genetic recombination. Like A-T, gonadal abnormalities are a feature of the syndrome. Men with BS have azoospermia, and female homozygotes tend to have irregular menses as well as early menopause. However, some women with BS have borne children (GERMAN 1993). Diabetes has been reported in older BS homozygotes (MORI et al. 1990; GERMAN 1993). BS homozygotes typically express average or near-average intelligence, although learning disabilities are frequent and there are occasional BS homozygotes with moderate mental retardation (GERMAN 1995). Like A-T homozygotes, the personality of BS homozygotes tends towards an innate optimism. Major congenital malformations are not seen in BS homozygotes, although they have a distinctive appearance and high-pitched voice due to their small, dolichocephalic skulls, malar hypoplasia, protuberant ears, and small mandibles (GERMAN 1995).

2.7.5 Spontaneous Genetic Instability

Genetic instability is a hallmark of the BS phenotype. Beginning with GERMAN'S initial observations of an increased frequency of chromosome rearrangements, (GERMAN 1964; GERMAN et al. 1965), a variety of spontaneous chromosomal aberrations have been observed in cells from BS homozygotes, including SCEs, quadriradials, telomere associations, chromosome breaks, chromosome gaps, and micronuclei (reviewed in KUHN and THERMAN 1986; COHEN and LEVY 1989). SCEs and quadriradials (chromosome aberrations that involve recombinational exchanges between homologous chromosomes) are especially frequent in BS homozygotes (e.g., CHAGANTI et al. 1974). Multiple cell lineages express chromosomal instability in BS, including lymphocytes, lymphoblasts, keratinocytes and fibroblasts (COHEN and LEVY 1989).

Spontaneous genetic instability in BS homozygotes also has been documented by molecular and immunologic analyses. Studies of the glycophorin locus in erythrocytes found 50- to 100-fold elevations in frequencies of conversion of M and N alleles to hemizygosity and homozygosity in cells from BS homozygotes (KYOIZUMI

et al. 1989; LANGLOIS et al. 1989). BS T lymphocytes have significant elevations in their spontaneous frequencies of *HPRT* mutations (EVANS et al. 1983), and tandem repeats rearrange more frequently in BS lymphoblasts than controls (GRODEN and GERMAN 1992). Increased frequencies of spontaneous mitotic chromosomal re-combination have been documented in BS T lymphocytes in vivo (GRODEN et al. 1990) and in B lymphoblasts and fibroblasts in culture (BRAINARD et al. 1991; KUSUNOKI et al. 1994). Extrachromosomal recombination also is elevated (BUBLEY and SCHNIPPER 1987), suggesting that generalized hyperrecombination is an in-trinsic feature of BS. Finally, it should be noted that the *BLM* locus itself is a target for interchromosomal recombination in BS cells, with intralocus rearrangements restoring a wild-type gene and normal genetic stability (ELLIS et al. 1995b).

2.7.6 Abnormal Responses to DNA Damage

Bloom Syndrome homozygotes are not as sensitive to DNA-damaging agents as A-T homozygotes or FA homozygotes. However, there is in vivo and in vitro evidence for decreased resistance to the killing effects of these agents. As indicated above, BS homozygotes can be more sensitive to therapeutic radiation and che-motherapy than normal individuals. Cells from BS homozygotes exhibit sensitivity in culture as well. Most BS cells are moderately sensitive to the killing effects of ethyl methanesulfonate (EMS) (ARLETT and HARCOURT 1978; KREPINSKY et al. 1989), N-ethyl-N-nitrosourea (KURIHARA et al. 1987a), and 5-fluorouracil (LONN et al. 1990). Many BS cells are sensitive to X-rays (ARLETT and HARCOURT 1980; WEICHSELBAUM et al. 1980; HALL et al. 1986; COSTA and THACKER 1993) and UV light (KREPINSKY et al. 1980; ISHIZAKI et al. 1981), although the degree of sensitivity is variable, with some BS cells expressing essentially normal resistance to these agents and to double-strand DNA breaks induced by restriction enzyme treatment (COSTA and THACKER 1993).

Another part of BS phenotype is a low threshold for the induction of chro-mosomal aberrations by many DNA-damaging agents. Following treatment with UV light (KREPINSKY et al. 1980; KURIHARA et al. 1987b), ionizing radiation (EVANS et al. 1978; KUHN 1980; AURIAS et al. 1985a), EMS (KREPINSKY et al. 1979), or bromodeoxyuridine (BrDU) (HEARTLEIN et al. 1987), cells from BS homozygotes typically express significantly more chromosome breaks, SCEs and/or quadriradials than controls. In contrast, the cross-linking agents MMC and DEB do not induce excess SCEs in BS cells (SHIRAISHI and SANDBERG 1978; AUERBACH and WOLMAN 1979), even though BS cells are sensitive to the killing effects of MMC (ISHIZAKI et al. 1981).

Although abnormalities in DNA repair enzymes have been documented in BS cells (see below), BS cells do not have major functional defects in the several DNA repair pathways that have been examined to date (GERMAN and SCHONBERG 1980; COHEN and LEVY 1989; EVANS and BOHR 1994). Damage-induced cell cycle checkpoints have not been extensively studied in BS. However, the G_1/S checkpoint is triggered normally after UV irradiation in BS fibroblasts (VAN LAAR et al. 1994), despite impaired induction of p53 following irradiation in some BS cell lines (LU

and LANE 1993). The p53-independent, X-ray-induced, S phase checkpoint also appears to be intact (YOUNG and PAINTER 1989).

2.8 *BLM* Gene

Apparent abnormalities in the structure, activity, or expression of a variety of enzymes involved in DNA metabolism have been found in BS cells, including DNA ligase I (CHAN et al. 1987; WILLIS and LINDAHL 1987), topoisomerase II (HEART-LEIN et al. 1987) thymidylate synthetase (SHIRAISHI et al. 1989), uracil-DNA gly-cosylase (VOLLBERG et al. 1987; SEAL et al. 1988), N-methylpurine DNA glycosylase (DEHAZYA and SIROVER 1986), O^6-methylguanine methyltransferase (KIM et al. 1986), and SOD (NICOTERA et al. 1989). However, the recent positional cloning of the gene responsible for BS indicates that these abnormalities are, at best, epi-phenomena.

Unlike A-T and FA, a single complementation group defines BS (WEKSBERG et al. 1988). Four years ago, McDANIEL and SHULTZ (1992) used microcell-medi-ated chromosome transfer to demonstrate that chromosome 15 complements the high spontaneous SCE phenotype of BS cells in culture, suggesting that the single locus for BS lay on chromosome 15. This observation facilitated the work of German's group, who subsequently linked the BS locus to chromosome 15q26.1 markers (GERMAN et al. 1994), using homozygosity mapping in inbred families (LANDER and BOTSTEIN 1987). They subsequently isolated a gene from 15q26.1 that was mutated in multiple BS homozygotes (ELLIS et al. 1995a). The gene, termed *BLM*, codes for a 4.4-kb RNA transcript. It is homologous to the *S. cerevisae SGS1* and *E. coli recQ* genes, members of the DExH family of DNA/RNA heli-cases, which also include the mammalian DNA repair genes *XPB*, *XPD*, and *ERCC6* (ELLIS et al. 1995a).

Mutations in *SGS1*, the most closely related gene to *BLM* , share phenotypic similarities with BS homozygotes. The phenotype of *sgs1* null mutants includes slow growth, poor sporulation, increased mitotic nondisjunction, missegregation at meiosis, and high frequencies of both inter- and intra-chromosomal homologous mitotic recombination (GANGLOFF et al. 1994; WATTS et al. 1995). Sgs1p normally interacts in vivo with the topo II and Top3p topoisomerases and *sgs1* mutants suppress the phenotype of *top3* mutants (GANGLOFF et al. 1994; WATTS et al. 1995), suggesting that the Sgs1p helicase functions together with these topoisomerases in an in vivo complex. The BLM protein may interact with the human homologues to the yeast topo II and Top3p topoisomereases (TSAI-PFLUGFELDER et al. 1988; AUSTIN et al. 1993; FRITZ et al. 1996) since the topoisomerase activity of BS cells is abnormally low following exposure to BrDU (HEARTLEIN et al. 1987).

The sequence and phenotypic similarities between *SGS1* and *BLM* described above suggest that these two genes normally perform similar functions. It is not known at the present time what these functions might be. However, as suggested by GANGLOFF et al. (1994) for Sgs1p, the putative BLM helicase may help resolve intertwined strands at replication forks during DNA replication. This hypothesis is

supported by observations of DNA synthesis abnormalities in BS cells (HAND and GERMAN 1975; OCKEY and SAFFHIL 1986; LONN et al. 1990). The BLM helicase also may facilitate separation of chromosomes at mitosis and meiosis, as well as unwind strands so as to allow transcription and repair complexes access to damaged DNA. Finally, as discussed below, BLM could help maintain genetic stability by functioning as an anti-recombination protein.

2.9 BS Phenotype as a Manifestation of Somatic Genetic Instability

GERMAN (1993) has speculated that the growth retardation seen in BS homozygotes is the result of their tissues containing abnormally small numbers of cells. This could be the result of a defective BLM helicase interfering with DNA synthesis and/or chromosome separation, causing a prolonged cell cycle and slow growth both in vivo and in vitro. Alternatively, GERMAN (1993) has proposed that a model for BS in which growth retardation and other phenotypic abnormalities of BS homozygotes are the result of the deleterious effects of genetic instability. In this somatic mutation model for BS, growth retardation is secondary to lethal mutations and/or uniparental disomies resulting from somatic recombination, immune deficiencies are caused by disturbances in immune gene recombination, male sterility is due to disruption of crossing-over during meiosis, altered pigmentation, and other minor developmental anomalies are secondary to increased genetic damage, and cancer is the end result of a generalized increase in genetic instability.

2.9.1 How Defects in the BLM Helicase Might Promote Genetic Instability

Defects in the BLM helicase could lead to genetic instability via one or more of several different routes:

1. Inability to resolve intertwined strands during replication may lead to DNA breaks, which are highly recombinogenic.
2. The BLM helicase may be involved in decatenating sister chromatids at mitosis and homologous chromosomes at meiosis. If so, *BLM* mutations could lead to an impaired ability to separate homologous DNA strands at cell division, thereby causing increased aneuploidy as well as chromosome breakage.
3. Wild-type BLM protein may directly inhibit genetic recombination, either by disrupting nascent heteroduplex formation during the strand invasion phase of recombination or by promoting the parental resolution of Holliday structures. In this scenario, absence of a functional BLM helicase would allow more nascent recombinational events to proceed to completion, resulting in an increased frequency of SCEs, quadriradials and other homologous recombinational events.
4. Finally, like the RAD3 DNA helicase of *S. cerevisiae* (SUNG et al. 1987), BLM protein normally may unwind DNA so as to facilitate access to damaged DNA by repair complexes. Loss of BLM function in BS homozygotes would limit the access of repair complexes to sites of DNA damage, and the resultant increase in

unrepaired DNA lesions would contribute to the mutagen sensitivity, chromosome aberrations and genetic instability seen in these individuals.

2.9.2 Genetic Instability and Cancer in BS Homozygotes

The lack of a DNA helicase in the somatic cells of BS homozygotes presumably increases their frequency of spontaneous and induced chromosome aberrations, mitotic recombination, and loss of heterozygosity. The result is a loss of control of genetic integrity and an unusually rapid accumulation of the multiple genetic changes in tumor suppressor genes and oncogenes necessary to give rise to tumors. The wide variety of common tumors that occur in young BS homozygotes provides strong support for the hypothesis that acquisition of a genetic instability phenotype is a key step in oncogenesis. The immune deficiencies seen in BS homozygotes, as well as their high risk of leukemia and lymphoma, suggest that the BLM helicase may play a role in immune gene rearrangement as well as support the idea that the initial production of strand breaks and other DNA damage is a rate-limiting step in tumor development, even in genetically unstable cells.

3 What Is the Effect of Germ Line Heterozygote Mutations on Cancer Risk and Genetic Instability?

As detailed in the previous sections, a great deal of progress has been made in the last few years towards understanding the molecular pathology of the chromosome instability syndromes. These advances may help us address an important practical consideration, i.e., the extent to which mutations in the chromosome instability syndrome genes contribute to the development of cancer in the general population.

In evaluating the potential impact of the chromosome instability syndrome genes on the overall human cancer risk it is useful to consider what effects germ line mutations in these genes might have on the incidence of familial and sporadic cancers. As summarized previously, people who are homozygous for germ line mutations in chromosome instability syndrome genes are at high risk for a variety of tumors. However, these individuals represent a very small fraction of the total number of patients with cancer (see below). On the other hand, the heterozygote carriers are much more common. If they are at increased risk to develop cancer, then they might account for a significant proportion of cancer patients. The following section reviews published data concerning cancer risk and other phenotypic abnormalities in chromosome instability syndrome heterozygotes and discusses their likely contribution to the total burden of familial and sporadic cancers.

3.1 Are Chromosome Instability Syndrome Heterozygote Carriers at Risk for Cancer?

Individuals who carry germ line mutations in certain genes that affect genetic stability are known to have a high cancer risk. For example, both humans and mice who are heterozygotes for mutations in *p53* have a high incidence of lymphoma and other cancers (BIRCH et al. 1994; HARVEY et al. 1995), and patients with germ line mutations in the *hMLH1* and *hMSH2* mismatch repair genes are at increased risk for colon and endometrial cancers as well as other solid tumors (reviewed in LYNCH and LYNCH 1994). Given these examples, one might expect that heterozygote carriers of mutations in the *ATM*, *BLM*, or FA genes would be at increased risk to develop cancer.

As summarized below, the published literature supports the conclusion that *ATM* heterozygotes face an approximately fourfold higher risk of breast cancer than normal individuals. They may also have an elevated risk for other tumors. In contrast, existing evidence for any increased cancer risk in FA heterozygotes is slim, and almost non-existent for carriers of *BLM* mutations.

3.1.1 Ataxia Telangiectasia

Direct support for an increased cancer risk in A-T heterozygotes comes from studies of the relatives of A-T homozygotes. An increased incidence of cancer amongst relatives of A-T homozygotes was first noticed by REED et al. (1966). In 1976, SWIFT et al. reported a study of 27 A-T families in which relatives of A-T patients suffered an excess of deaths from cancer over that expected in the general population (59 deaths observed vs. 42.6 expected, $p < 0.02$). A subsequent retrospective study of 110 additional families confirmed and expanded the original findings (SWIFT et al. 1987). This was followed by a 1991 prospective study of 1420 individuals in 161 A-T families (SWIFT et al. 1991) which found that the rate ratios of all cancers in blood relatives when compared to spouse controls were 2.5 for men and 2.1 for women. The ratios for obligate heterozygotes were higher (3.9 for males and 2.7 for females). Relative risk estimates for heterozygotes were calculated to be 3.8 for males and 3.5 for females ($p < 0.005$).

In SWIFT's studies, the increased occurrence of cancer in blood relatives of A-T patients was particularly striking for breast cancer, although there also were excess numbers of lung, ovarian, pancreatic, bladder, and stomach cancers (SWIFT et al. 1987, 1991). SWIFT's conclusions that A-T heterozygotes have an increased relative risk for breast cancer have been supported by two smaller studies. In a survey of 67 A-T families in Great Britain by PIPPARD et al. (1988), researchers found an excess of breast cancer in the parents of A-T homozygotes, as did a study of eight Norwegian families by BORRESEN et al. (1990). The observed increases in breast cancers amongst blood relatives of A-T homozygotes were due primarily to an excess of cancers in women under 55 and, in the largest survey (SWIFT et al. 1991), 25% of female blood relatives with breast cancer had two independent cancers. Although these data sets are not large, the excess numbers of cancers, shift in the age of onset

towards younger women, and elevated frequency of multiple primary tumors are what might be expected for individuals with a hereditary predisposition to develop breast cancer.

In a recent meta-analysis, EASTON (1994a) reviewed the published family studies and found that the combined data supported estimates of a relative risk of 3.9 (95% confidence interval of 2.1–7.2) for developing breast cancer in female A-T heterozygotes (see Table 1). EASTON (1994a) concludes that these studies provide compelling evidence for an increased risk of breast cancer in A-T heterozygotes. The case for an increased risk of breast cancer in A-T heterozygotes is further supported by unpublished work of SWIFT (1996) in which polymorphic markers linked to the *ATM* locus were used to identify heterozygotes in A-T families. This method of analysis avoids some of the ascertainment bias problems inherent in previous studies. Of the 27 A-T family members with breast cancer that have been analyzed to date, 21 were found to be A-T heterozygotes.

These same family studies do not present as strong a case for the proposition that A-T heterozygotes face an increased risk of developing cancers other than breast carcinoma. In EASTON's meta-analysis (1994a), the combined data sets gave a relative risk of 1.9 (95% confidence interval of 1.5–2.5) for all cancers in A-T heterozygotes. However, as EASTON points out, the four published surveys are inconsistent regarding the overall risk of cancer. SWIFT's retrospective and prospective studies of American A-T families predict 2.6–3.6 increases in the relative risk of developing cancer in A-T heterozygotes, while the smaller British and Norwegian surveys estimate that the overall risk of developing cancer is essentially normal in these individuals. Resolution of the discrepancy between these studies may require both addition of new families to the study populations as well as identification of A-T heterozygotes within these families by molecular analysis.

The DSN model for A-T summarized above presumes that p53 is downstream of ATM in one or more pathways that mediate cellular responses to DNA damage. Given the assumption of this and other models that ATM and p53 are in the same signal transduction network (ENOCH and NORBURY 1995; KASTAN 1995; MEYN 1995), it is useful to compare the spectrum of tumors seen in heterozygotes and homozygotes for *ATM* and *p53* mutations.

Table 1. Estimated relative risks of cancer in A-T heterozygotes (modified from EASTON 1994a)

| Study | Breast cancers | | | Other cancers | | |
	Observed/expected (A-T blood relatives)	Relative risk to A-T heterozygotes Mean	Range	Observed/expected (A-T blood relatives)	Relative risk to A-T heterozygotes Mean	Range
SWIFT et al. (1987)	27/20.5	6.8	2.0–22.6	111/103	2.6	1.4–4.7
SWIFT et al. (1991)	23/7	5.1	1.5–16.9	68/29	3.5	2.0–6.1
BORRESEN et al. (1990)	6/2.4	3.9	1.26–12.1	14/20	0.77	0.32–1.85
EASTON (1994a)	2/0.17	1.3	0.33–5.2	25/24.5	1.0	0.59–1.7
	58/30	3.9	2.1–7.2	218/179	1.9	1.5–2.5

As can be seen in Table 2, both A-T homozygotes and *p53*-null mice have a predilection for immune system tumors. KASTAN et al. (1992) suggests that an abnormal response to DNA strand breaks may be responsible for the high incidence of lymphoid tumors in both cases. In the DSN model, this abnormal response would be the inability to trigger the ATM-dependent DSN and activate its cell cycle checkpoints. Breast cancer is common in both Li-Fraumeni family members and A-T blood relatives, yet is relatively infrequent in A-T homozygotes or *p53*-null mice. Death at an early age from other causes may reduce the apparent occurrence of breast cancer in homozygotes. Alternatively, these differences may reflect phenotypic differences between the heterozygote and homozygote states of these two genes.

The spectrum of tumors seen in A-T homozygotes is not identical to that in *p53*-null mice. For example, leukemias are the second most common tumor in A-T homozygotes but are not seen in *p53*-null mice, while sarcomas are a common tumor type in *p53*-null mice but are rarely seen in A-T homozygotes. The latter may reflect a functional difference between the two genes rather than a difference between the two species, since sarcomas are frequent in human *p53* heterozygotes (e.g., affected Li-Fraumeni family members).

3.1.1.1 Etiology of A-T Heterozygotes' Cancer Risk

Analogous to germ line mutations in the *Rb* gene predisposing to retinoblastoma, A-T heterozygotes could be at increased risk to develop cancer solely because they have only one functional *ATM* allele to inactive before they lose ATM-dependent damage responses and become genetically unstable. Alternatively, their increased cancer risk could be due, in part, to *ATM* mutations not being completely recessive. If cells from A-T heterozygotes are not phenotypically normal, then inherent genetic instability and mutagen sensitivity would directly facilitate the accumulation of somatic mutations necessary for tumor development. As detailed in Section 3.2.1, this possibility is supported by multiple studies demonstrating that *ATM*

Table 2. Tumor spectrum in *p53* and *ATM* homozygotes and heterozygotes

Tumor type	Genetic background (source)				
	Li-Fraumeni family members	$p53^{+/-}$ mice	A-T blood relatives	$p53^{-/-}$ mice	A-T homozygotes
Lymphoma (%)	–	31	5	55	56
Leukemia (%)	6	–	4	–	24
Sarcoma (%)	18	54	–	23	–
Breast carcinoma (%)	26	2	20	2	2
Brain tumor (%)	12	3	–	4	3
Other tumors (%)	38	10	71	18	15
Tumors analyzed (*n*)	231	60	138	90	107
Source	MALKIN (1993)	HARVEY et al. (1995)	SWIFT et al. (1987)	HARVEY et al. (1993)	SPECTOR et al. (1982)

heterozygotes have subtle abnormalities in their responses to DNA damage and their ability to maintain genomic stability.

3.1.2 Fanconi Anemia

In 1951 BAUMANN suggested that leukemia occurs unusually often in relatives of FA homozygotes, and, in a 1959 survey of 49 families with at least one FA homozygote, GARRIGA and CROSBY found leukemia in four "unaffected" relatives, a rate of one case of leukemia in a relative for every 12.2 FA homozygotes. They compared this rate to a previously reported rate of one case of leukemia in a blood relative for every 450 patients with leukemia and conducted that "the rate of leukemia in the Fanconi families seems to be astonishingly high," suggesting that this was due to "some hereditary fault." Subsequently, FANCONI (1967) wrote that "in the families with F.A., leukemia and other myelopathies are more frequent than in normal families." Two subsequent reviews of leukemia in FA homozygotes noted an apparent increased incidence of leukemia in blood relatives but offered no formal analysis thereof (DOSIK et al. 1970; SCHROEDER and KURTH 1971).

The putative link between FA heterozygote carriers and malignancy was formally investigated by SWIFT in 1971, who conducted a retrospective analysis of cancer in eight FA families. In this study, there were 27 cancer deaths observed in blood relatives of FA homozygotes, compared to the 17.4 expected ($p < 0.05$). There were excess deaths from leukemia (three observed, one expected), colon cancer (five observed, two expected), and stomach cancer (four observed, two expected). Cancer deaths were predominantly in the 50- to 74-year-old age group, however, and there was only one case of multiple primary tumors. SWIFT concluded that FA heterozygotes had threefold increase in their relative risk of cancer and that they might represent 5% of individuals with acute leukemia.

Nine years later, SWIFT et al. (1980) published an expanded study that included 1206 blood relatives from 25 FA families of diverse ethnic backgrounds. Forty-eight cancer deaths occurred in the blood relatives compared to 56.2 expected, and only three blood relatives had leukemia compared to 4.5 expected. There were no significant differences between cancer rates in blood relatives and controls, although there was a higher than expected number of tumors for four sites: gastric cancer (six observed, 4.9 expected), bladder cancer (seven observed, 4.8 expected), breast cancer (17 observed, 13.5 expected), and lung cancer (three observed, 0.6 expected). SWIFT et al. concluded that there was no overall excess of cancers in either FA blood relatives or obligate FA heterozygotes, although the question of increased risk of bladder, stomach, and breast cancer among FA heterozygotes was unresolved.

Subsequent to SWIFT et al.'s 1980 report, a smaller epidemiological study was conducted by POTTER et al. (1983), who surveyed 129 family members of nine index cases of FA. Seven malignancies were observed, as opposed to the 10.4 expected. Five of seven tumors occurred in individuals over age 60, and there were no leukemias. The authors concluded that there was no evidence for increased cancer risk in FA heterozygotes.

These studies suggest that, despite their abnormal responses to DNA-damaging agents (see Section 3.2.2), FA heterozygotes face little, if any, increase in their relative risks of developing cancer when compared to the population at large. However, these surveys cannot exclude the possibility that specific FA genes or mutant alleles of those genes might predispose to cancer in heterozygotes while the majority of FA mutations do not. The report of HILL (1976) is suggestive of this possibility. He described a remarkable inbred family in the Scottish Hebrides in which 13 of 21 immediate relatives of a FA homozygote had developed cancer, including three first cousins whose malignancies presented before age 10.

3.1.3 Bloom Syndrome

In an early report, GERMAN (1974) noted the occurence of five malignancies in 82 parents of BS homozygotes entered in the Bloom's Syndrome Registry. The tumors included colon, breast, and gastric carcinomas, as well as melanoma and myeloid dysplasia. Since then, formal analyses of cancer risk in BS heterozygotes have not been published, although one may be in the near future, using the families entered into the Bloom's Syndrome Registry (GERMAN 1995). GERMAN, who has had contact with the largest number of BS patients and families, states that he does not believe that BS heterozygotes have an increased cancer risk.

3.2 Phenotypic Abnormalities in Chromosome Instability Syndrome Heterozygotes

3.2.1 A-T Heterozygotes

3.2.1.1 Spontaneous Genetic Instability in A-T Heterozygotes

If A-T heterozygotes have an increased cancer risk because *ATM* mutations are not completely recessive, then one might expect increased genetic instability in cell from A-T heterozygotes. As summarized previously, spontaneous genetic instability in A-T homozygotes manifests itself in a variety of ways including increased chromosome aberrations, elevations in mitotic recombination rates (MEYN 1993), high mutation frequencies (BIGBEE et al. 1989; COLE and ARLETT 1994), increased aneuploidy (AGUILAR et al. 1968). Studies of these manifestations of genetic instability in A-T heterozygotes have yielded mixed results.

Several early studies found that lymphocytes and fibroblasts from A-T heterozygotes had modest, but statistically significant, elevations in their spontaneous frequencies of chromosomal aberrations (COHEN et al. 1975; OXFORD et al. 1975). In addition ROSIN et al. (1989), using micronuclei formation as a measure of chromosome breakage, documented that the average frequency of in vivo chromosome breaks in epithelial cells exfoliated from the oral mucosa of 26 A-T heterozygotes was threefold higher than that of 29 controls.

However, increased spontaneous chromosomal instability in A-T hetero-
zygotes has not been confirmed by all investigators. Several studies found no ob-
vious differences between A-T heterozygotes and controls (e.g., NATARAJAN et al.
1982; NAGASAWA et al. 1985). A recent examination by SCOTT et al. (1994) is typical.
Studying lymphocytes from 27 heterozygotes, 14 homozygotes, and 66 age-matched
controls, they found no significant differences between A-T heterozygotes and
controls in the frequency of spontaneous chromosome aberrations.

Studies of individual genetic loci in small numbers of obligatory heterozygotes
(parents of A-T patients) have not documented increased spontaneous instability
either. For example, in the glycophorin study cited earlier, 23 obligatory A-T
heterozygotes did not have significant elevations in their frequency of GPA
hemizygous or homozygous variant cells (BIGBEE et al. 1989). More recently, COLE
and ARLETT (1994) compared the frequencies of spontaneous *HPRT* mutants
among circulating lymphocytes of 16 A-T homozygotes and 19 A-T heterozygotes
to those of age-matched controls. The mutation frequency in cells from A-T
homozygotes averaged 11-fold higher than controls, but there were no significant
differences between heterozygotes and controls.

3.2.1.2 Abnormal Responses to Induced DNA Damage in A-T Heterozygotes

When compared to cells from A-T homozygotes, heterozygote cells have similar,
but more subtle, abnormalities in their responses to induced DNA damage. These
abnormalities include decreases in survival following exposure to ionizing radiation
or radiomimetic drugs, increased sensitivity to damage-induced chromosome and
chromatid aberrations, post-irradiation DNA synthesis abnormalities, and pro-
longed G_2 arrest (CHEN et al. 1978; PATERSON et al. 1979; KIDSON et al. 1982;
KINSELLA et al. 1982; KOHN et al. 1982a; SHILOH et al. 1982b; ARLETT and
PRIESTLEY 1985; LITTLE and NAGASAWA 1985; PATERSON et al. 1985; NAGASAWA
et al. 1985, 1987; ARLETT et al. 1989; ROSIN et al. 1989).

3.2.1.3 Radiosensitivity and the Effects of Dose Rate

The sensitivity of cells from A-T heterozygotes to the killing effects of ionizing
radiation and radiomimetic drugs is mild compared to that of A-T homozygotes.
Consequently, it sometimes has been difficult to distinguish between A-T hetero-
zygotes and controls when studying the cytotoxic effects of ionizing radiation
delivered at typical dose rates. For example, in the first report of radiation sensi-
tivity of A-T heterozygotes, PATERSON et al. (1979) found that the radiation re-
sistance of primary fibroblast lines from three of five A-T heterozygotes was
intermediate between control cells and A-T homozygotes' cells. KINSELLA et al.
(1982) were unable to confirm these findings when they studied six A-T hetero-
zygotes, but NAGASAWA et al. (1985) found intermediate radiosensitivity in fibro-
blasts from six different A-T heterozygotes. Similar variability has been reported
for the radiosensitivity of peripheral lymphocytes from A-T heterozygotes (e.g.,
COLE et al. 1988).

Attempts to determine the sensitivity of A-T heterozygotes to radiation-induced chromosomal aberrations have also yielded mixed results. Using high dose-rate irradiation, several groups have reported normal levels of radiation-induced chromosome aberrations in both lymphocytes and fibroblasts (BENDER et al. 1985; NAGASAWA et al. 1985; JONES et al. 1995), while there have been two reports of increased sensitivity to chromatid aberrations induced in G_2 in A-T heterozygote fibroblasts (PARSHAD et al. 1985; SHILOH et al. 1986).

Decreasing the rate of irradiation for control cells improves their survival but does not appreciably affect the survival of cells from A-T homozygotes (PATERSON et al. 1984). Hence, low dose-rates of ionizing irradiation magnify differences between the radioresistance of control cells and A-T homozygotes' cells. In order to reveal subtle defects in radiation response, several groups have studied the survival of A-T heterozygotes cells after low dose-rate irradiation, reasoning that low dose-rate irradiation might accentuate differences in survival between control cells and A-T heterozygotes' cells. This indeed appears to be the case, in that multiple reports using low dose-rate irradiation all have demonstrated statistically significant differences in survival between lymphocytes and fibroblasts from groups of A-T heterozygotes and controls (PATERSON et al. 1985; WEEKS et al. 1991; WAGHRAY et al. 1992; HANNAN et al. 1991, 1994; WEST et al. 1995). For example, WEEKS et al. (1991) used low dose-rate irradiation to study clonal survival of primary fibroblasts from controls, A-T heterozygotes and A-T homozygotes. They found that D_{10} values for ten strains of control fibroblasts averaged 797 cGy, while D_{10} values for nine A-T heterozygotes averaged 496 cGy, and D_{10} values for three A-T homozygotes averaged 165 cGy. The differences between controls and A-T heterozygotes was highly significant ($\chi^2 = 11.14$, $P = 0.0009$). Several of these reports also examined the effects of high dose-rate irradiation on survival. For example, PATERSON et al. (1985) found that there was little difference between the survival of control and A-T heterozygote skin fibroblasts when given a single acute dose of γ-rays (1.1 Gy/min) but that the survival of controls and A-T heterozygotes was significantly different if exposed to chronic low dose-rate irradiation (0.005 Gy/min).

Decreasing the rate of irradiation also protects control cells from radiation-induced chromosomal aberrations, thus magnifying the sensitivity of A-T heterozygotes' cells to radiation-induced chromosomal damage (WAGHRAY et al. 1992; JONES et al. 1995). SCOTT's group (SCOTT et al. 1993; JONES et al. 1995) and others (WAGHRAY et al. 1992) have taken advantage of this effect to demonstrate that, when compared to controls, A-T heterozygotes have statistically significant elevations in their frequencies of radiation-induced chromosomal aberrations. For example, in a recent study by JONES et al. (1995), yields of dicentric chromosomes in lymphoblasts following 3 Gy γ-rays delivered at a high dose-rate (1.7 Gy/min) averaged 61.6 for 32 control individuals, 75.2 for eight A-T homozygotes, and 60.4 for 19 A-T heterozygotes. When low dose-rate irradiation was used (0.0031 Gy/min), the average yields of dicentric chromosomes dropped to 63 for the A-T homozygotes, 41.5 for the A-T heterozygotes, and 36.0 for the controls. Differences in the responses of heterozygotes and controls was statistically significant only for low dose-rate irradiation ($p < 0.05$).

3.2.1.4 DNA Repair Anomalies in A-T Heterozygotes

Further molecular evidence that *ATM* mutations are not completely recessive comes from studies of radiation-induced double-strand breaks. BLOCHER et al. (1991) used pulsed field gel electrophoresis to examine the number of residual double-strand breaks remaining in genomic DNA following exposure of fibroblasts to low-dose X-rays and found that, on average, cells from five A-T heterozygotes had 50% more residual breaks than controls. This was lower than the threefold elevation seen in A-T homozygotes but suggests that carriers of *ATM* mutations have subtle defects in their repair of certain double-strand breaks. This observation is further supported by work from PANDITA and HITTELMAN (1994) which showed that, using high-dose irradiation and premature chromosome condensation, four A-T heterozygote lymphoblastoid lines sustained increased levels of chromosome damage when compared to controls, even though they had normal survival as well as induction and repair of DNA double-strand breaks following high-dose irradiation.

3.2.1.5 Cell Cycle Checkpoints in A-T Heterozygotes

A cardinal feature of the cells of A-T homozygotes is their lack of cell cycle checkpoints. Although it may lie at the heart of genetic instability in A-T, this aspect of the A-T phenotype has not been thoroughly investigated in A-T heterozygotes. There have been no published studies testing the G_1/S checkpoint in actively growing cells from A-T heterozygotes. However, fibroblasts from A-T heterozygotes undergo an abnormally prolonged delay in entry into S phase following irradiation in G_0 (RUDOLPH et al. 1989).

Several reports have examined the S phase checkpoint in A-T heterozygotes. SHILOH et al. (1982b) found that the average degree of inhibition of DNA synthesis following the exposure of fibroblasts from three A-T heterozygotes to the radiomimetic mutagen neocarzinostatin were smaller that normal but did not find the differences statistically significant. In a more recent study by HANNAN et al. (1994), peripheral lymphocytes and primary fibroblasts from three A-T heterozygotes all had statistically significant defects in the S phase checkpoint, as measured by the presence of "radioresistant" DNA synthesis following exposure to 4 Gy X-rays. However, YOUNG and PAINTER (1989) found that fibroblasts from two obligate A-T heterozygotes had a normal S phase checkpoint following irradiation, and WIENCKE et al. (1992) did not find radioresistant DNA synthesis in lymphocytes from A-T heterozygotes. This suggests that, like other facets of the phenotype, not all A-T heterozygotes may express a defect in their G_1/S checkpoints.

The initial G_2/M checkpoint may be intact in A-T heterozygotes since both primary fibroblasts and peripheral lymphocytes from A-T heterozygotes temporarily pause in G_2 following irradiation (YOUNG and PAINTER 1989; SCOTT et al. 1994). However, several studies have indicated that fibroblasts and lymphoblasts from most, but not all, A-T heterozygotes have a tendency towards delayed G_2 arrest following radiation that is similar, but less marked, than that seen in A-T

homozygotes (BATES and LAVIN 1989; RUDOLPH et al. 1989; LAVIN et al. 1992, 1994).

3.2.1.6 Dominant Negative *ATM* Alleles as an Explanation for the A-T Heterozygote Phenotype

A molecular explanation of the phenotypic abnormalities of most A-T hetero-zygotes could be that many *ATM* mutations are not completely recessive, but rather function as weak, dominant negative mutations. ATM proteins may need to form complexes with themselves or other proteins in order to properly function in the damage surveillance signal transduction network. Mutant ATM proteins that are missing key domains (e.g., the PI-3 kinase region) still may participate in complex formation but yet fail to function, thereby inactivating the complex and perturbing the cellular response to DNA damage. The result is a dominant negative phenotype. Those remaining A-T heterozygotes who express no phenotypic ab-normalities may harbor null mutations of *ATM*, which would function as com-pletely recessive alleles and have no effect in the heterozygote state.

3.2.2 FA Heterozygotes

As a group, FA heterozygotes express some of the same phenotypic abnormalities as FA homozygotes but in an attenuated form. In vivo abnormalities in obligate FA heterozygotes include statistically significant increases in the frequency of minor skeletal malformations as well as mildly elevated fetal hemoglobin levels (PETRIDOU and BARRETT 1990). In addition, blood relatives of FA homozygotes appear to have an excess number of major genitourinary and hand malformations (GMYREK et al. 1965; ALTAY et al. 1975; WESHIMER and SWIFT 1982). As discussed above, some FA heterozygotes also may express increased cancer risk.

3.2.2.1 Genetic Instability in FA Heterozygotes

A few studies have found statistically significant differences between the sponta-neous frequencies of chromosome aberrations in FA heterozygote cells and con-trols (CERVENKA and HIRSCH 1983; PORFIRIO et al. 1983; KORKINA et al. 1992). However, most studies found no significant differences between the two groups (e.g., COHEN et al. 1982; MARX et al. 1983; ROSENDORFF and BERNSTEIN 1988). FA heterozygotes do not manifest increased in vivo spontaneous mutation frequencies at either the *HPRT* or the glycophorin locus, (SALA-TREPAT et al. 1993) but KORKINA et al. (1992) found elevated amounts of 8-hydroxy-2'-deoxyguanosine in the genomic DNA of FA heterozygotes, suggesting that they sustain more ROS damage than normal individuals. Lymphocytes from FA heterozygotes express a prolonged G_2 phase when grown in culture, although their cell cycle defect is not as pronounced as that of FA homozygotes (DUTRILLAUX et al. 1982; MARX and SMITH 1989). In addition, caffeine increases the frequency of spontaneous chromosome aberrations in FA heterozygotes to a greater extent than controls (PINCHEIRA et al.

1988), suggesting that a subtle spontaneous genetic instability in FA heterozygotes can be uncovered by loss of G_2 damage-sensitive checkpoint.

In contrast to spontaneous genetic instability, multiple studies have found that, as a group, cells from FA heterozygotes are significantly more sensitive than controls to the clastogenic effects of MMC, HN_2, 8-MOP, and DEB (AUERBACH and WOLMAN 1978; BERGER et al. 1980; AUERBACH et al. 1981; ALIMENA et al. 1983; MARX et al. 1983; PORFIRIO et al. 1983; DALLAPICCOLA et al. 1985; ROSENDORFF and BERNSTEIN 1988). For example, AUERBACH and WOLMAN (1978) found that, following exposure to 10 ng/ml DEB, chromosome breakage in lymphocytes from FA heterozygotes was fourfold greater than that of controls but much less than that induced in cells from FA homozygotes. However, in every study except one (MARX et al. 1983), some controls and FA heterozygotes had responses that overlapped each other, with some reports not even finding group differences (e.g., COHEN et al. 1982; CERVENKA and HIRSCH 1983; ROSENDORFF and BERNSTEIN 1988).

The overlap in behaviour between FA heterozygotes and controls may be the result of genetic heterogeneity. The one study that did not find any overlap between FA heterozygotes and controls in the frequency of DEB-induced chromosome aberrations was a study of 54 heterozygotes and 42 controls from an inbred South African Afrikaner population, where cases of FA most likely are caused by a single FA mutation from a common ancestor (MARX and SMITH 1989). Other studies have found a correlation between the severity of the phenotype within families. For example, WUNDER and FLEISCHER-REISCHMANN (1983) reported that all hetero-zygotes from three FA families were sensitive to the cytotoxic effects of 8-MOP, while all heterozygotes from three other FA families had normal cellular survival following 8-MOP exposure. DEGAN et al. (1995) found a marked variability in the amount of 8-hydroxy-2'-deoxyguanosine present in the DNA of FA parents but found that this variability was strongly correlated with the amount of 8-hydroxy-2'-deoxyguanosine in the DNA of their affected homozygote children.

3.2.3 BS Heterozygotes

Studies of cells from obligate BS heterozygotes have found normal or near-normal genetic stability, suggesting that *BLM* mutations are completely recessive at the cellular level. These analyses have, in general, focused on chromosome aberrations. Cytogenetic studies have found no differences between BS heterozygotes and controls in the frequency of spontaneous SCEs, quadriradials, or other chromo-some aberrations (CHAGANTI et al. 1974; BARTRAM et al. 1976; GERMAN et al. 1977b; KUHN and THERMAN 1979; ROSIN and GERMAN 1985; HOJO et al. 1995). In studies of MN variants at the glycophorin locus in erythrocytes that detected greater than 50-fold elevations in variant frequencies in BS homozygotes, the fre-quency of variants in three of four BS heterozygotes tested was normal and the fourth near normal (KYOIZUMI et al. 1989; LANGLOIS et al. 1989). An in vitro study of micronuclei formation in epithelial cells from BS heterozygotes found normal frequencies of micronuclei (ROSIN and GERMAN 1985), while a study of micronuclei formation in BS heterozygote fibroblasts grown in culture found threefold increases

in spontaneous micronuclei frequencies (FRORATH et al. 1984). The same study did not find increased susceptibility to the induction of micronuclei by mutagens, however.

3.3 What Proportion of All Cancers Occur in Heterozygote Carriers of Chromosome Instability Gene Mutations?

In a 1981 review of the etiology of cancer, DOLL and PETO wrote: "At present, the relevance of genetic susceptibility to the common types of cancer remains obscure." Since then, the inherited components of cancer risk have been much more clearly defined as more than 20 genes responsible for specific familial cancer syndromes have been isolated (reviewed in SANKARANARAYANAN and CHAKRABORTY 1995). The impact of germ line mutations in these genes on the overall incidence of cancer is variable. For example, germ line mutations in *p53* convey a very high risk of developing certain cancers, but the population frequency of *p53* heterozygotes is so low (~0.002%), that only a small portion of human cancers are the result of germ line mutations in this tumor suppressor gene (FORD and EASTON 1995). In contrast, germ line mutations in the *BRCA1* and *BRCA2* genes together account for most cases of familial breast cancer and 4%–8% of all breast cancers, affecting ~500 000 women in the United States alone (ROWELL et al. 1994).

    As with other cancer susceptibility genes, the contribution of germ line mutations in the chromosome instability syndrome genes to the overall incidence of cancer is dependent both on the penetrance of heterozygous mutations and on mutant allele frequency. In the following section, estimates for the heterozygote carrier frequency for A-T, FA, and BS mutations will be reviewed, and the potential impact of these germ line mutations on the overall incidence of cancer discussed.

3.3.1 Prevalence of Heterozygote Carriers in the General Population

3.3.1.1 Ataxia Telangiectasia

Taken as a whole, published clinical and laboratory studies reviewed in Section 3.1.1 support the conclusion that A-T heterozygotes are a subpopulation of individuals at increased risk to develop breast and, perhaps, other cancers. Given this conclusion, the frequency of A-T heterozygotes in the general population becomes an important public health consideration. The recent isolation of the *ATM* gene makes direct measurement of carrier frequency possible. However, until such studies are completed, we have to rely on estimates of the frequency of A-T heterozygotes in the general population based on the incidence of diagnosed A-T homozygotes and pedigree analysis of A-T families.

The first published estimate of the prevalence of A-T homozygotes was one in 40 000 live births, based on a survey of elementary school children in Los Angeles (BODER and SEDGWICK 1963). SWIFT et al. (1986), in a retrospective epidemiological study, calculated a lower limit for the mutant gene frequency in the United States Caucasian population of 0.17%, based on the incidence of ascertained cases. Analysis of the incidence of affected cousins in several large pedigrees from the same data set gave a most likely gene frequency of 0.7%, with 95% confidence limits of 0.12%–2%. In a survey of British children born between 1969 and 1976 with A-T, PIPPARD et al. (1988) calculated an incidence of one in 100 000 at birth. In a later study, WOODS et al. (1990) ascertained the prevalence of A-T in the British West Midlands, estimating a birth frequency of one in 300 000. CHESSA et al. (1994), used Dahlberg's formula, together with data from the Italian national registry for A-T patients and Vatican records for consanguineous marriages, to calculate the mutant ATM gene frequency among Italians. Their calculated mutant gene frequency, $q = 1.2\% \pm 0.65$, is the highest published estimate and implies an incidence at birth of one in ~7000, suggesting that ATM mutations may be unusually prevalent in the Italian population.

As shown in Table 3, one can use gene frequency estimates to estimate the proportion of individuals in the general population who are heterozygote carriers of ATM mutations. However, it should be noted that ascertainment of A-T homozygotes in retrospective surveys is incomplete, and gene frequencies based on the prevalence of ascertained cases are almost certainly underestimates. Given the practical difficulties in ascertainment, it is reasonable to assume that a significant proportion of A-T homozygotes were not identified during these surverys. However, published estimates that are based on pedigree analysis appear high. For example, pedigree analysis of the SWIFT et al. data implies that 90% of A-T homozygotes were not ascertained in this survey, an unlikely event. The true

Table 3. Published estimates of mutant ATM gene frequency

	BODER and SEDGWICK (1963)	SWIFT et al. (1986)	SWIFT et al. (1986)	PIPPARD et al. (1988)	WOODS et al. (1990)	CHESSA et al. (1994)
Number of A-T patients ascertained	?	231	231	61	11	73
Population surveyed	Los Angeles (US)	United States (Caucasian)	United States (Caucasian)	England	West Midlands (England)	Italy
Basis of estimate	Prevalence in survey	Prevalence in survey	Pedigree analysis	Prevalence in survey	Prevalence in survey	Pedigree analysis
Mutant ATM gene frequency (%)	0.5	0.17	0.7	0.32	0.18	1.21
Frequency of mutant ATM homozygotes (A-T patients)	~1/40 000	~1/346 000	~1/20 000	~1/97 000	~1/310 000	~1/7 000
Frequency of mutant ATM heterozygotes[a] (A-T carriers)	~1/100	~1/300	~1/70	~1/156	~1/277	~1/42

[a]Calculated assuming Hardy-Weinberg equilibrium for a single gene disorder (i.e., no genetic heterogeneity).

gene frequency probably lies between the high and low estimates derived from retrospective population surveys and pedigree analyses.

Previous estimates of the frequency of heterozygote carriers generally have assumed the existence of multiple A-T genes (genetic heterogeneity), based on the finding of at least four A-T complementation groups, as defined by heterodikaryon fusion analyses (JASPERS et al. 1988) and the assumption that each complementation group represented a separate gene. Accordingly, published carrier frequency estimates are higher than they would have been if calculated assuming a single gene disorder.

Although multiple complementation groups represent independent genes for FA and xeroderma pigmentosa, the A-T complementation groups A/B, C, D, and E recently were found to be caused by mutations in the *ATM* gene (SAVITSKY et al. 1995). In light of this finding that a single gene is responsible for essentially all classic cases of A-T, the published estimates of heterozygote carrier frequency need to be revised downwards, as has been done in Table 3. These revised carrier frequencies range from 0.34% to 2.42%, with a reasonable average of about 1%.

These estimates, together with the data summarized above that suggest that carriers of *ATM* mutations are at an approximately fourfold increased risk for breast cancer, lead to the conclusion that roughly 3%–5% of women with breast cancer are heterozygotes for mutations in the *ATM* gene. This estimate is similar to that of EASTON (1994a) and suggests that, in the United States, ~7000 female heterozygote carriers of *ATM* mutations develop breast cancer each year. As to the possible contribution of heterozygote *ATM* carriers to other types of cancer, the epidemiological and laboratory data reviewed in Sections 3.1.1 and 3.2.1 suggest a slight predisposition to gastric carcinomas and other malignancies, but the epidemiological data are not consistent (see Section 3.1.1), and any estimate based on these published studies would have a great deal of uncertainty.

3.3.1.2 Fanconi Anemia

Fanconi anemia has been reported in all major ethnic groups (ALTER 1994a) but is not as common as A-T, with the combined incidence of FA homozygotes from all complementation groups estimated to be ~1/350 000 (SWIFT 1971; SCHROEDER et al. 1976; SWIFT et al. 1980; SASAKI 1989). Given the existence of at least three and perhaps as many as five different FA genes, the combined heterozygote carrier frequency for all FA genes may be as high as one in 130. Specific ethnic groups may be at higher risk. For example, a recent survey of 3100 Jewish individuals for *IVS4* and *322delG* mutations in the *FACC* gene found a carrier frequency for the *IVS4* mutation of 1/89 in Ashkenazi Jews (VERLANDER et al. 1995), and ROSENDORFF et al. (1987) has estimated a 1/77 carrier frequency for the South African Afrikaners FA mutation, presumably due to a founder effect in this isolated population.

The studies summarized in Section 3.2.2 indicate that many FA heterozygotes express, to a lesser degree, the phenotypic abnormalities seen in FA homozygotes, including an increased sensitivity to the induction of chromosomal aberrations by bifunctional DNA cross-linking agents. These data suggest that at least some FA

mutations are not completely recessive and predict that many FA heterozygotes may have a mild degree of genetic instability in vivo. A consequence of this in vivo genetic instability might be an increased incidence of cancer. However, given the results of the few epidemiological surveys of FA families that have been conducted (see Sect. 3.1.2), this increase is likely to be minimal for most FA mutations. As a result, despite the fact that the carrier frequency of all FA mutations approaches that of *ATM* mutations in the general population, it is likely that the contribution of FA heterozygote carriers to the overall incidence of cancer is low. The possibility remains that a few FA mutations may carry a significant risk of cancer for heterozygote carriers, e.g., the FA family reported by HILL (1976). However, the contributions of such mutations to the overall cancer burden are likely to be minor.

3.3.1.3 Bloom Syndrome

Bloom Syndrome occurs in many different ethnic groups (GERMAN and PASSARGE 1989) but is considerably rarer than A-T and FA, with the United States incidence of BS homozygotes estimated to be 1/6 330 000 (GERMAN and TAKEBE 1989). A study of BS in Israel documented increased occurrence in Ashkenazi Jews and estimated the homozygote gene freqency in that population as 1/60 000, with a carrier frequency of ~1/125 (GERMAN et al. 1977a).

The studies reviewed in Section 3.2.3 indicate that cells from BS heterozygotes have normal or near-normal genetic stability, suggesting that *BLM* mutations are completely recessive at the cellular level. This is consistent with anecdotal reports that BS heterozygotes are not at a high risk to develop cancer (GERMAN 1995). Given the lack of evidence for increased genetic instability and cancer in BS heterozygotes and their relatively low carrier frequency, the impact of germ line mutations in the *BLM* gene on the overall incidence of inherited cancers would appear to be minimal or non-existent.

3.3.2 Do Germ Line Mutations in the Chromosome Instability Genes Play a Role in Familial Cancers?

Cancer is a common enough condition that occurrence of the same cancer in more than one first-degree relative frequently occurs by chance. However, it has long been recognized that familiar clustering of cancer can be the result of germ line mutations in tumor suppressor genes. For example, p53 is responsible for many cases of the Li-Fraumeni cancer predisposition syndrome, and hereditary non-polyposis colon cancers (HNPCC) are due to loss of function mutations in the *hMLH1*, *hMSH2*, *hpMS1*, and *hpMS2* genes that control the repair of DNA mismatches (FISHEL et al. 1993; LEACH et al. 1993; BIRCH et al. 1994; BRONNER et al. 1994; NICOLAIDES et al. 1994; PAPADOPOULOS et al. 1994). Given that these genes which control genetic stability have been implicated in familial cancer, one might expect that the mutations in one or more chromosome instability syndrome genes also would be associated with autosomal dominant familial cancer predisposition syndromes. However, there is little to support this possibility and, in the case of

ATM, several studies suggest that the gene does not play a major role in familial breast cancer.

3.3.2.1 *ATM* Mutations and Familial Breast Cancer

In a recent study, Cortessis et al. (1993) examined 64 families in which two or more individuals had had premenopausal bilateral breast cancer. They tested for possible linkage of the 11q23 marker *DRD2* to dominant transmission of breast cancer predisposition in these families. When all families were included in the analysis, there was strong evidence against linkage (i.e., a LOD score of –6.08 at $\Theta = 0.00001$). Even if families with linkage to 17q21 or a family history of ovarian cancer were excluded, there still was no evidence for linkage to DRD2. However, given that *DRD2* is ~7 cM from the *ATM* gene, this study could not, by itself, definitively exclude linkage to the *ATM* gene itself.

WOOSTER et al. (1993) typed five 11q markers in individuals from 16 families in which there were at least two affected sisters with cancer before the age of 45, together with at least one other relative with breast cancer. Families with individuals affected with ovarian cancer were excluded so as to eliminate families in which *BRCA1* mutations may be responsible for cancer predisposition. Multipoint linkage analysis of these families yielded a calculated LOD score of –1.34 for the linkage between the *ATM* locus and breast predisposition in these families. The authors concluded that *ATM* mutations do not make a major contribution to familial breast cancer.

Since surveys of A-T families consistently show an increased relative risk of developing breast cancer in *ATM* heterozygotes, one might expect that mutations in the *ATM* gene would play a role in familial breast cancer. Yet the studies of breast cancer families summarized above did not demonstrate linkage to the *ATM* locus on 11q23. As EASTON (1994a) has pointed out, both studies actually support the same conclusion: *ATM* mutations do not play a major role in familial breast cancer.

The apparent contradiction between studies of familial breast cancer families and A-T families can be resolved if one considers the penetrance of heterozygous *ATM* mutations. Using the published data for the occurrence of breast cancer amongst blood relatives of A-T homozygotes, EASTON (1994b) has calculated the risk of developing breast cancer for A-T heterozygotes to be 11% by age 50 and 30% by age 70. As summarized in Table 4, this is a relatively low penetrance compared to the *BRCA1, BRCA2,* or *p53* genes. One effect of this low penetrance is that first-degree relatives of A-T heterozygotes should have only a modest increase in their relative risk of developing breast cancer. As a result, *ATM* mutations should account for relatively few "breast cancer families," which, by the usual definition, are families in which clusters of multiple individual cancers have occurred.

EASTON et al. (1993) have estimated that, given their low penetrance, *ATM* mutations should be responsible for only 3.5% of familial breast cancers, while *BRCA1* and *BRCA2* mutations together account for ~90%. However, because

Table 4. Risk of developing breast cancer in heterozygote carriers of selected genes

Germ line nutation	Heterozygote carrier frequency (%)	Risk of breast cancer by age 50 years (%)	70 years (%)	Proportion of all breast cancer patients < 50 years (%)	> 50 years (%)	Proportion of familial breast cancer patients (%)	Sources
No mutation		1.4	5				(CLAUS et al. 1991)
ATM	~1	11	30	7	2	3.5	(EASTON 1994b)
BRCA1	~0.2–0.3	50	85	5	1	~50	(EASTON et al. 1993)
BRCA2	~0.5?		63			40	(SANKARANARAYANAN and CHAKRABORTY 1995)
p53	~0.002	40	90	1	< 1		(FORD and EASTON 1995)
HRAS	~4–6	3	8				(KRONTARIS et al. 1993)

ATM mutations are more frequent in the general population than mutations in these highly penetrant genes, germ line *ATM* mutations may give rise to many "sporadic" tumors. The net result is that *ATM* mutations may be responsible for at least as many cases of breast cancer in the general population as germ line mutations in *BRCA1, BRCA2,* or *p53.*

4 Do Somatic Mutations in the Chromosome Instability Genes Play a Role in Sporadic Tumor Development?

Cancer is thought to be the end result of a multistep process that includes the acquisition of mutations in multiple genes that control cellular behaviour (NOWELL 1976; VOGELSTEIN and KINZLER 1993). As such, cancer can be considered to be a somatic genetic disease in which a population of cells undergoes clonal evolution as the result of sustaining multiple genetic changes that activate oncogenes and inactivate tumor suppressor genes. For certain childhood tumors (e.g., retinoblastoma), as few as two mutations may be sufficient to give rise to a tumor (reviewed in Goodrich and Lee 1990). However, for common adult cancers, three to six mutations may be necessary to acquire the phenotypic characteristics of a fully malignant tumor (LOEB 1991; VOGELSTEIN and KINZLER 1993).

Somatic human cells normally maintain tight control of genomic stability, with spontaneous mutation rates calculated to be $1.2–1.4 \times 10^{-10}$ mutations/base pair per cell generation (summarized in LOEB 1991). This has led to the hypothesis that loss of normal control of genomic stability is a necessary step in carcinogenesis that allows clonal populations of cells to accumulate a sufficient number of mutations to acquire a fully malignant phenotype (NOWELL 1976; LOEB 1991). This hypothesis is supported by observations that genetic instability is expressed by most tumors (LOEB 1991; HARTWELL 1992; LANE 1992; VOGELSTEIN and KINZLER 1993) and that

genes involved in maintaining genetic stability frequently are found to be inactivated in tumor cells (e.g., *p53*, *hMLH1*, *hMSH2*, *hpMS1*, and *hpMS2*).

Given the loss of control of genomic stability in chromosome instability syndrome patients, one might expect that the chromosome instability syndrome genes would, like *p53* and the DNA mismatch repair genes, play key roles as guardians of the genome. If so, then loss of function of the chromosome instability syndrome genes may be a frequent event in the development of sporadic common cancers. With the cloning of the *ATM*, *FACC*, and *BLM* genes, it should be possible to provide definitive answers to this question in the near future. However, published studies of loss of heterozygosity of genetic markers in sporadic tumors already provide indirect evidence that at least some of the chromosome instability syndrome genes function as tumor suppressor genes that are targets for inactivation during the development and progression of common sporadic cancers.

4.1 Loss of Heterozygosity Studies

Loss of heterozygosity (LOH) studies use RFLPs, microsatellites, and other genetic polymorphisms to map regional deletions and amplifications of chromosomes in tumors. Regional alterations in the genome are frequent events in tumorigenesis, presumably because they can unmask recessive mutations in tumor suppressor loci (CAVENEE et al. 1983). Specific tumors tend to have consistent patterns of genomic alteration. For example, regions with frequent LOH in breast cancer include 1p, 3p, 11p15, 11q, 16q, 17p, 17q, and 18q (reviewed in GUDMUNDSSON et al. 1995). The consistency of these genomic alterations in tumors has facilitated the mapping and subsequent cloning of several tumor suppressor genes that play major roles in the development of common cancers. These studies also can be used to indict candidate genes in the development of specific malignancies as a prelude to further study of their potential as tumor suppressor genes.

4.1.1 11q23 LOH and the *ATM* Gene

The best case that chromosome instability syndrome genes are targets for inactivation during the development of common cancers comes from multiple studies that have found that loss of heterozygosity at 11q22-23 loci is a frequent event in sporadic breast, ovarian, colon, and lung cancers as well as malignant melanoma.

4.1.1.1 Breast Cancer

Several studies have documented that loss of heterozygosity at 11q22-23 is a frequent finding in sporadic breast cancers. For example 40% of a combined total of 182 sporadic tumors examined in four independent studies had lost heterozygosity at *D11S35*, a microsatellite locus ~8 cM centromeric to the *ATM* locus (CARTER et al. 1994; HAMPTON et al. 1994a; GUDMUNDSSON et al. 1995; WINQVIST et al. 1995).

Although most LOH studies provide only a very rough molecular estimate as to the location of the relevant locus that is lost during tumor development, several groups have attempted to specify an interstitial location for the 11q23 locus that is commonly deleted in sporadic breast carcinomas. In an analysis of 62 sporadic breast cancers, Carter et al. (1994) found that 39% of the tumors had lost 11q23 markers, with the common overlapping region of LOH having *D11S35* and *D11S29* as the centromeric and telomeric limits, respectively. This narrowed the 11q23 region of LOH to an ~30 cM region that includes the A-T locus. Based on an independent analysis of 44 breast cancers. HAMPTON et al. (1994a) defined the telomeric limit for deletion as proximal to *APOC3*, 3 cM centromeric to *D11S29* (UHRHAMMER et al. 1994). In a more recent study, TOMLINSON et al. (1995) found breast cancers that had LOH at *NCAM* but not *DRD2*. This moves the telomeric limit for the common region of LOH to 6 cM distal of the *ATM* locus. Finally, using polymorphic markers tightly linked to the *ATM* locus, NEGRINI et al. (1995) found that 50% (14/28) of tumors examined had lost heterozygosity at *D11S1818*, a marker that lies less than 1 cM telomeric to *ATM* (UHRHAMMER et al. 1994). NEGRINI et al. (1995) concluded that the region of minimal 11q23 deletion in these tumors was an ~9 cM area that included *ATM* and excluded another 11q23 candidate tumor suppressor gene, *ALL-1*.

Inactivation of the *ATM* locus may be an early event in the development of many breast carcinomas in that KORETH et al. (1995) found that 23% of in situ breast carcinomas had 11q23 LOH. Loss of *ATM* function may be an indicator of a poor long-term prognosis, since 11q23 LOH has been associated both with metastatic disease (KORETH et al. 1995) and with decreased survival after metastasis (WINQVIST et al. 1995). Interestingly, CARTER et al. (1994) found a correlation between LOH at 17p13 and 11q23 in the tumors they analyzed, suggesting that loss of both *ATM* and *p53* frequently occurs in the same tumor (although compare GUDMUNDSSON et al. 1995). Loss of both of these tumor suppressor genes could explain the poor prognosis seen in breast cancers with 11q23 LOH in that the resultant tumor would be both genetically unstable from a lack of multiple cell cycle checkpoints and resistant to treatment due to the loss of p53-dependent DNA damage-induced apoptosis.

4.1.1.2 Other Tumors

Although evidence for regional 11q23 chromosomal loss involving the *ATM* locus is strongest for breast cancers, regional deletions involving 11q23 also have been documented for several other solid tumors. For example two cytogenetic studies of colorectal cancers found frequent deletions of 11q in these tumors (MULERIS et al. 1990; KONSTANTINOVA et al. 1991). A combined cytogenetic and molecular study of 39 colorectal carcinomas by KELDYSH et al. (1993) found that 59% of the tumors had loss of 11q material, with the smallest region of overlap being 11q22-23. GUSTAFSON et al. (1994) studied colorectal carcinomas for LOH at the *DRD2* locus, a marker approximately 8 cM telomeric to *ATM* (UHRHAMMER et al. 1995). They found that 34% of 68 tumors had undergone LOH at *DRD2*, a proportion that was

higher than their background for random markers (8%-23%) but lower than that seen for the 68% LOH seen for the *p53* region of 17p. Taken together, these studies suggest that loss of *ATM* function may occur in many, but not the majority, of colon carcinomas.

Malignant melanoma also may be a cancer in which LOH at 11q23 is a common event. TOMLINSON et al. (1993) examined 52 malignant melanomas for LOH at four candidate regions, including 11q23, 17p13.3, 17p13.1, and 17q22. A high frequency of LOH occurred only at the 11q23 marker (*D11S29*), where 66% of the tumors showed LOH. In a subsequent study of 24 melanoma patients, HERBST et al. (1995) used microsatellite repeats to define a critical region of 11q23 LOH for melanoma that includes the *ATM* locus. However, given that the 11q23 critical region defined by HERBST et al. is 51 cM wide, it is far from certain that the *ATM* locus is a target for genetic loss in melanoma.

Cervical carcinomas have a high degree of karyotypic instability, but the instability is non-random, with many breakpoints clustered around 11q23-q25 (SREEKANTAIAH et al. 1991). HAMPTON et al. (1994b) studied 32 cervical carcinomas and found that 52% had LOH of the 11q23 marker *D11S144*. BETHWAITE et al. (1995) also examined cervical carcinomas for 11q LOH and found that 16 out of 53 tumors had lost heterozygosity at the *D11S29* locus, which lies telomeric to *ATM*, while preserving heterozygosity at 11p13 loci.

To date, there have been two reports of 11q23 LOH in ovarian and lung cancers. IIZUKA et al. (1995) analyzed 79 lung cancers and found that 11q24 LOH was a frequent event. This finding was confirmed by a recent study from Croce's group which examined 76 adenocarcinomas of the lung and found LOH of 11q in 63%, with three distinctive regions affected by loss, including a 4-Mb critical region of minimal loss that encompasses the *ATM* gene (RASIO et al. 1995). Ovarian tumors also have been examined for 11q23 LOH (WEITZEL et al. 1994; PEJOVIC 1995), but the evidence of an association between 11q23 LOH and the development of ovarian cancer is relatively weak. For example, WEITZEL et al. (1994) examined LOH at multiple loci in a series of 27 primary ovarian cancers and found high frequencies of loss (>50%) at markers near the *APC* gene on 5q21 and *BRCA1* locus on 17q21 but only 29% LOH at the 11q23 marker *D11S533*.

These LOH studies provide broad, but indirect, support for the idea that loss of *ATM* function plays a role in the development of many common tumors. With the recent isolation of the *ATM* gene, confirmation of these LOH studies should be forthcoming in the near future, and a more accurate picture of the role of *ATM* gene inactivation in tumorigenesis will emerge.

4.1.2 LOH and FA Genes

There is little evidence to suggest that LOH involving the *FACC* gene is a frequent event during tumor development. Bladder carcinomas, large cell lymphomas, and esophageal carcinomas all have been shown to undergo LOH for 9q, but recent fine mapping demonstrates that the critical regions of loss for these tumors center around 9q34, distal to the *FACC* locus (MORI et al. 1994; CHAGANTI et. al. 1995;

HABUCHI et al. 1995). LOH involving the region of 9q containing the *FACC* gene, 9q22.3, is a frequent event in basal cell carcinomas and medulloblastomas (AL-BRECHT et al. 1994; SHANLEY et al. 1995). However, interpretation of LOH studies involving 9q22.3 is complicated by the fact that three potential cancer predisposition genes are within several megabases of each other at 9q22.3: the *FACC* gene, the gene for xeroderma pigmentosum group A (*XPAC*), and the Gorlin syndrome gene (MORRIS and REIS 1994). Basal cell carcinomas and medulloblastomas are characteristic tumors of Gorlin syndrome (BALE et al. 1994), and LOH at 9q22 is frequently found in those basal cell carcinomas and medulloblastomas that occur in Gorlin syndrome patients (ALBRECHT et al. 1994; SCHOFIELD et al. 1995; SHANLEY et al. 1995; LEVANAT et al. 1996). Fine mapping has demonstrated that, for at least some basal cell carcinomas, the area of loss does not include the *FACC* gene (SHANLEY et al. 1995). Taken together, these observations suggest that the gene at 9q22.3 that is a target for inactivation in basal cell carcinomas and meduloblastomas is the Gorlin syndrome gene, not *FACC*.

Loss of heterozygosity for the region of 16q that contains the *FA(A)* gene, 16q23-q24, is a frequent event in sporadic breast carcinomas (DENG et al. 1994; HARADA et al. 1994; ITO et al. 1995; SKIRNISDOTTIR et al. 1995). In addition, 16q24 LOH has been reported to be frequent in hepatocellular carcinomas (YUMOTO et al. 1995). The critical region for LOH at 16q23-24 has not been as narrowly defined as the critical region for LOH at 11q23, but these studies are consistent with the possibility that the *FA(A)* gene is a specific target for inactivation during the development of breast and liver carcinomas and suggest that further investigation is warranted into the possibility that inactivation of the *FA(A)* gene occurs during the development of these tumors.

Many sporadic solid tumors have been shown to exhibit frequent LOH for loci on 3p, including breast carcinomas (CHEN et al. 1994), cervical carcinomas (KOHNO et al. 1993; KARLSEN et al. 1994; MULLOKANDOV et al. 1996), esophageal carcinomas (MORI et al. 1994; OGASAWARA et al. 1995), non-small cell lung carcinomas (reviewed in THIBERVILLE et al. 1995), mesotheliomas (LU et al. 1994), and head and neck squamous carcinomas (EL-NAGGAR et al. 1993; PARTRIDGE et al. 1994). The critical regions for these tumors range from 3p13 to 3p26, suggesting that there are multiple tumor suppressor genes on the short arm of chromosome 3. The microcell-mediated chromosome transfer experiments that resulted in the initial mapping of the *FA(D)* locus to 3p did not permit assignment of a specific map location for *FA(D)* (WHITNEY et al. 1995). Consequently, it is not yet possible to critically evaluate which, if any, of the sporadic tumors that demonstrate 3p LOH might inactivate the *FA(D)* gene during the course of tumor development.

4.1.3 15q LOH and the *BLM* Gene

Although one study found that nine out of 30 (35%) sporadic breast carcinomas demonstrated loss of the 15q (DEVILEE et al. 1991), the 15q26 region that contains the *BLM* gene does not appear to be a part of the genome that undergoes frequent LOH in sporadic tumors. Molecular analysis of the *BLM* gene in tumor tissue may

yet provide evidence for the inactivation of *BLM* during tumorigenesis. However, at this time, LOH studies suggest that *BLM* is not a frequent target for inactivation during tumor development.

5 Cancer Screening and Treatment

5.1 Lessons for Cancer Screening

As discussed above, A-T and FA heterozygotes may, in aggregate, represent several percent of the general population. Because many of them may have abnormal responses to DNA damage, A-T and FA heterozygotes may represent a group of individuals whose risks for developing cancer differs from that of the general population. Much needs to be learned before cancer screening issues in A-T and FA carriers can be fully resolved. However, our knowledge of A-T has progressed to the point where several points regarding cancer screening in A-T heterozygotes can begin to be addressed.

5.1.1 Screening for A-T Heterozygotes

Since A-T heterozygotes are at an increased risk to develop cancer and may have adverse reactions to cancer therapy, it would be useful to have a screening test to identify this high-risk subpopulation. The published studies reviewed above support the conclusion that groups of A-T heterozygotes, *on the average*, express more spontaneous genetic instability and greater sensitivity to the toxic effects of ionizing radiation than groups of control individuals. Multiple attempts have been made to develop screening tests for A-T heterozygotes. However, reliable screening tests for identifying A-T heterozygotes based on functional abnormalities have yet to be developed due to consistent overlap between the most radioresistant A-T heterozygotes and the most radiosensitive "normal" individuals (e.g., WEEKS et al. 1991).

This overlap may be due to a combination of two factors. First, A-T heterozygotes with apparently normal radioresistance may carry null mutations or other *ATM* alleles that do not interfere with the function of the remaining normal *ATM* gene but, instead, act like true recessive mutations. Second, given the population frequency of A-T heterozygotes (Section 3.3.1.1), it is likely that some radiosensitive normal individuals actually may be heterozygote carriers of *ATM* mutations, while others may carry mutations in other genes that affect radiation resistance.

Cloning the *ATM* gene has opened up the possibility of developing molecular screening tests for A-T heterozygotes. However, initial analyses of *ATM* mutations in A-T homozygotes suggest that there are many "private" mutations scattered throughout the gene (RANDAL 1995b; SAVITSKY et al. 1995; R.A. Gatti 1996, personal communication). Given the large size of the *ATM* cDNA and the existence of multiple different mutations, it may be difficult, using current molecular technology,

to develop a molecular screening test for A-T heterozygotes that will detect enough *ATM* mutations to be clinically useful. However, since many of the known mutations appear to code for protein truncations, it may be possible to develop screening tests that detect truncated ATM protein fragments. Protein truncation screening would not identify true null alleles. However, if dominant-negative alleles play a role in the cancer risk of A-T heterozygotes, then truncation screening may be of practical use since it could identify those *ATM* alleles that code for abnormal proteins that function as dominant-negative alleles.

5.1.2 Mammography and Radiation-Induced Breast Cancer in A-T Heterozygotes

Annual mammograms generally are recommended for women under age < 50 who are at high risk to develop breast cancer (KERLIKOWSKE et al. 1993; VOGEL 1994). A-T heterozygotes would appear to be a high-risk group that could benefit from such screening. However, in SWIFT et al.'s (1991) prospective analysis of cancer in blood relatives of A-T homozygotes, there was a significant correlation between breast cancer and a history of one are more fluoroscopic examinations of the chest, back, or abdomen (p < 0.005), with a relative risk of 5.8 for those individuals with a history of radiation exposure. This led SWIFT et al. (1991) to suggest that physicians consider alternatives to mammography in A-T heterozygotes. It should be noted, however, that SWIFT currently is neither for nor against mammographic screening for A-T heterozygotes (RANDAL 1995a).

The recommendation against mammography provoked much discussion and some criticism of the study's methodology by members of the cancer genetics, epidemiology, and radiology communities (e.g., BOICE and MILLER 1992; GRAY 1992; KULLER AND MODAN 1992; RANDAL 1995b). In particular, several individuals pointed out an apparent inconsistency between the relative risk of cancer seen in the irradiated group and their incremental exposure to radiation (BOICE and MILLER 1992; LAND 1992). Given that the women with breast cancer in the study already had 40–50 mGy cumulative lifetime exposures from background radiation, the incremental exposures from diagnostic radiation (1–9 mGy) appeared to be much too low to account for the 5.8-fold increase in relative risk seen in the irradiated group of A-T blood relatives. Although the study population was the largest group of A-T families yet analyzed for cancer risk, there still were only small numbers of blood relatives and spouse controls that developed cancer. To date, there have been no further published epidemiological studies that have studied links between radiation exposure and cancer risk in A-T heterozygotes. However, new light may be shed on this controversy now that investigators are using *ATM* gene analysis to reexamine this question (RANDAL 1995b).

Another way to approach this issue is to consider what conclusions can be drawn from published estimates of the risks of induced cancer from mammography in the general population and from the studies of the radiation responses of A-T heterozygotes reviewed above. Typical radiation exposures to the breasts from mammography are in the range, on average, of 2–3 mGy (MILLER et al. 1989; BOICE and MILLER 1992), compared to the 1–2 mGy per year exposure from background

radiation sources (NATIONAL RESEARCH COUNCIL 1990). Based on long-term studies of Hiroshima and Nagasaki survivors, as well as smaller studies of women exposed to diagnostic and therapeutic radiation, it has been estimated that, for the general population, the risk of breast cancer from radiation doses of 10 mGy is ~ 1 in 10 000 per year (NATIONAL RESEARCH COUNCIL 1990). NORMAN and WITHERS (1993) have used these figures to calculate 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 A-T heterozygotes to radiation-induced genetic alterations averages about 25%–60% higher than that of controls (e.g., JONES et al. 1995). As pointed out by NORMAN and WITHERS (1993), this suggests that the lifetime risk of breast cancer in A-T heterozygotes from mammography is less than twofold higher than controls, perhaps 2%–3%. This compares to an estimated lifetime risk of developing breast cancer in A-T heterozygotes of > 30% (EASTON 1994b). Given that annual mammography reduces the mortality of breast cancer through early detection by ~ 30% or more (RENNERT 1991), the benefits of annual mammography would appear to outweigh the risk of mammography-induced cancer in A-T heterozygotes by age 40, if not earlier.

5.2 Lessons for Cancer Treatment

A recurring problem in oncology is how to devise an effective treatment regime that can be safely tolerated by the cancer patient. A-T and FA genes, like other genes that control genetic stability, may be frequent targets for inactivation during tumorigenesis. Loss of function of these genes decreases the genetic stability of the malignant cells but at the same time affects their sensitivity to radiation and/or chemotherapeutic agents. For example, inactivation of *ATM* could play a role in the sensitivity of many tumor cells to induction of apoptosis by ionizing radiation, radiomimetic chemicals, and topoisomerase inhibitors (DIVE et al. 1992).

   Although much remains to be learned, it eventually may be possible to develop individualized treatment strategies that are tailored to exploit the phenotypic differences between a patient's malignant cells and their normal somatic cells that are caused by loss of function of chromosome instability syndrome genes or other tumor suppressor genes. For example, sporadic tumors that have lost *ATM* function may, like cells from A-T homozygotes, be quite sensitive to the induction of apoptosis by ionizing radiation and radiomimetic drugs, while those tumors that have inactivated their FA genes may become sensitive to killing by MMC and other cross-linking agents. New drugs also could be explicitly screened for their ability to exploit the differences between malignant cells and normal tissues in DNA repair, cell cycle checkpoint control, and cytokine regulation (MARSHALL 1996).

5.2.1 Chromosome Instability Homozygotes Are at Risk
for Adverse Treatment Reactions

Although their overall numbers are small, A-T, FA, and BS homozygotes make up
a subpopulation of cancer patients with relatively bleak prognoses due in part to
their poor responses to therapeutic radiation and chemotherapy (see Sections 2.1,
2.4, and 2.7). Care must be taken to modify standard regimes so as to minimize
iatrogenic damage, but reduced therapeutic regimes can be tolerated by these in-
dividuals (e.g., EYRE et al. 1988; PASSARGE 1991; ALTER 1994a).

5.2.2 Chromosome Instability Syndrome Heterozygotes as Subpopulation at Risk for
Adverse Reactions to Treatment

Given the abnormal responses of A-T and FA heterozygotes to DNA-damaging
agents, they could, in theory, represent a significant fraction of cancer patients who
have adverse responses to radiation and chemotherapy. Experimental evidence is
currently lacking for this possibility in FA heterozygotes; however, studies of
radiosensitive cancer patients and A-T heterozygotes provide indirect support for
this hypothesis.

5.2.2.1 A-T Heterozygotes and Radiosensitive Cancer Patients

In theory, sufficiently high doses of ionizing radiation could eliminate any tumor.
However, in practice, the therapeutic radiologist must limit radiation treatment so
as not to induce unacceptable damage to surrounding normal tissues. Un-
fortunately, apparently normal individuals vary widely in their radiosensitivity
(LITTLE et al. 1988; GENTNER and MORRISON 1989; TURESSON 1990). As a result,
radiation treatments are typically mild enough so as to prevent adverse reactions in
the occasional radiosensitive individual. A consequence of this approach is that
many patients are given less radiation than they could reasonably tolerate, thus
diminishing the probability that their radiation therapy will be curative.

It has been proposed that the radiosensitive "tail" of the population dis-
tribution of radiation responses may actually represent a subgroup of individuals
who are radiosensitive as the result of mutations in specific genes (NORMAN et al.
1988). If such individuals could be identified and given modified therapy, then the
efficiency of radiation therapy for the majority of cancer patients could be sig-
nificantly improved by giving them more intensive radiation without the fear of an
unacceptable number of adverse reactions (NORMAN et al. 1988; WEST et al. 1991)

For these reasons, multiple attempts have been made to correctly identify
radiosensitive cancer patients using in vitro assays that measure the radiosensitivity
of fibroblasts, lymphocytes, or lymphoblastoid cells. The results have been mixed.
Most studies have demonstrated a group correlation between in vitro radio-
sensitivity and adverse reactions to radiotherapy (LOEFFLER et al. 1990; BURNET et
al. 1992; LAVIN et al. 1994; JONES et al. 1995; RAMSAY and BIRRELL 1995). How-
ever, this has not always been the case for studies using survival following high-

dose irradiation as their measure of radiosensitivity (e.g., BEGG et al. 1993). As with studies of A-T heterozygotes, those in vitro studies that either have focused on cell cycle abnormalities or used low dose-rate irradiation have shown the most promise in identifying which cancer patients have in vivo radiosensitivities.

For example, Lavin et al. (1994) found that 20% of 104 unselected breast cancer patients had lymphocytes with radiation-induced G_2 delays in the A-T heterozygote range, compared to 8% of lymphoblasts from 24 age-matched controls ($p < 0.001$). In this study, an abnormally high fraction of cells in G_2 correlated with more severe clinical reactions to radiation, as well as a family history of breast cancer and more aggressive tumors. More recently, WEST et al. (1995) determined the survival, after low dose-rate irradiation, of lymphocytes from 13 breast cancer patients who had had severe early or late tissue reactions to radiation therapy. The average D_0 for this group of over-reactors was similar to that for 13 A-T heterozygotes. Both groups were significantly more sensitive than a group of 25 controls without cancer as well as eight breast cancer patients who had had no adverse radiation reactions ($p < 0.01$). The same investigators also were able to find statistically significant differences between over-reactors and controls using low dose-rate irradiation and radiation-induced chromosome aberrations (JONES et al. 1995). Unfortunately, as with the case of A-T heterozygote identification, a small degree of overlap between the in vitro radiosensitivity of over-reactors and controls precludes the clinical application of these functional assays at this time.

Taken as a whole, these reports indicate that intrinsic radiosensitivity may cause some individuals to suffer adverse reactions to therapeutic radiation. In these studies, cells from cancer patients with clinical radiosensitivity had in vitro responses to radiation that were similar to those of cells from A-T heterozygotes. This has led several investigators to suggest that some radiosensitive cancer patients may be unusually sensitive to radiation therapy because they are A-T heterozygotes (LAVIN et al. 1994; JONES et al. 1995). However, SWIFT (1994) has suggested, based on anecdotal evidence, that A-T heterozygotes do not have marked in vivo sensitivity to therapeutic radiation. This issue may have to be resolved by molecular analysis of the *ATM* gene in selected radiosensitive cancer patients.

6 Conclusion

The three diseases discussed above demonstrate the critical role that genetic instability plays in the development of cancer and illustrate the wide range of nuclear and cytoplasmic processes that can affect the stability of the genome. As discussed in this review, a great deal of progress has been made in the last few years towards understanding the molecular pathology of these chromosome instability syndromes, with recent efforts focusing on cloning the genes responsible for these syndromes. Now, with the identification of several of the genes, we are entering a new era of intense study that will focus on the pathobiology of these syndromes as

well as the role played by the chromosome instability syndrome genes in DNA metabolism, cell cycle checkpoints, genetic instability, and programmed cell death. These studies should test the predictions of our current models for the chromosome instability syndromes, further our understanding of basic biological processes, shed light on the origin and development of cancer, and improve our diagnosis and treatment of this formidable disease.

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Genetic Alterations in Human Tumors

K.R. Cho and L. Hedrick

1 Introduction

At the level of the cell, cancer is essentially a genetic disease. Studies from multiple disciplines, including molecular biology, somatic cell genetics, genetic epidemiology, tumor virology, chemical carcinogenesis, and others, have converged over the last few decades to provide many fundamental insights into the genetic basis of cancer. These studies have shown that the genesis of malignant tumors is dependent on the accumulation of a series of DNA alterations that generally occur somatically, but can also be inherited in the germ line. These alterations have a cumulative effect on many important cellular processes that become dysfunctional in cancers, including growth control, differentiation, and interactions of the affected cell with its microenvironment.

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The genetic alterations responsible for tumor development and progression must, by some mechanism, confer a growth advantage to the cells in which they accumulate. To date, alterations have been shown to occur in three specific classes of cellular genes: oncogenes, tumor suppressor genes, and genes involved in DNA repair. The products of oncogenes function in a positive way to promote tumorigenesis. Their normal cellular counterparts, called proto-oncogenes, encode proteins that are important regulators of many aspects of cell growth. Thus, the activating mutations that generate oncogenic alleles are effectively "gain-of-function" mutations. In contrast, mutant tumor suppressor genes lose their ability to regulate growth; that is, mutations in these genes generally result in "loss of function." The proteins encoded by both proto-oncogenes and tumor suppressor genes have a myriad of functions, in part reflected by their varied locations in the cell, including the nucleus, cytoplasm, inner cell membrane, and cell surface. More recently, mutations in a third class of genes have been found to occur in human tumors. These genes encode proteins involved in the recognition and repair of damaged DNA. As with tumor suppressor genes, loss-of-function mutations appear to underlie the role of DNA repair genes in tumorigenesis. However, such mutations do not appear to directly affect growth control. Rather, their inactivation presumably causes an increased rate of mutation in oncogenes and tumor suppressor genes in affected cells.

The identification and characterization of specific genetic abnormalities in human tumors have confirmed many well-established hypotheses of tumorigenesis, revealed many new facts about tumor cells, and have led the way to a more thorough understanding of the pathogenesis of human cancer. Moreover, our knowledge of specific genetic changes in cancer cells is already being put to practical use with respect to the diagnosis, assessment of prognosis, and treatment of cancer patients.

It is not our purpose to provide a comprehensive review of all of the various mutations that have been described to date in human cancers. The field of cancer genetics is moving so quickly that such a categorization would certainly be out of date in a short period of time. Instead, we hope to accomplish the following: (a) to review the mechanisms by which proto-oncogenes are frequently activated in human tumors, each with illustrative examples – these mechanisms include point mutation, gene amplification, chromosomal translocation, and rearrangement; (b) to review the mechanisms by which tumor suppressor genes are frequently inactivated in human tumors, including point mutation with loss of the remaining wild-type allele (often detected as losses of heterozygosity, LOH); (c) to review some of the recent studies of inherited cancer syndromes, with emphasis on mutations in genes involved in the recognition and repair of damaged DNA; and (d) to briefly discuss some genetic alterations in cancer cells that do not necessarily target tumor suppressor genes or oncogenes, such as aneuploidy and DNA methylation. Our emphasis will be less on the molecular mechanisms through which these alterations arise than on the manner in which our understanding of these genetic alterations is impacting on the diagnosis and management of cancer patients today.

2 Genetic Alterations Frequently Affecting Oncogenes

Much of our knowledge of oncogenic alleles in human cancer is derived from studies of the transforming genes of RNA and DNA tumor viruses. Despite the fact that numerous proto-oncogenes were identified by such studies, only a relatively small subset of these cellular genes has been found to be frequently targeted by somatic mutations in human cancers. Although the great majority of mutations that give rise to oncogenes occur somatically, germ line transmission of dominant transforming genes (specifically, mutated *RET* genes) has recently been described in patients affected by the cancer syndromes multiple endocrine neoplasia (MEN)2A and (MEN)2B (SANTORO et al. 1995).

Several approaches have been used to identify human oncogenes. For example, chronic transforming retroviruses can alter the structure and/or expression of proto-oncogenes by integrating into or near these genes. Proto-oncogenes activated by this type of insertional mutagenesis include c-*MYC*, c-*ERBB*, c-*MYB*, c-*MOS*, *INT*1, and *INT*2, among others (reviewed in PERKINS and VANDE WOUDE 1993). Other oncogenes, such as H-*RAS*, were identified by transfer of tumor-derived DNA into non-tumorigenic cells. Yet additional oncogenes were identified by the characterization of non-random chromosomal abnormalities such as translocation breakpoints, extrachromosomal elements known as double-minute chromosomes, and novel banding regions identified in cytogenetic analyses. Tumor cell DNAs clearly contain many mutated genes, some that directly contribute to tumorigenesis and others that are altered as innocent bystanders in genetically unstable tumor cell. How might these "bystanders" be differentiated from genes causally involved in tumor development? Simply stated, the identity of a gene as an oncogene can be tested by introducing the candidate gene into normal cells and observing the cellular response. If the cell acquires at least some attributes of a cancer cell, then the candidate gene may be considered an oncogene (BISHOP 1985). Although there are 50 000–100 000 genes in the human genome, only about 50 genes identified thus far fit this functional definition. Of these 50, only 20 or so have been found to be mutated in human tumors (WEINBERG 1994). Specific examples of oncogenes activated in human tumors are described below.

2.1 *RAS* Genes Are Activated by Point Mutation

DNA transfer techniques were used to identify the first oncogenic *RAS* allele from a human bladder carcinoma cell line (KRONTIRIS and COOPER 1981; PERUCHO et al. 1981; SHIH and WEINBERG 1982; DER et al. 1982; PARADA et al. 1982). In these experiments (Fig. 1), DNA from the human bladder carcinoma cell line EJ (also known as T24) was introduced into immortalized but contact inhibited rodent fibroblasts. Those cells receiving transforming sequences from the human tumor DNA underwent morphological changes and loss of contact inhibition ("focus formation"). Recovery and characterization of the human tumor-derived sequences
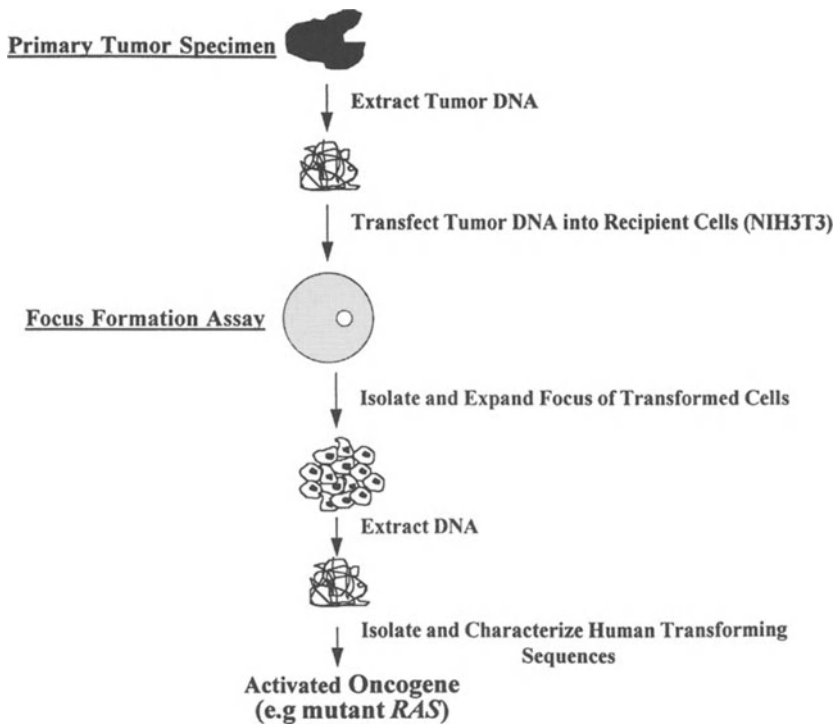
Fig. 1. DNA transfection assay for transforming sequences (oncogenes). DNA is isolated from a primary human tumor specimen or from cultured tumor cells. The DNA is then transfected into immortalized rodent cells that exhibit contact inhibition in culture (e.g., NIH3T3 cells). If the tumor DNA contains oncogenic alleles, the growth of cell(s) taking up the transforming sequences may fail to be inhibited by cell-cell contact, resulting in a "focus" of highly refractile cells that can be isolated and further expanded into a large population of transformed cells. The specific human tumor-derived DNA sequences responsible for transformation of the rodent cells can be identified by repeating this process with DNA isolated from a focus. In each round of such serial transfection and focus formation assays, the human transforming sequences are enriched with each round. This DNA transformation assay has successfully identified several oncogenes, most notably oncogenic *RAS* alleles

mediating this transformation resulted in the discovery of the first activated oncogene in human cancer, H-*RAS*. Further analysis of the activated *RAS* sequences established that the H-*RAS* allele from the bladder cancer cell line differed from the normal H-*RAS* gene by a single nucleotide substitution (point mutation) at codon 12 (Capon et al. 1983). Numerous additional studies identified point mutations in one of the three highly related *RAS* genes (H-*RAS*, K-*RAS*, or N-*RAS*) in approximately 10%–15% of human tumors (reviewed in Barbacid 1987). *RAS* gene mutations are even more common in certain cancer types, such as adenocarcinomas of the colon, pancreas, thyroid, and lung. The mutational spectrum and functional significance of *RAS* gene mutations have been reviewed in detail (Bos 1989). Overall, mutations at codon 12 of the K-*RAS* gene are most common, although mutations at codons 13 and 61 also occur. There is some evidence that

environmental agents may contribute to the induction of the point mutations. For example, the majority of *RAS* gene mutations are G to A transitions at the second G of a GG pair (Bos 1989). This type of mutation is characteristic of those caused by alkylating agents.

What is the functional significance of these point mutations? Each of the *RAS* genes encodes a 21-kD guanine nucleotide-binding protein (p21). The ras proteins function in the transduction of growth and differentiation signals from activated transmembrane receptors to downstream protein kinases (reviewed in Boguski and McCormick 1993; Lowy and Willumsen 1993; McCormick 1993; Bourne et al. 1990). Clearly, single nucleotide substitutions can have any of several consequences for the proteins encoded by genes altered by this type of mutation. Some mutations are missense; that is, they result in the substitution of one amino acid for another. Other point mutations, leading to the substitution of a stop codon for one encoding an amino acid and thereby creating a truncated protein, are called nonsense mutations. Point mutations can also occur in splice site recognition sequences, leading to the incorrect splicing of the RNA transcript (e.g., "exon skipping"). Such splice junction mutations can result in a frame shift that leads to truncation of the protein or may delete the portion of the protein encoded by the "skipped" exon. The oncogenic mutations at codons 12, 13, and 61 of the *RAS* genes are missense mutations, as might be expected for mutations that result in a gain of function. The RAS proteins encoded by these mutant genes have reduced ability to hydrolyze guanosine triphosphate (GTP) to guanosine diphosphate (GDP), primarily via their decreased ability to interact with the GTPase-activating protein called RAS-GAP. Hence, the mutant ras protein remains in the GTP-bound or activated state, no longer switching between the active (GTP-bound) and the inactive (GDP-bound) form. This scenario underscores the concept that proto-oncogenes do not lie dormant in the cell with the sole purpose of promoting tumorigenesis when activated. Rather, proto-oncogenes encode proteins that have important regulatory properties in normal cells and the mutant (oncogenic) proteins have altered function.

RAS gene mutations have been studied quite extensively in both pre-malignant (adenomas) and malignant colorectal tumors (Vogelstein et al. 1988). K-*RAS* or N-*RAS* mutations are present in approximately 50% of adenomas greater than 1 cm and in a similar percentage of carcinomas. In contrast, *RAS* mutations are relatively uncommon in small (< 1 cm) adenomas. These findings suggest that *RAS* gene mutations may be, at least in part, responsible for the progression of small adenomas to larger, more clinically aggressive lesions. However, it should be emphasized that *RAS* mutations are not always associated with malignant potential, as they have been identified in hyperplastic polyps and aberrant crypt foci (Jen et al. 1994; Pretlow et al. 1993), lesions that have not clearly been shown to progress to colorectal cancer.

Our knowledge of *RAS* gene mutations has already been applied to the assessment of prognosis for patients with certain types of cancers. For example, K-*RAS* mutations are present in about 30% of adenocarcinomas in the lung (Bos 1989) and patients with tumors harboring mutant K-*RAS* genes have been shown

to have a poorer prognosis than those with tumors containing wild-type K-*RAS* genes (Slebos et al. 1990). *RAS* mutation does not appear to be a useful prognostic marker in colorectal or other carcinomas in which *RAS* mutations are frequently present. Preliminary studies also suggest that the detection of *RAS* gene mutations may prove to be useful as an adjunctive tool in the early diagnosis of pancreatic and colorectal carcinomas since K-*RAS* gene mutations have been detected in the stool of patients with these tumor types (Sidransky et al. 1992; Caldas et al. 1994) as well as in fine-needle aspiration specimens of pancreatic cancers (Urban et al. 1993; Shibata et al. 1990). Given the prevalence of *RAS* gene mutation in specific types of cancer as well as cancers in general, there is great interest in further studying *RAS*-dependent growth regulatory pathways. In particular, the identification of agents that inhibit the altered signaling of a mutant ras protein may ultimately lead to new treatment strategies for cancer patients.

2.2 *NEU* and *MYC* Genes Are Frequently Amplified in Certain Human Tumors

Cytogenetic studies of cancer cells have provided many of our insights into the genetic abnormalities associated with human cancers. In fact, several oncogenes were identified because they are contained within DNA sequences amplified in tumor cells. Such amplified DNA sequences are detected in karyotypic analyses as double-minute chromosomes (DMs) and novel banding regions known as homogeneously staining regions (HSRs). These DMs and HSRs typically contain 20 to several hundred copies of a specific chromosomal region. Selection for and maintenance of these amplified DNA sequences is thought to be driven by the increased copy number and concomitant increased expression of target gene(s) within the larger amplicon. Because the amplified region of DNA usually involves several hundred thousand base pairs, in some tumors more than one oncogene may be contained within the amplified segment (Khatib et al. 1993). The mechanisms of gene amplification are discussed in detail by Tlsty (this volume).

The c-*MYC* proto-oncogene is the cellular homologue of a gene present in several highly oncogenic avian retroviruses. The c-*MYC* gene and its closely related cellular homologues N-*MYC* and L-*MYC* are frequently amplified in certain tumor types. For example, about 30%–40% of small cell carcinomas of the lung have amplification of c-*MYC*, N-*MYC* or L-*MYC* (DePinho et al. 1991). *MYC* gene amplification is also observed, albeit less frequently, in some other types of epithelial cancers, including carcinomas of the breast, colorectum, and cervix (De-Pinho et al. 1991; Little et al. 1983; Ocadiz et al. 1987; Baker et al. 1988). Amplification of *MYC* genes has also been identified in non-epithelial tumor types. Examples include c-*MYC* amplification in promyelocytic leukemia (Collins and Groudine 1982; Dalla-Favera et al. 1982) and N-*MYC* amplification in a subset of glioblastomas and neuroblastomas (Brodeur et al. 1984; Fuller and Bigner 1992; Rasheed and Bigner 1991). N-*MYC* gene amplification is observed more often in patients with high-stage neuroblastomas, and patients with neuro-

blastomas that show N-*MYC* amplification have a much poorer prognosis than patients whose tumors lack this genetic alteration (BRODEUR et al. 1984; SCHWAB and AMLER 1990). Similarly, amplification of *MYC* genes in small cell lung carcinoma also seems to be associated with disease progression (GARTE 1993). The correlation between *MYC* amplification and prognosis of patients with neuroblastomas and small cell carcinomas of the lung demonstrates additional clinical settings in which identification of specific genetic abnormalities impacts on the management of cancer patients.

How do the *MYC* genes normally participate in the regulation of cell growth? Recent studies have shown that each of the myc proteins dimerizes with a partner protein called max (BLACKWOOD and EISENMAN 1991). The resultant heterodimers then function as transcription factors by binding specific DNA sequences. The three *MYC* genes, although closely related, each have a unique pattern of expression during development and show subtle functional differences in in vitro assays. Moreover, amplification of *MYC* genes in certain tumor types does not appear to be interchangeable; for example, neuroblastomas show frequent amplification of N-*MYC*, but not of c-*MYC* or L-*MYC*. Taken together, these findings suggest that each *MYC* gene product functions to regulate expression of its own set of downstream genes, which are presumably involved in cell proliferation. Such specific target genes have yet to be identified.

The *NEU* gene (also known as *HER*-2 or c-*ERB*B-2) is another proto-oncogene that is frequently activated in human tumors through gene amplification and concomitant overexpression of its mRNA and protein. *NEU* amplification occurs most frequently in carcinomas of the breast, ovary, and stomach (SLAMON et al. 1989; ZHOU et al. 1988; PAIK 1992). The presence or absence of *NEU* amplification has also been found to be of prognostic significance in patients with node-negative breast carcinomas. Studies have shown that patients whose tumors showed *NEU* amplification and/or overexpression had a poorer clinical outcome than patients whose tumors did not demonstrate this genetic change (SLAMON et al. 1989; PRESS et al. 1993).

The *NEU* gene encodes a cell surface protein quite similar to the epidermal growth factor receptor (EGFR). The neu protein has a single membrane-spanning domain and a tyrosine kinase functional domain in the cytoplasmic portion. Like the *EGFR* gene, the *NEU* gene is believed to encode a peptide hormone receptor. In fact, it appears that EGFR and neu can form heterodimers, allowing neu activity to be modulated by EGFR agonists such as epidermal growth factor (EGF) and transforming growth factor-α (TGF-α) (DOUGALL et al. 1994). Numerous studies are underway to identify specific neu ligands, to understand the role of neu in growth regulation, and to elucidate the role of *NEU* gene overexpression in the pathogenesis of certain cancers. Ultimately, identification of *NEU* gene amplification and overexpression may find clinical utility not only in the assessment of prognosis of cancer patients, but perhaps in their treatment. In experimental model systems, antibodies directed against the extracellular domain of neu have been shown to inhibit tumor cell growth both in vitro and in animal tumor models (DOUGALL et al. 1994; DREBIN et al. 1988).

2.3 Other Oncogenes, Such as c-*ABL* and *BCL*-2, Are Activated by Chromosomal Translocations

Cytogenetic studies have revealed several recurring and highly consistent chromosomal translocations in certain tumor types. With the improvement of cytogenetic methods and the introduction of molecular biological tools, several oncogenes at or spanning the chromosomal breakpoints have been identified (reviewed in RABBITTS 1994). Cloning and characterization of these breakpoints have revealed some general principles underlying the functional consequences of alterations of the affected genes. There appear to be two major results of chromosomal translocations: (a) the gene encoding a T cell receptor (TCR) or an immunoglobulin (Ig) comes to lie near a proto-oncogene, resulting in overexpression of the proto-oncogene's mRNA and protein; or (b) the chromosomal breaks occur within two specific genes, resulting in a fusion gene encoding a chimeric protein with altered function. The fused genes often encode transcription factors such that the translocations result in altered transcription of downstream target genes in the affected tumor cells. Translocations affecting TCR or Ig genes and rearrangements creating fusion genes are frequently identified in hematopoietic malignancies. In solid tumors, the most common consequence of chromosomal translocation is the formation of chimeric proteins.

The *BCL*-2 (B cell lymphoma-2) gene was identified because it was found to be activated by a translocation between the Ig heavy chain locus on chromosome 14q and sequences on chromosome 18q. This translocation juxtaposes the *BCL*-2 gene on chromosome 18q near the regulatory elements at the Ig locus (TSUJIMOTO et al. 1985; CLEARY et al. 1986), causing deregulation of *BCL*-2 gene expression and overproduction of *BCL*-2 mRNA and protein. This type of translocation is typically noted in a subset of non-Hodgkin's lymphomas, namely the follicular lymphomas, which are of B cell origin. Detection of this translocation (traditionally by Southern blot analysis) has been used as an adjunctive tool in the diagnosis of these malignancies, particularly in complicated cases. Until relatively recently, the classification of hematopoietic malignancies was based exclusively on the morphology of the neoplastic cells. As might be expected, proper classification of leukemias and lymphomas was largely dependent on the skill of the pathologist and was limited by problems with reproducibility. Molecular DNA analyses detecting chromosomal translocations typical of each tumor type have contributed greatly to our understanding and appropriate classification of lymphoproliferative disorders in general. More recently, polymerase chain reaction (PCR)-based methods have been developed to assist in establishing the primary diagnosis and to detect minimal residual disease in patients with several types of hematopoietic neoplasms (reviewed in BORDEN et al. 1993; POTTER et al. 1993; YUNIS and TANZER 1993; GRIESSER 1993).

Simply stated, cancers are excessive accumulations of tumor cells which presumably result from an imbalance in cell proliferation and cell death, or some combination of both. Unlike many other oncogenes, which function to increase the percentage of cells in the population that are proliferating, *BCL*-2 appears to

contribute to tumorigenesis by protecting tumor cells from apoptosis (programmed cell death). Early clues supporting this notion were provided by studies showing that survival of interleukin-3-dependent lymphoid cells is prolonged in the setting of constitutive expression of *BCL*-2, even though there is no concomitant increase in the number of cells proliferating (VAUX et al. 1988). In addition, T cells expressing a *BCL*-2 transgene display sustained viability and resistance to lymphotoxic agents (SENTMAN et al. 1991; STRASSER et al. 1991). The mechanisms through which *BCL*-2 mediates the suppression of apoptosis remain incompletely defined; however, it appears that *BCL*-2 may regulate antioxidant pathways and cause a decrease in the production of reactive oxygen species (HOCKENBERY et al. 1993; KANE et al. 1993). The function of bcl-2 protein may be modulated by its interaction with other cellular proteins such as bcl-x, bax, and r-ras (reviewed in REED 1994).

Although some proto-oncogenes are activated by juxtaposing them near genes that normally undergo rearrangement, many others are activated by translocations resulting in gene fusion, which results in the expression of a chimeric protein of altered function. An example of such a gene fusion is provided by the translocation between chromosomes 9 and 22 observed in the great majority of chronic myelocytic leukemias. In this translocation (known as the Philadelphia chromosome) the proto-oncogene c-*ABL* on chromosome 9q is translocated to a 5-kb region on chromosome 22q called the breakpoint cluster region (*BCR*) which contains a transcription unit (HEISTERKAMP et al. 1983; BARTRAM et al. 1983; DE KLEIN et al. 1982). The consequence of this translocation is fusion of the promoter and 5' sequences of the *BCR* locus with c-*ABL*. The 8.2-kb fusion mRNA predicts a novel fusion protein of 210 kDa (COLLINS et al. 1984; KONOPKA et al. 1984). Assays detecting the bcr-abl fusion protein have been used as adjunctive tools in the diagnosis of chronic myelogenous leukemia. A second type of Philadelphia chromosome is observed in a small percentage of patients with chronic myelogenous leukemia and a subset of patients with acute lymphoblastic leukemia (DE KLEIN et al. 1986). In this translocation, the break on chromosome 22 falls within a *BCR* intron that is outside the main breakpoint cluster region. The resultant *BCR-ABL* fusion mRNA is 7.0 kb and encodes a 185-kD chimeric protein. Patients whose leukemic cells exhibit this variant Philadelphia chromosome have been shown to have a worse prognosis than those with the typical 9–22 translocation (PERKINS and VANDE WOUDE 1993).

The mechanisms through which the bcr-abl fusion protein contributes to leukemogenesis are not clearly understood. The normal c-*ABL* gene product is a member of the tyrosine kinase family and presumably participates in signal transduction. It appears that *BCR*-encoded sequences in the chimeric protein function to activate the kinase activity, perhaps through direct binding of the *ABL* kinase regulatory domain to an SH2-binding region in *BCR* (PENDERGAST et al. 1991; LUGO et al. 1990).

3 Genetic Alterations Frequently Affecting Tumor Suppressor Genes

Several tumor suppressor genes have been discovered in the last few years. Although study of these genes lags behind oncogene research by at least a decade, it is already clear that their inactivation contributes substantially to cancer causation. In fact, cancers seem to arise when there is disruption of the delicate balance between the actions of the proteins encoded by the growth-promoting proto-oncogenes and the growth-controlling tumor suppressor proteins. The cell fusion studies by HARRIS and colleagues provided the initial basis for the concept that the ability to generate a tumor is essentially a recessive trait at the cellular level (reviewed in HARRIS 1988). These investigators found that fusion of malignant murine tumor cells with non-tumorigenic murine cells often resulted in hybrids that were non-tumorigenic (Fig. 2). When passaged extensively in culture, the suppressed hybrids often reverted to tumorigenicity, and these tumorigenic revertants were found to have lost chromosomes when compared to their non-tumorigenic counterparts. Studies of hybrids generated by fusion of human tumor cells with normal cells yielded similar results. The observation that chromosome losses were asso-
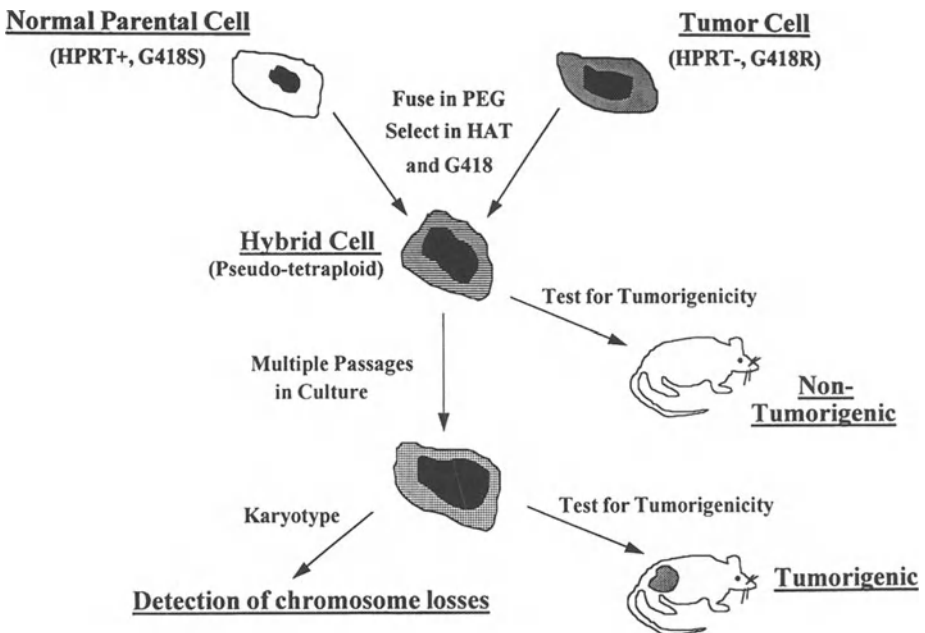
Fig. 2. Somatic cell genetic evidence for tumor suppressor genes. Somatic cell hybrids can be generated by fusing malignant cells with non-tumorigenic cells under the appropriate conditions. Hybrids are identified by growth in media that selects for cells containing the genetic complement of both parental cells. These pseudotetraploid cells are often non-tumorigenic when injected into mice. However, when passaged extensively in culture, the hybrid cells often revert to tumorigenicity. These tumorigenic revertants show losses of specific chromosomes when compared to the non-tumorigenic hybrids

ciated with reversion to tumorigenicity suggested the hypothesis that specific chromosomes, or even single genes on specific chromosomes, might be responsible for the tumor suppression. This hypothesis was confirmed by subsequent experiments showing that introduction of single specific chromosomes into certain tumorigenic cells resulted in suppression of tumorigenicity (WEISSMAN et al. 1987; OSHIMURA et al. 1990; TRENT et al. 1990). However, some specificity regarding the ability of a particular chromosome to suppress tumorigenicity of specific tumor types was noted. For example, tumor suppression was observed following introduction of chromosome 11 into cervical carcinoma cells or rhabdomyosarcoma cells, while chromosome 11 failed to suppress the tumorigenicity of some neuroblastomas and renal cell carcinomas (OSHIMURA et al. 1990; SHIMIZU et al. 1990).

While these cell fusion experiments suggested a critical role for the inactivation of cellular genes in tumor formation, concurrent epidemiological and genetic studies provided additional evidence supporting the existence of tumor suppressor genes. Amongst these, the studies of KNUDSON (1971, 1985) are of particular importance since they laid the groundwork for many of the concepts and techniques used to identify and characterize tumor suppressor genes and the mechanisms by which they are inactivated in both germ line and somatic cells.

Retinoblastomas are relatively uncommon malignant eye tumors affecting children. They occur sporadically in many patients, but are also observed in some families in a pattern consistent with autosomal dominant inheritance. KNUDSON analysed the age-specific incidence of retinoblastoma and proposed that two mutagenic events were required for the development of retinoblastoma, both in sporadic and inherited cases (the so-called two-hit hypothesis). In patients with the inherited form of the disease, one mutation was inherited and therefore present in all cells of the affected individual, including developing retinoblasts. A second mutation occurring in any of these retinoblasts would then lead to the development of a retinoblastoma. In those patients with the sporadic form of the tumor, both mutations must be acquired somatically and must occur in the same developing retinoblast. The low probability that two separate somatic mutations would occur in the same retinoblast is consistent with the low prevalence of retinoblastoma in the general population. In contrast, patients with the inherited form of the disease typically develop one or more retinoblastomas depending on the frequency of the second mutational event(s). KNUDSON subsequently proposed that the two mutations were likely to inactivate the same gene rather than two different ones. Thus, although predisposition to retinoblastomas is inherited by the affected individual in a dominant fashion, mutations in the putative disease gene are predicted to be recessive at the cellular level, such that both copies of the same gene need to be inactivated for a tumor to develop. Cytogenetic studies provided the first clues to the localization of the retinoblastoma predisposition gene to chromosome 13 (FRANCKE 1976). Interstitial deletions of band q14 of chromosome 13 were observed in the normal (non-neoplastic) cells of a subset of patients affected by the inherited form of retinoblastoma. Other studies provided evidence for strong genetic linkage between the esterase D locus at chromosome 13q14 and the retinoblastoma predisposition locus (SPARKES et al. 1983).

3.1 Tumor Suppressor Genes Are Often Inactivated by Loss of Heterozygosity

Somatic cells contain two copies of each autosomal chromosome, one inherited from each parent. Probes detecting DNA sequence polymorphisms allow investigators to distinguish between the maternally and paternally derived copies of particular DNA sequences in both normal and neoplastic cells. Such molecular biological tools were subsequently used to identify frequent losses of one parental copy of chromosome 13 in retinoblastomas (FUNG et al. 1987) regardless of whether they were inherited or sporadic. These losses, termed losses of heterozygosity (LOH), were detected by analysis of chromosome 13 DNA polymorphisms in matched pairs of tumor and normal DNA. LOH, also known as allelic loss, was found to occur through a number of different mechanisms (Fig. 3) including (a) chromosome non-disjunction with reduplication of the remaining chromosome; (b) chromosome non-disjunction without reduplication of the retained chromosome; and (c) mitotic recombination. In the inherited tumors showing chromosome 13 LOH, it was determined that the retained copy of chromosome 13 was always derived from the affected parent, suggesting that the LOH was effectively "unmasking" a pre-existing recessive mutation at the putative tumor suppressor gene locus. Taken together, these studies served to support KNUDSON's two-hit hypothesis and demonstrated that similar genetic mechanisms could account for both inherited and sporadic cases of certain cancers.

Cloning of the retinoblastoma susceptibility gene (*RB*1) was facilitated by the identification of a chromosome 13 marker that detected rearrangement and/or homozygous loss (loss of both copies) of chromosome 13q14 sequences in Southern blot analyses of some primary retinoblastomas (DRYJA et al. 1986). These findings ultimately led to the identification of the *RB*1 gene, a large gene containing 27 exons and spanning over 200 kb of genomic DNA (FUNG et al. 1987; LEE et al. 1987; FRIEND et al. 1986). Once cloned, investigators were able to more definitively study both inherited and somatic *RB*1 mutations. Although gross deletions and rearrangements of the *RB*1 gene are observed in a subset of retinoblastomas, most tumors lack gross alterations of the gene (DUNN et al. 1989; YANDELL et al. 1989; GODDARD et al. 1988). Rather, the majority of mutations inactivating the *RB*1 gene are more subtle, including point mutations, small insertions, and deletions. The effects of such mutations are discussed below.

3.2 Tumor Suppressor Genes Are Often Inactivated by Point Mutations and Other Subtle DNA Alterations

As described in the section on oncogenes, point mutations in tumor suppressor genes can have any of several consequences for the proteins encoded by the mutant genes. The mutations affecting *RB*1 typically result in premature truncation of the rb protein. Premature truncation of proteins may be caused by any of several
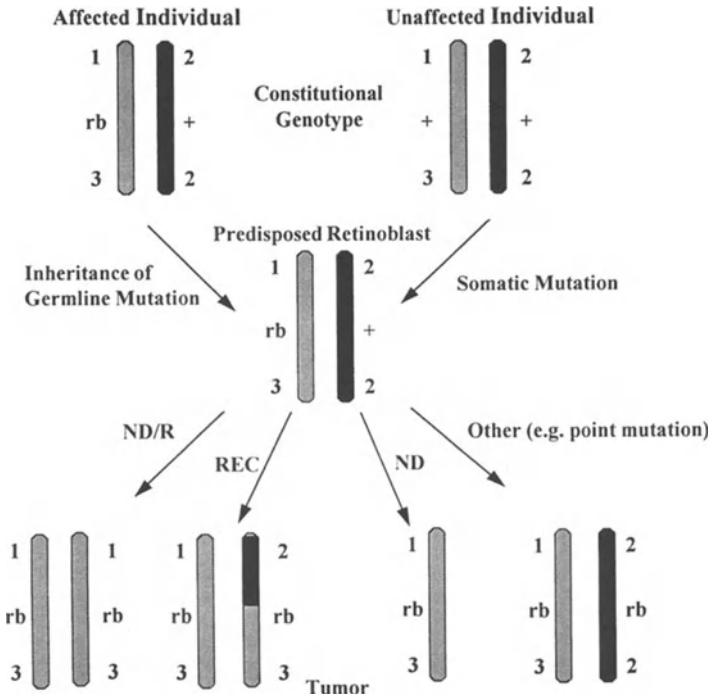
Fig. 3. Mechanisms that result in loss of heterozygosity for alleles at the retinoblastoma predisposition (*RB1*) locus. In the inherited form of the disease (*top left*), the affected patient has inherited one mutant *RB1* allele (*rb*) and one wild-type *RB1* allele (+). Thus, all of the cells in the affected patient, including the retinoblasts, contain one wild-type and one mutant allele such that the constitutional genotype at the *RB1* locus is (rb/+). In contrast, the constitutional genotype of the unaffected patient (*top right*) is +/+ (i.e., both alleles are wild-type). The two copies of chromosome 13 in non-neoplastic cells can be distinguished using polymorphic DNA markers flanking the *RB1* locus (these various polymorphic alleles are designated by *numerals*). In patients with the inherited form of the disease, a retinoblastoma arises from a predisposed retinoblast after inactivation of the remaining wild-type *RB1* allele. Patients with the sporadic form of retinoblastoma require somatic inactivation of both *RB1* alleles in the same developing retinoblast in order for a tumor to form. Several genetic mechanisms have been found to somatically inactivate wild-type *RB1* alleles during tumor development. These include: chromosome non-disjunction and reduplication of the remaining chromosome (*ND/R*), mitotic recombination (*REC*), non-disjunction (*ND*) without reduplication of the remaining chromosome, and other more localized mutations that inactivate the remaining *RB1* allele (*Other*)

different types of mutations in the corresponding DNA. For example, individual nucleotide substitutions may change a codon from one encoding an amino acid to one specifying translation termination (so-called nonsense mutations). Small insertions or deletions within the coding portion of the gene interrupt the open reading frame and often cause a frameshift, such that the DNA downstream of the insertion or deletion specifies incorrect amino acids followed by an early translation termination codon. Mutations affecting the splicing recognition signals at the intron-exon boundaries within a gene can also result in premature protein truncation. Such mutations cause the affected exons(s) to be skipped and other exons to be

incorrectly spliced together. This aberrant splicing may also result in a frameshift, with the same consequences for the protein as outlined above. *RB*1 is not the only tumor suppressor gene known to be frequently inactivated by mutations that cause early protein truncation. The *APC* (adenomatous polyposis coli) tumor suppressor gene on chromosome 5q is also typically inactivated by such mutations (NISHISHO et al. 1991; GRODEN et. al. 1991). Germ line mutations of *APC* have been identified in the great majority of patients affected by familial adenomatous polyposis or its variant, Gardner's syndrome. Moreover, 5q LOH and/or mutations of the *APC* gene are observed in the majority (60%–70%) of sporadic colorectal adenomas and carcinomas, suggesting that inactivation of *APC* is of critical importance in the development of epithelial colorectal tumors in general and not only in polyposis patients (POWELL et al. 1992). It is not difficult to envision that prematurely truncated proteins are likely to be inactive, particularly if they are small or trun-cated early. Investigators have capitalized on the fact that *APC* is typically in-activated by nonsense mutations in developing assays that screen for truncated APC proteins in colorectal tumors and non-neoplastic cells from patients at risk for familial adenomatous polyposis (POWELL et al. 1993). Such approaches are partic-ularly advantageous since they obviate the need for laborious DNA-based sequence analyses of *APC*, which is a rather large gene.

Tumor suppressor genes may also be "inactivated" by missense point muta-tions, that is, mutations that cause substitution of one amino acid for another. The effects of missense mutations on tumor suppressor protein function are best illus-trated by a brief review of the spectrum of mutations identified in the *p53* gene, the most commonly mutated gene known to date in human cancers. This gene lends itself well to mutation analysis since (a) it is relatively small (11 exons, 393 amino acids); (b) point mutations are distributed over a large region of the gene, allowing correlation between specific mutations and effects on protein function; and (c) the mutations are diverse (e.g., compared to those affecting the *RAS* genes), permitting more extensive inferences to be made regarding etiologic mechanisms. The *p53* gene encodes a 53-kD phosphoprotein that appears to function primarily in transcrip-tional regulation, although it may have other activities (VOGELSTEIN and KINZLER 1992; FRIEND 1994). The p53 protein contains three major functional regions, an amino terminal acidic domain with transcriptional activation function, a central hydrophobic core domain that is a primary determinant of p53 conformation and sequence-specific DNA binding activity, and a carboxy terminal domain containing the basic nuclear localization sequence and sequences mediating homo-oligomer-ization of p53 protein. The five distinct regions of p53 sequence that are highly conserved are found within the DNA binding and transactivation domains. Not suprisingly, the *p53* gene mutations that have been characterized in human tumors cluster within these conserved regions, particularly in the portions of the gene encoding the DNA-binding domain. Thus, the vast majority of mutant *p53* alleles that have been studied inactivate the DNA-binding activity of the p53 protein.

The spectrum of *p53* gene mutations in human cancers has been extensively reviewed (GREENBLATT et al. 1994). Missense mutations are most common in the conserved mid-region (79% of mutations) but are uncommon in the amino and

carboxy termini (23% of mutations), where nonsense mutations predominate. This observation suggests that single amino acid substitutions in the amino and carboxy terminal domains do not typically alter p53 protein conformation or function to the same degree as similar changes in the hydrophobic mid-portion. In fact, the most commonly mutated amino acids in the DNA-binding domain are close to the protein-DNA interface as determined by the recently described crystal structure of the p53 protein (CHO et al. 1994).

Evaluation of the *p53* mutational spectrum has also provided some clues and confirmed previous suspicions regarding the etiology of certain cancer types. A particularly illuminating example is provided by analysis of *p53* gene mutations in basal cell and squamous carcinomas of the skin. Development of these tumors has been clearly linked to UV light exposure. UV radiation acts as a physical mutagen that produces distinctive pyrimidine dimers that result in characteristic tandem mutations (usually CC $\rightarrow$ TT transitions) if left unrepaired. These tandem mutations, while rare in other tumor types, are common in both cutaneous basal cell and squamous carcinomas – directly incriminating UV light as the cause of these mutations in skin cancers (BRASH et al. 1991).

p53 mutation analysis has also found several clinical applications. For example, some studies have addressed the value of *p53* mutation as a prognostic factor in various tumor types. Expression of mutant p53 protein has been implicated as an independent prognostic indicator in carcinomas of the breast, stomach, colorectum, bladder, and lung (non-small cell types) (reviewed in DOWELL and HALL 1994). Other studies have used *p53* mutation analysis to screen body fluids for recurrent cancers (e.g., urine for recurrent bladder cancer) (SIDRANSKY et al. 1991), and to evaluate surgical margins of resection for small foci of tumor undetected by standard histopathological assessment (BRENNAN et al. 1995). Clearly, evaluation of *p53* mutations in human tumors has many diagnostic, prognostic, and therapeutic implications.

4 Mutations of DNA Mismatch Repair Genes and the Mutator Phenotype

Most adult human solid tumors, when properly analyzed, have been shown to have multiple mutations (FEARON and VOGELSTEIN 1990). This finding strongly supports the concept that human tumors develop due to the acquisition and subsequent accumulation of multiple mutations. LOEB (1991) has cogently argued that the spontaneous mutation rate in human cells is not high enough to account for the number of mutations thought to be necessary for the development of neoplasia. Thus, he hypothesized that the establishment of a "mutator phenotype" may be a necessary and early step in the formation of human tumors. This mutator phenotype was defined as an increase in the mutation rate of affected cells, thus predisposing them to the accumulation of genetic alterations necessary in the

pathogenesis of neoplasia (LOEB 1991). Several classes of genes were proposed that, when mutated, could generate the mutator phenotype. One of the suggested classes of genes included genes encoding DNA mismatch repair proteins.

Recently, the convergence of several different lines of scientific investigation have culminated in the identification and cloning of several human DNA mismatch repair genes (FISHEL et al. 1993; LEACH et al. 1993; PAPADOPOULOS et al. 1994; BRONNER et al. 1994). Moreover, it has been shown that these DNA mismatch repair genes are mutated in some human tumors and in the germ line of individuals affected by certain types of inherited predisposition to cancer. The story unfolded through an unforeseen combination of studies aimed at understanding human disease and fundamental processes in microorganisms and provides an extraordinary example of the potential impact of basic science on medical research.

Simultaneously, investigators studying both inherited and sporadic colorectal cancers noted alterations in the length of microsatellite sequences in DNA from tumor cells when compared to DNA isolated from the same patient's normal cells (IONOV et al. 1993; THIBODEAU et al. 1993; AALTONEN et al. 1993; PELTOMAKI et al. 1993b) (Fig. 4). Several groups were working to understand the genetic basis of an inherited family cancer syndrome called hereditary non-polyposis colorectal cancer (HNPCC). HNPCC is an autosomal dominant disease with three or more family members affected in two successive generations, including at least one affected individual under the age of 50 years. The most common tumors found in affected individuals are colorectal and endometrial carcinomas. Experiments revealed that DNA isolated from virtually all of the tumors from affected HNPCC family members showed instability at multiple microsatellite loci (AALTONEN et al. 1993). The groups studying sporadic colorectal carcinomas simultaneously noted instability of microsatellite DNA sequences (THIBODEAU et al. 1993; IONOV et al. 1993) in tumor DNA. Unlike tumors from individuals affected by HNPCC, in which nearly all of the analyzed tumors showed instability, only approximately 20% of the sporadic colorectal tumors studied demonstrated the phenotype. The phenomenon was referred to as microsatellite instability (MI) or replication error (RER). Initially, the significance of microsatellite instability and its potential role in tumorigenesis were unclear.

Shortly after the identification of microsatellite instability in human tumors, it was reported that mutations in DNA mismatch repair genes in *Saccharomyces cerevisiae* cause a 100–700-fold increase in the instability of simple dinucleotide repeat sequences in vivo (STRAND et al. 1993). In addition, previous work in *Escherichia coli* and *S. cerevisiae* had shown that defects in the DNA mismatch repair system could result in instability of simple repetitive DNA sequences (reviewed in MODRICH 1991). These observations provided a critical link between microsatellite instability and defects in DNA mismatch repair that set the stage for the identification of the human genes. Within several months a human homologue of the bacterial and yeast DNA mismatch repair genes was cloned using a PCR-based strategy that capitalized on DNA sequences conserved among the known microbial genes (FISHEL et al. 1993; LEACH et al. 1993). Shortly thereafter, three additional human homologues were cloned using a similar strategy, aided in part by the
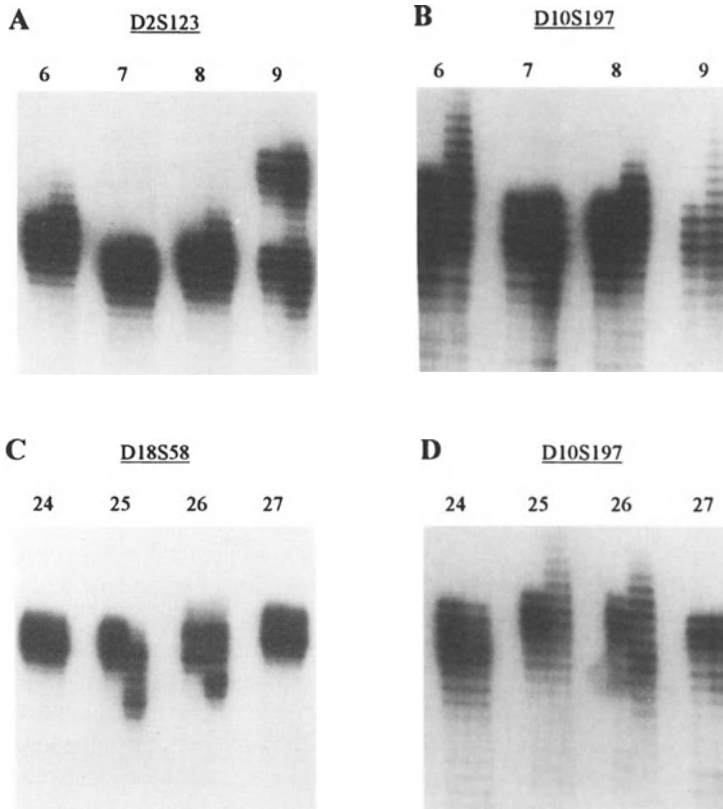
Fig. 4A–D. Assays for microsatellite instability. The polymerase chain reaction is used to amplify microsatellite sequences in tumor (T) vs. normal (N) DNA from the same patient. Tumors demonstrating microsatellite instability show alterations in the length of microsatellite sequences in the DNA from tumor cells when compared to matched normal DNA. The *panels* show several different normal-tumor DNA pairs assayed for microsatellite instability at different chromosomal loci (*D2S123*, *D10S197*, and *D18S58*). Cases 6, 8, 9, 25 and 26 exhibit the microsatellite instability phenotype

identification of conserved sequences in the data base of expressed sequence tags (dbEST) (BRONNER et al. 1994; PAPADOPOULOS et al. 1994).

The cloned human DNA mismatch repair genes have been named *hMSH2*, *hMLH1*, *hPMS1*, and *hPMS2*, in keeping with their microbial homologues. Each human DNA mismatch repair gene has been assigned a chromosomal location by physical mapping methods. The location of each gene has been shown to correlate with the location of the genes responsible for HNPCC as determined by genetic linkage analyses. The four genes, *hMSH2*, *hMLH1*, *hPMS1*, and *hPMS2*, are located on chromosomes 2p, 3p, 2q, and 7q, respectively. Studies of large families have determined that HNPCC is a genetically heterogeneous disease with some families demonstrating linkage to either chromosome 2p and 3p and other families showing linkage to the other chromosomal locations (PELTOMAKI et al. 1993a;

LINDBLOM et al. 1993; NYSTROMLAHTI et al. 1994). The combination of the linkage and physical mapping data provided further evidence for the role of DNA mismatch repair genes in the pathogenesis of neoplasms arising in the setting of HNPCC. It has since been shown that germ line mutations occur in one of the four known human mismatch repair genes in the great majority of individuals affected by HNPCC (FISHEL et al. 1993; LEACH et al. 1993; BRONNER et al. 1994; PAPADOPOULOS et al. 1994; NICOLAIDES et al. 1994). As expected, the specific gene mutated in each family is located on the respective chromosome to which the disease locus demonstrates genetic linkage.

Concurrent with the analyses of mutations in HNPCC kindreds, in vitro functional studies were performed that provided further evidence for the role of DNA mismatch repair genes in the production of microsatellite instability in human tumor cell lines. Using a microsatellite instability assay similar to that used in *S. cerevisiae,* investigators showed that the rate of instability of a dinucleotide repeat was a least two orders of magnitude higher in a colorectal carcinoma cell line derived from an MI-positive tumor compared to a cell line derived from a tumor that was MI negative (PARSONS et al. 1993). Such MI-positive colorectal carcinoma cell lines were also shown to be deficient in an early step in DNA mismatch repair, lending further credence to the role of DNA mismatch repair in microsatellite instability in human tumors. Moreover, a lymphoblastoid cell line derived from an affected HNPCC individual was able to appropriately direct mismatch repair in vitro, supporting the hypothesis that, at the cellular level, mismatch repair genes act in a recessive manner, requiring the inactivation of both alleles for expression of the phenotype. Accordingly, somatic mutations in the DNA mismatch repair genes have been identified in the tumors of affected HNPCC individuals. Affected members of HNPCC families thus inherit one mutant copy of the specific DNA mismatch repair gene responsible for the disease in their family and somatically acquire the mutation in the remaining normal allele. This situation is very similar to other familial cancer syndromes caused by inherited mutations of tumor suppressor genes, including familial adenomatous polyposis, and the inherited forms of Wilms' tumor and retinoblastoma. More recently, a subset of HNPCC patients were found to have widespread microsatellite instability not only in their tumor DNA, but also in the DNA of their non-neoplastic cells (PARSONS et al. 1995). This phenomenon occurred only in patients with certain germ line mutations of *hPMS2* and *hMLH1* not in patients with mutant *hMSH2* or *hPMS1* genes. These data suggest that some *hPMS2* and *hMLH1* mutations may confer dominant negative activity to the mutant proteins such that a mismatch repair defect is manifest when only one of the two copies of these genes is mutant.

How do microsatellite instability and associated mutations in DNA mismatch repairs genes predispose cells to the development of neoplasia? This question was addressed, in part, by looking for mutations in known cancer genes in MI-positive tumors from individuals with HNPCC. Many of the genetic alterations that underlie the development of colorectal carcinoma have been well characterized and are easily analyzed. Studies of MI-positive colorectal cancers showed that mutation frequencies of *p53*, K-*RAS*, and *APC* are similar to those found in sporadic forms

of colorectal cancer, the majority of which are MI negative (AALTONEN et al. 1993). This finding supports the idea that tumors arising in HNPCC share similar pathogenetic mechanisms to colorectal tumors occurring in the sporadic form. Furthermore, it may explain why individuals who inherit a defective DNA mismatch repair gene so frequently develop tumors, as defects in the DNA mismatch repair system may lead to a marked increase in the rate at which mutations occur in genes that give rise to the neoplastic phenotype (e.g., tumor suppressor genes and oncogenes). Therefore, cells of individuals who inherit one mutant copy of a DNA mismatch repair gene and undergo somatic inactivation of the remaining allele will rapidly accumulate mutations in the critical genes that promote tumorigenesis.

The DNA mismatch repair system has been well characterized in microorganisms where it has been shown to detect and repair mispaired bases present in the cellular genome (MODRICH 1991). Thus, it is of obvious importance in controlling the fidelity of DNA replication by decreasing the number of mutations that become fixed in the genome following each round of DNA synthesis. When the DNA mismatch repair system is lacking, the rate at which mutations accumulate is increased (COX 1976). Although the DNA mismatch repair system has been much less well characterized in mammalian cells, there is substantial data supporting the hypothesis that its functions are very similar to those described in microorganisms. In addition to the genetic and molecular studies described above, biochemical studies have demonstrated that purified hMSH2 protein binds to mismatched microsatellite DNA sequences (FISHEL et al. 1994). Furthermore, as microsatellite DNA sequences are quite prone to errors during DNA replication, it follows that these sequences would demonstrate numerous alterations in the absence of an intact DNA mismatch repair system. These studies suggest that, in mammalian cells, microsatellite instability may simply serve as a marker of the mutator phenotype, and the neoplastic process is produced by an increased rate of mutations in oncogenes and tumor suppressor genes.

Since the initial finding of microsatellite instability in sporadic and inherited colorectal cancer, many studies have shown that microsatellite instability occurs in a wide variety of presumably sporadic tumors, including sporadic endometrial carcinomas. Like sporadic colorectal cancer, microsatellite instability has been found in approximately 20% of sporadic endometrioid endometrial cancers (BURKS et al. 1994; RISINGER et al. 1993). This observation is of special interest in light of the fact that endometrial cancer is the second most common tumor type occurring in HNPCC and that the endometrioid variant of endometrial cancer has other pathogenetic features in common with colorectal carcinoma.

To date, only limited studies have addressed the underlying genetic defect that generates instability of microsatellite sequences in sporadic cancers. One study thoroughly analyzed all four of the known human DNA mismatch repair genes in DNA isolated from transformed lymphoblastoid cell lines of ten patients with presumably sporadic MI-positive colorectal cancers (LIU et al. 1995). A germ line mutation was detected in only one case (mutation of hMLH1). The identified mutation was a nonsense mutation resulting in a severely truncated protein. Furthermore, no normal transcript was identified in the cell line generated from this

patient's tumor. In retrospect, this patient, although not meeting the criteria for HNPCC, had two other family members with colorectal and endometrial cancer and the patient was less than 50 years old. Thus, it is possible that this patient is a member of an HNPCC family that had not been recognized previously as such. The investigators went on to study cell lines derived from MI-positive colorectal cancers arising in patients without significant family cancer histories. Only three of the seven cell lines harbored mutations in the four known DNA mismatch repair genes, all in *hMLH1*, and both copies of the gene were mutated somatically. A somewhat similar situation is seen in MI-positive sporadic endometrial carcinomas. Eight MI-positive primary endometrial carcinomas have been studied for the presence of mutations in all four known DNA mismatch repair genes (KATABUCHI et al., 1995). Only one tumor had a detectable somatic mutation and no germ line mutations were identified. The somatic mutation, in the *hMSH2* gene, is a splice site mutation that causes an exon to be skipped. The aberrant RNA processing creates a frameshift that results in a premature termination codon and, in turn, a truncated protein. Based on these results, it appears that germ line mutations in the known DNA mismatch repair genes in patients with presumably sporadic MI-positive colorectal and endometrial cancer are uncommon and that microsatellite instability in many sporadic forms of these cancers must be due to mutations in genes that have yet to be identified.

These results have significant ramifications for the use of MI assays in screening the general population for predisposition to colorectal cancer. When microsatellite instability was first identified in a subset of presumably sporadic colorectal cancers, it was thought that it might be useful in identifying potential members of HNPCC families. Since microsatellite instability assays are technically straightforward and fast, they appeared to offer a potentially cost-effective method for generalized screening. However, the recent data discussed above indicate that germ line mutations in the four known DNA mismatch repair genes in patients without a documented family cancer history are, at best, rare. Based on these results, it appears that generalized screening for microsatellite instability is not warranted. However, screening young patients with presumably sporadic colorectal cancers may be worthwhile, especially if the patient has a family member with colorectal cancer.

Finally, it is clear that additional genes exist that give rise to microsatellite instability. These genes may encode additional proteins involved in DNA mismatch repair, but it is likely that they may also encode proteins involved in replication, recombination, and other types of DNA repair processes. Obviously, the search for such genes is currently being vigorously pursued. In addition, if a mutator phenotype is required for the development of neoplasia then microsatellite instability must be only one manifestation of such a phenotype, as the vast majority of cancers lack instability of microsatellite DNA sequences. Thus, the nature of the mutator phenotype in the majority of tumors remains a mystery that will undoubtedly be a major focus of future investigation.

5 Changes in DNA Methylation Are Observed in Cancer Cells

DNA methylation involves covalent modification of DNA and as such can be considered a structural alteration of DNA in tumor cells. Changes in DNA methylation have been shown to influence several cellular processes that affect both DNA integrity and function, processes that go awry during tumorigenesis. Over the past several years, data have accumulated suggesting that changes in DNA methylation may play a role in tumor development and/or progression (reviewed in LAIRD and JAENISCH 1994). In mammals, DNA methylation occurs at the fifth carbon position of cytosine residues within CpG dinucleotides (ADAMS and LINDSAY 1993; GARDINER-GARDEN and FROMMER 1987). Although most of the CpG dinucleotides in the human genome are methylated, CpG dinucleotides are approximately fivefold underrepresented in the genome overall. Furthermore, CpG dinucleotides and 5-methylcytosine residues are unevenly distributed in the genome. Approximately 1%–2% of the human genome consists of "islands" of non-methylated CpG-rich stretches of DNA that are typically 1–2 kb in length located at the 5′ end of genes in association with the promoter. These non-methylated CpG islands are associated with all known housekeeping genes and with a number of genes that are expressed in a tissue-dependent fashion. Although housekeeping genes are ubiquitously expressed and thought not to be regulated by DNA methylation, several studies suggest an inverse correlation between DNA methylation and tissue-specific expression of certain genes, with the strongest correlation observed between gene activity and promoter hypomethylation (YEIVIN and RAZIN 1993). In general, methylated genes are inactive, while non-methylated genes are active. Thus DNA methylation provides a potential mechanism with which to inactivate specific genes (e.g., tumor suppressor genes) during tumorigenesis. Results of studies of cells in culture suggest that gene silencing by methylation can provide a selective advantage for cell growth (ANTEQUERA et al. 1990). Unfortunately, our understanding of the cellular processes regulating de novo methylation activity remains incomplete. Currently, only one DNA (cytosine-5) methyltransferase has been identified in human cells (YEN et al. 1992; BESTOR et al. 1988), and its major function appears to lie in maintenance of methylation status rather than de novo methylation.

Tumor cells exhibit both global changes in DNA methylation as well as alterations of methylation patterns in individual genes. Global hypomethylation has been reported in several tumor types including colorectal carcinoma (GOELZ et al. 1985), cervical carcinoma (KIM et al. 1994), seminomas (PELTOMAKI 1991), and others (RAO et al. 1989), and, in some cases, the degree of hypomethylation correlates with tumor progression (KIM et al. 1994; GAMA-SOSA et al. 1983). Hypomethylation of specific proto-oncogenes such as c-MYC, c-FOS, H-RAS, K-RAS, ERB-A1, and BCL-2 has also been observed, particularly in hepatocellular carcinomas and leukemias (reviewed in LAIRD and JAENISCH 1994).

Tumor cells also show regional increases in DNA methylation. For example, BAYLIN and his colleagues have found hypermethylation hot spots on chromo-

somes 3p, 11p, and 17p in several different types of human tumors (DE BUSTROS et al. 1988; MAKOS et al. 1992, 1993a, b). Moreover, other studies suggest that specific genes may be inactivated by hypermethylation in certain tumor types. For example, the *RB* tumor suppressor gene may be inactivated by hypermethylation in sporadic retinoblastomas (OHTANI-FUJITA et al. 1993; SAKAI et al. 1991), and, more recently, hypermethylation of regulatory sequences at the *GSTP*1 (glutathione S-transferase Pi-1) locus was detected in all human prostatic carcinomas studied but not in normal prostatic tissues or in tissues with benign prostatic hyperplasia (LEE et al. 1994). Expression of *GSTP*1 mRNA and protein was concomitantly reduced in the carcinomas. These findings may lead to several clinical applications. For example, since *GSTP*1 hypermethylation is the most common genetic alteration yet described in prostate cancer (in virtually 100% of cases), detection of this change (and/or loss of GSTP1 protein expression) may serve as an adjunctive tool in the diagnosis of prostate cancer. Also, the GSTP1 protein is an enzyme that is in part responsible for the detoxification of certain carcinogens. Expression of GSTP1 and related enzymes is in turn induced by certain foods such as cruciferous vegetables, suggesting a prostate cancer prevention strategy based on diets rich in foods that induce enzymes capable of compensating for loss of GSTP1 function.

In addition to the regional and global methylation changes noted in human cancers, it has long been recognized that CpG dinucleotides are hypermutable. That is, the estimated mutation rate of CpG dinucleotides is 10–40 times greater than that of other dinucleotides. The excess of mutations identified at CpG dinucleotides is attributable to transitions of CpG to TpG or CpA (SVED and BIRD 1990), many via spontaneous deamination of cytosine and especially 5-methylcytosine. Data are also accumulating suggesting that, in some cases, deamination may be facilitated by certain cellular enzymes, including a 5-methylcytosine-DNA glycosylase and/or the known DNA methyltransferase (reviewed in LAIRD and JAENISCH 1994).

6 Aneuploidy Frequently Characterizes Tumor Cells

Aneuploidy may be defined as an abnormal state in which the total DNA content of the aneuploid cells differs from the DNA content characteristic of that species, through the gain or loss of variable numbers of chromosomes or chromosome fragments. Normal somatic cells are diploid, that is, they contain a pair of each haploid ($n = 23$) chromosomes. In and of itself, diploidy does not imply normal chromosome structure or arrangement. Chromosomal losses may be balanced by chromosomal gains elsewhere in the genome. Similarly, aneuploidy is not observed only in malignant and pre-malignant cells. Certain forms of aneuploidy, including Klinefelter's syndrome, Turner's syndrome, and several trisomies, are compatible with near-normal growth and development. Aneuploidy may also arise from the gain of complete chromosomal sets. DNA indices (defined as the measurable ratio of abnormal to normal cellular DNA) up to 3.0 have been reported; however, the

maximum DNA content that is compatible with cell survival remains unknown. The biological significance of aneuploidy has been recently reviewed (REW 1994).

Up to 75% of all tumors analyzed display aneuploidy. One feature of aneuploid tumors is that they typically contain only one or a few stable aneuploid populations of cells. In flow-cytometric analyses, the DNA profile of the aneuploid cells is typically superimposed on the DNA profile of diploid cells within the tumor. This diploid population usually represents a mix of non-malignant bystander cells (e.g., stromal cells, inflammatory cells, endothelial cells, etc.) and diploid tumor cells.

Several studies have assessed the value of aneuploidy as a marker of biological aggressiveness in various tumor types. Overall, conflicting views have emerged regarding the utility of ploidy data in predicting prognosis or directing clinical management. These different conclusions most likely reflect a combination of true biological differences between different series of tumors and technical differences in data collection and analysis. Thus far, the identification of aneuploidy in and of itself has proven to be of relatively little clinical utility. In general terms, while diploidy has been associated with a more favorable prognosis in some types of tumors (e.g., colorectal, breast, and prostatic carcinomas), many purely diploid tumors behave very aggressively.

It should be emphasized that diploidy and aneuploidy are not two distinct states, but part of a continuum from very subtle DNA changes to gross chromosomal abnormalities. In this context, the definition of aneuploidy may be somewhat arbitrary, since subtle but functionally important chromosomal alterations may escape detection with the laboratory techniques that are currently available. Given these caveats, it remains unclear whether there is a causal relationship between aneuploidy and cancer or whether aneuploidy simply reflects an epiphenomenon in genetically unstable tumor cells.

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