ADVANCES IN

GENOME BIOLOGY

Editor: RAM S. VERMA

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GENETICS OF SEX DETERMINATION

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Editor: *RAM S. VERMA*
Division of Genetics
The Long Island College Hospital-
SUNY Health Science Center
Brooklyn, New York

Volume 1. UNFOLDING THE GENOME
Volume 2. MORBID ANATOMY OF THE GENOME
Volume 3A GENETICS OF HUMAN NEOPLASIA
Volume 3B GENETICS OF HUMAN NEOPLASIA
Volume 4. GENETICS OF SEX DETERMINATION
Volume 5. GENES AND GENOMES
Volume 6. MITOCHONDRIAL GENOME

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DEDICATION

To Harold L. Light whose distinct leadership and enthusiastic support has been a continuous source of inspiration to the editor of this series.

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PREFACE

The *Genetical Theory of Natural Selection* by R.A. Fisher (1930) dictated that sexual dimorphisms may depend upon a single medelian factor. This could be true for some species but his suggestion could not take off the ground as gender in *Drosophila* is determined by the number of X chromosomes. Technical advances in molecular biology have revived the initial thinking of Fisher and dictate that *TDF* or *SRY* genes in humans or Tdy in mice are sex determining genes. The fortuitous findings of XX males and XY female, which are generally termed sex reversal phenomenon, are quite bewildering traits that have caused much amazement concerning the pairing mechanism(s) of the pseudoautosomal regions of human X and Y chromosomes at meiosis. These findings have opened new avenues to explore further the genetic basis of sex determination at the single gene level.

The aim of the fourth volume, titled *Genetics of Sex Determination* is to reflect on the latest advances and future investigative directions, encompassing 10 chapters. I have commissioned several distinguished scientists, all preeminent authorities in each field to shed their thoughts concisely but epitomize their chapters with an extended bibliography. Obviously, during the past 60 years, the meteoric advances are voluminous and to cover every account of genes, chromosomes, and sex in a single volume format would be a herculean task. Therefore, I have chosen a few specific topics which may be of great interest to scientists and clinicians. The seasoned scientists who love to inquire about the role of genes in sex determination should find the original work of

these notable contributors very enlightening. This volume is intended for advanced students who want to keep abreast as well as for those who indulge in the search for genes of sex determination.

I owe a special debt of gratitude to the many distinguished authors for having rendered a valuable contributions despite their many pressing tasks. Almost 400 pages reflect professionalism and scholarship with their own impressive styles. The publisher and the many staff members of JAI Press deserve much credit. I am very thankful to the many secretaries who typed the manuscripts of the various contributors.

Ram S. Verma
Editor

GENETICS OF SEX DETERMINATION: AN OVERVIEW

Ursula Mittwoch

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The determination of sex is not by inheritance, but by the combined effect of external circumstances.[1]

The force of experimental evidence has now become irresistible that sex-determination must be treated as a form of heredity; and evidently the cytological facts provide a good basis for its analysis.[2]

I. INTRODUCTION

The two citations introducing this overview are by Edmund Wilson and were published in 1896 and 1911, respectively. The intervening period of 15 years saw not only the rediscovery of Mendel's laws,[3–5] but also the discovery of sex chromosomes, in which Wilson himself played a principal role. The difference in the viewpoints illustrated in the above citations is a reflection of a difference in outlook which distinguishes the twentieth century from its predecessor.

Toward the end of the nineteenth century, there was a shift in paradigm which resulted in environmental causes losing some of their preeminence of assumed agents in health and disease, while genetic factors began to come to the fore. "The world is beginning to perceive," wrote Galton,[6] "that the life of each individual is in some real sense a prolongation of those of his ancestry. His character, his vigour, and his disease, are principally theirs." This shift in people's perception regarding the origin of physical characteristics is nowhere more evident than in the attempt to answer the question as to what causes the difference between the sexes.

During the nineteenth century the view prevailed that sex was determined by environmental conditions, of which the most important was thought to be nutrition of the mother, while the effect of temperature was thought to be less important, even though evidence was cited purporting to show that more boys were born in winter (reviewed in reference[7]). On the other hand, the increasingly systematic collections of quantitative data that became available toward the end of the century showed that in different mammalian species the ratio of male to female births is a characteristic figure, which would vary only slightly, if at all, by environmental conditions.[8] It also became evident that large samples were needed before valid conclusions about sex ratios could be drawn, and that many earlier theories could be discounted for being based on insufficient data. At the close of the century, Cuénot[9] was able to dismiss the idea that sex was determined during gestation. The stage was thus set for the discovery of the sex chromosomes.

Sex chromosomes were originally discovered in insects[2] and were soon looked for in man, although definitive results were not obtained until the second half of the twentieth century (see Section IV). Since then, intensive attempts have been made to isolate sex-determining genes located on these

chromosomes. At the same time, it has become evident that sex chromosomes are not universally present in all animals. In amphibians, hermaphroditism, that is, the development of eggs and sperm in the same individual, are relatively common, and differences in temperature can influence sex differentiation.[10] More recently it has been shown that, among reptiles, sex is determined by the temperature of incubation in a variety of lizards, turtles, and crocodilians.[11] At the close of the twentieth century it seems appropriate to review the current state of the subject of sex determination and differentiation in its historical setting, and to search for clues about the way that our viewpoint may become modified as we approach the twenty first century.

In the same way as sexuality needs to be understood in terms of the developmental processes that gave rise to it, a proper appreciation of the genetics of sex determination requires some knowledge of the historical landmarks that have preceded present-day paradigms.

II. CLASSICAL GENETICS OF SEX DETERMINATION

Our present-day understanding of the genetic basis of sex determination originated in studies of the cellular processes underlying spermatogenesis in insects. More than 100 years ago, Henking,[12] in Göttingen, described the cell divisions of the spermatocyte in the plant bug, *Pyrrhocoris apterus*, and illustrated the two products of a meiotic division, one containing 11 chromosomes, and the other containing an additional large element, labeled "X." The implication that as a result of this meiotic division two different types of sperm would be formed caused McClung[13] to suggest that the two classes of sperm would be expected to give rise to two classes of individuals in about equal numbers, and, surely, the most obvious distinction between individuals is that of sex? The extra chromatin element, or "accessory chromosome," which a few years later received the name "X chromosome," McClung[13] thought to be male-determining, enabling the germ cells to pass beyond the slightly differentiated egg cell to the highly differentiated sperm cell.

Although the idea that sex is determined by a chromosome rather than by external conditions met with considerable scepticism, two investigators obtained further evidence in its favor. Nettie Stevens[14] investigated females as well as males and found that in the common mealworm, *Tenebrio molitor*, both sexes had the same chromosome number, but in males one chromosome was smaller than the others, which led her to conclude that "it seems certain that an egg fertilized by a spermatozoon which contains a small chromosome must produce a male, while one fertilized by a spermatozoon containing ten chromosomes of equal size must produce a female." This was confirmed by E.B. Wilson[15] at Columbia University who found that in insects of the order Hemiptera there were two types of dimorphic sperm: in some species, one

chromosome is present in one class and absent in the other, while in other species, the two classes differed in the size of one of the chromosomes. The larger chromosome became known as the "X," and the smaller as the "Y" chromosome,[16] and both as "sex chromosomes."[17] Evidently, sperm containing an X chromosome gave rise to females, while those with a Y, or a missing sex chromosome, produced males. Thus McClung had been right in principle, though wrong in detail.

The discoveries linking male and female sex to a difference in chromosome constitution were striking but hardly amounted to proof. Rather than causing sex differentiation, the different chromosomes in males and females might merely represent a secondary sexual difference. A particular difficulty arose from the fact that, whereas cytological studies indicated that the male was the heterogametic sex, the first example of sex linkage was worked out in the current moth, *Abraxas grassulariata*,[18] a member of the Lepidoptera. In this species, individuals of a special color variety are usually female, and breeding experiments showed that it was the female who carried two different sex determinants. Experiments on canaries and chickens pointed in the same direction.[19]

The introduction of the fruitfly, *Drosophila melanogaster*, was an important step in resolving this paradox. The chromosomes of this species were investigated by Stevens,[20] who found two X chromosomes in females and a single X in males; the Y chromosome in *Drosophila* was first described by Bridges.[21] The pattern of sex linkage proved to be different from that found in *Abraxas*. The first mutant, discovered by T.H. Morgan,[22] was a male with white eyes. When mated to his red-eyed sisters, this male produced 1,237 offspring, all red-eyed, apart from three white-eyed males (see below), while large numbers of white-eyed males reappeared among the offspring of red-eyed F1 flies. The mode of transmission of the gene for white eyes suggested that it was situated on the X chromosome.

The exceptional white-eyed male offspring of the white-eyed father actually helped to strengthen this hypothesis. Bridges[23] explained the occurrence of such individuals by assuming that occasionally the sex chromosomes failed to disjoin during oogenesis, so that some eggs would contain two X chromosomes and others none. On fertilization, eggs with two X chromosomes could give rise to either XXX or XXY females, and those lacking a Y chromosome to XO females or possibly XO males, the former carrying their father's X chromosome. Cytological investigations confirmed that certain aberrant females had XXY sex chromosomes, while males that had inherited their fathers' sex-linked characters were XO.[21,23] These results provided strong evidence both for the chromosome theory of inheritance and for the role of sex chromosomes in the determination of sex.

The findings that XO flies are male, while XXY flies were female, ruled out the possibility that the Y chromosome in *Drosophila* might have a male-

determining function. Although XO flies appeared to be normal males, they proved to be sterile. Stern[24] investigated Y chromosomes with various deletions and found that male fertility required the presence of different regions on both the long and the short arm of the Y chromosome. These regions were called "fertility factors".

The failure of the Y chromosome to confer maleness led to the conclusion that sex in *Drosophila* is determined by the number of X chromosomes, two X chromosomes giving rise to a female, while a single X chromosome per cell results in a male. This simple relationship needed modification, however, when it was found that two X chromosomes in conjunction with three sets of autosomes gave rise to intersexes with a mixture of female and male characteristics.[25] This implied that it was the ratio of X chromosomes to autosomes that determined the sex of a fly.

The interpretation advanced by Bridges[25] was that the X chromosome carried genes with a tendency for femaleness, while genes on the autosomes tended to produce male characteristics. In this way the theory of sex determination became incorporated into the theory of genic balance, that had originally been put forward to explain the fact that flies lacking the small chromosome No.4 could be distinguished by their phenotypic appearance.[26,27] The theory of genic balance is based on the view that each character results from the interaction of all the genes in the entire genome. Some of the genes will have the effect of increasing the character in question ("plus genes"), while others ("minus genes") work in the opposite direction, diminishing the character. Each of these genes participates in producing a joint effect, although for any particular character some genes are more effective than others.

Following the discovery by H.J. Muller[28] that chromosomes could be broken by X-rays, Dobzhansky and Schultz[29] set about trying to localize female-determining genes on the X chromosome of *Drosophila melanogaster*. They found that by adding pieces of X chromosomes to triploid flies with two complete X chromosomes, the development of the intersexes was shifted in a female direction. If the added piece was sufficiently long, fertile females could result, and there was no particular part of the X chromosome that was essential for this purpose. These results seemed to rule out the existence of a single female-determining gene, and they were interpreted as indicating the presence of a large number of such genes situated along the length of the X chromosome. The paradigm summarizing the classical genetics of sex determination in *Drosophila* could be summarized by the following equations where X = number of X chromosomes and A = number of sets of autosomes:

$X/A = 1$ results in female development
$X/A = 0.5$ results in male development
$X/A = 0.67$ results in intersexual development.

A. Molecular Genetics of Sex Determination in *Drosophila*

In recent years, molecular studies have led to the isolation of four X-linked "numerator genes," *sisterless-a,-b,-c,*and *runt*, but only one autosomal "denominator gene," *deadpan.*[30] Both numerator and denominator genes encode transcription factors, controlling the transcription of the *Sex-lethal* (*Sxl*) gene, which must be active in female development, and inactive in male development. If the X:autosome ratio is 1, the level of gene product produced by these genes will cause *Sxl* to be switched on and the embryos to embark on the developmental pathway of females.[31] The fact that only one denominator gene could be identified has led to the conclusion that the "male determining" function of the additional set of autosomes in triploid intersexes is caused by non-specific effects such as cell size as much as by specific genes.[30]

III. CLASSICAL MODELS OF SEX DETERMINATION OTHER THAN IN *DROSOPHILA*

Notwithstanding the predominance of *Drosophila melanogaster* in genetic research, this species was not the only one that yielded data on the genetics of sex determination. Flowering plants were one of the earliest classes of organisms in which sex-determining mechanisms were investigated and found, and the red and white campions, *Silene dioica* and *S. alba*, proved particularly favorable. The genus had previously been known under the names of *Melandrium* and of *Lychnis*.

The sex chromosomes of *Silene* were discovered independently by Winge[32] and by Blackburn.[33] Male plants have an unequal sex chromosome pair, which is the largest in the complement, and the Y chromosome is larger than the X. A number of investigators produced tetraploid plants by colchicine treatment.[34–37] Tetraploid plants with XXXX chromosomes were female, and those with XXYY or XXXY chromosomes were male, although in Westergaard's[36] strain the latter contained the occasional hermaphrodite flower. Various deletions of the *Silene* Y chromosome allowed Westergaard[38] to divide the differential section into three portions: whereas plants lacking either portion proximal to the pairing section were males, those lacking the distal portion were found to be hermaphrodite, which led to the conclusion that this portion suppresses the formation of female parts of the flower. A well-known curiosity is that plants infected by the smut fungus, *Ustilago violacea*, develop male flowers in the absence of a Y chromosome; however, instead of pollen, their anthers are filled with the spores of the fungus[38].

The sheep's sorrel, *Rumex acetosella*, is a subgenus of flowering plants providing another example of a strongly male-determining Y chromosome.[39] It comprises four species which are diploid, tetraploid, hexaploid, and

octoploid respectively. In tetraploid species, female plants are XXXX and male plants XXXY, and in hexaploid species females have six X chromosomes and males have five X and one Y chromosome.

Lepidoptera, in which the female is the heterogametic sex, are another group of organisms that were the object of active research into the genetics of sex determination. This applies particularly to the gypsy moth, *Lymantria dispar*, in which intersexes occur relatively frequently. Goldschmidt[40,41] found that intersexes occurred regularly among the offspring of different races. While crosses between Japanese females and European males resulted in normal males and females, the reciprocal cross gave raise to intersexual females. These and other results were interpreted on the assumption that the sex of a moth is determined by the relative strength of, or balance between, a female-determining factor, inherited from the mother, and a male-determining factor located on the Z chromosome, and that the strength of these determinants differ in different races.

We see that during the first half of the twentieth century the evidence accumulated from a variety of organisms indicated that each individual carries on its chromosomes a mixture of male and female-producing determinants which are distributed by different chromosomal mechanisms in such a way that the determinants of one or the other sex are normally, but not invariably, in a clear majority. But what is the situation in humans and other mammals?

IV. THE HUMAN Y CHROMOSOME

Before the advent of modern techniques of chromosome preparations in the 1950s, work on human chromosomes was mainly based on sectioned material. Attempts to study human chromosomes date from the ninteenth century, and the first investigator to draw attention to the sex chromosomes seems to have been Geyer in 1910, who concluded that the sex-determining mechanism was of the XO type (reviewed in reference[42]). Other investigators came to the same conclusion, but Painter[43–45] provided strong evidence for the existence of a Y chromosome in the spermatocytes of man, the American opossum (*Didelphis virginiana*), and the capuchin monkey (*Cebus sp*), including an illustration of the X and Y chromosomes in end-to-end association during metaphase of the first meiotic division. Although prior to the availability of modern techniques of cytogenetics the evidence could not be regarded as conclusive, by the middle of the century the weight of the evidence pointed to the existence of a Y chromosome in the human species.[46]

From 1960 onward the subject of human chromosomes became transformed by two new techniques: the ability of growing human cells in tissue culture removed the formerly extreme difficulty of obtaining suitable material, while various improvements in cytological technique, particularly the use of

hypotonic pretreatment, did away with the crowding together of human and other mammalian chromosomes and made their analysis into an exact science. By the time the participants of the Denver Conference published their recommendations for standardizing the representation of the 22 human autosomes and of the X and Y chromosomes[47]—even though the individual distinction of the latter two had to await the introduction of fluorescent staining 10 years later[48]—abnormal chromosome constitutions were already being discovered.

Two syndromes of abnormal sexual development that were immediately investigated by means of the new techniques were Klinefelter's and Turner's syndromes, in both of which unusual findings regarding sex chromatin status were already available. In Turner's syndrome—females with infantile genitalia and streak gonads in addition to certain somatic abnormalities—sex chromatin bodies were absent,[49,50] as in normal males; and two years later, at least six groups of investigators found that in Klinefelter's syndrome—males with small testes which lack spermatogenesis, and an increased incidence of mild mental retardation—sex chromatin bodies were present (reviewed in reference[42]), as in normal females, suggesting that both syndromes might be due to "sex reversal." This idea had to be modified in 1959, when chromosome analysis showed that the diploid number in three patients with Turner's syndrome was 45, containing a single X chromosome and no Y,[51,52] while patients with Klinefelter's syndrome had 47 chromosomes, which were interpreted to contain XXY sex chromosomes.[53,54] These early results indicated that both syndromes were due to abnormalities of the sex chromosomes, and they further suggested that the human Y chromosome was strongly "male-determining"; and this conclusion was further strengthened when it was found that even patients with four X chromosomes and a Y were male. These patients, however, show marked signs of sexual underdevelopment. A review of the first four cases to be described stated that of the eight testes, only one was descended[55].

In a review of the available findings of sex chromosome abnormalities published in 1963, Ford[56] concluded that "the Y chromosome carries one or more genes or sectors which normally determine that the primordial gonad shall become a testis and that subsequent development shall be in the direction of the male phenotype." Apparent exceptions to this rule included patients with XY gonadal dysgenesis and with testicular feminization, whose phenotype demonstrated that the possession of a Y chromosome is not enough to ensure the development of testes, or their ability to impose a male phenotype, and patients with true hermaphroditism, most of whom had an apparently normal female sex chromosome constitution. Basically, according to Ford,[56] two hypotheses could account for the development of ovarian and testicular tissue in the same individual: either the cells of the two gonadal types are genotypically distinct, as a result of mutation or nondisjunction, or all the cells are genotypically alike, and male and female-determiners are present in both XX

and XY chromosome sets but are so delicately balanced that chance local embryonic events determine which shall prevail.

Some years later, the finding by Jacobs and Ross[57] of two female patients with streak gonads, who had an abnormal metacentric chromosome interpreted as isochromosome of the long arm of the Y, led the authors to the important conclusion that the male-determining region of the Y chromosome is located on the short arm.

V. SEX-CHROMATIN BODIES AND X-INACTIVATION

Sex-chromatin studies are of interest, both because they were an overture to the study of chromosomes, and because the findings provided important pointers leading to an understanding of the behavior of X chromosomes. The discovery of sex-chromatin bodies is generally placed in 1949, with the publication by Barr and Bertram[58] of their finding of a nuclear body that could be seen in the neurons of female, but not male cats, using routine Nissl staining. As a matter of fact, a sex chromatin body, present in females and absent in males, had been described several years earlier in the spruce budworm, *Choristoneura [Archips] fumiferana,*[59] but publicity went to the cats. Soon it was found that the body was also detectable in other tissues and was also present in human females. The first patient in whom it was demonstrated was a true hermaphrodite,[60] and the finding of "female nuclear sex" in the cells from a skin biopsy created a strong impetus for examining other patients with abnormalities of sexual development. This in turn resulted in a search for simpler techniques for demonstrating sex chromatin bodies, and just two years later two groups showed that sex chromatin bodies can be detected in cells scraped off the buccal mucosa.[61,62] Findings in patients with Klinefelter's and Turner's syndromes revealed that sex chromatin bodies were not always associated with female sex, and when chromosome studies became available, it emerged that they were related to the number of X chromosomes present, that is, their number was one less than the number of X chromosomes present in the nucleus. The eponymous term "Barr body" came into use, being both shorter and sexually neutral.

The numerical relationship between Barr bodies and X chromosomes provided strong support for the idea, first formulated by Susumu Ohno and collaborators,[63] that a Barr body originates from a single X chromosome. Having developed the technique of autoradiography, J.H. Taylor[64] showed that the chromosomes in cultures of Chinese exhibited asynchrony of replication, and it soon became evident that one X chromosome in females replicates its DNA later than the rest, and that, if more than two X chromosomes were present in a cell, all but one were late replicating.[65]

At the same time, Mary Lyon formulated the single active X hypothesis,[66] implying that sex-chromatin formation and late replication are characteristics

of X chromosomes that have become genetically inactivated, a fate that befalls any but the first X chromosome present in a cell. That only one X chromosome is active in any cell has since become a fundamental tenet in mammalian genetics, and the phenomenon continues to impinge on new areas of research.[67] The mechanism by which X inactivation is brought about will be discussed in Chapter 5.

VI. SEX CHROMOSOMES IN OTHER SPECIES

Progress in human chromosomes soon spread to other mammalian species, and the XX/XY mechanism was found in the large majority of those investigated.[68] Furthermore, an increasing number of examples of individuals with sex chromosome abnormalities provided evidence that the male-determining power of the Y chromosome is of general application throughout the mammals. The existence of female XO mice was first deduced on the basis of linkage results in 1959 by Russell et al.,[69] and cytologically verified by Cattanach,[70] and the same authors[71,72] reported on their findings of sterile male XXY mice in 1961. This chromosome constitution also occurs in male tortoise-shell cats, though often in conjunction with other cell lines.[73]

There are, however, mammalian species that deviate from the usual XY mechanism. Some, like the wood lemming, *Myopus schisticolor*, contain fertile XY females, in addition to XX females.[74] The same applies to several South American field voles of the genus *Akodon*, in which the Y chromosome was found to contain multiple copies of the *Sry* and *Zfy* genes.[75] One of the most bizarre mechanisms is found in the mole vole, *Ellobius lutescens*. Its chromosome constitution was established by Matthey,[76,77] who found 17 chromosomes in both sexes; during male meiosis, a univalent was transmitted to one-half of the spermatids. Thus males and females appear to have an XO sex chromosome constitution,and the search for Y-chromosomal sequences has so far been unsuccessful.[78]

VII. SEX REVERSAL

Originally, the term "sex reversal" implied that a male, originally determined as such, can change into a female, and vice versa.[79] This would include sex changes occurring with age, for instance in fish, which function first as males and later as females (protogynous hermaphroditism), or the sex change may occur in the opposite direction (protandrous hermaphroditism). In more recent usage, the term is applied to individuals whose phenotypic sex appears to be in contrast to the sex chromosome constitution. According to Burgoyne,[80] XX sex reversal implies the presence of XX Sertoli cells, and XY sex reversal the presence of XY follicle cells. Many examples of XX males

and XY females are known in different mammalian species, including the human one.

Breeuwsma[81] made a study of intersexuality in domestic pigs (*Sus scrofa*), in which individuals showing varying degrees of sexual ambiguities occur relatively frequently. This results in a significant amount of economic loss, since affected animals are sterile, and their meat is often unsuitable for consumption because of "boar" flavor. The sex chromosome constitution is XX, and the external genitalia tend to show an enlarged clitoris, sometimes forming a penis. Most gonads are ovotestes or testes, and the derivatives of both Wolffian and Müllerian ducts are usually present. (see Figure 1).

In the goat (genus *Capra*), the *Polled, P*, mutation behaves as an autosomal dominant in inducing hornlessness, while homozygous females are masculinized to varying degrees.[82,83] A recent investigation of an XX, *PP* goat with male habitus, social behavior, and libido, who had ambiguous external genitalia and bilateral testes, proved negative for the presence of *Sry* and *Zfy*.[84] Absence of Y-chromosomal DNA sequences also seems to be a feature of most human patients with true hermaphroditism[85] and of a minority of patients diagnosed as "XX males."[86] On the other hand, in sex-reversed male XX mice, a condition originally thought to be due to an autosomal dominant gene, *Sxr*, transmitted through the father,[87] it is now known that *Sxr* is a piece of Y-chromosomal material that crosses over from the Y to the X chromosome in the father, thus giving rise to a sex-reversed XX offspring.[88] These males are sterile because of breakdown of spermatogenesis, and in addition their genito-anal distance is reduced,[89] implying that male sexual differentiation is adversely affected.

Other species with XX sex reversal include the mole, *Talpa occidentalis*, in which individuals with ovotestes can function as fertile females.[90] The *Sry* and *Zfy* genes were found to be absent.

Sex-reversal in the opposite direction, resulting in XY individuals exhibiting failure of masculinization and varying degrees of female development can occur, likewise, either with or without the involvement of male-determining genes, in humans as well as in mice. Most patients with XY gonadal dysgenesis have an intact Y chromosome, although a minority have a deletion or a mutation of the *SRY* gene.[91]

In mice, several different mechanisms are known to give rise to XY females and hermaphrodites. Eicher and collaborators[92] found that a Y chromosome from the *Mus domesticus poschiavinus* subgroup is unable to induce complete testicular differentiation when present on the genetic background of the inbred C57BL/6J strain. Impaired testis differentiation within the background of this strain has been traced to the absence of a 2.3-kb*Taq*I band on certain *domesticus*-type Y chromosomes.[93] Another type of XY female mouse is caused by a deletion in chromosome 17, and it was postulated that the deleted piece contains an autosomal sex-reversing gene *Tas*.[94] Other female XY mice carry

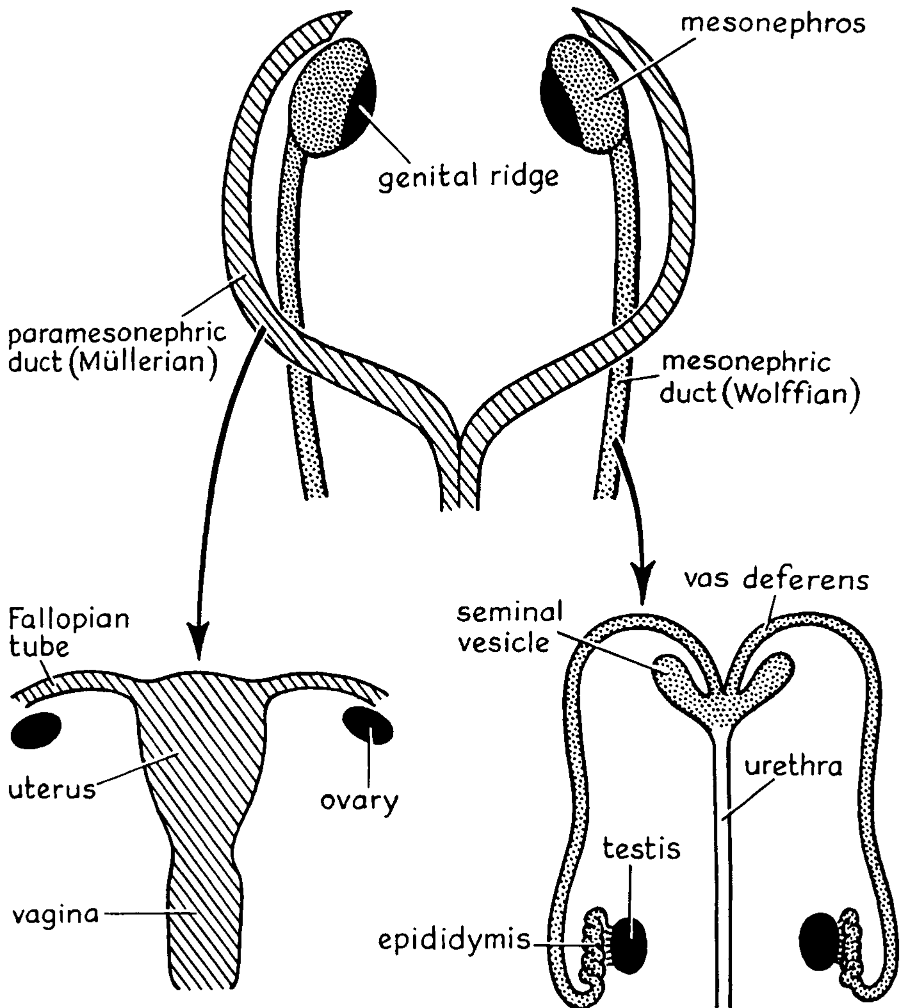

Figure 1. Differentiation of a male or female phenotype from a bipotential genital ridge and a double set of ducts. If the genital ridge differentiates into a testis, its hormonal secretions will demolish the Müllerian duct and induce the Wolffian duct to develop into vas deferens, seminal vesicle and epididymis. If testis development does not occur and an ovary is formed, in the absence of hormonal secretions, the Wolffian duct regresses and the Müllerian duct differentiates into Fallopian tubes, uterus and upper part of the vagina.

the Y^{tdym1} chromosome,[95] which contains a deletion including the testis-determining gene. These females are fertile, as are another group of XY female mice, which carry deletions in the Y chromosome situated at some distance outside the testis-determining region.[96] The latter authors regard the disruption of *Sry* action by long-range position effect to be the most likely cause of the sex reversal.

For a better understanding of sex reversal, we need to look at the developmental processes by which the embryo assumes a male or a female phenotype.

VIII. DEVELOPMENTAL PROCESSES: SEX DETERMINATION AND DIFFERENTIATION

In mammals as in other vertebrates, the principal anatomical features distinguishing the sexes—testes, vasa deferentia, seminal vesicles, prostate, and external genitalia in males, compared with ovaries, Fallopian tubes (oviducts), uterus, vagina, and external genitalia in females—show marked differences in the adult, but nevertheless derive from common structures in the embryo. The somatic cells of testes and ovaries originate from genital ridges, which form in apparently identical fashion on the mesonephroi of XX and XY embryos, and the primordial germ cells migrate into these ridges from an extra-embryonic site.[97] At this time the embryos forms two duct systems: the Wolffian (or mesonephric) duct, capable of developing into the male reproductive tract and associated structures, and the Müllerian (or paramesonephric) duct, with the potential of becoming the female reproductive tract.[98] A major task of developmental genetics is to unravel the causes that convert the seemingly bisexual embryo into an individual of one or the other sex, the process to which the terms "sex determination" and "sex differentiation" are applied.

In recent years these terms have been variously defined by different authors. For instance, Eicher and Washburn,[99] describe the process of the development of the gonads into either testes or ovaries as either "primary (gonadal) sex determination" or as "primary sex differentiation," whereas Goodfellow and Darling[100] define "sex determination" as the process resulting in either testis or ovary formation, and "sex differentiation" as the process subsequent to gonad formation. Subsequently, Peter Goodfellow and Robin Lovell-Badge[101] wrote that "Sex determination is the process of choosing either the male or the female sexual differentiation pathway". It may be appropriate, therefore, to consider the background of this terminology.

In 1939, Benjamin Willier[102] wrote as follows:

> In a consideration of the development of sex, two related problems present themselves for analysis: (1) The zygotic determination of sex, and (2) embryonic sex differentiation. By

> zygotic determination of sex is meant the primary determining constitution established in the fertilized egg by the union of the gametes ...; that is the nuclear determiners or genes which decide whether male or female sex characters shall develop. ... By sexual differentiation is meant the origin or expression of sex differences during ontogeny. More specifically, it deals with the origin and differentiation of the male and female form of the rudiments of all sex characters. At first the rudiment appears in generalized form, being laid down in the same manner in all individuals, whether determined as male or female. Subsequently they acquire the specific characteristic of peculiar to each sex.

Likewise Frank Lillie[103] distinguishes between sex determination, which he regarded as chromosomal in most cases, and sex differentiation, which in higher vertebrates is largely under the control of the sex hormones and only to a lesser extent under direct genetic control. Whereas in insects such as *Drosophila*, castration appears to have no effect on sexual characteristics, castration exerts a profound effect on the sexual phenotype in mammals and birds.

In this overview, we shall confine the term "sex determination" to the primary mechanism, which in mammals is chromosomal and particularly related to the presence or absence of a Y chromosome and specific DNA sequences that cause the development of either male or female sexual characteristics, whereas "sex differentiation" describes the processes by which the genetic blueprint gives rise to these characteristics.

Lillie[104] himself had played a major role in establishing the hormonal theory of sex differentiation through his interpretation, independently with Keller and Tandler,[105] of the origin of the bovine freemartin—the sterile female partner of a pair of cattle twins. The ovaries of the sterile heifer twins are much reduced and may even contain sterile testicular tissue, and the ovarian duct derivatives are absent.[104] Freemartins occur only if the twins share a common blood supply, and the partial masculinization of the female was assumed to be caused by testicular hormones secreted by the male fetus. But why does the bull calf escape serious sexual anomalies?

IX. ASYNCHRONY OF DEVELOPMENT AND ASYMMETRY OF HORMONE PRODUCTION BY FETAL TESTES AND OVARIES

It has long been known that testes differentiate earlier than ovaries. In an extensive study of the development of human gonads, Joseph Gillman[97] found that the differentiation of the indifferent gonad into a testis occurs typically in embryos with a crown-rump measurement of 14-16 mm, whereas the ovary only begins to resemble the adult organ late in development (twelth to sixteenth week), when the fetus has a crown-rump measurement of 120-40 mm. In other words, the testis develops precociously, whereas the development of the ovary is comparatively delayed.

Experiments by Alfred Jost[106-108] demonstrated a fundamental asymmetry of the role played by fetal testes and ovaries in inducing the development of the male and female tracts. When rabbit embryos were castrated before the time of testis differentiation on day 15, a female reproductive tract developed independently of the chromosome constitution. If the experiment was performed two days later in genetically male embryos, the development of the Müllerian duct was already inhibited, and there was some development of prostatic buds, but the external genitalia were still female. In order to achieve complete masculinization, the testes had to remain *in situ* until day 24, when a male phenotype was firmly established. These results indicated that secretions by fetal testes act to suppress the Müllerian duct and to promote the development of the Wolffian duct, whereas the converse situation results merely from the absence of testes and does not require the presence of fetal ovaries.

It became evident that some but not all of the fetal testicular secretions could be reproduced by the administration of testosterone. Exogenous androgens were found to stimulate the Wolffian duct derivatives but did not exhibit any inhibitory effect on the Müllerian duct,[108] thus raising the question of whether two different substances might be involved, one stimulating and the other inhibiting. The assumption that a second hormone was produced by the fetal testis, Müllerian inhibiting substance, or anti-Müllerian hormone, proved correct when Nathalie Josso and her collaborators[109] identified such a compound in a high molecular weight protein fraction produced by fetal Sertoli cells. Many important recent advances made on the nature of anti-Müllerian hormone and its role in sex differentiation will be described in Chapter 21.

The differentiation of the gonad itself remained seemingly unaffected by experimental procedures, and this raised the question regarding the mechanism by which the Y chromosome induces the indifferent gonad to develop into a testis. At one time Jost[110] expressed the view that testis development resulted from an accelerated rate of growth of the gonadal rudiment.

> I have come to the conclusion that the simplest explanation of gonadal differentiation would accept that some mechanism—perhaps the production of a special local hormone—correlated with the presence of a Y chromosome in the male, triggers an early and rapid development of the testis in the rudimentary sex organ, which otherwise would follow the slow pattern of development characteristic of the ovary.[110]

However, Jost did not pursue this theme. He subsequently investigated the time and manner of appearance of the seminiferous cords in the developing testis, which, he said, "is held to represent the earliest difference between males and females. It has been known for a century that testes differentiate much earlier than ovaries."[111] The first histological sign of testicular differentiation is the appearance of Sertoli cells, which proceed to encompass the primordial germ cells. In embryos of the rat, a few Sertoli cells were first seen at 13 days

and 7-9 hours after fertilization. These become more numerous, aggregate, and form seminiferous cords.[111]

Regarding the question by what mechanism the Y chromosome causes the indifferent gonad to develop the histological characteristics of the testis, Jost[112] in one of his last publications, concluded that "The testis-determining gene(s) might well play no direct role in any of the cellular processes resulting in testicular differentiation, but rather 'turn on' other genes possibly residing on different chromosomes and expressed according to the particular testicular chronology."[112] Time, evidently, is of the essence.

X. DIFFERENTIAL GROWTH OF FETAL TESTES AND OVARIES

Twenty years earlier, I had addressed the question of how sex chromosomes might function in the last chapter of my book *Sex Chromosomes,*[42] published in 1967, adding that any hypothesis on this topic was bound to be speculative. Because of the inconclusiveness of earlier hypotheses regarding sex-determining genes, the hypothesis that I put forward was not based on conventional genes giving rise to phenotypes that segregate in Mendelian ratios, but that the sex chromosomes might give rise to quantitative phenotypic differences that resulted in two qualitatively different classes, called "quasi-continuous variation" by Grüneberg,[113] or, in the terminology of Sewall Wright,[114] "threshold dichotomy." The fact that the sex chromosomes exhibit the properties of heterochromatin to a large extent seemed to support this hypothesis, since there had been an ongoing discussion about a possible relationship between heterochromatin and quantitative variation, caused by minor genes, or "polygenes," which unlike the familiar major genes could not be identified from the phenotypes of the progeny.[115]

The above considerations gave rise to the more specific proposal that for male sex differentiation to occur, the gonad needed to reach a certain size by a particular stage in development, failing which the gonad would become an ovary. The effect of the Y chromosome was envisaged to increase the number of mitoses at a critical time, while a second X chromosome might retard the growth process.[116] The first investigation bearing on this question made by my colleagues and myself consisted of measuring gonadal volumes in embryos of the rat. We found that the volumes of XY embryos were significantly larger than those of their XX litter mates already at 13.5 days, when we were unable to detect any histological sex difference.[117] After presenting these results at a meeting of the Royal Society in London,[118] I received a thesis from the University of Lund by Johannes Lindh[119] showing that testes grow faster than ovaries in embryos of the rat and the golden hamster, *Mesocricetus auratus*, from the earliest developmental stages onward. An accelerated rate of growth of developing testes compared with ovaries has since been established in other

mammalian species investigated, including those of the mouse[120,121] and of human fetuses,[122] as well as in a marsupial, the grey short-tailed opossum, *Monodelphis domestica.*[123] In the last-named species there was evidence, however, that the growth of the XY gonad was inhibited if the embryo was retained inside the mother.

XI. TESTIS-DETERMINING GENES

While accelerated growth of the XY gonad might be expected to promote the early differentiation of the testis compared with that of the ovary, the thrust of the last 20 years has been to search for genes relating to phenotypic end-points rather than to processes that might lead to such end-points. At the same time the notion of what constitutes a gene has become more fluid.[124] Combining the developmental evidence of the pivotal role of the fetal gonad in inducing the masculinization of the reproductive tract, and the cytological evidence of the association of the Y chromosome with a male phenotype (and disregarding the possibility of more than one Y-chromosomal gene being involved in inducing the testicular pathway), the concept of a "testis-determining" gene located on the Y chromosome has become widely accepted as dogma; and this gene, named *TDF* in humans and *Tdy* in the mouse, was mapped to the respective Y chromosomes before any candidate genes were identified.

When, in 1975, Stephen Wachtel[125] and his associates proposed that the minor transplantation antigen, known as H-Y antigen, might be the product of the Y-chromosomal gene causing the indifferent mammalian gonad to develop into a testis, the idea met with an enthusiastic reception and the topic became the subject of an extensive literature.[126] The hypothesis was discarded, however, when, in 1984, Anne McLaren[127] and her associates developed a strain of mice in which sterile males occurred in the absence of H-Y antigen expression.

A few years later, another candidate gene for *TDF* came on the scene, when David Page[128] and collaborators isolated a gene thought to encode a zinc-finger protein, and named *ZFY*, which was located on the short arm of the human Y chromosome, at a distance of 140 kb from the pseudo-autosomal region. This gene, too, fell out of favor with the discovery of a number of facts thought to be incompatible with a testis-determining gene. Chief among these was the finding of four XX patients, three males and one true hermaphrodite, who carried Y-derived DNA sequences excluding *ZFY*[129]; and the authors argued that exchange of Y-specific DNA next to the pseudo-autosomal boundary redefines the sex-determining region. Subsequently, the *SRY* gene was isolated from a 35-kb region of the human Y chromosome,[130] and a corresponding gene, *Sry*, was cloned from the Y chromosome of the mouse.[131] This gene, on a 14-kb genomic fragment, was found to induce testicular differentiation in three

out of 11 transgenic XX mice.[132] The successful masculinization of a proportion of XX mice by a small fragment of the Y chromosome brought the search for the testis-determining gene to an end, at least as far as the Y chromosome is concerned.

However, failure of testis development also occurs in patients with certain malformation syndromes due to autosomal or X-chromosomal genes. One of these conditions is campomelic dwarfism, or campomelic dysplasia, a severe disorder of the skeleton and frequently of other structures, causing most patients to die in the neonatal period.[133] The gene causing this condition was found to be localized on chromosome 17q24.3-q25 and named *SRA1*,[134] referring to its effect on sex reversal. Recently it was cloned independently by two groups of investigators,[135,136] who found that it was a member of the *SOX* series of *SRY*-related genes, *SOX9*. The *SOX* genes, like *SRY*, encode a protein with high mobility group (HMG) domain.

Other non-Y-chromosomal genes are likely to be involved in XY sex reversal. One of these causes failure of testis development when present in double dose on the same X chromosome. It has been named *DSS*[137] (dosage sensitive sex reversal gene). Patients with Klinefelter's syndrome escape sex reversal because of one of their X chromosomes is inactivated.

XII. DIFFERENTIAL GROWTH IN XX AND XY EMBRYOS

Although it has been widely assumed that genetically male and female embryos develop in identical fashion until a switch acts on the hitherto bipotential gonad causing it to develop into either a testis or an ovary, it has become apparent that this is not so. In recent years evidence has accumulated that the sex chromosome constitution exerts its effect on the embryo before the gonads have differentiated and, indeed, long before the genital ridges are formed.

It was found that in rat embryos aged 12 days genetic males exceeded females in weight, protein, contents and the rate of uptake of tritiated thymidine,[138] and in 9-day-old mouse neurulae, most embryos with 0-5 somites had XX chromosomes, while XY embryos predominated in the 6-12 somite class.[139] A longitudinal ultrasound study by Fog Pedersen,[140] measuring crown-rump lengths and biparietal diameters of the skull showed that female fetuses between 8 and 12 menstrual weeks were one day behind the male fetuses, and that this difference rose to 6-7 days at term. Extrapolation of the curve led the author to suggest that the slightly lower growth rate of the female fetuses may already be encoded at conception by the sex chromosomes. This suggestion has now been confirmed.

Tsunoda and colleagues[141] divided mouse blastocysts into three groups according to their rate of development and implanted them into foster mothers. They found that most fast-developing blastocysts developed into males and

most slow-developing blastocysts into females, while the intermediate group consisted of similar numbers of males and females. Similar results were obtained by Zwingman and colleagues,[142] who flushed embryos from the oviduct on day one and cultured them to the blastocyst stage, when their genetic sex was determined by PCR. There was a strong correlation between fast development and presence of a Y chromosome.

These results raise the question of whether the advanced development of the genetically male blastocysts is due to a faster developmental rate of the XY embryo or to the earlier occurrence of fertilization by a Y-bearing sperm. This question was answered by experiments on bovine embryos fertilized *in vitro*.[143-145] These investigations showed that fast-developing blastocysts were predominantly XY and slow-developing blastocysts mostly XX, thus providing strong evidence that the sex chromosomes affect the developmental rate at an early stage. This effect has recently been demonstrated also in human embryos fertilized *in vitro*, when it was found that the likelihood of a live-born male was greater than for a female if, at the time of transfer, the embryo contained at least four cells.[146]

XIII. PROBLEMS IN SEARCH OF A SOLUTION

The exact causes for the difference in developmental rates between XX and XY embryos remain to be worked out. However, there is evidence that the genes of the sex-determining region, *Sry* and *Zfy*, are transcribed at the two-cell stage in mouse embryos,[142] and that *SRY* is transcribed in early human embryos,[147] thus raising the question whether these genes may have the property of growth factors. This would be in accordance with the finding by Burgoyne[148] of a Y-chromosomal effect in increasing the number of cells in murine blastocysts.

Although it was originally thought that the expression of the Y-chromosomal sex-determining gene is confined to the genital ridge in embryos, apart from the adult testis,[149] transcription has since been detected in human prostate adenocarcinoma and benign prostate hypertrophy,[150] as well as in a large number of human fetal and certain adult tissues.[151] This increases the likelihood of a more generalized function of the gene in embryonic growth and/or metabolism. Whether or not this is so will presumably become clearer once it becomes known which genes are affected by the product of the *SRY* gene.[100]

The supposition that the Y chromosome carries genes acting as growth factors could explain a number of problems that at present seem puzzling. For instance, the accelerated growth of the XY embryos can be simply explained as an early preparation to increase the chance of the developing gonad to reach the threshold of testicular differentiation,[152] a process that requires a Y-chromosomal input to accelerate the rate of development of the

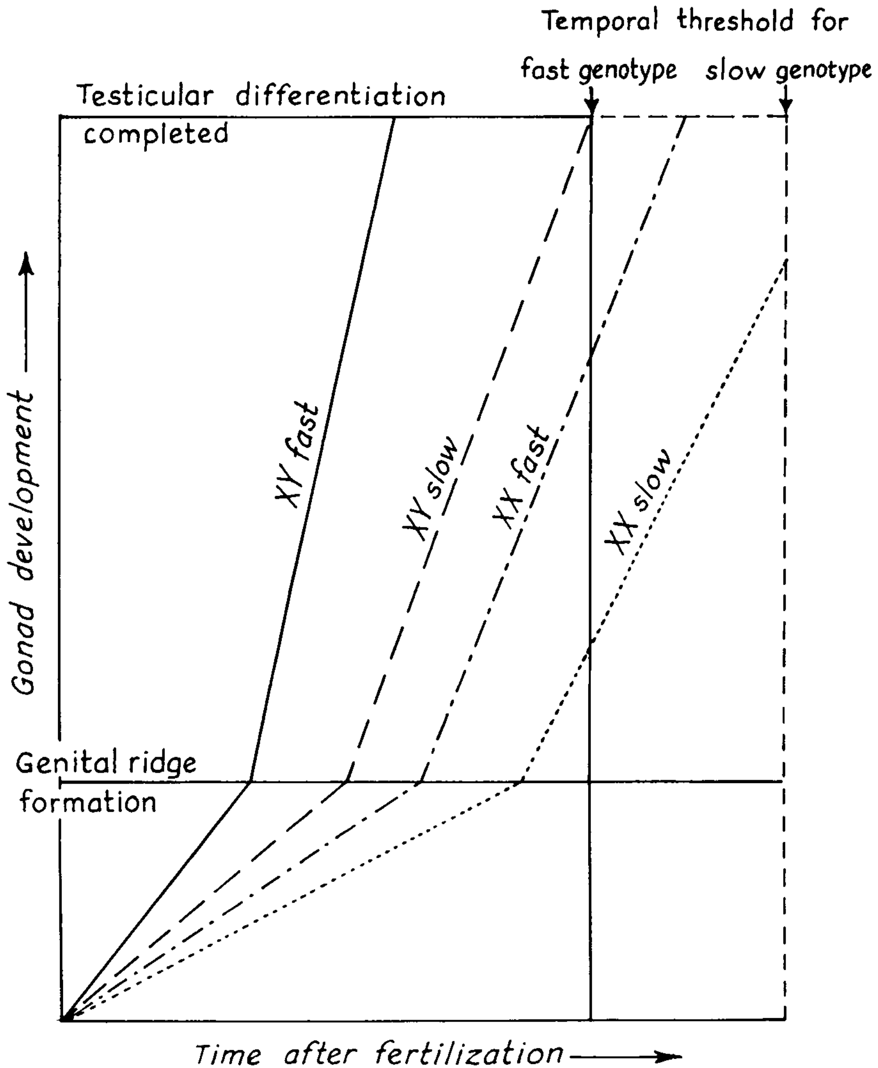

Figure 2. Model illustrating temporal and developmental thresholds for testicular differentiation in fast-growing and slow-growing genotypes. In the fast-growing XY embryo, testicular differentiation is completed well within the temporal threshold. In the slow-growing XY genotype, testicular differentiation is completed in time for the slow genotype, but only just on the threshold of the fast genotype. Hence a Y chromosome of a slow-growing genotype on a background of a fast-growing genotype results in frequent failure of testicular differentiation. Conversely, a fast-growing XX gonad may differentiate testicular tissue in a slow-growing genetic background, while a slow-growing XX gonad will differentiate into an ovary.

developing gonad in relation to the combined autosomal and X-chromosomal input. This model also helps us to understand the failure of the Y chromosome from the *poschiavinus* variety of *Mus musculus domesticus,* when on a background of autosomes and an X chromosome from C57Bl/6J, to ensure testicular differentiation;[92] this failure cannot be explained by the action of a single autosomal gene.[153]

In the model illustrated in Figure 2, two embryonic developmental rates, fast and slow, are each followed by two gonadal developmental rates, again fast and slow. It is assumed that a threshold dichotomy will ensure that for testis development to occur, the gonad will need to reach a given stage of development by a given time, failing which the gonad will become an ovary. In any one species, an XY chromosome constitution will result in fast growth of the embryo and gonads and hence testicular differentiation, while the presence of XX chromosomes will result in slower growth and ovarian differentiation; intermediate growth rates carry the risk of "sex reversal." Obviously, though, "fast" and "slow" are relative terms that need to be defined for each species. Since *poschiavinus* mice develop more slowly than C57BL/6J mice, their testes will also be expected to differentiate more slowly, and the threshold for testis development will be reached later. Hence a Y-chromosomal testis-determining gene would not accelerate growth sufficiently in relation to the autosomes and X chromosomes of C57BL/6J, which are attuned to a faster rate of development.

Differences in developmental rates could provide a link between the sex-determining system in mammals, in most of which the process of sex differentiation occurs inside the hormonal environment of the uterus, and the sex-determining systems in other vertebrates, in which the major part of development occurs outside the mother.[154] Temperature-dependent sex determinism in reptiles with be discussed in Chapter 11, while the genetic mechanism underlying the sex chromosomes of birds is in need of further investigation.

XIV. CONCLUSION AND OUTLOOK

The twentieth century has seen the discovery of sex chromosomes and the rise of genetics. Whereas the early geneticists tended to concentrate on mutations, many of which gave rise to defective enzymes and hence easily recognizable phenotypes, recent advances in molecular genetics have led to the isolation of an increasing number of genes that are active in development, but whose mode of action is not yet understood. These genes tend to interact with others, as well as with environmental factors, and for that reason show a greater resemblance to that part of the genotype that was previously referred to as "minor genes" than to "major" genes giving rise to discreet Mendelian ratios.

Whereas the latter were the only genes that could be methodologically handled in classical genetics, molecular genetics has the methodology to identify genes whose phenotypic effects are much more dependent on genetic background and environmental factors.

Just as sex differentiation is a gradual process during which the embryo assumes either a male or a female phenotype, our understanding of this process is likewise becoming modified as more experimental data become available. We are beginning to perceive a connecting link between the sex-determining mechanism in mammals and the environmentally-induced sex differentiation in different reptiles. On the basis of the many recent advances in sex determination described in the following chapters, we can be confident that our understanding of the subject in the next century will be biologically more meaningful than any model that is available today.

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THE TESTIS DETERMINING GENE, *SRY*

Michael O'Neill and Andrew Sinclair

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I. INTRODUCTION

Determination of sex is one of the most fundamental developmental events for any organism. It sets the course for the entire life history of an individual within a population. Indeed, the restrictions on behavior and physiology associated with sexual reproduction are what define populations as species. In humans, sexual development and sexual identity have a profound effect on the psychology of an individual and their interactions with the society in which they live. Elucidating the mechanisms of sex determination, therefore, has been among the highest priorities of biologists from a variety of disciplines; from geneticists to physiologists and clinical scientists. Decades of work on this topic has resulted in the identification of genes and a genetic hierarchy that have not only broadened our understanding of sex determination, but also have revealed some of the beauty and complexity of developmental gene expression.

Molecular genetic research into the mechanisms of sex determination has been particularly successful in the fruit fly; *Drosophila melanogaster*, the nematode; *Caenorhabditis elgans*, and more recently in mammals. In this chapter we will review the past four decades of research into mammalian sex determination which developed into the search for a Testis Determining Factor (*TDF* in humans and *Tdy* in mice) and ultimately led to the isolation of *SRY* (sex determining region Y gene). We will also present experimental evidence confirming that *SRY* is *TDF*. In addition, we will discuss; recent studies implicating *SRY* as an important regulator of gene expression, the search for its downstream target genes, and its place in the testis determining pathway.

While it can now be stated with certainty that *SRY* is the mammalian Testis Determining Factor (*TDF*), how it operates remains a mystery. It is the first and most important piece in a puzzle that will involve many more years of research. The first step in understanding mammalian sex determination took place nearly 50 years ago in series of technically difficult and insightful experiments conducted by Alfred Jost.

II. EARLY EXPERIMENTS IN MAMMALIAN SEX DETERMINATION

In 1947 Jost[1] discovered that, in mammals, the development of the testis is central to male development. Jost castrated sexually indeterminate embryonic rabbits *in utero* and observed that they all developed as females. These results indicated that the "default" sex is female and that male development must depend on the presence of a testis in the embryo. Thus in mammals, sex determination is equated with testis determination. Clinical observations more than 10 years later were to establish the essential role of the Y chromosome in this process.

In 1959 Ford and colleagues[2] noticed the abnormal karyotype of patients with Turner's syndrome. Affected individuals have an XO karyotype (hemizygous for the X and lacking the Y chromosome) and are invariably female and sterile. In that same year Jacobs and Strong[3] found that patients with Klinefelter's syndrome, who are phenotypically male, have an XXY karyotype. Taken together, these observations showed the number of X chromosomes was irrelevant to development of the female phenotype but that the Y was absolutely necessary for development as a male. Consequently, it was deduced that the Y chromosome must harbour a gene(s) necessary for development of the testis. In the years to follow, genetic analysis of sex reversed humans and mice led to the hypothesis of a single locus on the Y chromosome required for testis determination, namely, the Testis Determining Factor, *TDF*.

III. STRUCTURE OF THE HUMAN Y CHROMOSOME

All chromosome pairs undergo recombinational exchange of genetic material at meiosis. The *TDF* gene must, by its nature, escape this exchange and thereby remain exclusively on the Y chromosome and in the male genetic lineage. Burgoyne[4] discovered that the X and Y chromosomes pair over a small portion of their lengths during meiosis. This region, known as the *pseudoautosomal region* (*PAR*), must necessarily exclude *TDF*. The sex determining portion of the Y chromosome is separated from the *PAR* by a distinct delineation known as the *pseudoautosomal boundary* on the Y (*PABY*).[5] Even though *TDF* could be excluded from the *PAR*, there remained the bulk of the Y chromosome to which it could map. Initially, the lack of a well defined locus for *TDF* meant that virtually any sequence cloned from the sex-determining portion of the Y was a potential candidate for *TDF*. A gene that was Y-linked in all mammals was a particularly good candidate for *TDF*.

IV. EARLY CANDIDATES FOR *TDF*

The *H-Y* antigen[6], was found to be expressed on the surface of certain cells only in male mammals, mapped to the Y chromosome and was the first candidate for *TDF*, a position it retained for 10 years. It was not until 1984 when McLaren and colleagues[7] discovered male mice with testes which lacked *H-Y* antigen that it fell out of favor as a candidate for *TDF*. During this time another candidate was put forward, the *Bkm* repeat sequences[8]. This repetitive satellite sequence originally cloned from a snake, the banded krait, was found on the sex chromosomes of many mammals. Sequence analysis showed the *Bkm* satellite to be largely composed of tetranucleotides GACA and GATA[9]. However, the human Y chromosome was shown to lack *Bkm* sequences and

for this reason *Bkm* was abandoned as *TDF*[10]. It became apparent that *TDF* would be isolated only when a strictly defined sex-determining region on the Y chromosome was identified.

V. MAPPING THE HUMAN Y CHROMOSOME

Through the 1980s the rapid advance of molecular genetics and an increasingly large sex-reversed patient base allowed molecular biologists in collaboration with clinical scientists to finely map *TDF* on the human Y chromosome. With the refinement of genetic mapping, *TDF* could be precisely localized using DNA from sex reversed individuals. On rare occasions, aberrant exchange between the X and Y occurs beyond the *PAB*. Such aberrant meiotic exchanges can result in sex reversal either by transferring the testis determining portion of the Y to the X producing an XX male or by the deletion of this region from the Y resulting in an XY female (see Figure 1). By analyzing the extent of Y chromosome deleted in XY females, *TDF* was localized to the short arm of the Y chromosome (Yp)[11]. Finer localization of *TDF* was achieved in three phases: first, construction of a deletion map based on analysis of sex-reversed

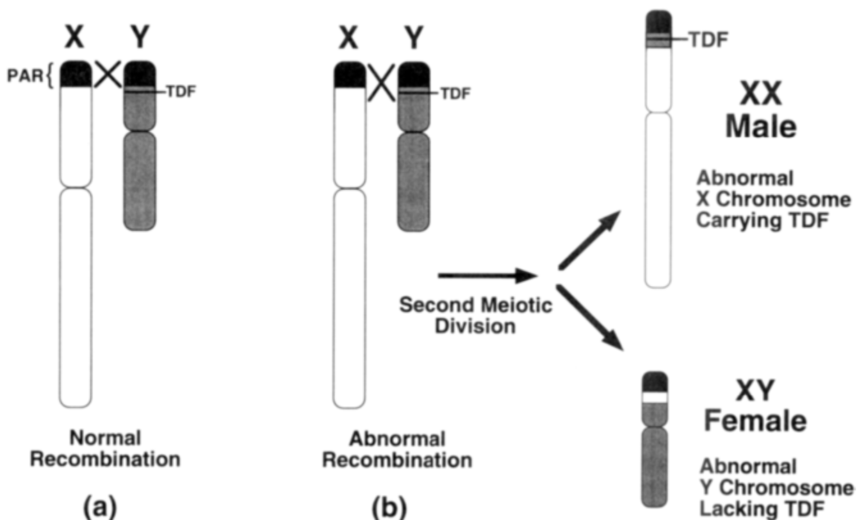

Figure 1. (a) Normal recombination between the X and Y chromosome, distal to the pseudoautosomal boundary. On rare occasions exchange occurs proximal to the pseudoautosomal boundary involving the *TDF* (b) If either gamete (c) produced from this aberrant recombination fertilizes the ovum the resultant fetus will be sex reversed.

XX males and XY females; second, a meiotic map of the pseudoautosomal region which allows ordering of loci within this region[12, 13]; and third, a long range restriction map linking the other two maps[14], stretching from the pseudoautosomal region across the *PABY* through the area delineated by the deletions.

The physical deletion map limited *TDF* to the most distal part of the Y-specific region, adjacent to the *PABY*. The meiotic map had identified the pseudoautosomal *MIC2* gene as the closest marker within the *PAR* to the sex-determining region defined by the deletion map[12]. The long-range restriction map linked the physical deletion map and the meiotic map, which are based on different principles. The long range restriction map identified a CpG rich island within the sex determining region of the Y chromosome[14]. CpG islands are often associated with expressed genes and therefore this newly discovered Y-specific island was the best place to start the search for *TDF*.

VI. WALKING TO *TDF*

Using this mapping information, chromosomal walks were initiated simultaneously in the laboratories of Peter Goodfellow and David Page. Goodfellow's group chose *MIC2* as their starting point to walk across the pseudoautosomal boundary toward the Y-specific CpG island in the sex-determining region. Contiguous overlapping genomic segments covering the entire region were cloned and restriction mapped. Page's group started their walk on the opposite side (centromeric) to the CpG island identified by Pritchard and colleagues[14]. Page's group also used a deletion map comprised of two patients, an XY female (WHT 1013) and an XX male (LGL 203). This deletion map delimited *TDF* to a 150 kb region of Y specific chromatin within the vicinity of the CpG island (see Figure 2). Overlapping walk clones from within this region of the Y chromosome were analyzed and revealed a gene, which they named *ZFY*, (Zinc Fingers Y) that seemed to fulfil the requirements of *TDF*[15]. *ZFY* has 13 zinc finger domains which are capable of binding to DNA, a nuclear localization signal and a transcriptional activator domain. *TDF* was expected to regulate the transcription of other genes because it was believed to act as a "switch" in a bifurcate developmental pathway. Such domains identify *ZFY* as a transcription factor. In addition, *ZFY* was found to map to the Y chromosome of all eutherian mammals examined, suggesting it played some critical role in male development.[15]

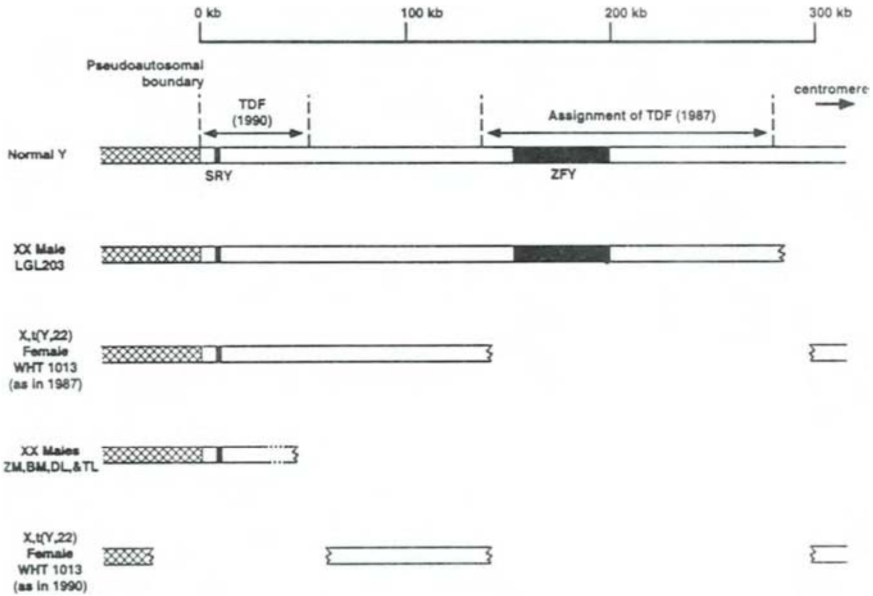

Figure 2. Schematic map of the distal portion of the Y chromosome containing the region of *TDF* as determined by deletions in XY females and in XX males with small regions of the Y. Of particular note is the reassignment of *TDF* from 1987 to 1990 (see text).

VII. *ZFY* EXPRESSION

A shadow was first cast on the notion that *ZFY* was *TDF* when a homologue was found on the X chromosome (*ZFX*)[16, 17]. The remarkable sequence similarity between *ZFY* and *ZFX* led to the hypothesis that sex was being determined by gene dosage. According to this hypothesis, X inactivation would result in females with only one active member of the pair (*ZFX*), while males would have two active genes (*ZFY and ZFX*). This hypothesis was dismissed when *ZFX* was found to escape X inactivation[16]. *ZFY* also had an expression profile inconsistent with a developmentally regulated gene, as it was shown to be expressed in a variety of different tissues, both fetal and adult[16, 18]. This pattern of expression is more often found in genes that have a general cellular function rather than a specific developmental role.

VIII. *ZFY* IS AUTOSOMAL IN MARSUPIALS

ZFY was shown to be Y linked in a wide variety of eutherian mammals and so it was logical to ask if it mapped to the Y chromosome in marsupials which

also have an absolute requirement for the Y chromosome in testis determination. Southern analysis confirmed that the marsupial genome had sequences homologous to *ZFY* but the marsupial homologues were not male specific. Mapping by *in situ* hybridization confirmed that *ZFY* was not on the marsupial Y chromosome but was located on the autosomes in two different species of marsupial.[19] This suprising result could be interpreted to mean that either marsupials had a different sex determining mechanism from eutherian mammals or that *ZFY* was not the testis determining gene.

IX. DEFINING A NEW SEX DETERMINING REGION EXCLUDING *ZFY*

Although, *ZFY* had many inconsistencies as a putative *TDF*, it was still the only gene found in the sex determining region delimited by the deletion map of the XX male LGL 203 and XY female WHT 1013. The XY female WHT 1013 had a Y:22 translocation and it was possible that she may have a more complex explanation for her sex reversal. Goodfellow and colleagues proposed that it might be possible that normal *TDF* was present in this patient but was inactivated by a position effect of the translocation. Additionally, some XX males had been shown to lack *ZFY*. This can be explained by the presence of downstream "gain-of-function" mutations leading to male development. Another possibility was that *TDF* lay distal to *ZFY*, between *ZFY* and the *PAB*. If this were the case then some of those XX males lacking *ZFY* must have Y specific chromatin distal to *ZFY*. Consequently, a panel of 14 *ZFY* negative XX males and intersexes (all of whom had testes) were examined by Goodfellow's laboratory for the presence of the Y chromosome boundary and markers between the boundary and *ZFY*. Among these XX males negative for *ZFY*, four (ZM, BM, DL, and TL) were found who had retained the Y specific region just proximal to the *PAB*[20] (see Figure 2). This result formally excluded *ZFY* as a candidate for *TDF* and, most importantly, redefined the sex-determining region on the Y chromosome. Therefore, it was possible to assign *TDF* to 35 kb of the Y specific region adjacent to the *PAB*.[21]. This implied a discrepancy between this newly defined sex determining region and that previously defined by Page and colleagues.[15] Subsequent, re-evaluation of patient WHT 1013 by Page and colleagues[22] revealed a second deletion of approximately 45 kb extending from the *PAB* into the newly defined Y-specific sex determining region (see Figure 2) .

The four XX males ranged in phenotype from a normal male with undescended testes, lacking germ cells, to a hermaphrodite with bilateral ovotestes and uterus. A number of hypotheses have been proposed to account for this phenotypic variation. It could be due to another gene on the Y chromosome that contributes to, but is not absolutely necessary for, testicular development.[23] Other possible

explanations include breakpoints on the Y chromosome that partially disrupt *TDF* expression, or the translocated Y fragment on the paternal X chromosome may be affected by spreading X-inactivation. Whatever the cause, phenotypic variation among sex reversed individuals is one of the major areas of current research in mammalian sex determination.

X. CLONING *SRY*, A NEW CANDIDATE FOR *TDF* ON THE HUMAN Y CHROMOSOME

In their chromosomal walk from *MIC2* into the Y-specific region Goodfellow's group had obtained overlapping lambda and cosmid clones of the now narrowly defined sex determining region. On the original walk they had not found any genes in this region as their sights were focused on the distant but alluring CpG island. The region was now carefully reexamined by subcloning the entire 35 kb into fragments approximately 4 kb in length. The subclones were then digested using frequent cutting restriction enzymes into segments from 500 bp to 1 kb. These short segments were then used as probes on Southern blots of male and female DNA from a variety of mammalian species. In this manner, 50 probes from the sex-determining region were analyzed. The objective was to find sequences that gave a unique, male-specific band in all mammalian species. To prevent hybridization to repetitive elements probes were blocked with total human DNA. Seven probes detected single Y specific bands in human DNA. Only one probe pY53.3 detected a single Y (male) specific band in human, bovine, and mouse DNA. The probe pY53.3 also detected a unique band in the four XX males who lacked *ZFY*. The gene detected by the probe pY53.3 was named *SRY* (for Sex determining Region Y) based on its location.[21]

Sequences homologous to *SRY* were detected on the Y chromosome in all of the eutherian mammals tested.[21] The mouse equivalent, *Sry*, was detected in *Sxr'* mice which carry the smallest known murine sex reversing segment on the Y chromosome. In addition, *Sry* was deleted from a mutant mouse Y^{tdym1} chromosome which was no longer sex determining and which lacks *Tdy*.[24] Unlike *ZFY*, *SRY* was subsequently found on the Y chromosome of metatherian (marsupial) mammals as well.[25] Here was a new candidate for *TDF* which so far fulfilled the expectations of map location and conservation on the Y chromosome of all mammals, yet nothing was known of its possible function or whether it was an expressed gene.

XI. IDENTIFICATION OF THE SRY CODING SEQUENCE

Two possible open reading frames (ORFs) were identified within the genomic sequence of *SRY*; one of 99 amino acids and another of 223. The two ORFs

overlap in different frames and read 5' to 3' from the centromere to the PAB.[21] When the nucleotide sequence of *SRY* was compared with the EMBL database no significant homologies emerged. The two potential open reading frames were then compared with the PIR protein database. The 99 amino acid sequence showed no significant homology to any sequence in the database. However, the 223 amino acid sequence revealed several proteins which showed homology over a 79 amino acid motif.[21] This motif, known as the HMG-box was originally identified in high mobility group proteins 1 and 2[26] and the human nucleolar transcription factor, hUBF.[27] Subsequently, *HMG-boxes* have been found in a variety of DNA-binding proteins, including several transcription factors.[28] This finding suggested that *SRY* may act as a transcriptional regulator of other genes in the sex determining pathway.

SRY's putative open reading frame of 223 amino acids could only be significant if it was conserved in all mammals. When it was compared to the sequences of the Y specific clones from rabbit[21] and mouse[24] only about 50% homology was found outside of HMG box motif. Strikingly, within the box, homology was approximately 90% . When marsupial Y specific clones were compared they too were very similar to the human *SRY* HMG box at about 85%.[25] The high degree of sequence conservation within the DNA-binding domain suggested that *SRY* was an expressed gene.

XII. PROOF: *SRY* IS *TDF*

SRY was an excellent candidate for *TDF* because it mapped to the smallest sex-determining region of the human and murine Y chromosome, its Y linkage was conserved in all mammals, including marsupials and it encoded a DNA-binding motif. Still, definitive biological evidence was needed to prove that it was in fact *TDF*. This proof came in two forms; (1) sex-reversing mutations in the *SRY* gene of XY female patients, and (2) sex reversed transgenic mice carrying the *Sry* gene alone.

XIII. SEX-REVERSING MUTATIONS IN *SRY*

If *SRY* was *TDF* then some of those XY females carrying the *SRY* gene might be expected to have mutations in their *SRY* gene. Consequently, *SRY* was subjected to molecular analysis in a number of XY females. Genomic DNA from XY females was PCR amplified using *SRY* specific primers and was assayed for mutations by single-strand conformation polymorphism (SSCP) and ultimately by DNA sequencing.[29] Initially, 50 males were used to establish the "normal" male SSCP pattern. Two XY females carrying *SRY* (AA and JN) showed band shifts in initial SSCP analysis. *SRY* was also analyzed from the fathers of these patients and while AA's father was normal, JN's father

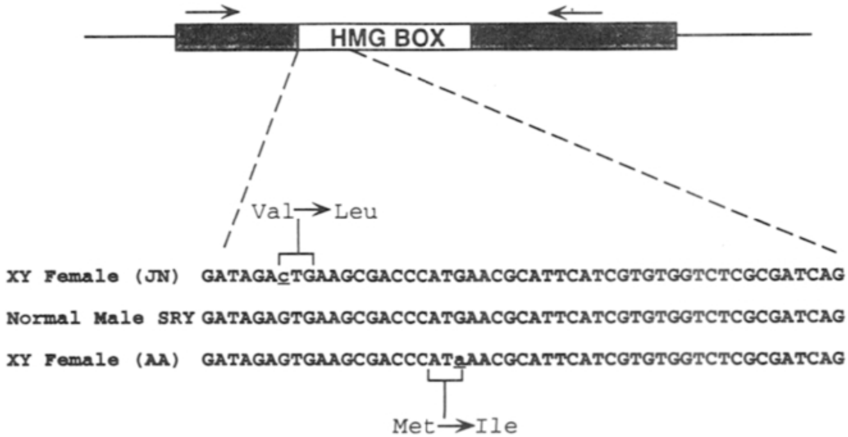

Figure 3. Boxed area shows SRY coding sequence with the DNA binding HMG box labeled. Arrows above boxed area show location of primers for PCR amplification of the fragment of the gene analyzed by SSCP. Enlarged region shows mutations in the HMG box of *SRY* in individuals AA and JN.

showed a band shift identical to hers. Subsequent cloning and sequencing of the *SRY* gene from AA and JN identified a mutation within the HMG box of *SRY* in each patient as well as one in the father of JN (Figure 3). The *de novo* mutation in the XY female AA indicates that mutations in *SRY* result in sex reversal and that *SRY* is required for normal testis development.

Since the initial studies more than 20 sex-reversing mutations have been identified in the *SRY* gene.[30] In all but one case, sex-reversing mutations occurred in the HMG box. Gel mobility shift assays studying five mutations (3 *de novo* and 2 inherited) showed that DNA-binding activity was abolished in four of the five and significantly diminished in one. All of the *de novo* mutations lacked any capacity to bind the *SRY* consensus sequence. The apparently inherited mutation of JN also completely lacked DNA-binding activity while another inherited mutation showed some ability to bind the target sequence.[31] While incomplete penetrance of the latter can be explained by diminished DNA-binding capacity, the incomplete penetrance in the case of JN and her father can be explained by a sex reversing inherited mutation which is influenced by genetic background. Most mutations detected in these studies were *de novo*. Interestingly, only about 15% of XY females in the studies showed mutations in the *SRY* open reading frame.[30] The remaining 85% of XY females may have either mutations outside of the coding region, perhaps in transcriptional control elements or carry mutations in genes downstream

of SRY. The one mutation found outside the HMG box is 3' to the DNA binding motif. This mutation is unlikely to affect binding but indicates a new region of the gene with functional importance.[32] In addition, McElreavey and colleagues have identified a large 1.8 kb deletion 5' to the *SRY* transcription unit in an XY female.[33] Presumabley, this deletion affects control sequences which regulate *SRY* transcription levels. Some sex reversed mice have also been shown to contain deletions outside the *Sry* transcription unit which abolish gene expression and also are likely to be affecting regulatory elements.[34]

XIV. SEX-REVERSED MICE TRANSGENIC FOR *Sry*

The identification of loss of function mutations in the *SRY* gene of XY females clearly shows that *SRY* is necessary for normal testis development. Proving *SRY* is alone sufficient to initiate testis development was not as straightforward. It was possible that XX males carrying a portion of the Y chromosome carried Y genes in addition to *SRY* that were required for testis development. To find out if the *Sry* gene, alone, could induce sex reversal in XX mice a 14.6 kb genomic fragment of mouse *Sry* was used to create transgenic mice.[35] This fragment was used in the hope that it included all the regulatory elements needed for correct expression. In addition, the fragment was sequenced and there were no genes other than *Sry* present. About 25% of XX mice carrying the *Sry* transgene were sex reversed. The presence of *Sry* in these mice led to development of a full male phenotype with small testes and display of normal male copulatory behavior. The transgenic mice are sterile, however, as they lack Y sequences necessary for proper spermatogenesis and two X chromosomes are known to be incompatible with male fertility. The lack of germ cells (which make up the bulk of the testis volume) in these mice accounts for the small size of the testes in these sex reversed animals and has similar histological features to the testes of human XX males. It is assumed that the failure of the 14.6 kb fragment to reverse sex in all cases is due to position effects diminishing expression of the construct dependent upon sites of integration. The successful sex-reversed transgenic experiments demonstrate that *Sry* is the only Y gene required for testis development and that other genes in the testis determining pathway are on the X or autosomes and by definition will be present in both males and females.

Transgenic mice were also produced using a human *SRY* gene construct but sex reversal did not occur even though the transgene was expressed in the genital ridge.[35] One possible explanation is that the mouse and human *SRY* genes differ outside the HMG box region and this may reflect functional species differences.

However, the production of sex reversed XX mice transgenic for *Sry* was proof that *Sry* alone is the only gene on the Y chromosome capable of initiating

a cascade of events resulting in testis formation. Consequently, it can be stated that *Sry* is *Tdy* in mice and *SRY* is *TDF* in humans.

XV. THE *SRY* TRANSCRIPT AND EXPRESSION PROFILE

Several unusual features about the *SRY* gene made difficult the task of characterizing its mRNA. *SRY* appears to be a single exon gene, which codes for a protein of 204 amino acids.[21,36] Northern blot analysis showed human *SRY* to be a 1.1 kb testis-specific transcript in adult testis.[21] Subsequent analysis showed the *SRY* transcript was 0.9kb.[36] Recent experiments have identified at least two transcription start sites used in human embryonic and adult tissues. Using 5' RACE-PCR and RNase protection assays, Vilain and colleagues[37] identified a transcription start site, utilized in adult testis, which was 91 bp upstream of the initiation codon . Clepet and colleagues[36] confirmed this start site as the major transcription start in adult testis by 5' primer walking PCR and identified another minor start at least 410 bp upstream of the major site. Additionally, Su and Lau[38] identified initiation sites at 78 and 137 bp upstream of the start ATG using primer extension analysis on mouse cell lines engineered to overexpress human *SRY*. The sites identified by Su and Lau have not been confirmed *in vivo* and may not reflect authentic start sites for *SRY* transcription in human tissues where the gene is normally expressed.

Initial experiments to localize and quantitate *SRY* message proved difficult because of the extremely low level of expression of the gene. Using RT-PCR on mouse embryonic RNA, Koopman and colleagues[39] detected *Sry* expression between 10.5 and 12.5 days *post coitum* (d.p.c.) with peak expression at 11.5 days. This expression profile for *Sry* precedes by one day the morphological differentiation from the genital ridge into a testis which occurs at 12 days d.p.c. Using RT-PCR (less than 40 cycles) *Sry* transcripts were not detected anywhere else in the fetal mouse or in adult tissues with the exception of adult testis. However, by using a high cycle number for the RT-PCR assay Clepet and colleagues[36] were able to detect low levels of SRY expression in several fetal and adult tissues outside the developing and mature gonad. The otherwise normal phenotype of XY sex-reversed females who lack *SRY* expression argues against a fundamental role for this gene outside the testis. *SRY* gene expression has also been detected in pre-implantation embryos but the significance of this is unclear.[40]

Localization of the *Sry* message to genital ridge was confirmed by *in situ* hybridization.[39] *Sry* expression was localized to the somatic portion of the developing testis as mice homozygous for the white spotting mutation (W^e) lack germ cells and yet still displayed *Sry* expression.[39] However, in adult mouse testis *Sry* expression seems to be germ cell dependent, primarily in pre- and post-meiotic germ cells.[39,41] Interestingly, the major transcript in adult mouse

testis appears to be circular.[42] This aberrant message is probably due to the unusual genomic structure of the mouse *Sry* gene. The coding region of *Sry* is surrounded by an inverted repeat spanning ~15 kb. The circular message which comprises > 95% of *Sry* transcripts in adult mouse testis is probably the result of splicing a stem loop structure formed by a primary transcript including at least part of the inverted repeat. This strange circular transcript is only seen in mice and represents a minor component of *Sry* transcription in embryonic testis and is unlikely to be translated since it is not associated with polysomes (Figure 4).

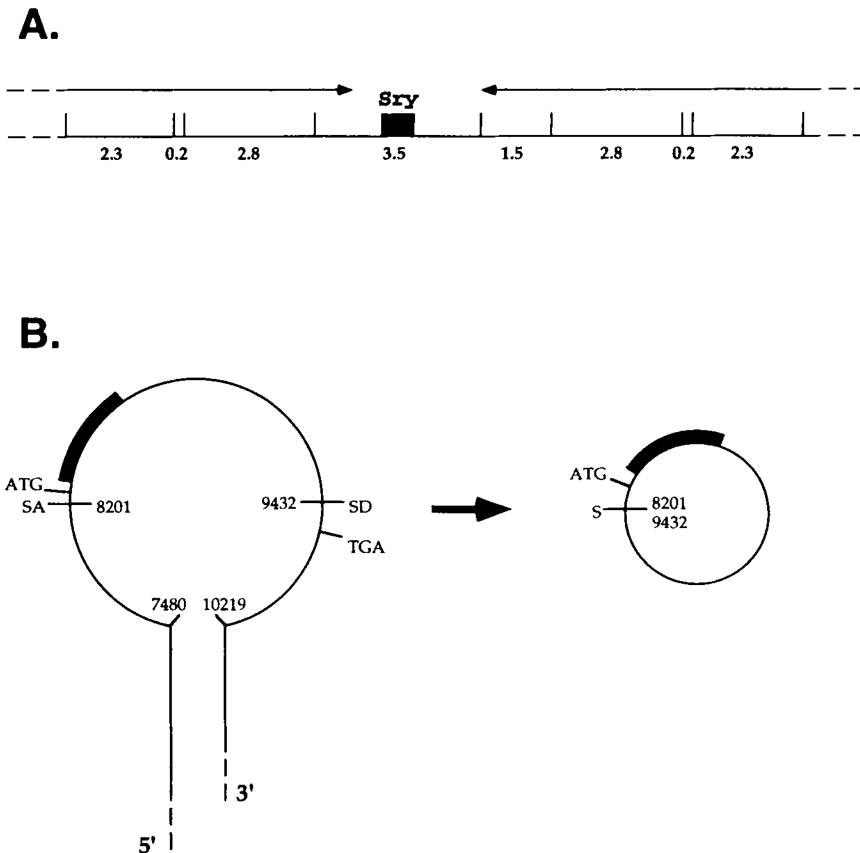

Figure 4. (A) Eco RI restriction map of the inverted repeat region surrounding mouse *Sry*. Transcription through this region in adult mouse testis leads to formation of a stem-loop structure (B) which is resolved by splicing into a circular transcript (reprinted with permission from Capel and colleagues[42] and *Cell*).

SRY is the testis-determining gene in mammals but it should be remembered that many other genes (not on the Y chromosome) act in conjunction with *SRY* to direct testis determination. The precise control of *SRY* expression and tissue specificity implies that other genes are regulating *SRY* expression "upstream" in the sex determining pathway.

XVI. *SRY* PROTEIN AND DNA BINDING

SRY protein has been shown to bind DNA in a sequence specific manner, and this binding activity is necessary for normal testis development.[31] Like other members of the HMG box family of DNA-binding proteins, SRY also has the ability to bind in a non-sequence-specific manner to cruciform DNA.[43] This latter activity's significance is not known, but may be a general feature of HMG box proteins. The sequence specific binding of SRY protein occurs by a novel mechanism. King and Weiss[44] showed by two-dimensional NMR spectroscopy that SRY protein binds by partial intercalation into the minor groove of its target sequence. They proposed that SRY protein inserts an a helix into a widened minor groove at the center of a sharp DNA bend. The ability of other HMG box proteins to bend DNA by sequence specific binding has been reported.[45] Interestingly, human and mouse *SRY* protein appears to bind DNA with slightly different dynamics.[46] Mouse *SRY* protein (mSry) shows more extensive major groove contacts with DNA and a higher degree of sequence specificity than human SRY protein (hSry). Additionally, mSRY induces a sharper bend (85°) than hSRY (65°) when bound to the same sequence. This bending of DNA suggests that SRY could bring otherwise distant regions of the genome into juxtaposition and so assist in bringing together components of the transcriptional machinery. SRY DNA binding and bending properties have been examined in an XY female with a mutation which results in a 100-fold reduction in binding without affecting DNA bending properties. Another XY female mutation shows a decrease in DNA binding but altered bending properties suggesting the two properties can be separated.[47]

Both Giese and colleagues[46] and Harley and colleagues[48] were able to identify a consensus binding site for human SRY which is: A/TAACAAT/A. This site has been identified in the promoter sequences of many genes including several candidate downstream targets of *SRY* including Mullerian Inhibiting Substance (*MIS*) and P450 aromatase. Both *MIS* and aromatase have crucial roles in sexual development. Aromatase converts testosterone to estradiol during female gonadal development and is down regulated in males. *MIS* is expressed from Sertoli cells at 12.5 d.p.c. in the mouse and induces regression of the Müllerian ducts during male development. As *SRY* and *MIS* show an overlap in expression it was suggested that *SRY* may activate *MIS*. However, Sertoli cell nuclear extracts have shown the presence of a factor which binds

to the *MIS* promoter but at a site different to the one bound by SRY.[49] Further work showed *SRY*-dependent activation of constructs containing the *MIS* promoter.[50] Mutation of the *MIS* promoter *SRY* binding site did not abolish activation *in vitro*, demonstrating that *SRY's* effect must be indirect and *MIS* activation requires other factors.

Another gene found to have potential binding sites for SRY is the c-fos relative fos-related antigen 1 gene (*fra-1*) which is expressed during spermatogenesis. Cohen and Sinclair[51] proposed that as both genes were expressed in the adult testis they may be interacting. To explore this hypothesis they used purified recombinant SRY protein and showed it bound strongly to one of these sites in gel mobility shift assays.[51] These investigators also showed that SRY protein significantly enhanced transcription from *fra-1* promoter constructs in cotransfection assays in CHO cells. The potential for *SRY* protein to activate transcription *in vivo* has yet to be demonstrated and caution must be exercised interpreting *in vitro* DNA/protein binding studies as these may not reflect the *in vivo* conditions.

There are potential *SRY*- binding sites in the 5' region of the *SRY* gene itself.[31,37] In total there are more than 10^5 sites in the human genome which conform to the consensus DNA-binding sequence recognized by SRY protein. Consequently, *SRY's* sequence specificity alone cannot account for its biological specificity.[30] Many features of SRY protein such as: sequence-specific binding, the nuclear localization of SRY protein and the activation ability of a glutamine/histidine rich region from the mouse protein are all consistent with a role in regulation of transcription.*52*

XVII. *SRY* AND THE BIG PICTURE OF TESTIS DEVELOPMENT

A better understanding of the genetic cascade leading to cellular changes in the construction of the testis will come with the characterization of genes which act upstream and downstream of *SRY*. So far, only a handful of genes have been shown to be expressed in the right place at the right time to implicate them in gonadal development. These genes include: the Wilm's tumor gene, *WT1*; Mullerian Inhibiting Substance, *MIS*; and a gene important in the differentiation and functioning of steroidogenic cells, *Ftz-F1*.

Ftz-F1 is an orphan nuclear receptor first identified in *Drosophila* as a gene regulating the expression of *fushi tarazu*.[53] The gene produces two transcripts one of which is *SF-1* (Steriodogenic Factor 1). *WT1* and *Ftz-F1* genes have been shown to play important roles in the establishment of the presumptive gonad, actually preceding the expression of *SRY*. Using gene knockouts targeting of the *WT1* gene resulted in mice deficient in *WT1* lacking gonads with female internal genitalia.[54] The same holds true for *Ftz-F1* knockouts. *Ftz-F1* null mice also lack gonadal tissue and develop female internal and

external genitalia.[55] *Ftz-F1* mice die soon after birth due to corticosteroid crisis because they also lack adrenal tissue. Transcripts of *WT1* and *Ftz-F1* can be detected in the genital ridge as early as nine d.p.c. before *Sry* is turned on at 10.5 d.p.c. *Ftz-F1* expression persists in males throughout the period of sex determination, but is turned off in females. In the adult, *SF-1* is expressed in all steriodogenic tissue: adrenal cortex , testicular Leydig cells, ovarian theca, granulosa cells, and corpus luteum.[56] (see Figure 5).

Recent experiments also suggest *Ftz-F1* may regulate the expression of the *MIS* gene. *SF-1* has been shown to activate transcription of genes encoding steroidogenic enzymes including Aromatase. A 180 bp region upstream of the *MIS* gene has been shown to be sufficient for Sertoli cell specific expression. This region contains a consensus binding site for *Sry* and several nuclear receptor half-sites. It appears unlikely that *Sry* directly regulates *MIS* since its expression is extinguished before that of *MIS*. However, the shared spatio-temporal profile of *SF-1* and *MIS* expression, the presence of nuclear receptor binding sites in the *MIS* promoter, and *SF-1's* demonstrated ability to bind

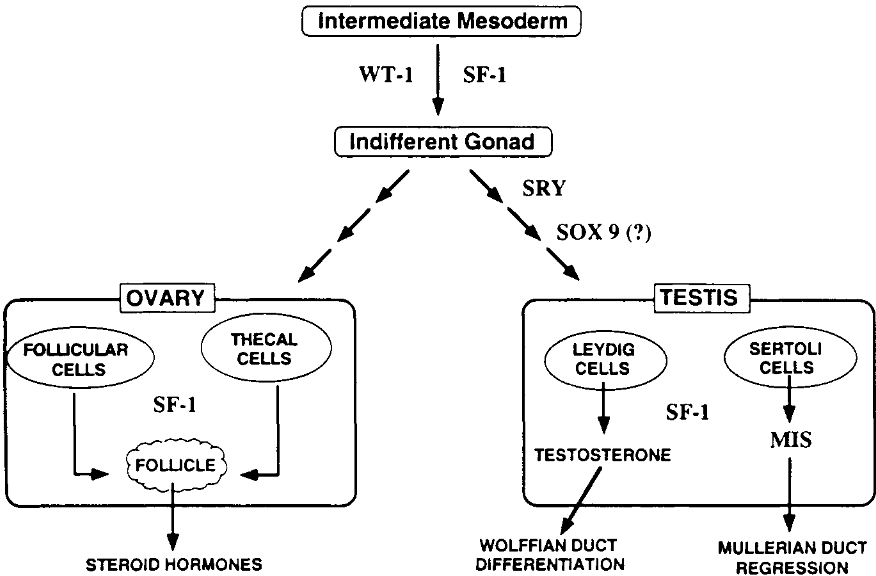

Figure 5. The cascade of mammalian gonadal development and the genes thought to be involved in differentiation of specific stages and specific cell types. *WT-1* and *SF-1* are expressed prior to the initiation of *SRY* expression and morphological sexual differentiation. *SOX-9* expression is essential in the developing testis, but it is not known whether it acts upstream or downstream of *SRY*. (See text for detailed discussion.)

these sites and activate the *MIS* promoter *in vitro* strongly suggest that *SF-1* regulates the expression of *MIS*[49] (see Figure 5). Studies by Haqq and colleagues[50] showed that mouse *SF-1* was not able to transactivate human *MIS* reporter constructs and further that *SF-1* exerted a repressive effect on *Sry* induced activation of *MIS* reporter constructs. This may be due to species differences in the DNA and cells used and may not necessarily represent the *in vivo* situation. Switching off *Ftz-F1* expression in females at the time of sex determination argues for the activation of a *Ftz-F1* repressor at this stage. Perhaps *Sry* represses this repressor in the male pathway or in some other way maintains *Ftz-F1* expression. These possibilities are currently being investigated.

As important as the timing of gene expression is the specific cell types in which they are expressed. While little is known about the elements of the genetic cascade in the sexually dimorphic development of the gonad, even less is known about the cellular interactions necessary for the induction and maintenance of that dimorphism. Some light has been shed on this aspect by studies on XX<->XY chimeric mice.[57,58] Depending on how the chimeras are constructed, the chimeric gonad can be comprised of different relative contributions of XX and XY cells, and the relative contribution determines the sexual phenotype of the gonad. All the cells of the fully developed gonad arise from only four, initially sexually bipotential, cell lineages. These are: steroidogenic cells (giving rise to either Leydig cells in males or theca cells in females); supporting cells (either Sertoli or follicle cells); connective tissue cells (either tunica or stromal cells); and germ cells (either spermatogonia or oogonia).

Chimeric studies indicate that *Sry* is acting only in the supporting cell lineage turning them into Sertoli cells.[57,58] *Sry* alone, however, is not sufficient to promote Sertoli cell differentiation from the indifferent supporting cell lineage. Like the other cell types that go into forming the gonad, the supporting cells are destined to become ovarian tissue by default. In XX<->XY chimeras supporting cells (including those that are XY) will give rise to follicle cells if the XY contribution to the soma is less than 25%. If it is greater than 25% all the cells, XX and XY, will form Sertoli cells indicating that the Sertoli cells stimulate their own differentiation in a paracrine fashion. Perhaps *Sry* stimulates this signaling between Sertoli cells. Another cell interaction necessary for Sertoli cell differentiation came from the observation that isolated genital ridges in organ culture fail to differentiate into testis cords.[59] A group of mesenchymal cells originating in the mesonephros migrate into the developing gonad at about 11.5 d.p.c. approximately 24 hours after the induction of *Sry* transcription. Again it is possible that these cells migrate in response to a signal induced by *Sry* expression in Sertoli cells.

XVIII. SOX GENES

Initial studies, using the *SRY* gene as a probe on a Southern blot of DNA from a variety of mammals showed the expected Y-specific band in all mammals but also identified a related gene family present on the autosomes and the X chromosome.[21] Subsequent work led to the isolation of four of these genes from a mouse embryonic library.[24] These genes showed a high degree of sequence identity within the box region to each other (78-98% amino acid homology) and to *SRY* (77-82% amino acid homology) and were consequently named *Sox* genes (*SOX* in humans) due to the presence of the *SRY*-related HMG box. Outside the box region, these genes did not demonstrate any sequence similarity. Together with *SRY*, the *SOX* genes represent a distinct family of transcriptional regulators with more than 100 members. The expression patterns of the *Sox* genes within the early mouse embryo suggested that, like *SRY*, they may have a particular role in the regulation of developmental processes.

One member of the *SOX* family, *SOX-9*, showed a high level of expression in the skeletal precursors of the developing mouse embryo[60] and in human embryonic (rete testis and seminiferous tubules) and adult testis.[61] In humans, *SOX-9* has been mapped to a narrowly-defined region encompassing the locus for the disease campomelic dysplasia (CD).[62,61] CD is a congenital syndrome which is characterized by skeletal and cartilage defects with angulation and bowing of the long bones. Patients with CD usually die within the first month of life. Along with skeletal abnormalities this disease is associated with a significant occurrence of sex reversal. Of 74 cases of CD that have been karyotyped, 24 were XX females, 14 were XY males, and 36 were XY females.[62] This data suggests that the gene mutated in these patients is involved in testis development.

Initial attempts to isolate the gene or genes responsible relied upon positional cloning strategies using sex-reversed CD patients carrying chromosome 17 translocations. This mapped the human autosomal sex reversal locus (*SRA1*) associated with the campomelic dysplasia locus (*CMPD1*) to human chromosome 17q24.1-q25.1.[63] A candidate gene, *SOX-9* had mapped to this region on 17q and when the expression and mapping data for *Sox-9* in mice became available this gene immediately became a strong candidate for *CMPD1*.[61,62,63] Subsequent analysis showed heterozygous mutations in XY female sex-reversed CD patients.[61,62] This confirmed *SOX-9* as the gene responsible for autosomal sex reversal and campomelic dysplasia. The phenotypes appear to be due to haploinsufficiency (i.e., loss of one copy of the gene) as there is no evidence for loss of both copies of the *SOX-9* alleles.[61,62] Not all mutations in *SOX-9* resulted in CD and sex reversal, and many more patients will need to be examined for mutations to separate the different functions. The *SOX-9* HMG box is 71% similar to the *SRY* box at the amino

acid level and binds to the same DNA target. In addition *SOX-9* has a glutamine and proline rich region at the C-terminal end of the protein similar to many other transcriptional activators[62]. All this evidence suggests that *SOX-9* plays a major role in mammalian testis-determining pathway.

Another member of the *SOX* family that may play a role in gonadal development is *SOX-3*. This gene maps to the X chromosome in eutherian mammals and marsupials.[64,65] Of all the *SOX* genes, *SOX-3* is most similar to *SRY* in sequence and may be the ancestral gene to *SRY*.[65] *Sox-3* transcripts are present in genital ridge at the same time as *Sry* and display approximately the same level of expression. All *SOX* genes bind to the same DNA sequence with about the same affinity.[64] It is formally possible that members of the *SOX* family and *SRY* could compete for the same binding sites and therefore complement each others' actions by regulating the same genes in sexual development. One hypothesis is that *SRY* acts as a repressor of *SOX-3* which may be an ovary determining gene. Since *SRY* seems only to be an HMG box with no other transcriptional activating domains, its presence in presumptive gonadal tissue could simply be to bind up available sites blocking *SOX-3* binding. There is, as yet, no experimental data to support this hypothesis, and X chromosome deletions encompassing *SOX-3* do not affect normal testis development.

XIX. FUTURE PROSPECTS

There can be no doubt that *SRY* is the mammalian testis determining factor. Normal *SRY* expression in the presumptive gonad is needed to initiate differentiation into a testis and the consequent development of a male phenotype. The upstream regulating elements of *SRY* gene expression and its downstream targets remains a mystery. *SRY* is undoubtedly affecting the transcriptional activity of downstream genes in the construction of the testis, but as yet none of these target genes has been identified. Nor is it known whether *SRY* is activating or repressing the expression of its targets. Indeed, the ease of finding the *SRY* binding site in the promoters of a wide variety of cloned genes will make the process of identifying these targets difficult. Furthermore, *SRY* appears to operate as the sex determiner only in mammals. Attempts to find *SRY* in birds and reptiles have been unsuccessful. For this reason, investigators are taking a variety of different approaches to elucidate the details of differentiation of the gonads. One approach is the positional cloning of autosomal loci known to cause sex reversal in humans. We, on the other hand, have broadened our focus and are searching for sex determining genes in lower vertebrates by assaying for sex specific transcripts in the embryonic presumptive gonads of both sexes. Given the high degree of morphogenetic conservation of gonadal development in all vertebrates, any conserved genes

cloned from lower vertebrates (e.g., birds and reptiles) may have significant roles in gonadal differentiation in mammals.

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SEX REVERSAL IN MAMMALS

Claude M. Nagamine

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I. INTRODUCTION

The mammalian gonads are derived from thickenings of the coelomic epithelium of the urogenital ridges. The transformation of the genital ridge into the fetal gonad is regulated by at least two genes: the Wilms' tumor suppressor gene *WT1*[1,2] and *Ftz-F1* which encodes the transcription factor steroidogenic factor-1, also known as Ad4 binding protein (SF1, Ad4BP).[3-8] In laboratory mice in which *WT1* expression was disrupted ("knocked out") by gene targeting in embryonic stem cells, the gonadal ridges initially form but almost immediately start to degenerate and are subsequently lost.[9] Knockout of SF1 affects gonad differentiation slightly later, after the gonadal ridge is already present. The gonads regress and neither ovaries nor testes are present at birth in SF1 knockout mice.[10]

Mammalian fetal gonads are initially indifferent and have the potential to develop into either testes or ovaries. Furthermore, all fetuses have two pairs of sex ducts: the mesonephric or Wolffian ducts, which give rise to the male epididymides, vasa deferentia, and seminal vesicles, and the Müllerian ducts, which give rise to the female oviducts (fallopian tubes), uterus, and upper vagina.

If the fetus has a Y chromosome, as in an XY karyotype, the gonads differentiate into testes. The locus on the Y responsible for testis determination was designated the testis determining factor (*TDF*) in humans and testis-determining, Y-linked (*Tdy*) in the laboratory mouse (*Mus musculus*). Although once believed to be involved in testis determination, it is now clear that the male histocompatibility antigen HY does not function in sex determination. Molecular and genetic data suggest that the high mobility group protein, sex determining region on the Y (*SRY*/*Sry*, human and non-*Mus*/*Mus* gene symbols) is the Y chromosomal gene that induces testis determination and therefor is allelic to *TDF*/*Tdy*.[11-13] *SRY* is hypothesized to trigger, either positively or negatively, a cascade of gene interactions that ultimately transforms the indifferent gonads into testes.

The classic experiments of Alfred Jost demonstrated that once testes are formed, the subsequent differentiation of the male phenotype is dependent upon at least two hormones.[14,15] The first, Müllerian inhibiting substance (MIS), also known as anti-Müllerian hormone or Müllerian inhibiting factor, is a glycoprotein of the transforming growth factor-ß superfamily.[16,17] *MIS* is produced and secreted by the Sertoli cells in the testes and induces the Müllerian ducts to regress. The second, testosterone, which is produced and secreted predominantly by the Leydig cells, induces the Wolffian ducts to differentiate into the male accessory sex ducts and glands, for example epididymides, vas deferens, and seminal vesicles. Both steroidogenesis and MIS expression are regulated by SF1, which, as noted previously, also play a role in the early development of the fetal gonad.[3,4,7,8] Testosterone, circulating via the blood stream, is converted to dihydrotestosterone by 5α reductase in the tissues of

the genital tubercle, genital folds, and genital swellings, inducing them to differentiate into the glans penis, the penile urethra, and scrotum, respectively.[18]

In the absence of a Y chromosome, as in an XX or XO karyotype, the gonads develop into ovaries. The absence of *MIS* allows the Müllerian ducts to survive and develop into the oviducts, uteri, and upper vagina. The absence of testosterone results in the degeneration of the Wolffian ducts except for a few mesonephric tubules that form the ovarian rete. For mammals, the female phenotype is, in computer terminology, the default pathway upon which male sexual differentiation is imposed. This is clearly demonstrated in SF1 knockout mice in which the fetal gonads regress prior to sexual differentiation. Both XX and XY mice lack gonads. Importantly, XY mice are completely femininized both internally (oviducts, uteri, and vagina) and externally with no evidence of masculinization.[10] Differentiation of the female genital tract and external genitalia is not dependent upon any estrogen stimulus since knockout of the estrogen receptor resulted in uteri and oviducts, albeit hypoplastic, and normal external genitalia.[19] The reader is referred to[20,21,22,23] for extensive reviews on mammalian sex determination and sexual differentiation.

Sex reversal is defined as the condition in which an individual has the karyotype of one sex but the gonads or gonadal tissues of the opposite sex. Individuals of XY karyotype with gonads consisting of ovaries or ovarian tissues have XY sex reversal; individuals of XX karyotype with gonads consisting of testes or with testicular tissues have XX sex reversal. Both XY and XX sex reversal can be associated with hermaphroditism. A *hermaphrodite* is defined as an individual in which both ovarian and testicular tissues are present either within the same gonad (ovotestis) or in separate gonads.

This definition excludes individuals who have the karyotype and gonads of the same sex (XX and ovaries, XY and testes) but the secondary sexual characters of the opposite sex, a condition often referred to as pseudohermaphroditism. Furthermore this chapter ignores hermaphroditism arising from sex chromosome mosaicism, for example, the XO/XY/XYY mosaics of BALB/cBm mice,[24] or chimerism.[25]

A useful strategy to attack the problem of identifying the genes and gene interactions involved in normal sex determination is to study cases of abnormal sex determination. Human and animal models of XY and XX sex reversal have contributed immensely, and are still contributing, to our understanding of mammalian sex determination and sexual differentiation. Indeed, studies on patients with XY and XX sex reversal were crucial to the identification and confirmation of *SRY* as the testis-determining gene on the Y.

II. XY SEX REVERSAL

XY sex reversal has been reported in humans, the laboratory mouse (*M. musculus*), the wood lemming (*Myopus schisticolor*), the varying and collared

lemmings (*Dicrostonyx* sp.), several species of South American field mice (*Akodon* sp.),[26,27] the horse (*Equus caballus*),[28] and *Microtus cabrerae.*[29]

A. XY Sex Reversal in Humans

XY sex reversal in humans can be divided into two classes based on gonadal histology: XY gonadal dysgenesis and XY hermaphrodites. This classification is admittedly artificial and does not correlate with etiology. In addition, XY sex reversal is associated with three syndromes (Xp duplications, campomelic dysplasia, and 9p deletions). The reader is referred to a recent review of XY and XX sex reversal in humans for additional details.[30,31]

It should be noted that the definition of "gonadal dysgenesis" includes any gonadal defect arising from abnormal embryonic development, for example, absence of gonads (agonadism or agenesis), infertile but recognizable gonads (dysgenetic testes), or ovotestes. However, gonadal dysgenesis is presently used in a more restricted manner, describing only conditions that give rise to streak gonads.[32] The latter, more restricted meaning is used here.

1. XY Gonadal Dysgenesis Syndrome

Polani (1981) estimates that an XY female occurs in 1:100,000 women and that XY gonadal dysgenesis represents only 2.9% of all gonadal dysgenesis.[33] The XY gonadal dysgenesis syndrome is divided into complete and incomplete forms.[34] The complete form of XY gonadal dysgenesis (Swyer's syndrome, pure gonadal dysgenesis) is defined as a phenotypic female with normal 46,XY karyotype, normal female internal organs, and female external genitalia, a height that is normal or slightly taller than average, absence of the stigmata of Turner's syndrome, and adult gonads that have degenerated to fibrous white streaks of tissue (streak gonads).[35,36] Streak gonads are characterized as dysgenetic ovaries if histological sections reveal dense whorls of connective tissue resembling the stroma in the cortex of the ovary, no ova, and the absence of seminiferous tubules. Ovarian rete tubules and pockets of hilar cells may also be present.[32] The uteri of patients with complete XY gonadal dysgenesis, although immature, are capable of carrying a pregnancy to term provided the patient receives the appropriate endocrinological support. Successful pregnancies, one a twin birth, have been reported in the literature.[37,38]

The incomplete form of XY gonadal dysgenesis syndrome is defined as a patient with a dysgenetic testis and a contralateral gonad consisting of a streak gonad, that sometimes contains rare primordial follicles, or the absence of a gonad. The internal sex organs and external genitalia are ambiguous with the degree of internal and external masculinization postulated to depend upon the fetal testis' ability to synthesize and secrete testosterone and MIS prior to dysgenesis.[39,40] Incomplete XY gonadal dysgenesis is sometimes classed

with patients having mixed gonadal dysgenesis (asymmetric gonadal dysgenesis).[40-42]

The streak gonads of XY gonadal dysgenesis patients are morphologically and histologically identical to streak gonads found in 45,XO (Turner's syndrome) females.[32,36] Studies on the ontogeny of the XO female gonad suggest that the streak gonad of the XY gonadal dysgenesis patient was initially an ovary. In human XO fetuses, the ovaries develop normally and are comparable to those of XX fetuses up to three months of gestation. After this gestational age, there is an increase in connective tissues and the formation of primary follicles is abnormal.[43,44] Degeneration of the ovary is variable among individuals and at puberty the XO gonad can range from streak gonads (common) to small but functional ovaries (rare). Cases of fertility have been reported in patients with Turners' syndrome.[45]

Direct evidence that the XY gonadal dysgenesis patient's streak gonads were initially ovaries comes from sporadic case reports. Cussen and MacMahon.[46] reported a three month XY female that at birth had gonads resembling underdeveloped ovaries. Biopsies of both gonads revealed ovarian stroma with numerous oocytes plus occasional primordial follicles. However, at three years 10 months the gonads had degenerated to streaks with only ovarian stroma. No testicular cords were identified. Indirect evidence also suggest that occasional patients with XY gonadal dysgenesis had functional ovaries past the pubertal period, similar to some XO females. A 17-year-old XY gonadal dysgenesis female who was evaluated for secondary amenorrhea was described by Russell and colleagues.[47] The patient had minimal breast development and irregular cycles of menses that started at age 14 suggesting normal, albeit suboptimal, ovarian function. Surgery revealed streak gonads that was confirmed histologically to lack germ cells and any evidence of testis development. Importantly, the streak gonads had no evidence of gonadoblastomas which are capable of steroidogenesis and therefore a potential source for estrogens.[48-50] These cases strongly suggest the XY streak gonads are initially ovaries that subsequently degenerate, the timing of degeneration varying among patients.

Because both XY and XO females have streak gonads, as opposed to ovaries, it has been suggested that two X chromosomes are required for normal development and maintenance of the human ovary.[51] Degeneration of the gonads is hypothesized to be due to hemizygosity of an X gene that normally escapes X inactivation and which is required for follicular development and/or maintenance of the germ cells.[36,52]

A major difference between the XY and XO streak gonads is that the XY streak gonad has a 25-30% incidence of developing gonadoblastomas, a rare germ cell tumor characterized by nests of cells containing germ cells and cells resembling immature granulosa or Sertoli cells.[32,50,53,54] Other germ cell tumors, for example, germinomas (dysgerminoma, seminoma), teratomas, teratocar-

cinomas, embryonal carcinomas, and endodermal sinus tumors (yolk sac tumors), may be associated with the gonadoblastoma.[49,55,56] The gene causing gonadoblastoma, designated *GBY* (gonadoblastoma locus on *Y* chromosome)[57] is mapped to the centromere or the long arm of the Y, proximal to probe DYS132.[58] The chromosomal position of *GBY* suggests that tumorigenesis is not linked to testis determination. Indeed, no correlation was found between mutations present in the testis-determining gene *SRY* and risk for development of gonadoblastomas.[59–61]

2. XY Hermaphrodites

XY hermaphrodites have ovarian and testicular tissues clearly present either within the same gonad (ovotestis) or on opposite sides of the same individual. XY hermaphrodites comprise 12-16% of all cases of hermaphroditism.[62,63]

The gonad distribution found in 75% of XY hermaphrodites is a right testis and left ovary or ovotestis. Bilateral ovotestes are rare.[62] This is in contrast to the gonads of XX hermaphrodites where the majority have an ovotestis and contralateral ovary or bilateral ovotestes. Therefore, the gonads in XY hermaphrodites are more masculine relative to the gonads in XX hermaphrodites. In human hermaphrodites, the right gonad tends to be the testis or is more masculinized relative to the left gonad.[62,63] For example, if an individual has an ovotestis and ovary, the ovotestis will be on the right.

The XY ovotestis has a clear boundary between the ovarian and testicular tissues.[63,64] In 80% of ovotestes, the ovarian and testicular tissues are arranged in an end-to-end fashion. The proportion of an ovotestis made up of ovarian versus testicular tissues ranges from almost entirely ovarian to almost entirely testicular. In 20% of ovotestes, the testicular tissue is small and present only at the hilar region of the gonad. Histologically, the ovarian tissue is normal except that fewer primordial follicles are observed. The seminiferous tubules lack germ cells and Sertoli cells are often present in the lumen of the tubules. The Leydig cells are hyperplastic.

Whether the gonad is associated with Wolffian (epididymis, vas deferens) or Müllerian duct derivatives (fallopian tube) correlates with the quantity of testicular tissues within the gonad. A large percentage of testicular tissues correlate with Wolffian duct derivatives being present. Van Niekerk[64] remarks that a fallopian tube and vas deferens do not coexist with the same gonad. In the mouse XY hermaphrodite, Müllerian and Wolffian duct derivatives can be associated with the same gonad.[65]

It is unclear why ovarian tissues survive in XY hermaphrodites but not in XY female patients. Simpson[32] surmises that many XY hermaphrodites may be cryptic XX/XY chimeras or XO/XY mosaics, a recognized cause of hermaphroditism, with the XX cell line not yet identified or lost.

3. Etiology

The etiology of XY sex reversal in humans is diverse. They can be divided into those that are Y-linked and those that are X or autosomal linked. In addition three syndromes that result in multiple somatic anomalies have also been associated with XY sex reversal.

3a. Y-linked: Mutations in SRY, *the testis-determining gene.* The SRY protein is divided into three domains: an amino terminus, the high mobility group (HMG) domain, and a carboxy terminus. The HMG domain is the DNA-binding domain of the SRY protein and is the *only* domain that is evolutionarily conserved among the various SRY proteins. The gene sequence encoding the HMG domain is called the HMG box.[66] Nuclear magnetic resonance spectroscopy data suggest that the HMG domain is comprised of three α-helices that are folded into the shape of an L.[66] The SRY amino and carboxy termini vary in size among species. In the laboratory mouse and several other species of rodents, the amino terminus consists of only two amino acids.[12,67] The function of the human SRY amino or carboxy termini have not been elucidated.

Based on studies in which the *SRY* locus, and not simply the DNA-binding high mobility group (HMG) domain, has been studied in detail, 19-33% of patients with complete XY gonadal dysgenesis harbor mutations in *SRY*.[60,68-70] Twenty-two *SRY* mutations have been reported at the time of this writing. The *SRY* mutations are divided into four groups: mutations in the HMG box, mutations outside of the HMG box, mutations in the putative *SRY* regulatory region, and deletions of Yp involving *SRY*.

Ninety-one percent (20/22) of the *SRY* mutations are in the HMG domain.[60,68-78] The mutations include 6 nonsense mutations resulting in premature stop codons at amino acids W70, Q74, K92, Q93, W107, Y127 (amino acids numbered according to Su and Lau,[79] 12 missense mutations resulting in amino acid changes, and two small deletions (1 amino acid at P108, 4 amino acids at E122) that result in frame shifts. Of the missense mutations, six (R62G, G95R, L101H, K106I, F109S, A113T) are in amino acids conserved among diverse HMG proteins.[66] and four (V60L, M64I, I90M, R133W) are in amino acids conserved among SRY proteins from eutherian (mouse, rabbit, primates) and metatherian (dunnart) species.[80]

Fifty-five percent (12/22) of the SRY mutations are *de novo*, that is, the mutations are absent in the father or male siblings of the XY sex reversed patient. For six mutations, whether the mutation was *de novo* could not be confirmed.

Three mutations (V60L, I90M, F109S) were familial and suggest variable penetrance of SRY expression.[69,77,78] In each case, an XY female and apparently normal XY males with the same *SRY* mutation were in the same pedigree (Figure 1). Obviously *SRY* in the father and male siblings of the XY female

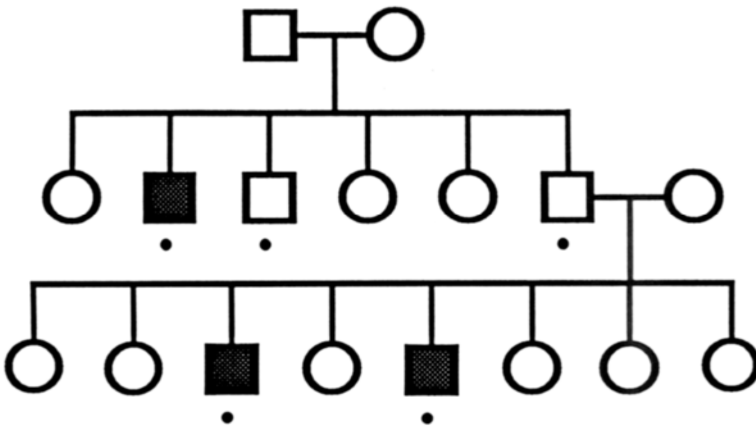


Figure 1. Familial complete XY gonadal dysgenesis that is Y-linked and due to a mutation in *SRY* with incomplete penetrance. Three XY females and two XY males, all of which are known to harbor the same *SRY* mutation, are present in this abbreviated pedigree adapted from Vilain et al. (1992).[77] Squares = XY karyotype, darkened squares = XY gonadal dysgenesis, circles = XX karyotype, • = XY karyotype known to carry the *SRY* mutation.

patient had functioned normally to initiate testis determination. It is hypothesized that the capability of the mutant SRY protein to induce testes may be at a critical threshold level. On certain genetic backgrounds or environmental or physiological conditions, the abnormal *SRY* is incapable of complete testis determination.[77] The positions of the *SRY* mutations in these familial cases do not provide any obvious clue as to why they have variable penetrance.

Affara and colleagues[60] reported a familial case in which two siblings have an identical nonsense mutation at Q74. The predicted SRY protein would have been severely truncated and it was unlikely that the encoded protein could function normally. Nevertheless, the sisters must have inherited their Y from their apparently normal father. It was hypothesized that the father was a gonosomal mosaic with two populations of spermatogonia, one harboring a normal, the other with a mutant, *SRY* allele. Unfortunately, the father was deceased and therefore the hypothesis could not be tested.

Two studies on the *SRY* status of XY hermaphrodites have been published. In the first, an *SRY* mutation was identified in an XY hermaphrodite using DNA isolated from histological sections of the patient's ovotestes.[74] The patient

was mosaic for two *SRY* alleles, a normal *SRY* and a mutant *SRY*. Peripheral blood DNA from the patient and father had the normal *SRY* allele. The mutant *SRY* had two point mutations. One was silent (no change in amino acid). However, the other was a missense mutation (L101H) in a conserved amino acid in the HMG domain. The authors proposed that the etiology of the hermaphroditism was a postzygotic somatic point mutation in *SRY* in cells giving rise to the gonad resulting in the loss or abnormal expression of *SRY* and the formation of an ovotestis.[74]

In the second study,[81] the DNA from an XY hermaphrodite had a normal HMG box. However, since only the HMG box was sequenced, the possibility remains that mutations may be present in other regions of the *SRY* gene.

Mutations in *SRY* outside of the HMG box have been reported in two cases. In the first case, Tajima and colleagues[59] identified two sisters with a nonsense mutation in the SRY carboxy terminus (L163) that would truncate the SRY protein by 41 amino acids (aa). The mutation identified by Tajima and colleagues may shed light on the function of the SRY carboxy terminus. In the mouse, the carboxy terminus is highly polymorphic and it has been hypothesized that this polymorphism may play a role in XY sex reversal.[82] Unfortunately, the father was unavailable for study so it is not known if the mutations were *de novo*, in which case the father would have been a gonosomal mosaic for the SRY mutation as in the case of Affara and colleagues,[60] or penetrance of the SRY mutation is variable as illustrated by the case of Vilain and colleagues.[77]

In the second case, McElreavey and collealgues[70] reported an XY gonadal dysgenesis patient with a large (25-50-kb) deletion that began about 1.7-kb 5' of the *SRY* transcription start site. The *SRY* transcription initiation start site was mapped to about -136 bp relative to the translation start site.[79,83-85] Denaturing gradient gel electrophoresis and DNA sequencing of the entire *SRY* open reading frame (ORF) failed to identify any mutation. *SRY* was not expressed in a lymphoblastoid cell line derived from the patient suggesting that the deletion did not result in ectopic expression. The promoter region of *SRY* is not well defined. It is reasonable to propose that the deletion removed a testis-specific enhancer(s) necessary for the correct timing and/or levels of expression of *SRY* during fetal development. For example, the enhancer controlling expression of zinc finger Y-1 gene, *Zfy1*, in the fetal testis is located 4.3-21 kb upstream of the transcription start site.[86]

Yp deletions. XY sex reversal due to deletions on Yp are rare[87-90] Some, if not all, of the deletions arise from abnormal meiotic X-Y pairing and crossing-over and are the counterpart of the condition giving rise to XX males (see below).[89] These Yp- patients show characteristics of Turner's syndrome.[89,91]

An XY female without the stigmata of Turner's syndrome and with a deletion of SRY has been reported.[92] The patient had a complex, reciprocal Y; 22

translocation (X,t(Y;22)Y) that resulted in at least two deletions on Yp, one including *SRY*.

SRY and incomplete XY gonadal dysgenesis. Patients with incomplete XY gonadal dysgenesis have not been studied as extensively for *SRY* mutations as complete XY gonadal dysgenesis. Two non-familial cases have been reported; both lacked *SRY* mutations.[59] A familial case of incomplete XY gonadal dysgenesis but which is X or autosomal-linked and not Y-linked is noted below. Because of the absence of Y-linkage, no *SRY* mutation is predicted and none was found.

It has been suggested that *SRY* mutations correlate only with complete XY gonadal dysgenesis and that incomplete XY gonadal dysgenesis is due to downstream genes in the testis differentiation pathway.[61,93] A more cautious approach is necessary given that penetrance of *SRY* mutations in some familial cases is not 100% (Figure 1). If these mutations can give rise to XY females and XY males, they may also give rise to intermediate levels of testis determination resulting in incomplete XY gonadal dysgenesis. Indeed cases have been reported in the literature in which complete XY gonadal dysgenesis and male pseudohermaphroditism (bilateral dysgenetic testes) are present in the same family.[94,95]

3b. X-linked or Male Limited Autosomal Dominant. Families have been described in which the incomplete[96] or complete[52,97–99] forms of XY gonadal dysgenesis have been reported over two generations with transmission of the syndrome occurring through *females* (Figure 2). Because the transmission is through XX females, it is unlikely the sex reversal is related to mutations on the Y. In one pedigree,[96] the mother was married twice and the children born from each father had incomplete XY gonadal dysgenesis (Figure 3). As predicted, DNA sequencing of almost the entire *SRY* ORF of four affected individuals failed to identify any mutations. Karyotyping did not reveal an Xp duplication, a known cause of XY sex reversal (see below).[96]

Simpson[100] cautions that the investigator should not overlook the possibility of cryptic 45,X/46,XY mosaicism in patients with complete or incomplete XY gonadal dysgenesis. Although females with 45,X/46,XY mosaicism usually have the stigmata of Turner's syndrome, this is not always true. A 45,X/46,XY female fetus with no Turner stigmata or other abnormality except clitoromegaly was reported by Chang and colleagues.[101] Percent mosaicism in 45,X/46,XY mosaics does not necessarily correlate with severity of somatic anomalies observed.[100–102] Furthermore, 45,X/46,XY mosaicism can be familial.[48] A careful examination for mosaicism will avoid the needless search for *SRY*, X, or autosomal mutations when none exist.

3c. Syndromes Associated with XY Sex Reversal. XY sex reversal is found associated with three syndromes that result in multiple congenital

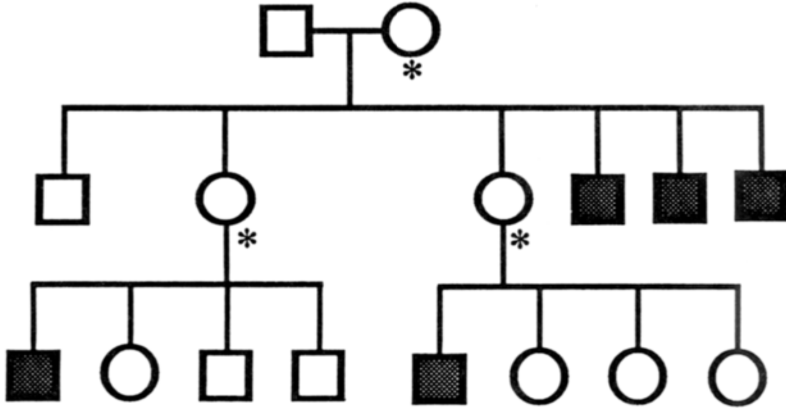


Figure 2. Familial complete XY gonadal dysgenesis transmitted through females. Squares = XY karyotype, darkened squares = XY gonadal dysgenesis, circles = XX karyotype, * = presumed carriers. Abbreviated pedigree adapted from Espiner et al. (1970).[98]

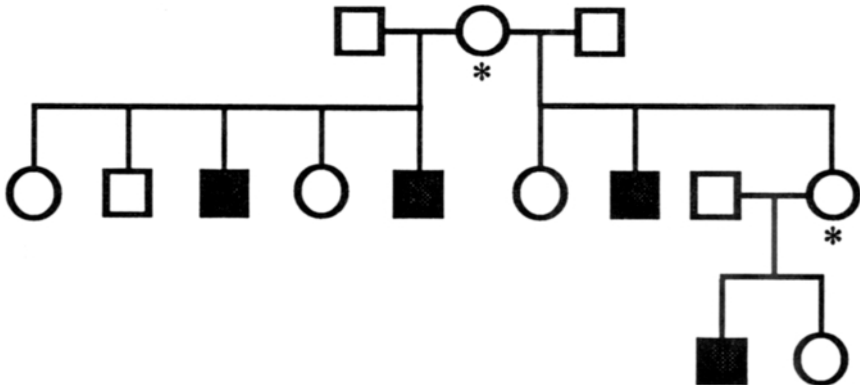


Figure 3. Familial incomplete XY gonadal dysgenesis transmitted through females. darkened squares = incomplete XY gonadal dysgenesis. Other symbols as in Figure 2. Abbreviated pedigree adapted from Fechner et al. (1993).[96]

abnormalities. These cases are informative since they suggest genes involved in testis differentiation, either upstream or downstream of *SRY* in the testis determination cascade. Furthermore these loci are prime candidates for X-/autosomal-linked XY sex reversal.

Duplications of Xp. Duplications of the short arm of the X chromosome [46,X,dup(Xp),Y] or translocations of Xp to the Y chromosome [46,X,der(Y)t(Xp;Y)] result in severe congenital craniofacial and cardiac defects. Important for the present context is that the gonads can vary from bilateral dysgenetic testes to bilateral streak gonads (XY gonadal dysgenesis).[103-109] A five-month old affected XY fetus examined by Bernstein and colleagues[108] was remarkable in having fetal ovaries with numerous primordial follicles. This contrasts with the degeneration of the XO fetal ovary after the first trimester.[44] The data suggest that a gene on Xp, when present in two copies, result in XY sex reversal. The dup(Xp) syndrome is sometimes referred to as Gardner-Silengo-Wachtel or genito-palato-cardiac syndrome.[109]

Not surprisingly, the *SRY* locus in dup(Xp) syndrome patients is normal as determined by Southern blot[104] and/or DNA sequence analyses.[106] Bardoni and colleagues[104] recently mapped the locus responsible for the XY sex reversal to a 160 kb region of the short arm of the human X (Xp21.2-21.3). The locus is adjacent to the adrenal hypoplasia congenita, glycerol kinase, and Duchenne muscular dystrophy genes. The gene was designated DSS (dosage sensitive sex reversal).[104] Other investigators have named the putative gene SRVX (sex reversal X)[103] or TDFX (testis determining factor on X).[106,110]

The dup(Xp) has no effect on XX carriers of the abnormal X. It is hypothesized that the gene responsible for the XY sex reversal is normally subjected to X inactivation since Klinefelter patients with an XXY karyotype, and which one of the two Xs is inactivated, have a male phenotype. It is only when the duplicated locus is associated with a Y chromosome, resulting in the abnormal X being active, that *DSS*, which is now being expressed at twice its normal dose, inhibits testis determination.

XY patients with *deletions* of the *DSS* locus are unambiguous males suggesting that SRY by itself is sufficient for testis determination.[104] Bardoni and colleagues.[104] proposes that *DSS* may function as a link between testis and ovarian differentiation and/or functions in ovarian differentiation. Ogata and Matsuo,[106] building on the regulatory cascade hypothesis for mammalian sex determination of McElreavey and colleagues,[111] propose that *DSS* inhibits downstream autosomal testis-differentiation genes, thereby permitting ovarian differentiation. In the regulatory cascade hypothesis, *SRY* suppresses an autosomal gene, designated Z, that normally inhibits male-specific genes.[21,111] In the current context, *DSS* is a candidate for Z.

In normal males, *SRY* suppresses *DSS*, ovarian differentiation is repressed, and testis differentiation occurs. In dup(Xp),Y patients, *SRY* is unable to completely suppress the double dose of *DSS* and ovarian differentiation

initiates. A corollary to this hypothesis is that a gain of function mutation of *DSS* would have the same effect as a duplication of *DSS* and result in XY sex reversal. This may be the etiology of XY females without *SRY* or Y mutations. Furthermore, a loss of function mutation of *DSS* may be the etiology of XX male and XX hermaphrodite patients in which no *SRY* gene is present (see below).[110]

Campomelic dysplasia. Campomelic dysplasia is an autosomal recessive disorder characterized by congenital craniofacial and skeletal abnormalities, the most characteristic being the bowing of the long bones of the legs (campomelic—Greek for "bent limbs").[112] XY sex reversal has been reported in some, not all, XY patients diagnosed with campomelic dysplasia.[112–115] The genetic locus causing campomelic dysplasia is mapped to 17q24.3-q25.1.[115] It is unclear if sex reversal is the result of a mutation involving one gene with pleiotropic effects or several closely linked genes. Because 17q duplications do not cause any of the characteristics of campomelic dysplasia, the phenotypic effects of campomelic dysplasia may arise from the loss of function of one or more genes.[115]

9p deletion. A few medical reports suggest deletions and translocations involving the short arm of chromosome 9, specifically 9p24, can cause sex reversal.[116,117] The patient described by Bennett and colleagues[116] was examined at two years eight months and had normal female external genitalia, uterus and oviducts, and bilateral streak gonads. Histological sections of the streak gonads were typical of the streak gonads of XY gonadal dysgenesis. Interestingly, the streak gonads were associated with some epididymal tubules and Wolffian duct remnants suggesting testicular development had occurred during fetal development. The patient's *SRY* gene was normal.

B. XY Sex Reversal in Mice

In the laboratory mouse, *Sry* is on the short arm of the Y (Yp). XY sex reversal in the laboratory mouse is divided into three classes based on their etiology: (1) deletion of *Sry* (XY̶),[118] (2) deletion of Yp sequences but leaving *Sry* intact,[119] and (3) the improper interaction of autosomal gene(s) and a *M. m. domesticus* Y chromosome (XYd, d=*domesticus*).[120] Unlike XY sex-reversal in humans, the XY sex reversed mouse has unequivocal ovaries; streak gonads do not occur. Furthermore, at least in XYd sex reversal, the ability of placing the sex reversal phenomenon on a defined genetic background by the use of inbred strains removes the variability arising from different background genes.

1. XY̶ Sex Reversal

The XY̶ sex reversed mouse strain originated from a chimeric male mouse constructed with embryonic stem cells that had been infected with a retrovirus.

The male sired XY females suggesting that testis determination was blocked. The mutation was hypothesized to be in the testis-determining gene on the Y (*Tdy*) and therefore was designated Tdy^{m1}; the Y harboring the putative mutation was designated Y^{Tdym1}, abbreviated as Ɏ.[118]

XɎ hermaphrodites do not occur.[118,121] The gross anatomy and histology of the adult XY ovaries (4 and 12 weeks *post partum*) do not differ from normal females except for fewer oocytes and a higher percentage of atretic follicles.

XɎ females are fertile and can transmit the Ɏ chromosome to their daughters. However, XɎ females have fewer and smaller litters relative to normal mice. Furthermore, many of the offspring from XɎ females are sex-chromosome aneuploids (XɎY males, XXɎ females, XO females) suggesting little or no pairing between the X and Ɏ chromosomes during female meiosis. XXɎ females are also fertile and, in contrast to XɎ females, they have a normal or close to normal reproductive lifespan and fertility.[118,122] This is not due to more oocytes in XXɎ females. The infertility of the XɎ females is attributed in part to the inefficiency of the Ɏ as a pairing partner during meiosis, resulting in meiotic failure and a higher incidence of oocyte atresia.[122]

The mouse *Sry* gene is present in 2.7 kb of unique sequences that is flanked on opposite sides by a large, nearly perfect, inverted repeat that extends for at least 15.5 kb in opposite directions. The etiology of XɎ sex reversal is an 11 kb deletion that removed the entirety of the *Sry* ORF.[123] The retroviral infection could not to be linked to the XY sex reversal.[118] The inverted repeat structure of the *Sry* locus may have contributed to the deletion of *Sry*.[123] Cytogenetic and Southern blot analyses suggest that other gross deletions or rearrangements of the Y were absent.[118,121,123]

2. *XY Sex Reversal Caused by Deletion of Yp Sequences*

An XY sex reversal model developed by Cattanach and colleagues gives rise to XY females and XY hermaphrodites. Etiology of the sex reversal is hypothesized to be due to loss of a repetitive sequence called Sx-1 on Yp that results in low or no expression of *Sry*. The deleted Y chromosome was designated Y^d.

The XY females and hermaphrodites were obtained by mating female mice to males that carry the sex reversed (Sxr) region, on their X, as opposed to Y, chromosome ($XSxr^a$/Y).[119,124] The sex reversed mutation (XY, Tp(Y)1Ct) is a duplication and transposition of Yp sequences, including *Sry*, to Yq and will be discussed in more detail in III B. What is important here is that the sex reversed region (Sxr^a) on the X chromatid can pair and recombine with Yp during male meiosis without involving the pseudoautosomal region. This "illegitimate" recombination may generate deletions along the length of Yp and allow the mapping of Yp genes.[119]

$XSxr^a$/Y males are produced in two steps. First, females heterozygous for the X-autosome translocation T(X;16)16H (T16H) are mated with males

carrying the Sxr^a mutation on their Y chromosome ($XYSxr^a$). During male meiosis, an obligate pairing and recombination transfers Sxr^a from one Y chromatid to an X chromatid. Although progeny inheriting Sxr^a are usually of male phenotype, $T16H/XSxr^a$ *females* develop due to preferential inactivation of the X carrying Sxr^a.[125,126] $T16H/XSxr^a$ females are subsequently mated with normal XY males. Fifty percent of the XY progeny are $XSxr^a/Y$ males.[124] Mating $XSxr^a/Y$ males with normal females resulted in a small percentage of the progeny being XY females. Three XY female strains carrying Y chromosomes from either BALB/c (Y^{d-1}) or B6 (Y^{d-2}, Y^{d-3}) have been reported.

The XY^d females are fertile and as in X¥ female mice produce a high percentage of aneuploid (XO, XXY^d, XYY^d) progeny arising from the failure of the X and Y chromosomes to pair during female meiosis and the subsequent independent assortment of the sex chromosomes into the oocyte. Similar to X¥ females, XY^d females have reduced fertility and XXY^d females have normal fertility.[119]

Unlike X¥ females, hermaphrodites are present in the Y^{d-2} and Y^{d-3} strains but not in the Y^{d-1} strain. About 10% of the XXY^d class develop as hermaphrodites. The hermaphroditism was not due to mosaicism arising from somatic loss of the Y^d chromosome. The hermaphrodites had an ovary and contralateral testis and ambiguous external phenotype. In addition, rare males heterozygous for X-linked genetic markers were observed. These were interpreted to be XXY^d males. Thus the phenotypes in XY sex reversal in the Y^{d-2} and Y^{d-3} strains run the gamut from XXY^d males to XXY^d hermaphrodites to XXY^d females.[119]

The goal of the genetic cross was to use illegitimate recombination between the Sxr^a region on the X and Yp to generate deletions along the length of Yp. Therefore, the most logical explanation for the sex reversal was a deletion of sequences critical for the normal expression or function of *Sry*. Indeed, expression studies using the reverse transcription-polymerase chain reaction assay (RT-PCR) on the Y^{d-1} line suggested that *Sry* was not expressed or expressed at low levels in XY^{d-1} embryos obtained at 11.5 dpc, the age of gestation when *Sry* is normally expressed at high levels. However, Southern blot analysis revealed no obvious deletions, insertions, or rearrangements at the *Sry* locus and about 36 kb of flanking regions.

The Y^d chromosomes were analyzed further either by Southern blots or PCR for 16 single copy loci specific for Yp and one marker that is repeated on Yq. No abnormalities were noted. Expression of *HY* was also normal.

However, probing Southern blots containing genomic DNA of XY^{d-1}, XY^{d-2}, and XY^{d-3} digested with the restriction enzyme *Pst* I with a Y-repeat probe, pSx1, revealed that all XY female lines had lost a variable number of a 1.5 kb Sx-1 fragment. The degree of sex reversal seen in each mouse line correlated with the number of Sx-1 fragments lost. The Sx-1 sequences are hypothesized

to lie between the centromere and *Sry* on Yp. The current hypothesis is that deletion of Sx-1 sequences brings Sry under the influence of the Y heterochromatin resulting in complete or partial silencing of *Sry*.[119]

3. XYd Sex Reversal

XYd sex reversal occurs when certain *M. m. domesticus* Y chromosomes (Yd, d=*domesticus*) are introduced into the C57BL/6 (B6) laboratory inbred mouse strain by backcrossing. The etiology of XYd sex reversal is complex and suggests an interaction between *Sry* and autosomal (or pseudoautosomal) testis-determining genes. Two of these putative testis-determining genes are testis-determining, autosomal-1 (*tda-1*)[127] and T-associated sex reversal (*Tas*).[128]

Testis-determining, autosomal-1. XY hermaphrodites and XY females occur if a Y from *M. m. domesticus*, a subspecies of the house mouse *M. musculus*, is backcrossed into B6.[120,129] First generation offspring (N1) of the founding cross (B6 ♀ x *M. m. domesticus* ♂) are normal (Figure 4). However, if one performs a backcross by mating an N1 ♂, which carries the Yd chromosome of its father, to a B6 ♀, in addition to normal XY males and XX females, one also obtains XY hermaphrodites and XY females. Subsequent backcrosses result in the loss of normal XY male progeny and the production of only XY females or XY hermaphrodites. After the initial report in 1982,[120] the sex reversal phenomenon was confirmed in other laboratories.[65,130-132]

One interpretation of the genetic data is that the sex reversal results from the testis-determining gene on the *M. m. domesticus* Y chromosome (Tdy^{Dom}) interacting abnormally, with a B6, recessive, autosomal or pseudoautosomal testis-determining gene. This putative gene was designated testis-determining, autosomal-1 (*tda-1*).[127,129] *M. m. domesticus* is hypothesized to carry a dominant allele of this gene (*Tda-1*) since all N1 XY fetuses developed into normal males. XY fetuses with a Yd chromosome and homozygous for *tda-1* (Tdy^{Dom}, *tda-1/tda-1*) develop either as hermaphrodites or XY females.

Attempts to map the chromosomal location of *tda-1* have not been successful.[133] However, during the attempts to map *tda-1*, another putative testis-determining gene that interacts with Tdy^{Dom}, *tda-2*, was identified on chromosome 12.[133]

T-associated sex reversal. *T*-associated sex reversal occurs when the dominant brachyury (*T*) alleles *T* hairpin tail (T^{hp})[134,135] or *T* Orleans (Tt^{Orl})[136] are introduced into B6 in association with the Y chromosome of the AKR strain.[128,137,138] XY fetuses with both T^{hp} and an AKR Y have ovotestes or ovaries.[128] XY fetuses inheriting Tt^{orl} and an AKR Y are more severely affected and all develop as XY females.[137] Both T^{hp} and Tt^{orl} are deletions on proximal

XYd Sex Reversal

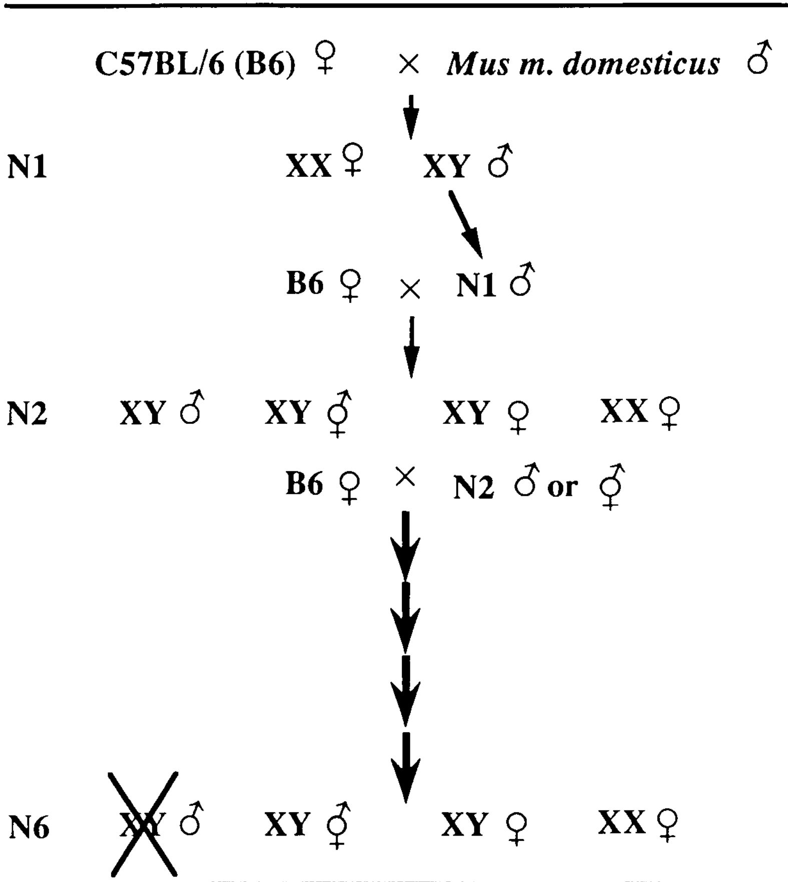


Figure 4. Mating scheme giving rise to XYd sex reversal. Note that by the N6 backcross generation, normal XY males are no longer found. Modified from Nagamine (1993).[172]

chromosome 17. The sex reversal is hypothesized to be caused by the hemizygous expression of a gene on the normal chromosome 17 demarcated by the overlap of the T^{hp} and Tt^{orl} deletions. This locus was designated *T*-associated sex reversal (*Tas*).[128,137,138]

The AKR Y is of *M. m. domesticus* origin.[130] Like *tda-1* sex reversal, the B6 genomic background and the presence of a *M. m. domesticus* Y

chromosome, in this case an AKR Y, are crucial for the phenomenon. *Tas XY* sex reversal does *not* occur when T^{hp} is introduced into the C3H/HeJ strain[128,138] nor when the AKR Y is replaced by the non-*domesticus* B6 Y.[137] Three conditions must be met for *Tas* sex reversal to occur: (1) the presence of the *domesticus* AKR Y chromosome, (2) a B6 genomic background with homozygosity of a recessive gene(s) (*tda-1?, tda-2?*), and (3) the presence of T^{hp} or Tt^{Orl}. Because both *tda-1* and *Tas* XY sex reversals require a *M. m. domesticus* Y chromosome and the B6 genomic background both sex reversals have been combined under the general heading of XYd sex reversal.

Syndrome. XYd sex reversal, as illustrated by the B6.Y^{Dom} strain, can vary in phenotype from XY females to XY overt hermaphrodites to XY "males."[65,130] There is a positive correlation between the phenotype of the adult and the amount of seminiferous tubules that develop in the fetal XY gonad.[65,139,140]

In normal mice, testis differentiation is first detected under the dissecting microscope at 12 days *post coitum* (dpc) by the presence of seminiferous cords and a tunica albuginea. Seminiferous cord differentiation initiates in the mid-region of the indifferent gonad and proceeds to the cranial and caudal poles of the gonad.[140] By 13 dpc seminiferous cords are well developed throughout the testis (Figure 5). In the B6.Y^{Dom} strain, testis differentiation occurs slowly. At 12 dpc a few XY gonads can be distinguished from XX gonads by a slight bulging in the mid-region of the gonad suggesting the initial stages in the differentiation of seminiferous cords. However, under the dissecting microscope, no seminiferous cords are obvious and a tunica albuginea is difficult to discern. That the fetuses with these gonads were indeed XY was confirmed by genotyping all fetuses for the Y chromosome gene *Zfy* using the polymerase chain reaction (PCR).

At 13 dpc, many of the XY gonads are still indistinguishable from XX gonads. A few can be found to have a mid-region bulge (Figure 6) or seminiferous tubules. It is only at 14 dpc that an XY gonad destined to be a testicular gonad or XY ovary can be identified. Only 50% of the XY fetuses develop any seminiferous cords.[140] The quantity of seminiferous cords that develop in a given gonad is variable even within the same fetus (Figure 7). The remaining XY gonads develop into XY ovaries and the fetuses into XY females.[65,129,141]

Three points can be made about the B6.Y^{Dom} XY gonads. First, the differentiation of the seminiferous tubules in the ovotestis is never random. Seminiferous tubules first form in the mid-region of the gonad then spread out to the cranial and caudal poles of the gonad. If testis differentiation is not completed, the undifferentiated tissues at the cranial and/or caudal poles develop into ovarian tissues.[65,120] The localization of testicular tissues to the mid-region and ovarian tissues to the cranial and caudal poles is not unique to XYd sex-reversed ovotestes but is also found in ovotestes arising from XO/XY mosaicism.[24,142-145]

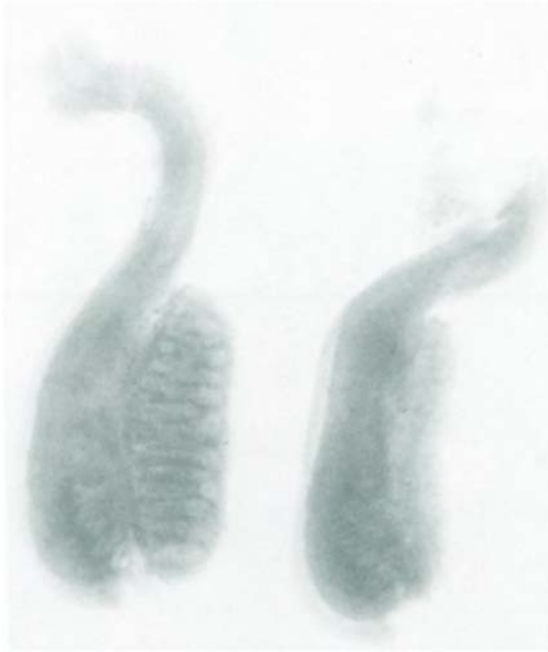


Figure 5. Fetal ovary (top) and testis (bottom) from the B6.Y^{FVB} strain. The gonads are from 13 dpc fetuses and are still attached to the mesonephros. The seminiferous cords are well developed and present throughout the testis.

Second, the differentiation of seminiferous tubules in a B6.Y^{Dom} XY gonad is highly variable among fetuses in the same litter or even between the two gonads in a fetus (Figure 7). The B6.Y^{Dom} strain has been backcrossed to the B6 strain for more then 26 generations; genetic variability among individual mice in the B6.Y^{Dom} strain is negligible. Therefore, the differences in seminiferous tubule development is not due to background genes.

Third, seminiferous tubule development is often better developed in the left gonad than the right. The net result is that the left gonad is usually more "masculinized" than the right.[129,130] For example, if two testicular gonads are present, the left gonad is apt to be larger and/or more normal in appearance. Similarly, in an overt hermaphrodite, the left gonad is usually the testicular gonad while the right is either an ovarian or ovotesticular gonad. This preponderance of testicular gonads being on the left is also seen in T16H/XSxr

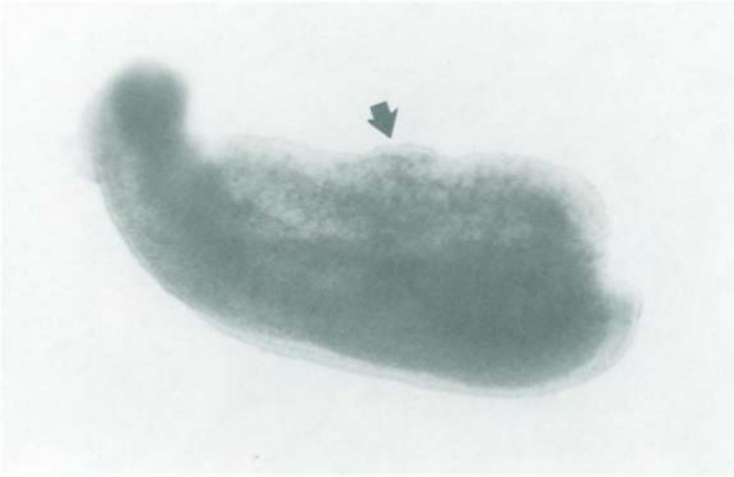


Figure 6. $B6.Y^{Dom}$ 13 dpc fetal XY gonad. The only evidence of testicular differentiation is a slight swelling in the mid-region of the gonad. No seminiferous cords are evident (compare to Figure 5). The fetus from which the gonad was obtained was genotyped by PCR and was shown to be XY.

hermaphroditic mice.[146,147] The simplest explanation for the laterality is that the left gonad develops at a slightly faster rate than the right in the mouse fetus. However, this view is compromised by hermaphrodites in the strains BALB/Gw[148] and BALB/cBm[142] which have an equal possibility of the right or left gonad being testicular. Hermaphroditism in BALB/cBm is due to mosaicism; this may play a role in the absence of laterality.

Adult $B6.Y^{Dom}$ gonads are categorized into three phenotypes based on gross anatomy: testicular gonads, ovotesticular gonads, and ovarian gonads.[65] It is reasonable to assume that the degree of seminiferous tubule development that occurred in the fetus determines whether the gonads develop into testicular, ovotesticular, or ovarian gonads. "Testicular gonads" resemble normal testes except they may be slightly smaller or abnormal in shape. Testicular gonads are associated with Wolffian duct derivatives (epididymides, vasa deferentia, seminal vesicles). Spermatogenesis is present in most testicular gonads and most of the $B6.Y^{Dom}$ mice with testicular gonads are fertile. However, histological sections also reveal that many testicular gonads harbor developing oocytes (Figure 8). Therefore, by definition, testicular gonads are ovotestes.[65] "Ovotesticular gonads" are small gonads with both Müllerian and Wolffian duct derivatives. Histologically they have ovarian tissues with oocytes and sterile seminiferous tubules (Figure 9). Ovotesticular gonads can occur in the

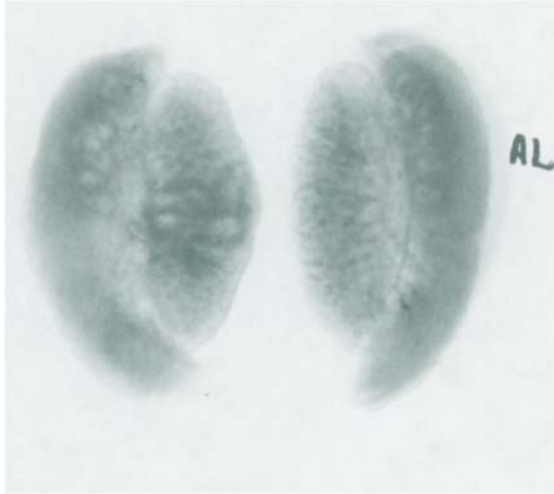
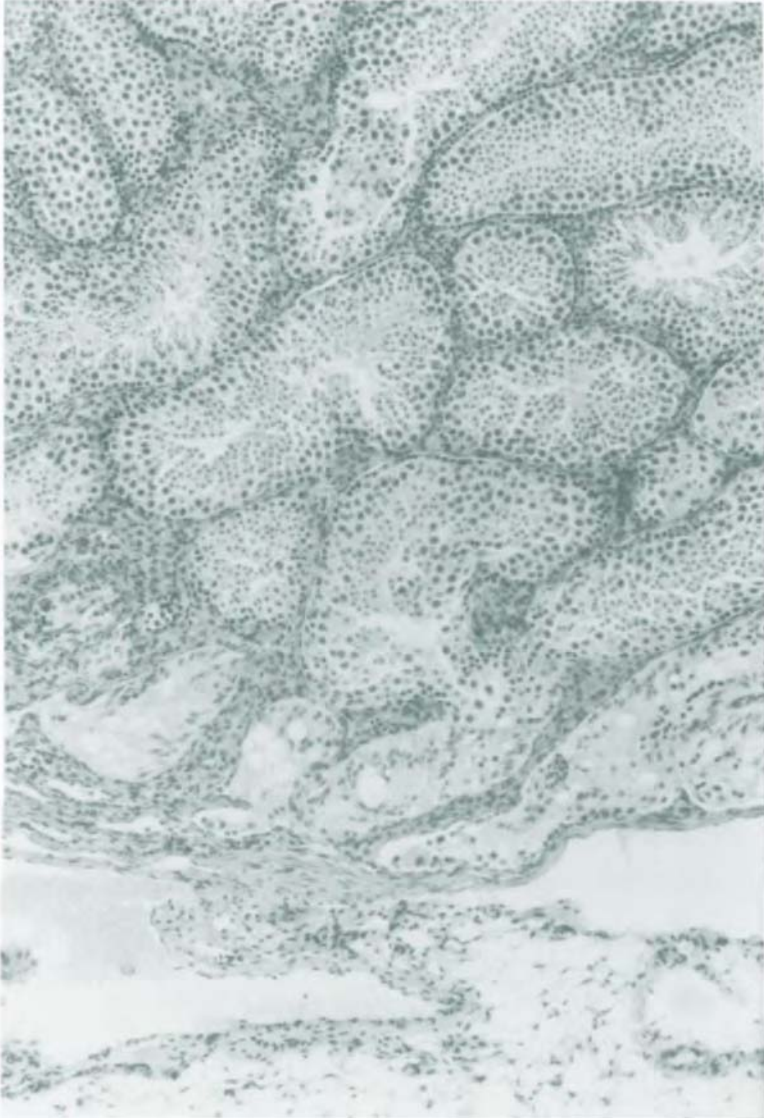


Figure 7. B6.Y^{Dom} 14 dpc gonads from the same fetus illustrating the large difference in testicular differentiation that is present in the two gonads. Only three seminiferous cords are seen in the mid-region of the gonad on the left. Ovarian tissues are present at the cranial and caudal poles of the gonad on the left and throughout the gonad on the right.

position of a normal ovary or may be partially descended. "Ovarian gonads" appear as normal ovaries both morphologically and histologically except that fewer oocytes are present (Figure 10). The ovarian gonads are normal at the gross anatomical and histological level and are capable of ovulation. However, unlike the XY and XXY^d females, females of the B6.Y^{Dom} strain are sterile. The exact cause of the sterility is unclear but may be due to three factors: an abnormal steroidogenic profile of the XYd ovarian gonad, the XYd oocyte being compromised with regard to its ability to develop into a viable embryo, and the persistence and proliferation of lutein cells.[65,141,149] In XX ovaries, oocytes in the medulla of the ovary are the first to undergo folliculogenesis. These oocytes subsequently undergo atresia and their follicular cells go on to differentiate into interstitial tissues that function in steroidogenesis. XYd ovarian gonads develop normally till about 16 dpc at which stage the oocytes in the medullary region degenerate prematurely, resulting in the medulla being devoid of interstitial steroidogenic tissues at birth. The premature loss of medullary oocytes in the fetal XY ovarian gonad is hypothesized to contribute



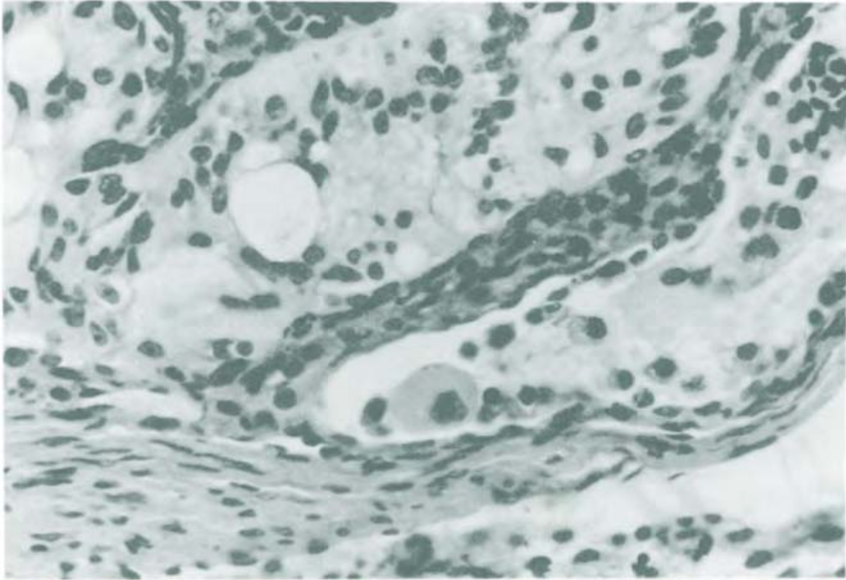


Figure 8. Histological section of a B6.Y^{Dom} XY testicular gonad illustrating the presence of normal spermatogenesis (8a) and a naked oocyte in the rete tubules (8b). By definition this gonad is an ovotestis despite its testicular appearance and the presence of spermatogenesis. Reprinted with permission from Nagamine et al. (1987).[65]

to an abnormal steroidogenic profile in the adult XYd female mouse.[141,149,150]

Oocytes in the cortex of the XYd ovarian gonad are not severely affected.[141] These oocytes undergo folliculogenesis and can be fertilized. However, they do not develop past the blastula stage.[141,151]

In the XX ovary, the corpora lutea degenerate following ovulation in virgin females. In the XY ovary, the corpora lutea do not degenerate but persist and undergo hyperplasia. The proliferating lutein cells break out of the corpora lutea, invade neighboring ovarian stroma and follicles, and become a major portion of the adult XYd ovarian gonad, displacing the remaining oocytes to the periphery of the gonad.[65] Oocytes in XYd ovarian gonads become increasingly rare after six weeks of age.

XYd sex reversal and interspecies hybridization. Because XYd sex reversal occurs when the *M. m. domesticus* Y is introduced into the laboratory mouse, it was proposed that the sex reversal is the result of the interbreeding of two different species of *Mus*.[133,137,152] However, not all *M. m. domesticus* Y chromosomes cause XY sex reversal (see below).[130,132,153,154] If XY sex reversal

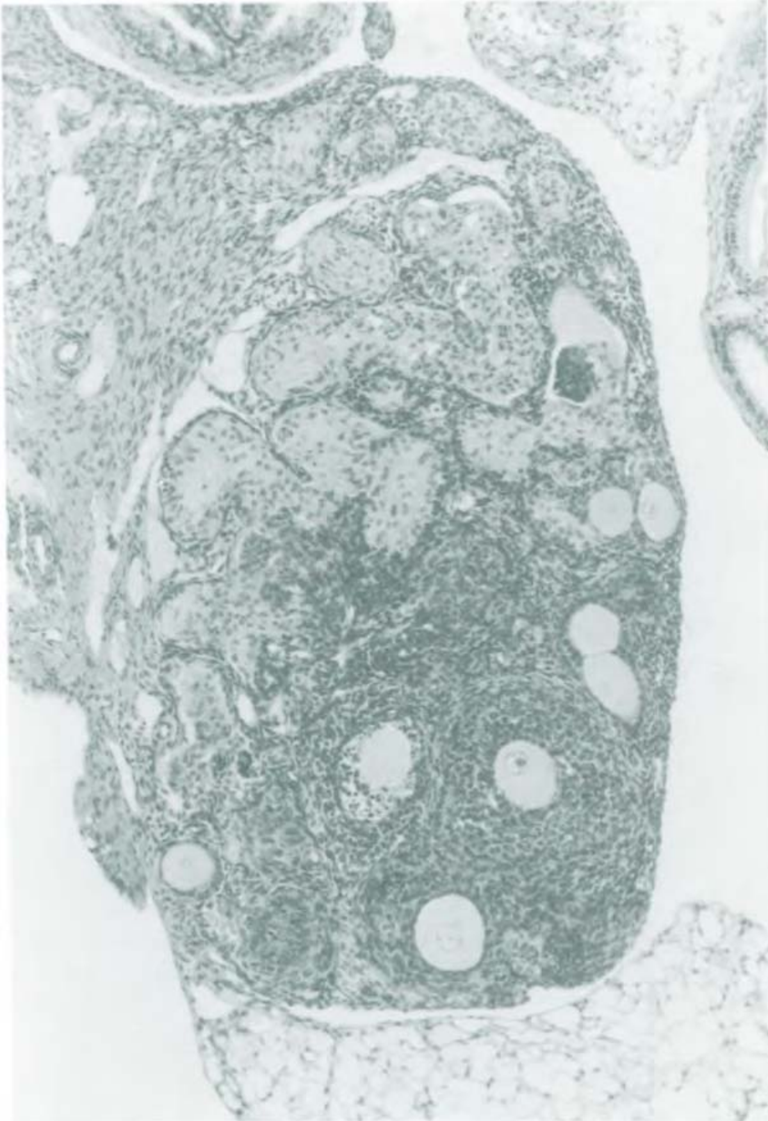


Figure 9. Histological section of a B6.Y^{Dom} XY ovotesticular gonad with sterile testicular tubules and ovarian stroma with developing oocytes. Reprinted with permission from Nagamine et al. (1987).[65]

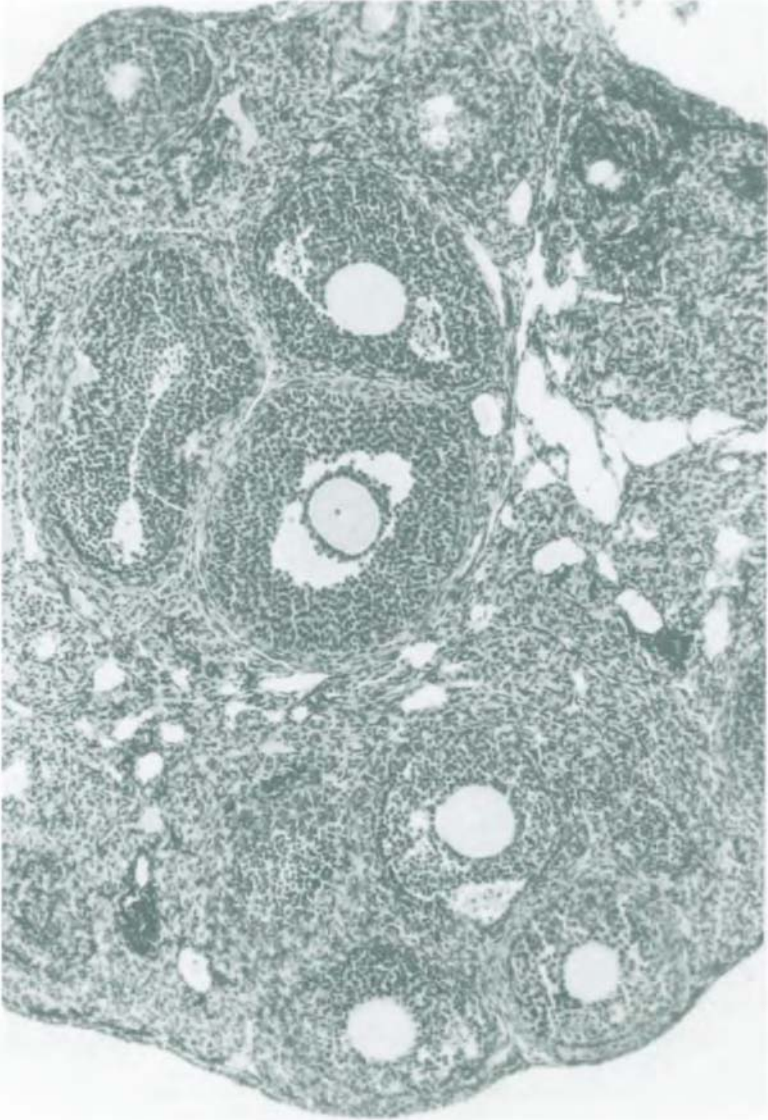


Figure 10. Oogenesis present in a B6.Y^{Dom} XY ovarian gonad. Reprinted with permission from Nagamine et al. (1987).[65]

is simply due to the incompatibility of genes between two different species, one would expect all *M. m. domesticus* Y chromosomes to cause XY sex reversal.

Furthermore, laboratory mice are not a distinct species or subspecies but a hybrid derived from different populations of the house mouse, *M. musculus. M. musculus* is recognized as a polytypic species comprised of four subspecies: *Mus musculus domesticus, M. m. musculus, M. m. castaneus*, and *M. m. bactrianus.*[155–157] In Japan, hybridization between *M. m. musculus* and *M. m. castaneus* resulted in a unique population, historically referred to as *M. m. molossinus.*[158,159]

Classical laboratory mouse strains were derived from, at minimum, *M. m. domesticus* and *M. m. molossinus.* Clear evidence for this is the presence in most laboratory mouse strains (including B6) of maternally inherited *M. m. domesticus* mitochondrial DNA,[160–162] a paternally inherited *M. m. molossinus* Y chromosome,[163,164] and nuclear genes derived from *M. m. domesticus.*[165–168] and *M. m. molossinus.*[169] About 25% of the laboratory strains have a *M. m. domesticus* Y.[170,171]

Nallaseth[152] contends that XYd sex reversal results from the *M. m. domesticus* Y chromosome being structurally unstable when introduced into B6. The sequence instability of the Y increases with backcrossing such that by the N7 backcross generation the Y is functionally inactive and incapable of testis-determination resulting in only sterile XY females being born.

On Southern blots, the author has not identified any difference in the Y chromosomes of B6.Y^{Dom} XY females, XY overt hermaphrodites, or XY male phenotypes with bilateral testicular gonads with regard to Y repetitive sequences (pY353/B), the Y-specific gene *Zfy*, or the testis-determining gene *Sry* using several restriction enzymes. Furthermore, the backcross generation of the B6.Y^{Dom} strain is > N26 with no evidence of going to extinction. More importantly, if hermaphroditism is the result of the loss of Y chromosomal sequences, then one would expect an overt hermaphrodite with an ovarian gonad and contralateral testicular gonad, to sire only XY females or additional overt hermaphrodites. In fact, overt hermaphrodites can have sons with bilateral testicular gonads.[172,173]

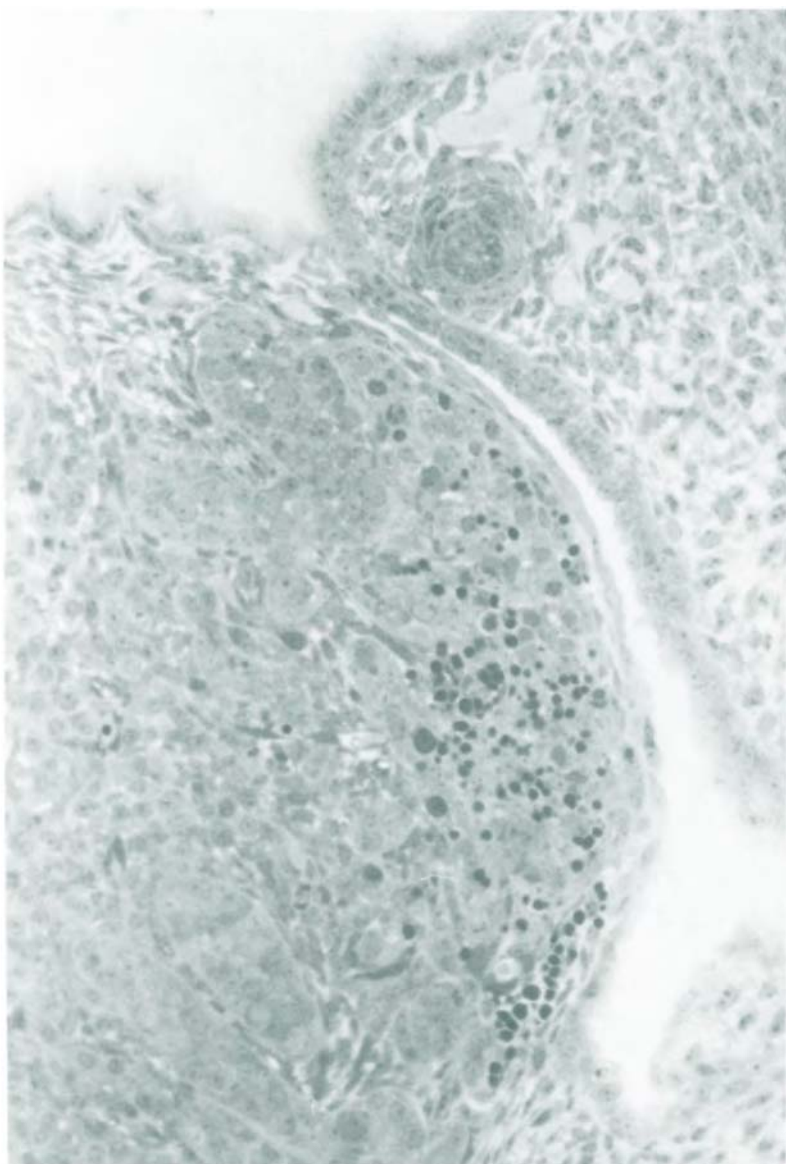
Yd chromosomes and XY sex reversal. As noted above, not all *M. m. domesticus* Y chromosomes cause XY sex reversal when placed on the B6 genomic background.[130,153] Yd chromosomes can be divided into three groups depending upon their capability of inducing normal testis differentiation in B6. The first group is exemplified by the Y from the Posch-1 strain. The Posch-1 strain originated from wild mice trapped in Tirano, Italy. Introducing the Posch-1 Y into B6 (strain designation = B6.Y^{Dom}) causes a severe form of sex reversal resulting in XY females and XY hermaphrodites at birth.[65,130] Yd chromosomes causing this severe form of sex reversal have been identified in

wild mice from diverse geographical locations in central Europe (northern, central and southern Italy, Switzerland, Croatia, Germany), as well as from *M. m. domesticus* that were inadvertently transported by humans to the new world.[120,129,132,174,175]

The second group is exemplified by the AKR Y. Backcrossing the AKR Y into B6 (strain designation = B6.Y^{AKR}) results in XYd sex reversal that is obvious in fetuses at ages 13 dpc (Figure 11). Morphologically and histologically the B6.Y^{AKR} fetal ovotestes are similar to that obtained from B6.Y^{Dom} with seminiferous cords being present in the mid-region of the gonad and undifferentiated regions at the cranial and caudal poles. XYd sex reversal in B6.Y^{AKR} is transient and the ovarian regions are lost as the seminiferous tubules continue to develop. From 14-16 dpc, the ovarian regions become increasingly difficult to identify and at birth, no evidence of hermaphroditism is seen either at the gross anatomical or histological levels.[130,153]

Histological sections of the affected regions of B6.Y^{AKR} gonads at 15-16 dpc reveal the absence of a tunica albuginea, the absence of seminiferous cords, and, most importantly, the presence of germ cells in the process of meiosis (Figure 12). The presence of meiotic germ cells is important since in the mouse, oocytes enter meiosis at 14-16 dpc. In contrast, spermatocytes enter meiosis only after birth. The presence of meiotic germ cells suggest that the tissues are following the ovarian pathway but are being overtaken by the growing testicular cords. The number of developing oocytes in the B6.Y^{AKR} ovotestes are considerably less than that found in B6.Y^{Dom} ovotestes and many of the germ cells appear to be degenerating. Furthermore, numerous round cell-like

Figure 11. B6.Y^{AKR} 13 dpc fetal gonad illustrating the severe delay in testicular development. A distinct tunica albuginea and seminiferous cords are present only in the mid-region of the gonad. The cranial and caudal poles are undifferentiated.



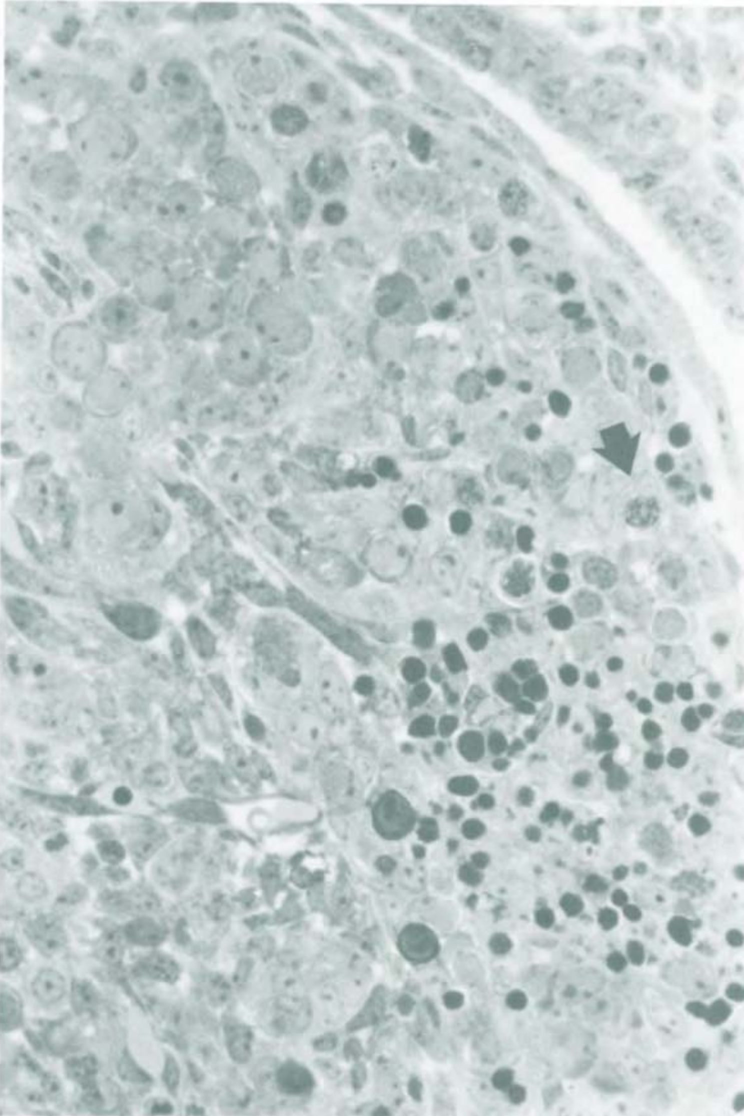


Figure 12. Histological section of the cranial pole of a B6.Y^{AKR} 15 dpc ovotestis. (a) A tunica albuginea is present over the testicular cords on the left but absent over the ovarian region. The ruffled appearance of the epithelium of the ovotestis is an embedding artifact. (b) Higher magnification of the same gonad as in (a). A germ cell in meiosis is present (arrow). The numerous, round dark staining bodies are surmised to be apoptotic germ cells.

bodies are often present in the same area (Figure 12). These stain intensely with toluidine blue suggesting that they are germ cells that had undergone programmed cell death. Toluidine blue stains apoptotic cells.[176]

It should be noted that designating the B6.Y^{AKR} XY gonads as ovotestes is controversial. Eicher and colleagues maintain that the B6.Y^{AKR} fetal gonads are not sex reversed but are only delayed in testicular differentiation.[137,177] The author maintains that sex reversal in B6.Y^{AKR} is partial and that other mutations, for example Tas, exacerbates the sex reversal such that hermaphroditism is seen at birth.[130] Credence to this viewpoint is the report that a deletion involving the dominant white spotting mutation (W^{19H}) can also exacerbate XYd sex reversal in B6.Y^{AKR} resulting in XY females and XY hermaphrodites at birth.[178,179] Additional markers for ovarian versus testicular tissues will help in resolving whether sex reversal is truly present.

The third Y is exemplified by the Y of the FVB/N strain. Backcrossing the FVB/N Y into B6 (strain = B6.Y^{FVB}), results in normal testis determination. No delay in testis determination is observed during fetal development at 13-14 dpc in B6.Y^{FVB} mice (Figure 5).[82] Yd chromosomes that have normal testis-determining capabilities in B6 have been identified in the SJL, SWR/J, SWV, ST/bJ, BUB/BnJ strains.[82,130,154]

The three groups of Yd chromosomes can be ranked in their ability to induce testes in B6 as follows: Posch-1 < AKR < FVB/N. These observations were confirmed by other investigators.[132,140,154]

XYd sex reversal in other mouse strains. XYd sex reversal is not restricted to B6. However, penetrance is highest in B6.[130] If XYd sex reversal is due only to the interaction of the *M. m. domesticus* Y and *Tda-1* or *tda-1* alleles, strains carrying the dominant *Tda-1* allele should produce normal F1 males when crossed to B6.Y^{Dom}. If they carry the recessive *tda-1* allele, then XYd sex reversal should occur.

AKR, BALB/c, C3H/An, and C3H/He females crossed to B6.Y^{Dom} male phenotypes resulted in 14-15 dpc fetuses with ovotestes. However not all XY fetuses were affected, the effect was transient, and no hermaphroditism was seen postnatally. This suggests that the XY sex reversal was less severe. Either these inbred strains have additional *tda-1* alleles or other genes ameliorate the XYd sex reversal by epistasis.[130] In contrast, SJL/J females crossed to B6.Y^{Dom} male phenotypes resulted in all F1 male fetuses being normal suggesting that this strain has the dominant *Tda-1* allele.[130]

Because transient XYd sex reversal as seen in B6.Y^{AKR} fetuses can confound the interpretation of the results, XYd sex reversal is presently being scored in newborn F1 mice, thereby identifying strains in which only the most severe form of sex reversal (XY overt hermaphrodites, XY females) occur. The cross C57BL/10 ♀ × B6.Y^{Dom} ♂ results in XY sex reversed newborn mice suggesting

that like B6, the C57BL/10 strain carries the recessive *tda-1* allele. However, while 50% of (B6 ♀ × B6.Y^{Dom} ♂) F1 XY progeny are XY females, only 9% of (B10 ♀ × B6.Y^{Dom} ♂) F1 XY progeny are XY females suggesting that penetrance of XYd sex reversal is lower in B10. The C57L/J strain also has XYd sex reversal but penetrance here is even lower. Only two (C57L/J ♀ × B6.Y^{Dom} ♂) F1 XY hermaphrodites and 1 XY female have been identified in more than 93 newborn mice (Nagamine, unpublished observations). Another related strain, C58/J, was reported to lack the *tda-1* allele[151].

The C57BL/6, C57BL/10, C57L, and C58 strains originated in 1921, by the brother-sister matings of a single male (#52) to two different females (#57, #58).[180] Progeny from #58 gave rise to the C58/J strain; progeny from female #57 gave rise to the C57 strains (C57BL/6, C57BL/10, C57L). Since the C58/J strain lacks the recessive *tda-1* allele, either the *tda-1* allele was a mutation in the original female (#57) or the *tda-1* allele was lost in the derivation of the C58/J strain.

The NZB/BINJ strain, which is unrelated to B6,[181] was also reported to support XYd sex reversal.[133] We have not observed any overt hermaphrodites or XY females in 50 newborn (NZB/BINJ ♀ × B6.YDom ♂) F1 mice (Nagamine, unpublished observations).

Etiology. One hypothesis for XYd sex reversal is a "timing-mismatch." According to this hypothesis, ovarian differentiation can occur in both XX and XY fetal gonads. However, in XY fetuses, SRY initiates testis differentiation prior to ovarian differentiation, preempting the development of ovarian tissues.[129,182,183] In XYd sex reversal, the initiation of testis differentiation is delayed, for example, either by the inability of *SRY* to correctly regulate the next gene in the testis differentiation cascade, by a delay in *Sry* transcription, and/or by a delay in *Sry* translation. In support of this hypothesis is the finding that testicular cords form about 14 hours later in XYd fetuses.[140,184]

RT-PCR data suggest that the delay in testis differentiation in XYd sex reversal is not due to a delay of when *Sry* is transcribed. *Sry* expression initiates normally in B6.Y^{Dom} [185] Interestingly, its expression is extended past the time when Sry normally turns off. In both B6 and B6.Y^{Dom} strains, all XY fetuses express *Sry* at 11 dpc. However, while *Sry* transcripts were absent from B6 XY gonads by 14 dpc, *Sry* transcripts were still present in B6.Y^{Dom} XY gonads. Furthermore, *Sry* transcripts were more likely to be detected in B6.Y^{Dom} XYd *ovaries* than in XYd *ovotestes*. This was attributed to XYd ovaries having either a slower turnover rate of *Sry* mRNA, an up-regulation of *Sry*, or a slower down-regulation of *Sry* transcription.[185]

The *Sry* locus of the B6.Y^{Dom}, B6.Y^{AKR}, and B6.Y^{FVB} Y chromosomes are grossly intact. No *Sry* RFLPs have been identified among these Y chromosomes using the restriction enzymes *EcoR* I, *Sst* I, *Taq* I, *Bam* HI,

Hind III, and *Msp* I.[186] Gubbay and colleagues (1992)[123] reached a similar conclusion.

The published *Sry* gene was cloned from the 129 mouse strain.[123] The 129 mouse strain has a *M. m. molossinus* Y chromosome and therefore the published sequence represents the *M. m. molossinus Sry* allele.[164] Cloning and sequencing of the *Sry* ORF of the $B6.Y^{Dom}$, $B6.Y^{AKR}$, and $B6.Y^{FVB}$ Y chromosomes identified five missense mutations, one of which was previously reported to be present in the HMG box,[163,187,188] two deletions, and one nonsense mutation.[82] The *molossinus* SRY protein is predicted to be 395 aa. The nonsense mutation identified in the *domesticus* Y chromosomes occurred in the region 3' of the HMG box, truncating the *domesticus* SRY protein to 230 aa. However, all but one of the mutations were shared among the *domesticus* Y chromosomes and therefore these mutations were not strong candidates for the cause of XYd sex reversal.

The only mutation that was not shared among the $B6.Y^{Dom}$, $B6.Y^{AKR}$, and $B6.Y^{FVB}$ Y chromosomes was a deletion in the region 3' of the HMG box. The deletion resulted in a polymorphism in the numbers of a CAG (glutamine) repeat. The deletion in the triplet repeat resulted in $B6.Y^{Dom}$ having 11, $B6.Y^{AKR}$ having 13, and $B6.Y^{FVB}$ having 12 glutamines. B6 and 129 both had 12 glutamines.[82] The variation in the number of glutamines in the carboxy terminus of SRY in addition to the mutations common to the *domesticus* SRY proteins may compromise the ability of the $B6.Y^{Dom}$ and $B6.Y^{AKR}$ SRY proteins. This is in keeping with the RT-PCR data suggesting that *Sry* transcription initiates normally in $B6.Y^{Dom}$.[185]

Are tda-1 *and* Tas *testis-determining genes?* *Tas* and *tda-1* were proposed as autosomal (or pseudoautosomal) testis-determining genes.[129,138] An opposing view is that *tda-1* and *Tas* cause sex reversal by nonspecifically retarding the growth of the XY gonad. This results in a delay of testis differentiation, allowing ovary differentiation to initiate.[178,183] In support of this is the observation that a deletion involving the dominant white spotting mutation (W^{19H}) exacerbates the transient XYd sex reversal seen in B6.Y*AKR* such that XY females and hermaphrodites are present at birth.[178,179]

It should be noted that within a litter of $B6.Y^{Dom}$ fetuses, the XY females are never runts. The author has been unable to predict which fetus will be an XY female, XY hermaphrodite, or XX female based on external characteristics. Nor are the kidneys, which also differentiate from the urogenital ridge, obviously smaller in XY females relative to XY hermaphrodites or XX females (Nagamine, unpublished observations). Therefore if there is a delay in development it is specific to the development of the XY gonad. Cloning of *tda-1* and *Tas* will resolve this question.

C. XY Sex Reversal in *Myopus* and *Dicrostonyx*

The wood lemming (*Myopus schisticolor*) and the collared and varying lemmings (*Dicrostonyx* sp.)are considered together because they are hypothesized to have XY sex reversals that are X-linked.

1. Myopus schisticolor, *the Wood Lemming*

The wood lemming was the first species in which fertile XY females were documented.[189] The wood lemming is a small, relatively uncommon, rodent of the taiga of Europe and Asia where it lives in mossy bogs and coniferous forests.[190] It is most closely related to the Norwegian lemming (*Lemmus lemmus*) which has normal sex determination. The collared and varying lemmings (*Dicrostonyx* sp.) which also have XY sex reversal (see below) are only distantly related. All three species are known to have periodic fluctuations in population density resulting in population explosions and crashes.

The most striking characteristic of the wood lemming is its sex ratio which is highly skewed to favor females (4 females:1 male).[189,191,192] The abnormal sex ratio does not vary much seasonally or yearly.[193] The preponderance of females is due to the presence of two types of females: those that give birth only to other females (F) and those that give birth to both males and females (MF).[192]

The MF females are of XX karyotype. In contrast, F females are XY. In contrast to the mouse, the XY females are completely fertile with no reduction in litter size. No abnormalities are present in the XY female's reproductive tracts or ovaries either at the gross anatomical or histological levels.[189,191]

Importantly, XY females produce only X-bearing oocytes despite their XY karyotype.[189,191,194] It has been hypothesized that during fetal development, a double non-disjunction occurs during the mitotic divisions of the primordial germ cells in the XY female fetus resulting in the doubling of the X and the loss of the Y.[189,191,194]

The XY sex reversal is X-linked. Cytogenetic analysis using G-banding identified two different X chromosomes. The first, designated the normal X, is found in some MF females (XX) and all males (XY). The second X chromosome, designated X*, has a short arm (Xp) that is about 7% smaller than the normal X and has a different G-banding pattern.[195] The X* chromosome is present in all F females (X*Y) and in some MF females (X*X) but never in males.[196] Therefore, there are three types of genetic females: XX which give rise to a 1:1 sex ratio of females to males, X*X which give rise to a 3:1 female: male sex ratio, and X*Y which give rise to all females.

The double non-disjunction mechanism is not foolproof. In about 9.3% of X*Y females the double non-disjunction mechanism either fails or is incomplete and, in addition to X*-, Y-, O-bearing oocytes, X*Y-bearing

oocytes are produced.[191,197] X*Y-bearing oocytes give rise to X*YY, and X*XY individuals.

The importance of these rare karyotypes is that their phenotypes give insight into the function of the X* chromosome. X*YY are fertile females suggesting a double dose of *SRY* cannot abrogate the dominant effect of the X* chromosome. The X*XY karyotype can give rise to three phenotypes: X*XY female, X*XY male, and X*XY hermaphrodite. The phenotype obtained depends upon whether the X or X* chromosome is subject to X-inactivation.[198] X*XY hermaphrodites are rare and only one has been reported. This hermaphrodite had a right testis and contralateral ovary.[197]

In rare cases, X*XX and X*O karyotypes have been observed, probably arising from meiotic non-disjunctions in X*X females or XY males, respectively. Both karyotypes are fertile females. One can conclude that the *Myopus* X* chromosome blocks the normal function of *SRY* in testis determination without affecting ovarian differentiation.

The G-banding pattern of the X* chromosome suggests that its origin was complex, involving an inversion and deletion.[195,196] Southern blot analyses have identified a *ZFX* RFLP that correlates with the X* chromosome.[199] The mutation giving rise to the *ZFX** allele is within the *ZFX** gene and not its flanking sequences.[199] It was not determined if the *ZFX** mRNA was abnormal. Northern blots containing RNA from tissues obtained from male and female animals harboring X or X* chromosomes suggest that the *ZFX** gene is not transcriptionally silent and that its mRNA is comparable in size to the normal *ZFX* transcript. Cloning and sequencing of the *ZFX* and *ZFX** cDNAs are necessary to determine if the *ZFX** RFLP correlate with a functional difference of the *ZFX* and *ZFX** proteins. It was concluded that either *ZFX** or a closely linked gene is involved in counteracting the *SRY* in X*Y females.[199]

2. *Dicrostonyx* sp., the Collared and Varying Lemmings

Dicrostonyx sp. inhabit the high Arctic tundra of North America, Europe, and Asia. The collared lemming (*D. groenlandicus*) is found in North America (Alaska, Canada, Greenland); the varying lemming (*D. torquatus*) is found in the tundra of Europe and Asia, and the Kamchatka peninsula.[190,200] Both species have XY females that can make up 30-40% of the female population.[200-202] XY hermaphrodites have not been reported in *Dicrostonyx*. In contrast to *Myopus*, the presence of XY females in a population of *D. groenlandicus* on Devon Island, Canada did not skew the sex ratio (50.3% males).[203]

Breeding studies have demonstrated that the mutation giving rise to XY females is X-linked and similar to that seen in *Myopus*.[204] In keeping with the *Myopus* terminology, the mutant X is designated X*. However cytogenetic

analysis with G-banding have so far failed to identify any difference between the normal and mutant X chromosomes in *Dicrostonyx*.[200,201,204,205]

Unlike *Myopus*, X*Y females produce both X* and Y oocytes, resulting in X*X female, X*Y female, and XY male progeny. YY young have never been found and are presumed to be inviable. The X*Y female is only slightly less fertile than an X*X or XX female, despite 25% of its progeny being YY and inviable. This paradox is explained by an increase in ovulation rate in X*Y females which compensates for the loss of the YY progeny.[201] Furthermore, the number of litters produced by an X*Y female is not significantly reduced relative to XX and X*X females.[201,202]

Cytogenetic studies in *Dicrostonyx* are complicated by the presence of Robertsonian translocations, microchromosomes, supernumerary (B) chromosomes, and possible pericentric inversions that can vary within a population.[200,206] Moreover, both the X and Y chromosomes are fused to autosomes and the autosome to which they have fused may differ among species and subspecies. For example, in *D. groenlandicus* the X and Y are fused to chromosome 15[200] while in *D. torquatus* the sex chromosomes are fused to chromosome 5. Furthermore, in one population of *D. torquatus*, a Y chromosome has never been identified.[202,205]

3. *Etiology*

XY sex reversal in *Myopus* and *Dicrostonyx* is X-linked. Ohno's Law states that the X chromosome is conserved *in toto* among all eutherian mammal species.[207] Therefore the DSS locus, which is the candidate for human dup(Xp) sex reversal, is a candidate for XY sex reversal in *Myopus* and *Dicrostonyx*. A gain of function mutation in DSS[110] would explain XY sex reversal in these species.

D. XY Sex Reversal in South American Field Mice (*Akodon* sp.)

The genus *Akodon* (Cricetidae, Rodentia) encompasses several species of small South American field mice.[190] XY females in *Akodon* was first reported in *A. mollis*.[208] Subsequently, Y-specific DNA probes demonstrated that the chromosome initially reported as a "grossly deleted X" and which was present in a percentage of *A. azarae* females was in fact the Y chromosome.[26,27] To date, three species are known to have XY females: *A. mollis, A. azarae*, and *A. varius*. Two additional species, *A. boliviensis* and *A. puer*, are suspected of having XY females based on cytogenetic data.[27,209] XY females are not characteristic of all *Akodon* species; XY females have not been identified in *A. dolores*, *A. xanthorrhinus*, and *A. molinae*.[210]

In both *A. mollis* and *A. azarae*, XX and XY females coexist. No hermaphrodites have been reported. The XY females in *A. azarae* are fully

fertile and, as in *Dicrostonyx*, produce both X and Y-bearing ova.[211] Matings between XY females and XY males give approximately a 2:1 sex ratio of females (XX, XY) to males (XY). Of 49 females born of XY females, 28 were XY, 18 were XX, one was XO, and two were XY/XO mosaics. No YY young were identified and they are presumed to be inviable. Similarly to *Dicrostonyx*, despite lethality of the YY karyotype, XY females have litter sizes (4.8 young/litter; $n = 20$ litters) equivalent to or greater than XX females (3.9 young/litter; $n = 21$ litters).[211] Counts of corpora lutea suggest that more ova are released from an XY ovary relative to an XX ovary (8.6 vs. 5.7) thus compensating for the loss of the YY progeny.[212]

XY sex reversal in *Akodon* is *not* X or autosomal-linked. In *Myopus* where XY sex reversal is clearly X-linked, X*X females give rise to X*Y females. In contrast, in *A. azarae*, no XY females were identified in 56 females born to XX females. Two of the XX females tested were daughters of XY females and therefore should have sired XY females if the sex reversal is X or autosomal-linked.[211] XY females are derived only from other XY females, presumably from the Y-bearing oocytes. The genetic data suggest two different Y chromosomes coexist in *A. azarae:* one that is capable of normal testis determination and found in XY males and a second that is present in XY females.[211,213]

One hypothesis for the etiology of XY sex reversal in *Akodon* is a null mutation of *SRY* on the Y of XY females rendering it inactive in sex determination.[212] However, molecular analysis of *SRY* and *ZFY* have failed to identify any polymorphisms indicating that the "inactive" Y of XY females is different from the "normal" Y of XY males.[27,210,213] Furthermore, molecular analysis of *SRY* from both normal males and XY females have not identified any mutations in XY females within a 157-bp segment of SRY corresponding to a part of the *SRY* ORF that includes the HMG box.[213] RT-PCR studies on the expression of *SRY* in the fetal gonads of XY females would be informative.

Multiple copies of *SRY* exist on the Y chromosome of *A. azarae* (2-3 copies) and A. mollis (3-4 copies).[213] However, several lines of evidence suggest that the multiple *SRY* genes are not the cause of the XY sex reversal: (1) multiple *SRY* genes are equally present on the Y from XY males,[213] (2) multiple *SRY* genes are present in *Akodon* species in which XY females do not exist,[213] (3) the neighboring gene *ZFY* is also amplified in many *Akodon* species,[27] and (4) *SRY* exists as two or more copies on the Y in several species of rodents of the Muridae families, none of which have XY females.[214,215]

Interestingly, an *SRY Pst* I RFLP was identified in *A. mollis.*[213] Southern blot analysis showed two males and one XY female giving only a single *SRY* band. In contrast, a second XY female had the same *SRY* band plus a second, smaller band. The RFLP data suggest that the *A. mollis* XY female population is heterogeneous and harbors two different Ys, one of which is similar (or identical) to that in males. It has been proposed based on breeding studies with

A. azarae that XY sex reversal is caused by a Y that is incapable of testis determination. If true, this Y must be clonally inherited and transmitted only to other XY females.[211] If correct, three hypotheses explain the *SRY* RFLP in *A. mollis*: (1) The *Pst* I RFLP occurred in a line of XY females, (2) inactivation of the Y occurred on more than one occasion in *A. mollis*; and (3) XY females are derived from both XY males and XY females in *A. mollis.*

E. XY Sex Reversal in the Domestic Horse (*Equus caballus*)

XY sex reversal in the horse was first reported in 1975 when it shown to be associated with infertility or low fertility.[216] Twelve percent (22/180) of mares with primary infertility and sterile ovaries were of XY karyotype.[217]

XY sex reversal in the horse results in a range of phenotypes: (1) XY females with fertile ovaries that are normal or slightly small in size, (2) XY females with small, sterile ovaries, (3) XY masculinized females with intra-abdominal ovotestes or testes, and (5) XY males that transmit the mutation.[218,219] Fifty-two percent (14/27) of XY sex reversed horses have small, sterile ovaries. One XY female, despite having small ovaries and an underdeveloped uterus, sired six young, one of which was an XY female.[220,221]

Pedigree analysis suggests that XY sex reversal in the horse has at least two different etiologies. The first is transmitted through females probably through an X-linked or autosomal male-limited dominant gene.[218] This would be comparable to X-linked or male-limited XY sex reversal in humans.

The second is Y-linked and paternally transmitted either through XY males carrying a Y chromosomal mutation (*SRY*?) with variable penetrance or through males that are gonosomal mosaics for Ys with normal and abnormal *SRY* alleles.[218] Both of these explanations for Y-linked XY sex reversal are plausible since data suggest that both occur in familial cases of XY gonadal dysgenesis in humans (see above).

Molecular genetic data on XY sex reversal in the horse is sparse. A preliminary *in situ* hybridization study with a snake recombinant DNA repetitive probe called Bkm (*b*anded *k*rait *m*inor satellite) that recognizes a tetranucleotide repeat sequence of GAT/CA on the Y of the mouse, revealed an inverse relationship between masculinization and the presence of Bkm-homologous sequences on the Y. This suggests that deletions of the Y may be present in XY females.[221] The data should be confirmed with more specific Y chromosomal probes, for example *SRY* or *ZFY*.

III. XX SEX REVERSAL

XX sex reversal has been reported in humans, the laboratory mouse,[222] the domestic goat (*Capra hircus*),[223] dogs (*Canis familiaris*),[224] and the domestic

swine (*Sus scrofa*).[225-229] In addition, XX sex reversal has been reported in the mole (*Talpa occidentalis*)[230] and llama (*Lama* sp.).[231]

A. XX Sex Reversal in Humans

The first XX sex reversed patient was reported in 1964.[232] XX sex reversal occurs in newborn males at a frequency of 1:20,000-25,000.[233] XX sex-reversed patients can be divided into two classes based on the presence (Y+) or absence (Y−) of Y chromosomal sequences. Approximately 80% of XX sex reversed patients are Y+.[234] The phenotype of Y+ XX sex reversed patients range from XX males to XX hermaphrodites. In general, Y+ XX sex reversed patients are phenotypic males ("XX males") with no genital ambiguities. If genital ambiguities are observed, there is a positive correlation between the amount of Y chromosomal material present and the degree of masculinization. Six Y+ XX hermaphrodites have been reported in the literature (reviewed in Fechner et al. [1994][235]).

Y- XX sex-reversed patients usually have ambiguous genitalia, hypospadias, cryptorchidism, and/or hermaphroditism. Secondary sexual characteristics range from male to female depending upon the quantity of testicular tissues present in the gonads. Y-XX sex-reversed patients without ambiguous genitalia have been documented.[236,237]

Familial cases of XX sex reversal (Y+ and Y−) are known with XX males and XX hermaphrodites sometimes being present in the same pedigree.[111,236,238-240]

The phenotype of Y+ and Y− XX sex-reversed patients are similar. XX males tend to be shorter than normal males and have normal body proportions. XX males are sterile. The testes of XX sex reversed infants possess spermatogonia but these degenerate before 10 years of age, the exact age of germ cell loss being variable.[237,238,241] The adult XX male testis is small, azospermic, with hyperplastic Sertoli and Leydig cells, and hyalinized tubules. The penis and scrotum are normal and gynecomastia often occurs at puberty. About 10% of XX males have hypospadias.

XX hermaphrodites tend to be shorter than XX males. The phenotype of the XX hermaphrodite ranges from male to female with most having ambiguous external genitalia at birth. Most of the male phenotypes have hypospadias and/or cryptorchidism and exhibit gynecomastia at puberty.[34,63] Ramsay and colleagues[242] report that the gonads of 37 XX hermaphrodites were composed of an ovary and contralateral ovotestis in 51.4% of the cases, bilateral ovotestes in 18.9%, and a testis and contralateral ovary or ovotestis in 24.3%. Similar results were obtained by van Niekerk[62] and Simpson.[63] The XX ovotestis is similar to the XY ovotestis at the gross anatomical and histological levels.

XX hermaphrodites with female phenotypes can be fertile. Two XX hermaphrodites that presented with an ovotestis and contralateral ovary became pregnant following removal of the ovotestis.[243,244]

It should be emphasized that the designation of a patient as an XX male in the literature should be viewed with caution, especially if the patient has ambiguous genitalia, hypospadias, or other genital abnormalities. Often ovarian tissues are localized within an ovotestis and these can be missed by gonadal biopsy. Indeed, a diagnosis of "XX male" being changed to "XX hermaphrodite" is not unusual once a more complete histological analysis is performed.[237,238]

Etiology

In humans, as in the mouse, the X and Y chromosomes undergo an obligatory pairing and recombination during meiosis in the male.[245] Pairing and recombination occur primarily at the telomeric region of the short arm of the X and Y chromatids in an area designated the pseudoautosomal region, so named because genes present in this region of the Y appear autosomal, and not sex-linked. A smaller region of exchange is present at the tip of the Y and X long arms.[246]

The human *SRY* locus is located in a 35 kb region of the Y that is proximal from the pseudoautosomal boundary of the Y (PABY).[247] Y+ XX sex reversal arises from the abnormal transfer of Y-specific sequences, including *SRY*, from the Y chromatid to the X chromatid during meiotic pairing and recombination. The abnormal X-Y interchange occurs at regions on the X and Y that are highly homologous.[234] Sperm bearing an X carrying *SRY* give rise to XX sex reversal.

The hypothesis that XX sex-reversed patients were derived from the translocation of Y chromosomal material to the X was first proposed in 1966[248] and has since been confirmed using genetic,[249] cytogenetic,[250] and molecular analyses.[251-260]

The Y chromosomal sequences that are translocated to the X are variable in length.[91,236,253,259,261] All but one case included the pseudoautosomal boundary of the Y (PABY) as would be expected if X-Y interchange is involved. The exceptional XX male had Y sequences from the proximal but not distal Yp and did not have sequences for *SRY* or PABY.[262] PABY is the minimal piece of the Y that would be transferred to the X if abnormal X-Y exchange had occurred.

XX males who harbor discontinuous segments of Yp have been reported.[236,253,263] It has been proposed that these exceptional XX males arise from fathers who are polymorphic for inversions on Yp.[253,263]

As noted above, in familial cases of Y+ XX sex reversal, both XX males and XX hermaphrodites have been identified.[238,264] Preferential inactivation of the X carrying *SRY* may decrease the number of cells expressing SRY resulting in XX hermaphrodites. Preferential inactivation of the X carrying Y-specific sequences was demonstrated *in vitro* for cells from two XX males and one XX hermaphrodite.[235,265]

In southern Africa, a high frequency of XX hermaphroditism have been reported in the Bantu-speaking black population.[62,242] The majority of the patients have ambiguous genitalia; XX males are rare. The cases are sporadic and do not cluster in any chiefdom. Furthermore, family history studies did not reveal any evidence of relatedness among the affected individuals. Although molecular analysis with Y probes on 10 XX hermaphroditic patients did not reveal any Y chromosomal material,[242] the presence PABY and *SRY* remain to be tested in these patients.

The etiology of Y- XX sex reversal is unknown. That Y- XX sex-reversed patients truly lack Y chromosomal material was demonstrated by the absence of PCR amplification of PABY and the absence of hybridization with recombinant DNA probes that recognize Y-specific sequences close to PABY.[240,260,266]

The regulatory cascade hypothesis for mammalian sex determination has been proposed to explain Y- XX sex reversal.[111] In this hypothesis, a loss of function mutation has occurred in an autosomal (or X-linked) gene (Z) that normally represses testis determination (or induces ovarian differentiation). A loss of function mutation will allow testis differentiation to occur in the absence of *SRY*. The loci linked to XY sex reversal (*DSS*, campomelic dysplasia, 9p) are all candidates for Z.

B. XX Sex Reversal in Mice: The Sxr Mutation

The sex-reversed mutation (Sxr) (XY, Tp(Y)1Ct) was identified in 1971[222] in a male mouse carrying an unbalanced, duplication of the X-autosome translocation, *flecked*. Breeding data using a coat-color marker gene and cytogenetic analysis showed that two of its sons were XX. The XX males had small testes and were sterile. However, two normal sons were found to carry Sxr and were used to propagate the mutation. The interested reader is referred to a recent review[267] for additional details on XX sex reversal in the mouse. The Sxr mutation has proven invaluable for studies on sex determination, spermatogenesis, X-inactivation, meiotic pairing and recombination, and mapping of Y genes.

Syndrome

The XX male mouse (XXSxr), in general, is comparable to XY males with regard to behavior, weight, and external genitalia.[222,268] Phenotypic differences that have been reported include a reduction in anogenital distance,[269] the failure for the head of the XXSxr epididymis to develop normal vasculature or an initial segment,[270,271] and abnormal bioregulation of the extracellular matrix within the testis and epididymides.[272]

The most prominent characteristic of XXSxr males is sterility. XXSxr males have small testes with hypoplastic tubules containing abnormal Sertoli cells

and an absence of all stages of spermatogenesis. The interstitial Leydig cells are hyperplastic.[222,273] No obvious feminization of the internal organs is present.

The cause of the sterility is the degeneration of the XX germ cells prior to and soon after birth. Testis development of the XXSxr male at 11.5-12 dpc (normal gestation period = 19-20 days) is normal.[274] XXSxr males have spermatogonia at 16 dpc. However, the spermatogonia degenerate just prior to and soon after birth and are gone by 10 days *post partum*.[222] Spermatogonia loss is attributed to the presence of two X chromosomes.

Two observations support the hypothesis that an XX karyotype is incompatible with spermatogenesis. First, XSxrO males have spermatogonia and spermatogenesis. XSxrO males are generated by mating XO females, which are fertile in the mouse, with XYSxr males. In contrast to XXSxr males, spermatogonia do not degenerate in XSxrO testes and spermatogenesis is present within the seminiferous tubules at puberty. However, spermatogenesis is abnormal due to the absence of a meiotic pairing partner for the X and only immotile spermatozoa with abnormally shaped heads are formed.[222,275-277] Second, in some XXSxr testes, tubules with spermatocytes undergoing meiosis have been identified. These were found to be of XO, not XX, karyotype. The XO spermatocytes are derived from spermatogonia that have lost one their two X chromosomes by non-disjunction.[278]

Because XXSxr males are sterile, the Sxr mutation is propagated through males carrying Sxr (XYSxr). XYSxr males are fertile but their testes are smaller compared to age-matched XY testes. Occasional XYSxr males are sterile or have reduced reproductive life spans.[268] The smaller testis size first becomes obvious at about 2.5 weeks *post partum* and is due to a 15-fold increase in degeneration of pachytene spermatocytes. The degeneration of the spermatocytes is linked to sex chromosome univalence.[275] The cause for the high frequency of X and Y chromosome univalence during meiosis is unclear but it is linked to the Sxr mutation.[279]

Two pieces of data suggest that testis development in the XXSxr fetus may not be completely normal. In the mouse fetus, oocytes enter meiosis at about 14 dpc; spermatocytes enter meiosis after birth. In 83% (15/18) of 15-17 dpc XXSxr gonads a small number of germ cells were found to be in meiosis suggesting that these germ cells are developing into oocytes.[280,281] Most were situated close to the mesonephros. In contrast, no meiotic germ cells were found in 51 XY or XYSxr and five XOSxr siblings. A variable number of these meiotic germ cells survive and develop into oocytes since 29% (6/21) of XXSxr testes studied histologically at 8-18 days *post partum* had one or more growing oocytes; none were found in 30 normal (XY or XYSxr) testes of the same age. The oocytes were found in seminiferous tubules (Figure 13) or within the rete testis.[280,281]

Second there was a report of an XXSxr hermaphrodite fetus with bilateral ovotestes that was identified in a study of 15-16 dpc XXSxr fetuses.[282] Additional study on the fetal development of XXSxr males is warranted.

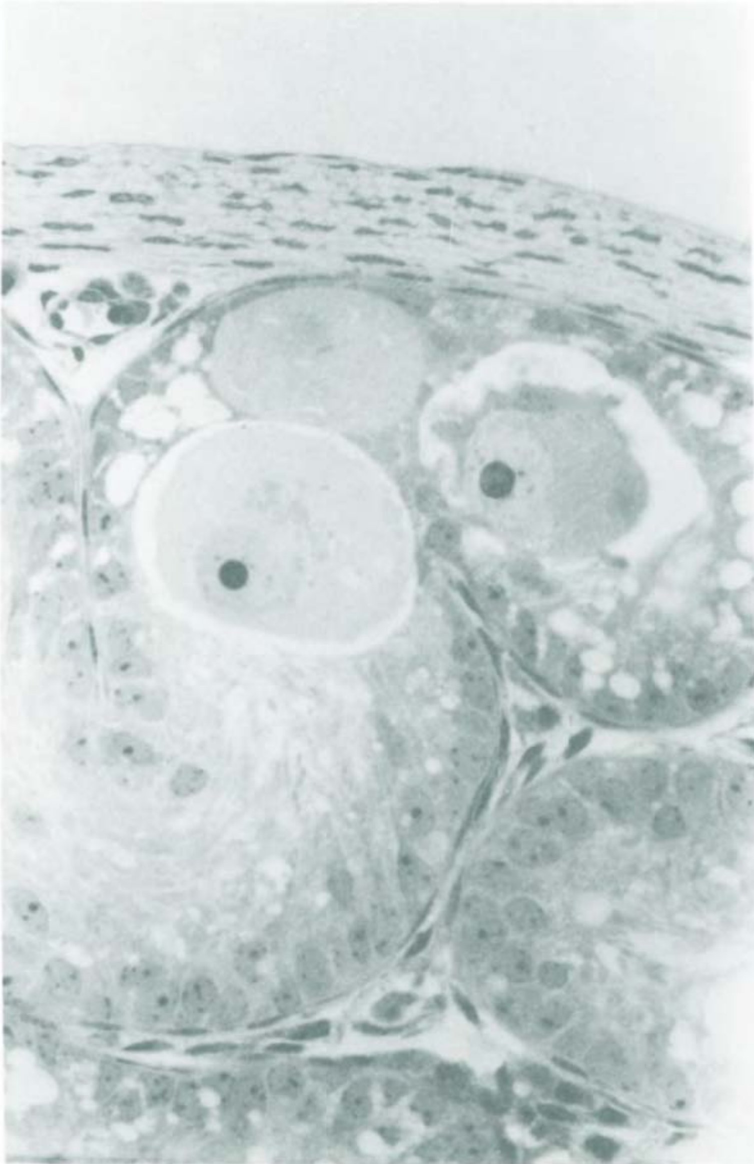


Figure 13. XXSxr male sterile testis. Three oocytes are present in a seminiferous tubule. Spermatogenesis is absent in the seminiferous cords.

Etiology

The mode of inheritance of Sxr suggested an autosomal dominant mutation. The observation that Sxr was never transmitted through a female suggested that penetrance was 100% and all XXSxr mice developed as males. However, attempts to map Sxr to an autosome by linkage analysis were unsuccessful.[268]

The reason for this failure became obvious in 1982[283] when Southern blot analyses and *in situ* hybridization were performed with the snake probe, Bkm. Although Bkm sequences are scattered throughout the mouse genome, some are concentrated on the mouse Y chromosome. Southern blot analysis using Bkm as a probe suggested that XXSxr male mice carried Y chromosomal material. Furthermore, the interpretation of *in situ* hybridization studies on XYSxr meiotic chromosomal spreads suggested that Bkm sequences were being transferred from a Y chromatid to an X chromatid during meiosis.[127,283,284]

The Sxr mutation arose from a duplication of about five megabases of the murine Y short arm, including the testis-determining gene *Sry*, and the subsequent transposition of this duplicated region to the telomere of the Y long arm, distal to the pseudoautosomal region (Figure 14a).[127,284-288]. The duplicated portion of the short arm is designated here as the "Sxr region." Because the Sxr region is distal to the pseudoautosomal region, meiotic X-Y pairing and recombination transfers the Sxr region from one Y chromatid to one X chromatid (Figure 14b). Four types of sperm are produced (YSxr, Y, XSxr, and X) and upon fertilizing an oocyte, these give rise to XYSxr carrier males, normal XY males, XXSxr males, and XX females, respectively.

Pairing and recombination during meiosis is obligatory and spermatocytes in which the Y fails to pair degenerate.[277,285,289] Genetic data confirming that the Sxr region was distal to the pseudoautosomal region was obtained by the linkage of Sxr to the pseudoautosomal gene steroid sulfatase.[290,291]

In addition to *Sry* and Bkm sequences, other genes and pseudogenes have been mapped to the Sxr region. These include *Hya*, a gene regulating expression of the male-specific, minor histocompatibility antigen HY,[286,287,292-295] the zinc finger-Y genes (*Zfy1, Zfy2*),[296-299] a homologue for the ubiquitin-activating enzyme E1 (*Ube1y1*),[300-302] pseudogenes of *Ube1y*,[303] *Smcy* (*s*elected *m*ouse *c*DNA from the *Y*),[304] and a spermatogenesis gene (*Spy*).[305-307]

XXSxr Females, XXSxr Hermaphrodites, Sxr^b

In female mice heterozygous for the X-autosome translocation T(X;16)16H (T16H), X-inactivation is not random and the normal X is preferentially inactivated.[308] XX mice heterozygous for both T16H and Sxr, T16H/XSxr, develop into one of three phenotypes: male, hermaphrodite, or female.[125,126,146,147] Which phenotype is obtained depends upon two factors:

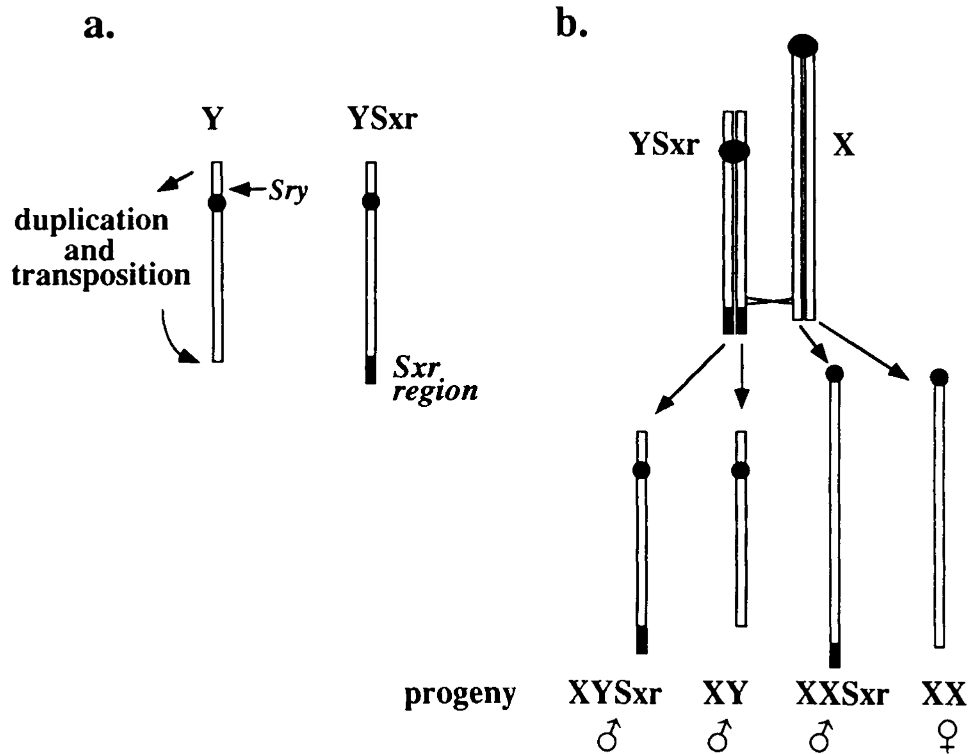


Figure 14. (a) Origin of Sxr region by a duplication and transposition of all or part of the short arm of the Y to the long arm, distal to the pseudoautosomal region.
(b) Cartoon illustrating the inheritance of Sxr.

whether X-inactivation has spread into the Sxr region to inactivate *Sry* and the percentage of cells in which *Sry* is inactivated.[267,294]

The T16H/XSxr females are fertile. T16H/XSxr females mated to XYSxr males give rise, in addition to other progeny, to viable XSxrYSxr males. XSxrYSxr males are fertile and when mated to normal females sire predominantly males and rare XO females who are derived from sperm carrying neither sex chromosome.[309]

An Sxr variant that lacked the male-specific antigen HY was identified in a (T16H/X ♀ × XSxrYSxr ♂) F1 female.[295]. The Sxr variant was named Sxr^b (= Sxr') and the original sex-reversed mutation was renamed Sxr^a.[310] At the gross anatomical level, $XXSxr^b$ males are indistinguishable from $XXSxr^a$ males. However, while $XOSxr^a$ males have spermatogenesis, XOSxrb males are severely compromised with fewer spermatogonia and only rare spermatocytes.[305,307] The Sxr^b variant has a deletion of > 900-kb in the Sxr region between the *Zfy2* and *Zfy1* genes. The deletion removed the genes *Hya, Smcy, Spy,* and *Ube1y1.*[311,312] In addition, the coding sequences of *Zfy2* and the promoter region of *Zfy1* were deleted resulting in the fusion of the promoter and 5' untranslated region (UTR) of *Zfy2* with the coding sequences of *Zfy1.*[313]

C. XX Sex Reversal in the Domestic Goat (*Capra hircus*): The Polled Mutation

Intersexed goats have been reported since 1913 and it was known that a high incidence of intersexes occur in the Saanen (11.1%) and Toggenburg (6%) goat breeds.[223,226] However, it was three decades later before the intersexuality was genetically linked to an autosomal dominant mutation causing hornlessness called polled (*P*).[314] The high incidence of sex reversal in Saanen goats can be attributed to selection for P; hornlessness is required for registration in the Saanen breed.[315] Subsequent cytogenetic studies demonstrated that polled intersexes had an XX karyotype (XX sex reversal).[223,226,315]

Heterozygous *P*/+ male and female goats are hornless and normal. In contrast, homozygous *P*/*P*, XX goats, in addition to being hornless, exhibit varying degrees of sex reversal ranging from XX males with sterile testes to hermaphrodites with female phenotype but intra-abdominal ovotestes. The more masculinized XX intersexes lack Müllerian duct derivatives (oviducts, uteri, vagina) and have well developed Wolffian duct derivatives (epididymides, vasa deferentia, seminal vesicles). As in XX male human patients and XXSxr male mice, germ cells are present in the XX gonads during fetal development (up to day 126 of the 150 day gestation period) but are absent after birth.[223,315-318]

One possible explanation for the XX sex reversal is the freemartin effect whereby an XX fetus connected to an XY twin via chorionic vascular

anastomoses is masculinized (reviewed in Jost et al. 1973[15]). This explanation is plausible since polled causes, in addition to hornlessness, a higher incidence of twins and triplets. Due to the mixing of the freemartin's and male co-twin's blood systems during fetal development, a freemartin is a chimera with both XX and XY lymphocytes. The vast majority of XX sex reversed polled goats do not have any evidence of blood chimerism suggesting that they are not freemartins. Furthermore, XX sex reversal is also seen in single births and when twinning is present, the XX sex reversal does not correlate with the sex of the co-twin.[223,315]

The polled mutation also affects some XY males. *P*/*P*, XY goats are hornless males with normal male phenotype. However, about 10-30% of *P*/*P*, XY males have infertility resulting from congenital occlusion of the caput epididymis.[315,319]

Southern blot and PCR analyses were negative for the Y-chromosomal genes *SRY* and *ZFY* in four *P*/*P*, XX intersexes.[316,317] The PCR data suggest that XX sex reversal is not due to a Y-autosome translocation.

The XX sex-reversed goat is a clear example of an autosomal gene causing XX sex reversal. The genetic and molecular data suggest that *P* or a closely linked autosomal recessive gene is causing XX sex reversal and that penetrance of the gene is variable. Cloning of the polled locus may identify one or more genes that regulate horn development, litter size, epididymal function, and XX sex reversal.

D. XX Sex Reversal in the Domestic Dog (*Canis familiaris*)

XX sex reversal has been reported for the American and English cocker spaniels, beagles, Chinese pug, Kerry blue terrier, Weimaraner, and German short-haired pointer.[320,321] As in XX sex-reversed goats, the phenotype is variable ranging from XX males with sterile testes to XX hermaphrodites with female external and internal characteristics but bilateral ovotestes or an ovotestis and ovary. Both XX hermaphrodites and XX males can occur in the same litter.[322]

XX males comprise only 10% of the affected cocker spaniels.[323] Of five XX males who have been described in detail, four were cryptorchid with testes that were intra-abdominal while one had a left scrotal testis and a right inguinal testis.[323,324] The external genitalia were abnormal (e.g., malformed or hypoplastic penis, relatively shorter genital-anal distance compared to normal males, presence of hypospadias, absence of scrotum). The XX testes were small and composed entirely of seminiferous tubules with vacuolated Sertoli cells and no germ cells; the Leydig cells appeared normal.

An important clue to the XX sex reversal may be that all had Müllerian duct derivatives (uteri or vagina) despite the gonads being composed completely of testicular tissues. Meyers-Wallen et al.[322] demonstrated that the XX testes

were capable of secreting functional MIS *in vitro*. The presence of Müllerian duct derivatives in XX males may be due to insufficient and/or inappropriately timed expression of MIS during fetal development, possibly arising from a delay in testicular development.[322]

The XX hermaphrodite cocker spaniel usually has a female external phenotype. The internal organs usually consist of bilateral ovotestes or an ovary and contralateral ovotestis and the gonads are present at the normal ovarian position.[323] Rarely, an ovotestis and testis are present.[323,324] In contrast to XX and XY hermaphrodites in humans and mice, laterality with regard to testis development was absent, that is testicular cords developed equally in the left or right gonad.[323]

The proportion of ovarian and testicular tissues in each ovotestis within a hermaphrodite is usually the same with the ovarian region being cortical and the testicular cords being present at the hilus of the gonad. If the testicular region is substantially large such that it reaches the surface of the gonad, its external surface was covered by a tunica albuginea. The degree of internal masculinization (presence of Wolffian duct derivatives) and external masculinization (clitoral enlargement, presence of an os clitoris) is variable. A positive correlation exists between the quantity of testicular tissues found in the ovotestes and the degree of masculinization.

Cytogenetic studies demonstrated that XX sex-reversed dogs are not mosaics or chimeras and have a normal 78,XX karyotype as determined by G-banding.[323,324] Although XX males are sterile, XX hermaphrodites can be fertile and function as a females.[224,324] One XX hermaphrodite gave birth to an XX male and a normal XX female.

A pedigreed family of American cocker spaniels that can be traced to a single male ancestor has been studied in detail.[224,322-324] In this well documented case, the XX sex reversal is familial and inherited as an autosomal recessive trait. Breeding experiments excluded fully penetrant X-linked or autosomal dominant and X-linked recessive mutations as the cause for the XX sex reversal.[323,324] The etiology of the XX sex reversal is hypothesized to be an autosomal recessive mutation with partial penetrance that induces sex reversal of the XX gonad. Molecular genetic studies for *SRY* and candidate X and autosomal testis-determining genes may prove informative as to the etiology of the XX sex reversal and confirm that the sex reversal is due to an autosomal recessive gene.

E. XX Sex Reversal in Domestic Swine (*Sus scrofa*)

Sex reversal in swine has been reported in the Western literature since the late ninteenth century and it was suggested as early as 1925 that intersexed swine were not cases of freemartins but XX sex reversal.[325,326] This was prophetic since XX sex reversal is indeed the primary cause of hermaphroditism

in domestic swine.[327] The incidence of intersex swine at slaughter is estimated at 0.1-0.2 %.[226,229]

The phenotype of the XX sex reversed pig ranges from XX males to almost normal females. Most are hermaphrodites with a female external phenotype but an enlarged clitoris. As in XX sex-reversed male dogs, a uterus is usually present, even in XX males.[327] Typically urination occurs in upward spurts allowing one to identify an XX hermaphrodite among normal females.[327-329]

XX males have sterile testes that are either abdominal or scrotal in position, and associated with epididymides, vasa deferentia, and seminal vesicles. The testes are invariably sterile and are composed of small seminiferous cords containing Sertoli cells but no spermatogonia and hyperplastic Leydig cells. Müllerian duct derivatives present in the XX male include oviducts, uteri, and/or vagina. XX males without any evidence of Müllerian duct derivatives have also been described.[228,330]

XX hermaphrodites have an enlarged clitoris, a vagina, long bipartite uterine horns, and bilateral ovotestes.[225] An epididymis and vas deferens plus oviduct was associated with an ovary in three hermaphrodites studied by Booth.[328] The testicular tissues usually occur in the medulla of the ovotestes and as in XX males are devoid of germ cells. The ovarian tissues occupy the cortex of the ovotestis and consist of ovarian stroma, developing oocytes, and corpora lutea. The right gonad is usually the more masculine of the two in hermaphrodites.[326,331]

Steroidogenesis in the testicular tissues and submaxillary gland of XX males and hermaphrodites is comparable to normal boars. A positive correlation exists between percentage of testicular tissues and degree of masculinization of the genital tract.[328]

Hermaphrodites with a female phenotype externally but a testis and ovary internally, can be fertile and give rise to normal litters.[225,331,332] In swine, the embryos distribute themselves evenly between the two uterine horns. In hermaphrodites, this still occurs and the uterine horn adjacent to an ovotestis or testis, although smaller than the uterine horn adjacent to an ovary, is not compromised in its ability to support fetuses.[333] It is clear that the presence of testicular tissues is not detrimental to ovarian function, fertility, and parturition.

The etiology of XX sex reversal in the pig is unknown. A recessive autosomal gene(s) that induces sex reversal is suggested from breeding studies.[229,330] A large pedigree giving rise to 25 intersexed pigs with a wide range of phenotypes suggest an autosomal recessive pattern of inheritance.[329] Molecular genetic studies for *SRY* or candidate X and autosomal testis-determining genes would improve our understanding of the etiology of XX sex reversal in swine.

IV. CONCLUSIONS

The molecular genetics of sex determination and sexual differentiation in the fruitfly *Drosophila melanogaster* and the nematode *Caenorhabdites elegans* are very complex. The same is undoubtedly true in mammals. The identification of *SRY* as the testis-determining gene on the Y is a milestone in our understanding of mammalian sex determination. However, it is unclear how the SRY protein acts to induce testis determination and the genes immediately upstream and downstream of *SRY* remain elusive. XY and XX sex reversal models have proven to be and will undoubtedly continue to play prominent roles in deciphering the molecular genetics of mammalian sex determination and sexual differentiation.

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NOTE ADDED IN PROOF

Candidate genes for dosage sensitive sex reversal were cloned from the DSS locus: *DAX1*, a member of the nuclear hormone receptor superfamily (*Nature* **1994**, *372* 635-641; 672-676), and several members of *MAGE* superfamily of tumor-associated antigens (*Mammalian Genome* **1995**, *6* 571-580; *Proc. Natl. Acad. Sci. USA*. **1995**, *92*, 4987-4991). The gene responsible for XY sex reversal in campomelic dysplasia was identified as the *SRY*-related gene *SOX9* (*Cell* **1994**, *79*, 1111-1120; *Nature* **1994**, *372*, 525-530).

Southern Africa XX hermaphrodite patients were found to be *SRY* negative (*AM.J. Med. Genetics* **1995**, *55*, 53-56). Similarly, XX males and XX hermaphraodites in the domestic dog and swine are *SRY* negative (*Mol. Reprod. Develop.* **1995**, *41*, 300-305; *Animal Genetics*. **1994**, *25*, 299-305).

MOLECULAR GENETICS OF X-CHROMOSOME INACTIVATION

Mary F. Lyon

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I. INTRODUCTION

Although X-chromosome inactivation in mammals was discovered as long ago as 1961[1,2] the mechanism by which it is brought about is still not understood. Nevertheless recent advances in molecular biology have brought considerable new insights into the phenomenon and there is reason to hope that major breakthroughs in knowledge may occur in the not too distant future.

X-chromosome inactivation has been well reviewed recently[2,3,4,5] and this review will concentrate mainly on the most recent findings.

The inactivation of a single X-chromosome in somatic cells of female mammals is regarded as a mechanism of dosage compensation, having the effect that chromosomally XX females and XY males both have a single dose of the products of X-linked genes. In the very early embryo both X-chromosomes of females and the single X-chromosome of males are thought to be active. The initiation of X-inactivation (XCI) occurs at the blastocyst to egg cylinder stage, and once it has occurred each X-chromosome maintains its active or inactive state throughout all further cell divisions in the life of that individual. An exception occurs during the development of female germ cells, in which a previously inactive X-chromosome is reactivated as the cell enters meiosis, so that in mature oocytes both X-chromosome are in the active state. Conversely, in male germ cells the X-chromosome becomes inactive at or just before the onset of meiosis.

In the embryo proper of eutherian mammals either the maternally (X^m) or paternally (X^p) derived X-chromosome may be inactivated in different cells. However, in the trophectoderm and primitive endoderm cell lineages of mice and rats the paternal X-chromosome is preferentially inactivated in all cells. This is an example of genomic imprinting.

The inactivation of one X-chromosome in females is achieved by a system of chromosome counting, such that one X-chromosome is maintained active per two autosome sets. The evidence for this comes from sex chromosome aneuploids, such as XO, XXY, XXXX, in which a single X-chromosome remains active no matter how many are present, and from triploids and tetraploids which may have two active X-chromosomes.

The onset of X-inactivation in the embryo is thought to be initiated from the X-inactivation center (XIC) on the X-chromosome. The evidence for the

existence of such a centre comes from mouse X-autosome translocations.[6] Only one of the two segments into which the X-chromosome is broken by such translocations is capable of undergoing inactivation. The interpretation is that only segments containing the XIC can be inactivated. Both X-chromosome imprinting and the counting mechanism are thought to act through the XIC.

After the initiation of XCI by the XIC, the signal for inactivation is thought to spread in both directions along the chromosome, and the mechanism of spreading, although unknown, is suggested to be different from that of initiation of inactivation. Spreading is followed by the maintenance or stabilization of inactivation through all subsequent cell divisions. This again is thought to be achieved by a different but unknown mechanism.

The inactivity of the X-chromosome is manifested by lack of transcription, and in addition the chromosome has other characteristic properties.[2] It shows asynchronous replication, with DNA replication commencing late in the S phase; it is condensed to form the sex chromatin body during interphase; it shows differential methylation of CpG islands; and it lacks acetylated histone 4.[7]

The mechanism of X-inactivation has mainly been studied in mice and humans, using both cultured cells and *in vivo* studies. In the mouse a system of particular interest is the use of embryonic stem (ES) cells and embryonal carcinoma (EC) cells. In a chromosomally XX EC cell line Martin et al.[8] showed that while the cells were maintained in a totipotent undifferentiated state both X-chromosomes remained active but when the cells were allowed to differentiate XCI took place. Chromosomally XX ES cells behave similarly.[9] Thus, by appropriate manipulation of EC or ES cells factors affecting the initiation of X-inactivation can be investigated.[10]

Marsupials have provided a further valuable source of material for study of XCI. In this group the phenomenon differs from that seen in eutherian mammals in two major ways. First, in contrast to the random inactivation of X^m or X^p in different cells of the embryo proper in eutherians, in marsupials there is preferential inactivation of X^p in all cells. Second, whereas in eutherians inactivation is highly stable and reactivation occurs at a very low level if at all, in marsupials reactivation occurs readily for some genes in cultured cells and for others *in vivo* in some tissues.[11]

II. X-CHROMOSOME ACTIVITY IN GERM CELLS

Whereas XCI in somatic cells is regarded as a system of dosage compensation, changes in X-chromosome activity in germ cells are believed to have other functions. The active state of chromatin is believed to be required for meiotic pairing and recombination (reviewed in McKee and Handel[12]). Thus, in mammalian females reactivation of the previously inactive X-chromosome is

needed to bring both X-chromosomes into the active state in order for meiotic pairing and recombination to occur. It is also thought that in the heterogametic male conversion of the sex chromosomes to the inactive state protects against potentially harmful non-homologous recombination between the X and Y-chromosomes.[12] In addition, the presence of unpaired or unsaturated pairing sites in the active state is thought to be deleterious to the viability of germ cells.[12,13,14] There is evidence that initiation of recombination is accompanied by double-strand DNA breaks (reviewed in Mckee and Handel[12]), and that if these are not repaired by homologous recombination harmful results are likely to ensue. Thus, the inactivation of the single X-chromosome of the male in germ cells is postulated to protect against both non-homologous recombination and the occurrence of harmful unrepaired double-strand DNA breaks. In addition there seems to be some harmful effect of incorrect X-chromosome dosage in germ cells, since in males with supernumerary X-chromosomes all germ cells die at the spermatogonial stage, that is, before the time of meiosis.[13]

In females, the reactivation of the inactive X-chromosome appears to occur at the onset of meiosis.[15,16] However, in male germ cells the timing of onset of inactivation is less clear. The X-chromosome appears to be late replicating in the pre-leptotene S phase (reviewed in Mckee and Handel[12]). In addition, Singer-Sam et al.[17] found that transcripts of the mouse *Pgk1* gene were already greatly reduced early in meiotic prophase, and that the shutting off of transcription probably began in the spermatogonial stage.

The inactive X and Y-chromosomes in the male germ cell are sequestered in the sex vesicle or sex body. Handel et al.[18] studied the formation of the sex body in mice with various rearrangements and other changes of the sex chromosomes. In all cases a sex body was formed, and in mice with X-autosome translocations both segments of the X-chromosome were included in it. Thus, in contrast to somatic XCI there is no evidence for the role of any control centre on the X-chromosome, such as the X-inactivation centre.

III. X-CHROMOSOME ACTIVITY IN EARLY EMBRYOS

The activity of both X-chromosomes in early female mouse embryos was demonstrated by Epstein et al.[19] who measured activity of the X-linked enzyme HPRT in blastocysts of known sex. This has been confirmed in later work but it appears that activity of X^m and X^p may not be equal. Studies have been conducted at the levels both of protein product and of transcription of mRNA. At the protein level activity of the product of the paternally derived *Pgk1* gene is not seen until six days of development, that is, after the time of onset of XCI.[20,21] However, at the transcription level, Singer-Sam et al.[22] showed that paternal *Pgk1* mRNA is in fact produced at the two-cell stage as is also *Hprt*

mRNA, and expression persists at the four-cell, eight-cell and blastocyst stages, although the level of mRNA produced by the paternal allele is lower than that of the maternal allele. Moore and Whittingham,[23] studying HPRT enzyme activity, also found expression of the paternal allele to be lower. Tam et al.[24] studied the behavior of a ubiquitously expressed X-linked *lacZ* transgene in early embryos, as judged by histochemical staining for *lacZ*. When on the X^m the transgene was first expressed at the 4-8 cell stage, whereas on the X^p expression was not seen until the 32-cell stage.

Thus, the general picture is that both X-chromosomes, X^m and X^p, are expressed before the time of X-inactivation in the female mouse embryo, but that activity of X^p may appear later and be at a lower level than that of X^m. This fits with the work of Thornhill and Burgoyne[25] who found that chromosomally X^pO mouse embryos were smaller than XX, which in turn were smaller than X^mO. This could be due to some reduced activity of X^p in early stages, but as the embryos were studied after X-inactivation, at 10.5 days, the effect could also be due to some inactivation of the X^p in the imprinted cell lineages, the trophectoderm and primitive endoderm.

In marsupials, no suitable experimental material comparable to that of the mouse embryo is yet known, and work on marsupial embryos is technically difficult. Hence, little data on X-chromosome activity in embryos exist. The earliest embryos studied were unilaminar blastocysts of the marsupial mouse *Antechinus stuartii*, in which a late replicating X-chromosome was already present.[26] In later embryos, only the maternal isozyme of α-galactosidase was produced in both embryo and yolk sac. In kangaroo embryos, *Macropus robustus*, which were examined rather later in development, all tissues from the embryo and extraembryonic membranes showed activity of the maternal allele of *G6pd* only, except for the yolk sac, in which both alleles were expressed,[27] indicating that X-inactivation was incomplete in the yolk sac. The American marsupial *Monodelphis domestica* has no known X-linked variants but sex chromatin bodies and a late replicating X-chromosome could be seen in the embryonic area and yolk sac of blastocysts.[28] Thus, evidence of the presence of an inactive X-chromosome was found in the youngest embryos studied and the possibility cannot be excluded that in marsupials the X^p is inactive from fertilization onward, having entered the zygote in an inactive state from the male germ cell, and that there is no stage with both X-chromosomes active. This is, however, merely a possibility.

IV. INITIATION OF X-INACTIVATION

A. Timing of X-inactivation

Takagi et al.[29] found the first indication of X-inactivation to be asynchronous replication of X^p in blastocysts at 3.5 days. The asynchronous chromosome

replicated early rather than late in S-phase and a switch to late replication occurred later in development.[30] Inactivation followed soon after in the primitive endoderm, and occurred in the primitive ectoderm, or epiblast, giving rise to the embryo proper, at around six days.[31] However, the recent work of Tam et al.[24] using mice transgenic for an X-linked *lacZ* transgene, suggests that inactivation may not be completed in all cells at this stage. They were able to study XCI in individual cells by observing *lacZ* expression. The percentage of cells showing expression declined gradually, rather than sharply, in different cell lineages, and some cells of the developing heart, notochord, and gut apparently did not undergo X-inactivation until 2-3 days after cells were allocated to these lineages. This is the first study in which the X-inactivation status of individual cells of embryos could be investigated, and presents a new picture. However, there must be an element of caution in interpreting the results, as it is known that some X-linked transgenes do not undergo inactivation normally.

B. Imprinting and X-chromosome Counting

Imprinting of the X-chromosome has been much studied in mouse embryos. Whether or not similar imprinting occurs in extraembryonic tissues of human females is not clear. Migeon and Do[32] and Migeon et al.[33] studied G6PD phenotypes in chorionic villi of heterozygous fetuses and reported equal expression of X^m and X^p, implying no imprinting. However, Harrison and Warburton[34] studying amnion, chorion, and chorionic villi, found predominant expression of X^m. Harrison[35] took this further by separating from chorionic villi the cytotrophoblast cells, derived specifically from the trophoblast lineage, in contrast to the stromal cells, which are of mesodermal origin. The cytotrophoblast cells showed strong preferential expression of X^m. Thus, it may be that in the human embryo, as in rodents, the trophoblastic component of the extraembryonic tissues shows imprinted preferential paternal X-inactivation, whereas the mesodermal component undergoes random inactivation.

Early studies of imprinting in mouse embryos indicated that the counting mechanism, maintaining one X-chromosome active per two autosome sets, could override the parental imprint. In chromosomally X^pO mouse embryos the single X-chromosome can remain active in the extraembryonic tissues, where X^p is typically preferentially inactivated.[36] Similarly, in diploid parthenogenetic embryos, in which all chromosomes are maternally derived, inactivation of one X-chromosome in yolk sac endoderm, as well as in the embryo itself, can take place.[37,38] However, more recent evidence indicates that counting does not completely override the imprint. In Endo and Takagi's[38] work the frequency of cells with a late replicating X-chromosome in the extraembryonic tissues of parthenogenotes was below normal. Shao and

Takagi[39] then found that embryos which had a supernumerary X-chromosome due to malsegregation of the translocation T(X;4)37H, and were of chromosomal type $X^mX^mX^p$ or X^mX^mY, had a subnormal frequency of late replicating X-chromosomes. Tada et al.[40] took this further by comparing X^mX^mY, X^mX^pY, and $X^mX^mX^p$ embryos found among the offspring of animals heterozygous for the Robertsonian translocation Rb(X.2)Ad and resulting from non-disjunction. Whereas X^mX^pY appeared normal and had a normal proportion of late replicating X-chromosomes, the X^mX^mY and $X^mX^mX^p$ embryos had a low frequency of late replicating X-chromosomes. The excessive X^m chromosome activity in the embryos was apparently harmful as the embryos were retarded and abnormal. In those cells with only a single late replicating X-chromosome it was of X^p origin, and such cells were deduced to be from extraembryonic tissues. Thus, it appears from these later results that the counting mechanism can only partially override the imprint which tends to maintain X^m chromosomes active in extraembryonic cells.

Further evidence concerning the counting mechanism has come from study of triploid and tetraploid mouse embryos. In digynic triploid mouse embryos, $X^mX^mX^p$ or X^mX^mY, either one or two X-chromosomes remained active in different cells.[41,42] In extraembryonic tissues, particularly the yolk sac endoderm, the proportion of cells with two active X-chromosomes was higher than in other tissues. Further, the inactive X-chromosome in yolk sac endoderm was predominantly X^p, whereas in embryonic tissues X^m and X^p were inactivated randomly. Cells of tetraploid embryos, formed by electrofusion of blastomeres at the two-cell stage, predominantly had two active X-chromosomes. The inactive X-chromosomes were mainly X^p in the endodermally derived tissue, and were randomly X^m or X^p in embryonic tissue.[43] Takagi,[44] studying near-tetraploid cultured cells formed by fusion of EC cells and lymphocytes, found that the pattern of X-inactivation was more variable than that found in embryos by Webb et al. It is not clear whether this was due to the unnatural conditions in these cell hybrids.

Thus, the evidence is that the imprinting and X-chromosome counting mechanisms interact, with the imprinting mechanism more prominent in the yolk-sac endoderm but the counting mechanism functioning more in the embryonic tissues. The mechanism of the counting effect is not known. It is possible that one or more specific autosomal loci are involved, but none has so far been identified. Thus, it is also possible that the effect is due to a more general effect of the total autosomal material present, perhaps via an effect on nuclear volume.

It is of interest to know whether a parental imprint persists in ES cells, while they are maintained in the undifferentiated state, and may undergo many cell divisions. The position is not yet clear. In differentiating ES cells heterozygous for marker genes Norris et al.[45] and Buzin et al.[46] found that parental imprints had been lost, so that roughly random XCI occurred. However, Tada et al.[47]

found imprinting to be still present with preferential inactivation of the X^P, in the mural region of embryoid bodies, although it was less evident in other regions. Possibly ES cell lines differ in respect to the persistence of imprinting.

C. The X-inactivation Center

Evidence for the existence of the XIC comes originally from mouse reciprocal X-autosome translocations (reviewed in Lyon[48]). Only one X-chromosome segment undergoes inactivation, on the basis of cytogenetic studies and from studies of X-linked gene products. The interpretation is that only segments carrying an XIC can undergo inactivation. The counting mechanism is suggested to block one XIC per two autosome sets and the X-chromosome with the blocked center remains active, as do segments of X-chromosome lacking an XIC.[6] In addition, some X-chromosomes with deletions fail to undergo inactivation and these are interpreted as lacking the XIC. Similar phenomena are seen in human X-chromosomes. By study of different translocations and deletions the XIC has been mapped to chromosome band XD in the mouse[9] and band Xq13 in man.

From this region in man, Brown et al.[49] cloned a gene with a unique expression pattern in that it was transcribed from the inactive (Xi) but not the active (Xa) X-chromosome. The gene was termed *XIST* (X-inactive specific transcript). A homologous gene, *Xist*, with a similar pattern of expression from the Xi only was cloned from the corresponding region in the mouse.[50,51] From the combination of its map position and its unique expression pattern *XIST*/*Xist* was considered as a strong candidate for a role in the X-inactivation center.

D. Evidence Concerning the Role of Xist

For *Xist* to have a causal role in XCI it must be expressed in the early embryo before the time of initiation of inactivity. Kay et al.[52,62] showed that although there was no expression in two-cell embryos, expression was present in pooled embryos from the four-cell stage onwards. In early embryos only the paternal allele was expressed and transcription of the maternal allele was not seen until the egg cylinder stage.

Expression of *Xist* was also studied in ES and EC cells. In undifferentiated ES cells Kay et al. found that *Xist* was not expressed, but *Xist* mRNA appeared when the cells were allowed to differentiate. Quantitative RT-PCR showed that the levels of *Xist* RNA reached in differentiated ES cells were only about 10% of those in adult kidney[46] and furthermore some ES lines showed no increase in the RNA on differentiation. In EC cells Tai et al.[53] found a low level of *Xist* RNA in undifferentiated cells. They considered this to indicate that the *Xist* gene was being expressed while both X-chromosomes were active, whereas

Buzin et al. attributed their similar finding in ES cells to the presence of a few differentiated cells in the culture.

Thus, both in the embryo and in cultured ES and EC cells the time of appearance of *Xist* transcripts precedes the onset of X-inactivation and is thus appropriate for *Xist* to have a causal role in the initiation of XCI. Moreover, in the embryo the imprinted expression of *Xist* when first seen, followed later by expression of either maternal or paternal allele, is again appropriate. An enigma is the very early appearance of expression. Although *Xist* expression was present at the four-cell stage of the embryo, an inactive X-chromosome is not seen until a few cell divisions later, at the blastocyst stage. Thus, the presence of *Xist* expression is not necessarily sufficient for X-inactivation. It is not yet clear whether some limiting threshold of expression must be reached, or whether some other factor is needed.

Using quantitative RT-PCR Buzin et al.[46] found that in 7.5-day embryos and adult female kidney the amount of *Xist* RNA was less than 2,000 transcripts per cell. They considered that these rates of transcription were too low to fit models of *Xist* action which involve *Xist* RNA coating the entire inactive X-chromosome.

In germ cells also the expression of *Xist* varies with X-chromosome activity. In the male, *Xist* expression is seen only in the testes, and specifically in the germ cells.[54,55,56] The *Xist* gene was not expressed in fetal testes,[56] which contain only primordial germ cells, but had appeared by the stage at which dividing spermatogonia were present and before meiosis, at the onset of which XCI is believed to occur. In female germ cells, by contrast, McCarrey and Dilworth[56] showed that expression of *Xist* was present at first but disappeared at the onset of meiosis, when reactivation of the Xi occurs. Thus, as in the embryo, expression of *Xist* is appropriate for it to have a causal role in XCI in germ cells, but such a role has not yet been conclusively shown. A feature of gene activity in spermatogenesis is that many genes are expressed, but so far the functional role of the expression of many of these genes is not known. It is possible that *Xist* also is a gene expressed during spermatogenesis without any clear function. It may be relevant that Kay et al.[52] claim the expression to be 1,000-fold lower in male germ cells than in the embryo.

E. Evidence from Humans on the Role of XIST

Evidence concerning a role for *XIST* in humans has come from individuals with chromosome anomalies. Human females heterozygous for a deleted X-chromosome usually have a fairly mild syndrome of malformations. However, some women with small marker or tiny ring X-chromosomes show a much more severe phenotype including mental retardation. Migeon et al.[57] and Wolff et al.[58] tested the small marker X-chromosomes from a number of such females for the presence and expression of *XIST*. In some the *XIST* gene was deleted

and in the remainder, although the gene was present, it was expressed either weakly or not at all. This suggested that the marker chromosomes were not undergoing inactivation, and that the severe phenotype was due to overexpression of X-linked genes. Migeon et al.[59] showed that genes on several tiny ring X-chromosomes were indeed transcribed. Leppig et al.[60] studied a female with a rearranged X-chromosome who provided information on the mapping of *XIST* relative to the XIC. The rearranged X-chromosome was always late replicating, and hence presumably inactive, and thus was assumed to carry the XIC. The *XIST* locus was shown to be present on the chromosome, and the breakpoint was located about 200-700 kb distal to *XIST*. This defines a new distal boundary for the XIC; previous studies had provided a proximal boundary about 500 kb proximal to *XIST*. Thus the evidence that *XIST* is correctly located to have a role in the XIC is strong. Interesting information was provided by a male patient carrying an X-chromosome with a duplication including the Xq13 region, known to bear the XIC, and shown to have a duplication of *XIST*. On the basis of its synchronous replication, and of the twofold higher expression of the X-linked gene *PGK1*, the duplicated X-chromosome was shown not to be undergoing inactivation.[61] Thus, it appears that for inactivation to occur two XICs must be present on different chromosomes.

F. Imprinting and Methylation of Xist

Kay et al.[62] and Norris et al.[45] have carried out studies on imprinting and methylation of the *Xist* gene. Methylation of cytosines in DNA has been proposed as a mechanism of imprinting[63] and in addition CpG islands in promoter regions of genes on the inactive X-chromosomes are differentially methylated. Hence it was important to know the methylation status of the *Xist* gene. Norris et al.[45] studied sites in the promoter and 5' region of the body of the gene. In somatic tissues of males these sites were fully methylated, whereas in females both methylated and unmethylated alleles were present. To identify whether the unmethylated alleles were present on the Xi, the authors made use of females heterozygous for the translocation T(X;16)16H in which there is non-random XCI with the translocated X-chromosome active in all cells. By breeding such females with recognizably different alleles of *Xist* on the two X-chromosomes, they showed that it was the allele on the inactive, non-translocated, chromosome that was unmethylated, whereas the allele on the active X-chromosome was fully methylated as in a male. In embryos, the embryo itself and the yolk sac mesoderm showed equal methylation of maternally and paternally derived alleles. However, in the yolk sac endoderm, in which there is preferential paternal X-inactivation, the paternal allele was completely demethylated. Thus the methylated state of the gene was correlated with lack of expression. In order to test whether methylation was also

associated with imprinting of *Xist* Norris et al. studied methylation in male germ cells. In the testis, in contrast to other tissues of the male, the *Xist* gene was mainly demethylated. This demethylation was a property of the germ cells, since in testes of mice lacking germ cells the gene was fully methylated. The time of appearance of demethylation was around the time of entry into meiosis, and once demethylation had occurred it persisted throughout spermatogenesis to the mature sperm stage. Thus, it is possible that this demethylation constituted the imprint on the *Xist* gene, but further work is needed to establish this. A feature of the demethylation of *Xist* in male germ cells was that methylation persisted at a small group of sites which the authors called the "Mlu cluster," but they were unable to establish the significance of this.

Some evidence concerning the interrelationship of imprinting and X-chromosome counting has come from the work of Kay et al.[62] on *Xist* expression in androgenotes, gynogenotes, and parthenogenotes. Embryos of these different uniparental types were produced by microsurgical methods. In androgenotes, with all chromosomes paternally derived, *Xist* was first expressed at the four-cell stage, as in normal embryos. In both gynogenotes and parthenogenotes, with only maternal chromosomes, however, *Xist* expression was not seen until later, at the morula to blastocyst stage. Thus, an imprinting effect on *Xist* expression is clearly seen in these uniparental embryos. A further striking point in the androgenotes concerned the counting mechanism. Whereas all gynogenetic and parthenogenetic embryos are chromosomally XX, androgenetic embryos can be XX, XY, or YY. The YY embryos would be expected to die early in development through lack of X-linked gene products. Kay et al. found that the proportion of androgenetic embryos at 4-8 cell stages expressing *Xist* was so high as to indicate that both X^pX^p and X^pY embryos were included among them, that is, the counting mechanism blocking the expression of one allele was not functional. This was further confirmed by studies of androgenetic embryos with distinguishable *Xist* alleles, in which some embryos expressed both alleles and must therefore have been XX, whereas others expressed only a single allele and thus may have been XY. In addition, some embryos genetically sexed as XY were shown to express *Xist*.

In parthenogenetic and gynogenetic embryos, although *Xist* expression was delayed relative to normal and to androgenetic embryos, it appeared at the morula or blastocyst stage. In gynogenotes with distinguishable *Xist* alleles equal proportions of expression of the two alleles were seen, suggesting, but not proving, that the counting mechanism was by this stage functional, and that each cell was expressing one or an other allele. Thus, the combined results from the various uniparental embryos suggest that the imprint on *Xist* expression is present in early cleavage stages and is lost as development proceeds, whereas the X-chromosome counting mechanism is absent at first and appears later. The question arises whether the counting mechanism is

functional at very early stages in normal embryos. This was not clear from the work of Kay et al.[52] since for the very early stages these authors used pooled embryos. However, at the blastocyst stage, when single embryos could be examined, four of 109 male embryos expressed *Xist*. Thus, the possibility remains that, at stages prior to the blastocyst, more male embryos may have been expressing *Xist*.

A further interesting point concerned the androgenetic embryos. Expression of *Xist* appeared at the four-cell stage as in normal embryos, but whereas in normal embryos such expression persists through development, in androgenotes it was down regulated at the morula stages and had almost disappeared by the blastocyst stage. The authors concluded that some other maternally-derived gene product is needed for continued *Xist* expression.

G. Possible Evolutionary Relationship of Imprinting and X-chromosome Counting

Whereas in eutherian mammals evidence of an X-chromosome counting mechanism, from polyploids and sex chromosome aneuploids, is clear, in marsupials there is almost no such evidence. Only very few aneuploids, XXY and XO, have been described, and no polyploids. The parental origin of the X-chromosomes of the aneuploids was not known,[11] and hence it is not clear whether X-chromosome activity in them depended on counting or on imprinting. In chromosomally normal animals single X-chromosome activity could be achieved by imprinting alone, since males are X^mY and would have an active X-chromosome and females are X^mX^p and would have one active and one inactive chromosome. It is possible that this is the mechanism of XCI in marsupials. An early suggestion was that in evolution somatic XCI was derived from an earlier inactivation of the sex chromosomes during male meiosis.[64,65] Inactivation of heteromorphic sex chromosomes during male meiosis has arisen in various disparate animal groups, and is obviously of important significance. The suggestion is that in primitive mammals XCI occurred first at male meiosis. The inactivity of X^p in sperm was then carried over into the new zygote and provided a starting point for somatic X-inactivation. The evidence concerning the *Xist* gene is in support of such a suggestion. The imprint on the *Xist* gene leads to the expression of the paternal allele very early in development. It is possible that early in evolution this led directly to inactivation of the X^p in somatic cells. In eutherians the X^p is reactivated and XCI is delayed until later in development, when the imprint is lost and replaced by a counting mechanism, resulting in random inactivation. It is possible that this delay in XCI is a later evolutionary development. In the primitive system the X^p may have remained inactive from fertilization, leading to the preferential paternal X-inactivation seen in present marsupials. The random XCI in eutherians is more stable than the system in marsupials,

resulting in better dosage compensation. It is possible that the greater stability of the eutherian system provided the selective advantage needed for its evolution, although at present it is not known whether there is any inherent association between random inactivation and stabilization of inactivity.

In present mammals the function of XCI seems clearly to be dosage compensation, and activity of additional X-chromosomal material is clearly harmful. However, the original role of XCI may have been different. In mouse embryos with supernumerary X-chromosomes, the extraembryonic tissues are poorly developed.[39,40] It is possible that in evolution inactivation of X^P favored the development of the extraembryonic tissues and was involved in the evolution of viviparity. It is interesting that in monotremes, which belong to the mammalian evolutionary lineage but are not viviparous, only vestiges of X-inactivation are seen.[11] Thus, the role of XCI in dosage compensation may have been added later, followed later still by a switch to random inactivation.

H. Sequence and Physical Mapping of *XIST* and *Xist* Genes

In the hope of obtaining further understanding of the role of the *XIST*/*Xist* genes, the sequences of both the human and mouse genes have been studied.[66,67] Both genes contain no substantial open reading frame, and the transcript is retained within the nucleus. In the human Brown et al. found that the transcript remained associated with the sex chromatin body, or inactive X-chromosome. Thus, the gene does not appear to encode a protein and may act through RNA. In both species the gene contains a number of tandem repeats. Comparison of several mammalian species revealed a degree of homology below that for typical protein coding regions.[68] However, the position of a major transcription start site was conserved, as also were differential methylation patterns in the 5' region of the gene on the active X-chromosome. Thus, knowledge of the sequence of *XIST*/*Xist* has not yet revealed its function.

Physical mapping in the vicinity of *XIST*/*Xist* has been undertaken in both human and mouse. In the human, a 2.6 Mb YAC contig has been constructed, covering the whole XIC region,[69] which was narrowed to 680-1200 kb. Several CpG islands were identified within the YAC contig, but none were in the XIC region. In the mouse, Heard et al.[70] developed a long-range restriction map of 1 Mb, containing a YAC contig of 550 kb, while Cooper et al.[71] constructed a physical map of 2 Mb in the neighborhood of *Xist*. As in the human, various CpG islands were present. The relative locations of the *RPS4X*/*Rps4x*, *PHKA1*/*Phka1*, and *XIST*/*Xist* genes were similar in the two species, but the *XIST*/*Xist* genes were oppositely oriented.[69] These physical mapping studies provide a framework for the identification of any other sequences in the region of the XIC that are involved in the control of XCI.

I. Relation of *Xist* and *Xce*, X-chromosome Controlling Element

Cattanach[72] has described a factor on the mouse X-chromosome, different alleles of which affect the probability of the X-chromosome bearing them becoming inactivated. Up to now four different alleles have been identified, carried in various inbred strains and the wild species, *Mus spretus*. The allele *Xce*^{c} is regarded as "strongest" in that the X-chromosome carrying it is most likely to remain active, followed by *Xce*^{b} and *Xce*^{a}. *Mus spretus* carries the recently discovered allele, *Xce*^{d}, which is stronger than *Xce*^{c}.[73] The *Xce* locus maps to roughly the same point as the XIC,[74] and in view of its effect is thought to represent part of the XIC. Therefore, connections have been sought between *Xist* and *Xce*. The expression of *Xist* varies in mice with different *Xce* alleles, being inversely related to the strength of the allele, that is, mice homozygous for the allele from *Mus spretus* show a lower expression of *Xist* than those with the allele *Xce*^{b}.[51] Furthermore, sequence differences have been found in the 5' region of *Xist*, in a T-rich spacer between repeats[68] in mice carrying different *Xce* alleles. Whether these sequence differences have any functional significance is not known. The sequence and expression differences suggest, but do not prove, that *Xce* effects might be a part of *Xist* gene function. However, results of mapping experiments with *Xce* and microsatellite markers in the neighborhood of the *Xist* gene have shown an apparent crossover between *Xce* and *Xist*.[75] Thus, the relation of *Xce* and *Xist* remains unclear.

V. SPREADING AND MAINTENANCE OF X-INACTIVATION

The spreading of inactivation from the XIC along the chromosome after initiation of XCI and the subsequent maintenance of inactivity throughout all further cell divisions are regarded as separate phenomena, probably with different mechanisms. Little is yet known about either process. However, ideas as to the mechanisms of each overlap, with certain features of the inactive X-chromosome, such as late replication and differential methylation, suggested as being involved in both spreading and maintenance of inactivity. Thus, it is more convenient to consider the two phenomena together.

Spreading is regarded as a long range cis-limited process, operating over relatively long distances in chromatin. The evidence for this comes from mouse X-autosome translocations. In these XCI spread from the X-chromosome segment carrying the XIC into attached autosomal material, and the resulting inactivation can lead to variegation for autosomal coat color genes. In addition, the inactivated autosomal segment shows late replication and differential Kanda staining. The distance of spread differs in different translocations, and from cell to cell in the same translocation.[6,76] It can extend over several chromosomal G-bands and thus probably over 30-40 Mb. Spreading of XCI

somewhat resembles position effect variegation in *Drosophila*, in which genes normally located in euchromatin undergo inactivation when translocated close to heterochromatin. However, this operates only over distances of around 1.5 Mb.[77] Similar spreading of inactivation into autosomal material is seen in human X-autosome translocations[78] and again the extent of spread is variable. This raises the possibility that there are features of autosomal chromatin that may impede the spreading mechanism. In attempts to investigate such features studies have been made of XCI in transgenic mice in which autosomal genes have been inserted into the X-chromosome. In the human, not all X-linked genes undergo XCI. An increasing number of human genes are now known which are transcribed from both the active and inactive X-chromosomes, and these genes have been studied in the hope of elucidating the basis of their resistance to the spreading or maintenance processes. By contrast, in the mouse very few genes are known which escape XCI.

In attempts to study the mechanism of maintenance and stabilization of XCI studies have been made of reactivation of the Xi. In the mouse spontaneous reactivation has been detected at a low level for a few genes, but otherwise in both mouse and human somatic cells spontaneous reactivation is not detected. Hence, many efforts have been made to induce reactivation experimentally.

In seeking mechanisms for both spreading and maintenance of XCI interest has centered on the various characteristic properties of the inactive X-chromosome. These include condensation during interphase to form the sex chromatin body, asynchronous, and typically late, replication, differential methylation of CpG islands of housekeeping genes, and the recently discovered lack of acetylated histone 4.

A. Escape from X-chromosome Inactivation

The subject of escape from XCI has recently been reviewed by Disteche.[79]

As XCI is regarded as a mechanism for dosage compensation it is to be expected that X-linked genes with a homologue on the Y-chromosome will escape inactivation, since this will leave XX and XY animals with effectively equal dosage. Thus, genes in the pseudoautosomal segment would not be expected to undergo inactivation. Some of the human genes that escape inactivation are indeed pseudoautosomal, but recently some genes well away from the pseudoautosomal region, and without Y-linked homologues, have been found to remain active on both X-chromosomes. These include the *ZFX* gene in medial Xp, *UBE1* in proximal Xp, and *RPS4X* in proximal Xq. All of these genes have normally inactivated genes lying on both sides. Thus, the spreading mechanism must have run past them. Either the non-inactivated genes resisted the signal for inactivation, or they resisted the stabilizing mechanism and were immediately reactivated. Thus, it seems that the features

leading to sensitivity or resistance of genes must be at the level of individual genes or groups of genes rather than large chromosomal regions. A possibility once entertained was that some form of stop signal prevented the spreading mechanism entering the pseudoautosomal region, and thus protected the genes there, but present evidence is against this. Mohandas et al.[80] studied an X-chromosome in which the distal end of Xq including the *G6PD* locus had been duplicated and transferred to distal Xp. The genes *MIC2* and *STS* in distal Xp, which normally escape inactivation, maintained their escape on this chromosome, whereas the *G6PD* locus lying beyond them underwent XCI. Similarly, in the mouse, in an X-chromosome in which the sex determining region of the Y-chromosome has been transferred to the distal tip of the X-chromosome, beyond the pseudoautosomal region, in the so-called *Sxr* mouse, the sex determining gene *Sry* and the HY antigen gene, *Hya*, can undergo XCI.[81,82,83] Thus, genes in the pseudoautosomal region must have some means of resisting inactivation.

In the mouse, most genes that escape XCI in humans are inactivated normally. Genes studied include the *Zfx, Rps4x,* and *Ube1x* loci. These are completely inactivated both *in vivo*[79] and in cultured cells.[84,85] The only exceptions to complete inactivation so far known in the mouse are the *Smcx* gene, which has a Y-homologue,[86] and perhaps the *Sts* gene, which also has a Y homologue, and in which the inactivation status may vary among strains.[87] This difference between mouse and human in escape of genes from inactivation probably explains the difference in phenotype of XO individuals in the two species. The XO mouse is a normal female whereas human XO females mainly die *in utero* with the survivors being malformed with Turner's syndrome. The normal development of the human female may require a double dose of those genes that escape inactivation.

Thus, the escape of genes from XCI in humans results in incomplete dosage compensation. It is possible that this may be selectively advantageous in maintaining useful sexual dimorphisms. Alternatively it may be that the evolution of XCI is not yet complete. It is postulated that XCI has evolved gradually as the genetic disparity between the X and Y-chromosomes has increased by the reduction of the genetic content of the Y-chromosome.[88] The limited spread of XCI in autosomes, and the resistance of some X-linked genes to inactivation, suggest that there may have been selection during evolution for the loss of sequences from the X-chromosome that result in resistance to XCI. The alternative would be that the X-chromosome carries specific sequences necessary for XCI but this seems unlikely, in view of the susceptibility of many autosomal genes and also transgenes (see below) to inactivation. Indeed, no specific X-chromosomal features have yet been found, but the possibility that such exist is not yet excluded. At present the most probable explanation seems to be that XCI spreads in autosomal material until it encounters some feature causing resistance to its spread. In different cells

carrying the same X-autosome translocation XCI may spread for varying distances[6,78] and hence it appears that resistance of autosomal material is not an all-or-none phenomenon.

B. Inactivation of X-linked Transgenes

Studies of XCI in mice with transgenes inserted into the X-chromosome have revealed a variety of effects with different transgenes. Inactivation may occur or not, and may be incomplete. The failure of a chicken transferrin gene to undergo inactivation[89] raised the question of whether certain features of a mammalian gene were needed for XCI. However, two bacterial genes, a tk*neo* gene and a *lacZ* gene, underwent XCI, and showed typical paternal XCI in extraembryonic tissues.[24,90] The *lacZ* gene also underwent typical inactivation, followed by reactivation, in female germ cells.[91] As mentioned earlier initiation of inactivation of the *lacZ* gene occurred slowly in some tissues, over a period of several days. Inactivation of an α-fetoprotein transgene occurred normally in the embryo proper, but did not occur in the extraembryonic tissues.[92] This suggests some difference in the mechanism of XCI in the various cell lineages. However, in its normal location the α-fetoprotein gene is strongly expressed in endoderm tissue and it is possible that this led to some override of the XCI mechanism in this tissue. A human α I collagen gene underwent XCI, but not in all cells[93] and other transgenes also have been subject to inactivation.[4]

On the basis of the results from transgenes and from the escape from XCI of endogenous X-linked genes Goldman[4,89,94] has suggested that the unit of sensitivity or resistance to XCI is a chromosomal domain of the order of 100 kb in size. The chicken transferrin transgene that escaped XCI consisted of several tandem copies extending over 187 kb and thus may have formed one or more domains that lacked an appropriate segment for XCI. All X-linked transgenes so far known to undergo XCI are smaller.[4] The domains are segments of DNA anchored to a protein matrix at a scaffold or matrix associated region (SAR or MAR) and forming independent loops.[4,94]

C. Reactivation of X-linked Genes

The subject of reactivation of X-linked genes, either spontaneously or after experimental treatments, has recently been reviewed by Gartler and Goldman.[95]

Reactivation of the entire inactive X-chromosome occurs normally in female germ cells, at the time of entry into meiosis, and presumably also on entry of the X^P into the new zygote at fertilization, this X-chromosome having become inactive during spermatogenesis.

In somatic tissues of adult eutherians XCI is very stable. Slight reactivation with increasing age is seen in mice heterozygous for a deficient allele of the

ornithine transcarbamylase gene, *Otc*. Mice were bred which carried the deficient allele on the translocated X-chromosome in the translocation T(X;16)16H, in which the translocated X-chromosome remains active in all cells. In the absence of reactivation all cells of the liver would be expected to stain negatively for *Otc*, but in fact small patches of *Otc* positive cells were found and these increased with age.[96] Similarly, Cattanach[97] bred mice which carried the wild-type allele of albino (or tyrosinase) in an insertion into the X-chromosome opposite the translocated X-chromosome of T16H. Without reactivation the mice were expected to be albino, but instead they carried small pigmented patches and darkened with age. Reactivation has also been claimed in mice carrying T16H and Mo^{blo}, a mouse homologue of Menkes' disease, but this is less clear.[98] No evidence of reactivation has been found with the mouse or human *Hprt* gene,[99,100] or with mouse *Pgk1*[101] or tabby (*Ta*).[102] It is suggested that those genes that show reactivation are tissue-specific and lack the CpG islands found in the promoter regions of housekeeping genes. Methylation of these CpG islands of housekeeping genes on the inactive X-chromosome is thought to provide a mechanism for stabilizing inactivation (see below).

In marsupials, XCI appears much less stable. Both the *G6PD* locus and the *PGK1* locus may show expression from both X-chromosomes in certain tissues of some species, the picture varying from species to species.[11] The *G6PD* gene may also be expressed from the inactive X-chromosome in cultured fibroblasts from species where this is not found *in vivo*. This expression from the Xi is generally regarded as due to reactivation, but the possibility that it is in fact due to escape from XCI is not excluded.[11]

Evidence suggests that embryonal cells of eutherians may lack some stabilizing system. In an embryonal carcinoma (EC) cell line Paterno et al.[103] obtained reactivation by treatment with the demethylating agent 5-azacytidine (5AC), a treatment which does not reactivate adult cells. However, if the cells were allowed to differentiate before treatment with 5AC, reactivation did not occur. This suggests there may be at least two mechanisms of stabilization, one involving methylation. Takagi[104] fused somatic cells with undifferentiated EC cells, and obtained reactivation of the inactive X-chromosome of the somatic cells, suggesting some factor concerned in reactivation present in the EC cells. Later, he obtained reactivation and also *de novo* inactivation after fusion of rat somatic cells with mouse EC cells.[105]

There is also some indication that cells of extraembryonic tissues may be more easily reactivated, since Migeon et al.[106] obtained complete reactivation of the Xi in human chorionic villus cells fused with a mouse cell line.

At present there is no evidence of a role for the *XIST*/*Xist* gene in maintaining inactivation. In adult females of human and mouse *Xist* is expressed in all tissues. However, X-chromosomes from which the gene has been lost remain inactive. Brown and Willard[107] devised a system in which they

could select for loss of the *XIST* gene from human hybrid cells. Chromosomes which had lost *XIST* maintained their inactivity. Furthermore, Rack et al.[108] found two instances of rearranged X-chromosomes in cells from human leukemias, in which the *XIST* gene had been lost. Again the altered X-chromosomes remained transcriptionally inactive and late replicating. Thus, the significance of the expression of *XIST*/*Xist* in adult tissues remains unknown.

Much work has been carried out on reactivation by use of the demethylating agent 5AC. This agent will induce localized reactivation of certain genes on the human inactive X-chromosome in somatic cell hybrids (reviewed in Gartler and Goldman[95]). However, with the exception of the EC cells mentioned above cultured cells not involved in hybrids with another species are only reactivated with great difficulty by 5AC. Thus, the formation of somatic cell hybrids in itself destabilizes the inactivation in some way. The effect of 5AC in causing localized reactivation is part of the evidence for a role of methylation in stabilizing inactivation, as discussed below.

VI. RELATION OF VARIOUS PROPERTIES OF THE INACTIVE X-CHROMOSOME

A. Lack of Acetylated Histone 4

Jeppesen and Turner[7] stained chromosomes with an antibody to acetylated histone 4 and showed that such acetylated histone was lacking from both human and mouse inactive X-chromosomes. It was, however, present on human tiny ring X-chromosomes which fail to undergo inactivation.[59] On the autosomes, acetylated histone 4 mapped to R-bands, believed to be rich in coding DNA and transcriptionally active. Regions of constitutive heterochromatin, such as centromeric regions, were devoid of acetylated histone. On the Xi a few small regions showed acetylated histone 4 staining, including the pseudoautosomal region. It is possible that these may be regions of escape from inactivation, but this is not clear.

Conversely, in *Drosophila*, where dosage compensation is achieved by hypertranscription of the single X-chromosome of the male, this male X-chromosome is strongly labeled with antibodies to acetylated histone 4 at hundreds of sites along its length. This histone is acetylated specifically at Lysine 16. It may be forming an association with the products of the *msl* and *mle* genes, which are similarly located at many sites. Possibly one of these proteins is an acetyltransferase, but this is speculation at present.[109,110,111] Thus, acetylated histone 4 is thought to be involved in the control of transcription, but the underlying mechanism of this is not known.[109] Its lack from the Xi is probably secondary to other factors affecting transcription.

B. Differential Methylation of Active and Inactive X-chromosomes

The extensive literature on differential methylation of active and inactive X-chromosomes has been reviewed by Grant and Chapman[3] and Riggs.[112,113,114]

On the Xi CpG islands in the 5' promoter regions of genes are extensively methylated in both human and mouse,[115] whereas on the Xa, as on the autosomes, such CpG islands are non-methylated. Pfeifer et al.[116,117] studied the promoter region of the human *PGK1* gene and found methylation at almost all of 120 sites on the Xi but not the Xa. By contrast the corresponding region of the mouse *Pgk1* gene was much less heavily methylated.[118] Similarly, the mouse *Hprt* gene showed only partial methylation of sites in its 5' CpG island promoter region.[119] However, study of anonymous DNAs containing CpG islands showed that in general such islands are methylated on the inactive mouse X-chromosome.[115]

Various lines of evidence indicate that this differential methylation has a functional role. The first is the evidence already mentioned that the demethylating agent 5AC can cause localized reactivation of the Xi in hybrid cells.[3,120] On reactivation, previously methylated sites in the reactivated genes became demethylated[116,121,122] whereas non-reactivated genes on the same chromosomes remain methylated. Further evidence is that genes that escape inactivation, including the human *MIC2* gene[123] and genes in the putative mouse pseudoautosomal region,[115] do not show differential methylation.

Thus, DNA methylation is thought to have an important role in XCI. However, XCI can occur without differential methylation of CpG islands. In marsupials 5' CpG islands are not methylated on the Xi.[11,124] In addition, in eutherians there is no evidence of differential methylation in germ cells, either in the female or in the male.[124,125,126,127] There is also evidence that the inactive X-chromosome of the extraembryonic tissues of eutherians does not undergo differential methylation. Kratzer et al.[128] found that DNA from the Xi of mouse extraembryonic tissues could transfect HPRT- cells, implying that this DNA was not modified, and similarly, Luo et al.[127] found no differential methylation in human chorionic villi. However, Grant et al.[125] found methylation of sites in the *Pgk1* and *G6pd* genes of mouse extraembryonic endoderm, and hence the evidence on this point is not clear.

Because XCI can occur without methylation of CpG islands, this is considered not to be the primary spreading mechanism. Methylation also appears to occur later than the first appearance of XCI. The *Hprt* locus on the mouse Xi did not show methylation until several days after XCI,[129] but the *G6pd* and *Pgk1* loci became methylated somewhat earlier.[125] Grant et al suggested that the methylation took time to travel along the chromosome from the X-inactivation centre. Similarly, in EC cells in which XCI was induced by allowing the cells to differentiate, methylation did not appear at the time of XCI.[130] The types of cells in which CpG island methylation of Xi is not

seen are those in which reactivation can occur, namely germ cells, extraembryonic tissues and marsupial somatic cells. Hence, methylation is regarded as a means of stabilizing inactivation. The original methylation is suggested to occur by means of a *de novo* methylase running along the chromosome. Thereafter, a feedback loop is maintained by a maintenance methylase, which at DNA replication methylates already hemimethylated sites.[112,113,114]

The mode of action of methylation is thought to be to prevent binding of transcription factors. Two proteins termed MeCP1 and MeCP2 bind to methylated DNA[131] and are thought to prevent the binding of other proteins. Pfeifer et al.[117,132] showed that the promoter region of *PGK1* on Xa was covered with various proteins, whereas the corresponding region on Xi was devoid of such proteins, and the DNA was coiled round nucleosomes. The gene for MeCP2 is X-linked[133] but this is not thought to be of significance.

Although CpG island methylation in general appears not to be an essential feature of XCI, the possibility is not excluded that methylation of specific sites might be part of the spreading mechanism. Some sites in the body of genes are preferentially methylated on the active, rather than the inactive, X-chromosome. Such sites are found in the human *HPRT* gene,[134,135] and in the *HPRT* and other genes of marsupials.[136,137] Whether these sites have any functional significance is not known.

In view of the demethylation of the *Xist* gene in male germ cells[45] and the probable importance of *Xist* in initiating XCI, methylation may also be involved in imprinting of the X-chromosome.

C. Asynchronous Replication of the Inactive X-chromosome

In contrast to the methylation of CpG islands, asynchronous replication of the Xi is found in all types of somatic XCI, in marsupials as well as in the extraembryonic and embryonic cell lineages of eutherians. It appears early in the sequence of events at the initiation of XCI, being seen first in four-day mouse blastocysts of about 40 cells.[29,138] In studies of localized or complete reactivation of the Xi, the association of asynchronous replication with inactivity is almost but not totally complete.[4] It appears that, in cases of reactivation or of escape from inactivation, individual genes may replicate synchronously, even though the Xi as a whole is still asynchronous. Selig et al.[139] developed a method for studying the replication of individual loci in interphase cells. This method was used by Boggs and Chinault[140] to show that the *HPRT*, *PHKA1*, and *FRAXA* loci, which undergo inactivation, together with *XIST*, expressed only from the Xi, showed asynchronous replication, whereas *RPS4X*, *ZFX*, and *ANT3*, which escape XCI, replicated synchronously. Similarly, in another study *HPRT*, *FMR1*, and *XIST* replicated asynchronously in fibroblasts, whereas *F8C*, which is not expressed

in fibroblasts, replicated late on both Xi and Xa.[141] In view of this close association between XCI and asynchronous replication, it is regarded as a fundamental part of the mechanism of XCI.[3,112,113,114] It is suggested that, like methylation, it could form a feedback mechanism for stabilizing inactivation, if early replication is required for transcription and transcription is needed for early replication.[114] If this is so, then eutherians have two mechanisms for stabilizing inactivation, methylation and asynchronous replication, whereas marsupials and eutherian extraembryonic lineages have only one such mechanism, perhaps explaining the lower stability of XCI in them.

The mechanism underlying asynchronous replication is not known. Takagi et al.[30] found that when asynchronous replication was first seen in mouse blastocysts the Xi replicated early rather than late, and a switch to late replication occurred later. Yoshida et al.[142] studied two mouse cell lines, derived from a T-cell lymphoma, that had a precociously replicating X-chromosome. They demonstrated that these chromosomes were indeed inactive. When these cell lines were fused with other cell lines the time of replication quickly changed from early to late in S phase. The authors suggested that late replication is maintained by some trans-acting factor, and that in the absence of this factor there is a switch to early replication. This still leaves open the question of what causes the Xi to respond to this postulated factor. At present adequate knowledge of mammalian replication origins seems not to be available.

D. Chromatin Structure and Condensation

The condensation of the Xi in interphase, to form the sex chromatin body, and also its dark staining at metaphase after hot hypotonic treatment, the so-called Kanda[6,31] staining, both imply some difference in its chromatin structure. This altered chromatin structure may be the underlying mechanism of XCI but, as with asynchronous replication, the mechanisms that bring it about are unknown. Indeed, the actual nature of the difference in chromatin is not clear.

The Xi in the sex chromatin body has a looped structure with the two telomeres in close apposition and lying near the nuclear membrane.[143] Gartler et al.[4] used electron microscopy to show that it had the shape of a metaphase chromosome, with the arms formed of loops, composed of strands, and surrounded by less dense loops. In contrast to the nuclear membrane location in normal cells, in somatic cell hybrids the Xi was often located centrally, and was less compact.[144] The authors suggested that this might partially explain the lesser stability of XCI in such cells.

A different aspect of chromatin structure was studied by Pfeifer and Riggs.[132] They studied the 5' region of the human *PGK1* gene and showed, by DNase I and other studies, that the Xa had several footprints protected from DNase I nicking, and indicating binding of proteins to the DNA. The transcription start site was free of footprints. The Xi had no such footprints, but had regions

of DNase hypersensitivity suggesting that the DNA was wrapped round particles, probably two nucleosomes. As a mechanism for stably maintaining the difference between Xa and Xi, they suggested that at replication transcription factors competed successfully with nucleosomes for binding to the Xa. These transcription factors then excluded both nucleosomes and methyltransferase. They further suggested that this difference between Xa and Xi resulted from the difference in replication timing.

An interesting suggestion concerning the mechanism of condensation of the Xi was made by Riggs.[112,113] He postulated that enzymes, resembling bacterial type I restriction enzymes, bind to the DNA and draw DNA toward themselves by a bidirectional reeling-in process. Unlike the bacterial enzymes, he suggests that the mammalian enzymes would have no cutting activity. Instead they would remain attached to the DNA and the reeling-in would cause the DNA to be thrown into loops, with a protein scaffold at the base of the loops. The bases of the loops would be the scaffold- or matrix-associated regions, SARs or MARs. Each enzyme would reel in DNA until it encountered the neighboring SARs. There might be different enzymes for different chromatin structures, such as G-bands and R-bands. For the inactive X-chromosome he suggests that some factor from the XIC passes along the loop-fastening proteins of the SARs and brings about a change in the conformation. This is in accord with the suggestion of Kay et al.[52] that the *Xist* gene is involved in bringing about a change in chromatin structure. However, since the product of the *Xist* gene is RNA, it does not provide a protein to pass along the DNA. Either the *Xist* transcript may complex with a protein, or transcription through *Xist* may provide a local change in conformation which enables the spreading mechanism to start.[52] The work of Buzin et al.[46] indicates that the quantity of *Xist* RNA is not adequate for it to coat the entire chromosome.

At present no mammalian proteins have been identified which might be involved in the spreading of XCI, although Abe et al.[145] found extra bands of non-histone nuclear proteins in EC cell lines with an inactive X-chromosome relative to those without. In *Drosophila*, however, proteins specifically associated with heterochromatin are known. In the phenomenon of position-effect variegation (PEV) genes normally located in euchromatin undergo inactivation when moved close to heterochromatin. The protein HP-1 is associated with heterochromatin and mutations in the gene for HP-1 affect the level of PEV.[146,147] Similarly, genes of the polycomb group, Pc-G, encode proteins involved in stable inactivation of genes. These proteins share a common motif with HP-1. They form multiprotein complexes which associate with chromatin, and are thought to affect its packaging.[148] Spreading of the effect of the multimeric complexes is thought to be limited by boundary elements in the chromatin.[77] Mammalian homologues of both HP-1 and the Pc-G genes are known.[148]

VII. CONCLUSIONS

The *Xist* gene is a strong candidate for a role in the initiation of XCI in the embryo. Experiments involving knockouts of the gene may provide evidence as to its function, and the results of such work will be awaited with great interest. There must be some mechanism which blocks one XIC, and the expression of its *Xist* gene, and maintains a single X-chromosome active. Perhaps this may become clearer when the function of *Xist* is known. So far there have been no reports of the finding of an *Xist* homologue in marsupials. This may mean either that the gene is present but poorly conserved, so that it does not react with eutherian probes, or that the gene is in fact absent from marsupials. Such an absence would imply that *Xist* is involved in aspects of XCI peculiar to eutherians. Eutherians and marsupials may indeed differ in the mechanism of initiation of XCI. The X^p is already inactive in the earliest marsupial embryos examined. It is possible that it enters the zygote inactive after becoming so during spermatogenesis and remains inactive throughout. If so there would be no need for a counting mechanism. Activity of a single X-chromosome could be obtained by imprinting alone. Conversely, in eutherians there is ample evidence that the X^p is active in the early cleavage stages and that a counting mechanism operates in bringing about XCI. This more complex system may have evolved through providing more effective dosage compensation, and by obviating the expression of deleterious mutant X-linked genes in heterozygous females.

The nature and sequence of the changes that convert the X-chromosome from the active to inactive state are still not clear. At an early stage in the process the Xi becomes condensed and also late replicating. Both of these features are likely to make the Xi inaccessible to transcription factors, and late replication probably creates a self-maintaining feedback loop. However, the underlying bases of both condensation and late replication are not clear, neither is it clear which of these is primary. The suggestion by Riggs[112] that condensation is brought about by enzymes reeling in DNA and forming the base of loops at scaffold attachment sites (SAR) is very interesting. It fits with the idea of Goldman[4,89,94] that sensitivity or resistance to XCI is determined at the level of chromosomal domains, each domain being formed from a loop attached to an SAR. Goldman considers that both replication timing and chromatin conformation are regulated individually. A model such as Riggs' implies that the Xi bears a set of proteins that bring about different conformation of SARs from that in active chromatin. Candidate proteins would include homologues of those associated with heterochromatin in *Drosophila*. Their attachment would be somehow mediated via the XIC and the transcription of *Xist*.

The methylation of 5' CpG islands seen in certain somatic cell lineages of eutherians is taken to be a later evolutionary development aiding in

stabilization of the inactive state. The possibility of methylation at specific sites having a more fundamental role cannot be excluded. The differential demethylation of the *Xist* gene in male germ cells suggests that such methylation has an important role in the imprinting of the X-chromosome.

The relation of the recently discovered lack of acetylated histone 4 to the other properties of the Xi is not yet clear. The acetylation of histone is clearly associated with transcriptional competence of chromatin, and its lack with absence of transcription, but the relation of this to conformation and replication of chromatin has still to be worked out. If the *Xist* gene is indeed shown to be a major player in the initiation of X-inactivation, and its function can be understood, then the chain of events among the other properties of the Xi in bringing about inactivation may become clear.

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SEX CHROMOSOME ABERRATIONS AND GENETIC CONSEQUENCES

Henry Anhalt and E. Kirk Neely

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I. DETERMINANTS OF SEX DIFFERENTIATION

A. Nomenclature of Sex Chromosome Aberrations

High resolution chromosomal banding has allowed for the identification of each human chromosome and the elucidation of complex chromosomal abnormalities and rearrangements. *Aneuploidy* describes a cell with the total number of chromosomes different from those normally found (i.e., 47,XXY or Klinefelter syndrome and 45,X or Turner syndrome). Mechanisms responsible for aneuploidy include nondisjunction or anaphase lag occurring during meiosis or mitosis. Nondisjunction occurs when homologous chromosomes fail to separate during anaphase, resulting in one daughter cell with too numerous chromosomes and one daughter cell with too few. Anaphase lag occurs when one chromosome is lost from one or both daughter cells as a result of poor alignment during metaphase.

The existence of two cell lines of different genetic origins in one individual is termed *chimerism*. Postulated mechanisms for this phenomenon include double fertilization of a binucleate ovum, fusion of two fertilized ova prior to implantation, and fertilization of an ovum and its polar body by separate sperm.[1] Two or more cell lines of different chromosomal constitution arising from a single zygote, for example 45,X/46,XX, is termed *mosaicism*. This post-mitotic abnormality may be responsible in many cases for an apparent lack of correlation between phenotype and genotype. With new X and Y chromosome probes, the contribution of sex-chromosomal mosaicism in phenotype-genotype discrepancy continues to be elucidated.

Structural abnormalities in chromosomes usually occur as a result of chromosomal breakage and consequent fragment rearrangement or loss of chromosomal material. Included in this category are isochromosomes, deletions, duplications, translocations, and ring chromosomes. *Isochromosomes* are chromosomes with almost mirror image arms around one centromere resulting from centric fission, that is, transverse rather than longitudinal division of the chromosome. They most likely arise from deletion close to the centromere with subsequent fusion of the remaining chromatids. This process is then followed by normal division of the chromosome and duplication of the abnormal chromatid pair.[2] This structural abnormality primarily affects the X and Y chromosomes and commonly results in a chromosome with two long arms. An *isodicentric* chromosome, for example 46,XXq(i), occurs when a chromatid breaks, and fusion occurs in a mirror-image like fashion about the two centromeres. Isodicentric X chromosomes usually occur as part of a 45,X mosaic karyotype. *Deletion* refers to loss of a piece of the chromosome. The symbol p- refers to loss of a part of the short arm of the chromosome and q- refers to loss of a part of the long arm of the chromosome. *Translocation* refers to an exchange of chromosomal material

between two chromosomes. *Ring* chromosome, for example a ring X (Xr), refers to a chromosome which has lost material from the telomeres giving rise to "sticky ends" which rejoin in the shape of a ring.

B. Sex Chromosomes and Gonadal Organogenesis

A cascade of events from establishment of genetic sex to the phenotypic appearance of the internal and external genitalia characterizes normal sexual determination and differentiation. Many autosomal and sex-chromosome genes are responsible for regulating the process. Through the actions of organizing factors, peptide hormones, gonadally-derived steroid hormones, and receptors on the developing primordial tissue, the undifferentiated gonad develops into a testis or an ovary. The undifferentiated gonad becomes a testis in the presence of the sex-determining region of the Y chromosome sequence (*SRY*). An ovary is formed if *SRY* is absent.

1. The Y Chromosome and Testicular Ontogeny

The Y chromosome is divided into a euchromatic region, which encompasses the short arm and a proximal portion of the long arm, and a genetically inactive heterochromatic region on the distal long arm as characterized by Q-stained preparations. Closer analysis of the euchromatic element of the Y chromosome reveals several regions of interest. One area known as the "pseudo-autosomal region" is located at the distal end of the short arm and is homologous with the distal end of the short arm of the X chromosome. During meiosis, the pseudo-autosomal region of the X and pseudo-autosomal region on the short arm of the Y chromosome recombine along this homologous region. This process allows for maintenance of sequence homology as well as ensuring proper distribution of sex chromosomes to the daughter cells.[3,4] The genes in this distal portion of the X and Y chromosomes are paired, are subject to recombination, and are not subject to inactivation, thus acting more like autosomal genes than other sex chromosome genes, hence the name.

Another region of interest within the euchromatic portion of the Y chromosome is the "Y-specific region". The Y-specific region, bordered by the proximal end of the pseudo-autosomal region, on one side and the proximal end of the heterochromatic region on the other contains numerous known and postulated genes. Zinc finger Y (*ZFY*) is located proximal to the sex-determining region on the short arm of the Y chromosome and is thought to regulate protein transcription.[5,6,7] Other genes localized to the euchromatic region of the Y chromosome include a gene which controls the expression of the H-Y antigen and the pseudogene for steroid sulfatase.[8,9,10] It is postulated that genes affecting the development of stigmata of Turner syndrome,

spermatogenesis, and height are also located in the euchromatic region of the Y chromosome.[11,12]

Early work by Eichwald and Silmser in 1955[13] demonstrated the presence of an antigen in male mice which caused rejection in females grafted with male tissue. Further serological assays established the presence of the "H-Y antigen" in multiple species. In mammalian, bird, and amphibian species the H-Y antigen is only expressed in males and is not detected in females. The fact that this antigen is so highly conserved among multiple species led Wachtel,[14,15] Ohno,[16] and others to suggest that the H-Y antigen was the testis determining factor (TDF).

Further studies of patients with a variety of sex chromosome aneuploidies, for example, 47,XYY, 48,XXYY, and patients with 46,XY testicular feminization, demonstrated a dose response increase in expression of the H-Y antigen with extra Y chromosomes.[14,15] Supporting H-Y antigen as the TDF was its serological presence in male patients with a 46,XX karyotype in the absence of a Y to X translocation.[14,15,16] However, McLaren[17] and Goldberg[18] demonstrated XX male mice lacking H-Y antigen in serological and cell-mediated cytotoxicity tests. The results of their observations challenged the concept that the H-Y antigen could in fact be responsible for the development of the testes, and thus the search began for an alternate genetic determinant of testicular differentiation.

Page and co-workers[5] attempted to attribute testicular differentiation of the bi-potential gonad to *ZFY*. This gene, located on the short arm of the Y chromosome, encodes for a protein with 13 Cys-Cys/His-His zinc fingers at the COOH terminal end.[6] Evidence that the *ZFY* gene was not responsible for testicular differentiation arose from the fact that 46,XX true hermaphrodites and 10 percent of 46,XX males are *ZFY* negative. It was these patients which led Palmer and associates to search for a nearby segment of Y material translocated with the pseudoautosomal region.[19] Furthermore, the finding of a homologous structure on the X chromosome (*ZFX*), and its failure to be X-inactivated,[20] left *ZFY* an unlikely candidate for the principal determinant of testicular differentiation.

The sex determining region of the Y (*SRY*) gene was finally identified in 1990.[21] This gene is located approximately 35-kb from the pseudoautosomal boundary, distal to the *ZFY* gene. After probing with the 2.1-kb clone pY53.3 derived from the testes determining region, male-specific sequences were found in numerous species including human, chimpanzee, rabbit, pig, horse, bovine, and tiger. The 2.1-kb segment was then sequenced and revealed two open reading frames. One of these frames revealed a higly conserved DNA binding motif found in the nuclear high mobility group proteins (HMG-1 and HMG-2 boxes). Northern blot analysis using the pY53.3 probe demonstrated the presence of RNA transcripts in human testicular tissue but not in male lung, kidney, or ovary. RNA transcripts of *SRY* are found in the urogenital ridge

of 11.5-day old male fetuses, but not in the liver or urogenital ridge of female embryos.[22] The transcript becomes less pronounced in testicular tissue with time, until it is ultimately undetected. The highly conserved nature of this gene in all mammalian species, its location on the Y chromosome and its time-dependent expression exclusively in testicular tissue, fulfilled the criteria for *SRY* as a regulatory gene and the putative TDF. The *SRY* HMG box binds to specific DNA sequences potentially regulating transcription of other "downstream" genes affecting testicular differentiation. Absence of *SRY* has been demonstrated in female patients with 46,XY karyotype. Additional experiments demonstrating single base pair substitutions in the conserved domain of the *SRY* in 46,XY women with gonadal dysgenesis lend further support to *SRY* as TDF.[23] Compelling proof that *SRY* is the testis determining factor comes from work by Koopman and colleagues producing transgenic XX sex-reversed animals.[24]

Since SRY was discovered, other genes have been identified that encode for proteins with HMG boxes.[25] Those that encode for proteins with greater than 60% similarity to *SRY* HMG box region have been termed SOX genes.

The developing fetus contains both female (Müllerian) and male (Wolffian) genital ducts. The Müllerian ducts have the potential to develop into fallopian tubes and uterus, while the Wolffian ducts have the potential to further differentiate into the vas deferens, epididymis, and seminal vesicles. In the third month of fetal life, either the Müllerian or Wolffian ducts complete their development, while the other genital duct undergoes involution. The existence of a Müllerian inhibiting substance (*MIS*) was first postulated by Jost in 1947.[26] Since that time, both rat and human *MIS* genes have been cloned and sequenced and the candidate receptor identified.[27] The gene maps to the short arm of chromosome 19. It is hypothesized that *SRY* may induce *MIS* transcription in the organizing testes which then causes Müllerian duct regression. *MIS* belongs to a family of peptide growth factors which includes TGF-β, inhibins, and activins, characterized by dimeric structure and seven conserved cysteine residues in the C-terminal domain. *MIS* is believed to exert its inhibitory action by altering membrane phosphorylation and inhibition of epidermal growth factor tyrosine kinase activity. In humans, *MIS* is produced by immature Sertoli cells by day 51 of gestation and is detectable by ELISA assay at low levels throughout adulthood.[28,29]

In summary, current understanding of testicular differentiation is that the *SRY* gene on the short arm of the Y chromosome affects downstream regulation of many X or autosomal genes which induce Sertoli cell development. Sertoli cell development in the bipotential gonad influences Leydig cell differentiation. Subsequent rises in testosterone and *MIS* levels lead to regression of the Müllerian structures and full differentiation of the male internal and external genitalia. In the absence of *SRY*, the human phenotype is female.

2. *The X Chromosome and Ovarian Ontogeny*

The X chromosome, which is much larger than the Y chromosome, has a pseudoautosomal region on the distal aspect of the short arm, as well as an X-specific region. Genes believed to reside in the pseudoautosomal region include a gene that affects expression of the H-Y antigen, genes that affect stature and genes that cause some of the somatic features associated with gonadal dysgenesis.[30,31,32] The long arm contains numerous genes which are subject to X inactivation and are responsible for X-linked conditions. The paracentromeric region contains the gene coding for the androgen receptor,[33,34] as well as the X inactivation center (XIC).[35] Inactivation of most of one X chromosome occurs during the twelfth-eighteenth day (late blastocyst) in the female human embryo. Only the female germ cells destined to become oocytes are excluded from this process. The random partial genetic inactivation affecting either a maternally or paternally derived X chromosome during interphase is commonly referred to as the Lyon hypothesis.[36]

The presence of two intact X chromosomes is a prerequisite to the formation of normally differentiated ovaries. In fact, deletion analysis has demonstrated that deletions on either the long or short arm may lead to abnormal gonadal differentiation, germ cell loss and oocyte degeneration, ultimately yielding streak gonads.[31,37,38] The occurrence of familial 46, XX gonadal dysgenesis would suggest that not only are two intact X chromosomes required for normal ovarian function, but probably there exist other downstream autosomal genes integrally involved with normal ovarian function. It is hypothesized that 46,XX pure gonadal dysgenesis is inherited as an autosomal recessive trait. X chromosome to autosomal chromosome translocations may also lead to gonadal abnormalities. Translocation of material from Xp13 through Xp26 leads to infertility in both males and females.[39,40]

Recently, duplication of a locus on the short arm of the X chromosome (Xp21), named the DSS locus (dosage sensitive sex reversal), has been identified as a cause of male to female sex reversal in patients with 46,XY karyotype and intact *SRY*.[41] The DSS critical region is located in a 160 kb region of Xp21 adjacent to the locus for congenital adrenal hypoplasia. This finding conclusively demonstrates that genetic determinants on the X chromosome also play a significant role in sex differentiation. In other words, duplication of the Xp21 segment in the face of normal *SRY* disrupts normal testicular differentiation. The possible role of DSS in 46,XY gonadal dysgenesis remains to be elucidated.

II. SEX REVERSAL: Y CHROMOSOME DELETIONS AND TRANSLOCATIONS

A. 46,XY Females (Swyer Syndrome)

Harnden and Stewart first coined the term "*pure gonadal dysgenesis*" in 1959 to describe a 19 year old phenotypic female patient with pubertal delay who had a 46,XY karyotype.[42] The definition has since been expanded to include any phenotypic female with either 46,XX or 46,XY karyotype who has streak gonads, normal stature, absent secondary sexual characteristics, and no stigmata of Turner syndrome.

Both familial and sporadic patterns of 46,XY pure gonadal dysgenesis have been described.[43] Internal genitalia are female, but the phenotype may be variable if the syndrome is partial. Axillary and pubic hair are usually scant. Gonadal neoplasms are frequent, especially in patients who are serologically positive for the H-Y antigen, and thus exploratory laparotomy is indicated. The most common tumors arising from the dysgenetic gonads of the 46,XY pure gonadal dysgenesis patients are gonadoblastoma and dysgerminoma. One must suspect the presence of an estrogen secreting gonadal tumor if breast development occurs at the normal age of puberty.[44] Plasma gonadotrophins are elevated and serum testosterone may be high for females, presumably as a result of secretion from the abnormal gonads. In contrast to 46,XY pure gonadal dysgenesis, gonadal neoplasms are rare in 46,XX pure gonadal dysgenesis. Sex of rearing in cases of incomplete 46,XY pure gonadal dysgenesis depends on age of diagnosis and degree of sexual ambiguity. Pregnancy can be sustained in 46,XY females by implantation of a fertilized ovum once the uterus is enlarged with sex steroids to accomodate the pregnancy.

XY females are usually not attributable to cytogenetic abnormality of the Y chromosome. Instead they are likely due to very small deletions or point mutations in *SRY*. XY females with documented Y short arm deletions, as demonstrated by banding or by DNA probes, frequently manifest features of TS. Deletions involving the *SRY* segment of the short arm of the Y chromosome have been identified in about 10% of sporadic 46,XY gonadal dysgenesis patients.[45,46] XYp- females reported by Rosenfeld et al.[47] and Magenis et al.[48] exhibited lymphedema and redundant neck folds, but not organ anomalies or short stature. Gonadoblastoma was detected in the latter patient and in a patient reported by Disteche et al.[45] In two other XY females evaluated by Levilliers et al.,[49] congenital lymphedema and nuchal skin folds were seen in association with exchange of paternal X(pter-p22.3) for the homologous Y region, that is, normal exchange of the pseudoautosomal region but extending proximally to p22.3. These studies, supported by another recent case[50] suggest that lymphedema (as well as loss of male sex determination) can result from loss of the distal short arm of the Y.

Simpson and co-workers theorized that 46,XY pure gonadal dysgenesis is a heterogeneous group of disorders inherited as either a X-linked recessive or male limited autosomal dominant trait.[51] Grumbach and Conte report a kinship in which a consanguineous mating resulted in two phenotypic female siblings, one with 46,XX pure gonadal dysgenesis and one with 46,XY pure gonadal dysgenesis.[52] This variable phenotypic expression may represent incomplete expression or heterogeneity of a mutant gene or multiple genes.

One 46,XY patient has been described with a point mutation in the putative DNA binding domain of the protein encoded by *SRY*.[23] Four patients have been described with short arm duplications encompassing the *ZFY* locus.[53] Vilain and co-workers described a family in whom a single base substitution in the *SRY* domain was noted.[54] 46,XY pure gonadal dysgenesis was documented by histology in the propositus, her female sibling and a paternal aunt, but suprisingly, the mutation was found in the histologically normal father of the propositus and a male sibling. This finding raised the possibility that although base substitutions in the *SRY* domain may result in pure gonadal dysgenesis, mutations in other X-linked or autosomal genes, or changes on the Y chromosome outside the *SRY* domain rendering the testis determining region inactive, may cause the same phenotypic changes.

Autosomal loci on 9p, 10q, and 17q have been implicated in XY sex reversal by chromosomal deletions.[55,56] 46,XY, but not 46,XX, gonadal dysgenesis is associated with campomelic dwarfism, thought to be an autosomal recessive, dominant or X-linked cause of lethal short stature.[57] Foster et al. found mutations in single alleles of SOX9, an *SRY*-related gene located 88 kb distal to a translocation breakpoint at chromosome 17q23-24, in six of nine sex-reversed campomelic dysplasia patients.[58] The work by Foster et al. lends support to the theory that an autosomal dominant mutation resulting in haploinsufficiency is responsible for sex reversal in this condition. The dosage-sensitive roles of the Xp21 duplication (DSS) and the SOX9 haplo-insuficiency autosomal genes have begun to illuminate the role that "downstream" genes play in sexual differentiation.

B. 46,XX Males

First described in 1964 by de la Chapelle et al., 46,XX males are similar in phenotype to classical Klinefelter syndrome (47,XXY) and share similar characteristic gonadal baseline and stimulated test results[59,60,61] (see later in this paper). Despite their similarities, there are some minor differences. Patients with 46,XX karyotype have a 10% chance of penile hypospadias due to inadequate testosterone production by the fetal Leydig cells in the first trimester. These patients are more likely to be shorter, have normal skeletal proportions, have smaller tooth crowns, and have less intellectual difficulty than classical Klinefelter syndrome patients.[61] Advanced maternal age does not

seem to be an etiological factor. The incidence is estimated to be one in 20,000 male births.

On gross examination there may be cryptorchidism, hypospadias, and small testes, a hallmark of 46, XX males. Histologically, the gonads are composed mostly of interstitial tissue with numerous Leydig cells. Prostate tissue and epididymal tissue are present and small dysgenetic testes are characteristic. Seminiferous tubules, composed primarily of Sertoli cells, are rare. Testosterone levels during puberty are abnormally low, estradiol levels are normal or increased, and gonadotrophin levels become elevated.[62] Testosterone production in the post-pubertal testes in response to human chorionic gonadotrophin (HCG) is markedly impaired.[63] Gynecomastia affects approximately one in three patients. Genital abnormalities and severe gynecomastia are more likely to occur in patients who are *SRY* negative.[61]

More than 150 cases of 46,XX males reported in the literature have contributed greatly to understanding the nature of sex determination and sex reversal.[64,65,66,67,68,69] The paradox of male phenotype with absent Y chromosome may be explained by the following theories. As discussed previously, the pseudoautosomal region of the X and the Y chromosome are homologous and undergo obligate crossover during meiotic pairing. Through either equal or unequal exchange of chromosomal material, a portion of the pseudoautosomal region of the Y chromosome and *SRY* gene may be translocated onto the X chromosome.[70,71] Gene linkage analysis and molecular probes designed to detect Y chromosomal material have proven this mechanism to be operative in 80-90% of 46,XX males.[72,73,74] Yet this mechanism would not explain the phenomena of 46,XX SRY negative males.[75] Plausible theories include loss of Y chromosomal material early in embryogenesis or occult Y chromosome mosaicism, in some series accounting for as many as 17% of 46,XX males.[76] Mutation in downstream autosomal genes or an X-linked gene leading to constitutive activation of the testis-determining cascade is perhaps the most intriguing explanation for 46,XX males who are *SRY* negative. This last theory of mutation in downstream genes is supported by the occurrence of 46,XX males in familial clusters.[60,61]

C. True Hermaphroditism

The presence of both ovarian and testicular tissue in one individual, existing in separate gonads or combined as an ovotestis, constitutes true hermaphroditism. An ovotestis is commonly found, and testicular tissue is more likely to be present on the right side. Patients often have ambiguous genitalia at birth and develop breasts at puberty if undiagnosed. In two series published by Niekerk and Retief[77] and Hadjiathanasiou et al.,[78] the most common karyotype was 46,XX (116/195 and 17/22, respectively). Other less common karyotypes are 46,XX/46,XY, 46,XY, 46,XY/47,XXY, and 45,X/46,XY.

The pathogenesis of true hermaphroditism in the absence of a Y chromosome is unclear. Many of the theories that are useful to explain 46,XX sex reversal can be applied here as well. Occult Y chromosomal material translocated onto the X chromosome or lost early in embryogenesis could explain the development of testes in the face of an undetectable Y chromosome. Y chromosomal material existing as part of a 46,XX/47,XXY mosaic karyotype confined to a small portion of cells in the gonad may explain the phenotypic variation limited to the gonad. And lastly, relative expression of TDF and constitutive mutations of "downstream" genes may yield varying phenotypes. Insight to the etiology of true hermaphroditism has been gained by discovering families in which both 46,XX true hermaphroditism and 46,XX sex reversal occur.[79,80] Autosomal sex reversing genes are suspected to be responsible for these observations, but we are left with the lingering question of why one common defect leads to two different phenotypes.

III. SEX CHROMOSOME POLYPLOIDIES

The incidence of sex chromosome abnormalities in live births has been reported in many papers with relatively small numbers of patients. In a large series, 34,910 live births in Denmark were analyzed for chromosomal abnormalities. Klinefelter syndrome (47,XXY, 46,XY/47,XXY, 46,XX(male), 48,XXYY) was found in 1/596 boys; XYY (47,XYY, 46,XY/47,XYY) in 1/894 boys; XXX in 1/1,002 girls; and Turner syndrome (45,X and mosaic forms) in 1/2,130 girls. The incidence of any sex chromosome abnormality in this large series was 1 in 448 live births.[81] It is important to note that most XXX and XXY patients are never ascertained, and prenatal and postnatal rates of diagnosis differ markedly.

A. 47,XXY (Klinefelter Syndrome) and Variants

Klinefelter syndrome, characterized by adequate masculinization but dysgenesis of the seminiferous tubules, is comprised of a heterogeneous population of patients and karyotypes. All patients have in common the presence of a Y chromosome in conjunction with more than one X chromosome (e.g., 47,XXY, 48,XXXY, 49,XXXXY). The additional X chromosomes may arise secondary to non-disjunction of the sex chromosomes during the first or second meiotic division in either parent. Thus a XY sperm could fertilize an X ovum, or a Y sperm could fertilize an XX ovum. Alternatively, this aneuploidy may arise as a result of mitotic non-disjunction. DNA analysis and X-linked markers concur that the extra X chromosomes are of maternal origin in roughly two-thirds of cases of Klinefelter syndrome.[82,83] There seems to be no racial or geographic segregation, and only advanced maternal age is

considered a risk factor for non-disjunction.[84,85] It is estimated that the incidence of 47, XXY karyotype in unselected newborns is approximately 1 in 600 live births.[86,87] Estimate of this karyotype in spontaneous abortions is roughly 0.1%, indicating that most of these fetuses survive to term.

Classical Klinefelter syndrome, corresponding to the karyotype 47, XXY and originally described in 1942 by Klinefelter and Rifenstein,[88] is characterized by strictly male phenotype, adolescent-acquired gynecomastia, small testes, hyalinization of the seminiferous tubules, azoospermia, and elevated gonadotrophins. At birth, many of these patients have smaller mean head circumference and lower mean birth weight than normal. Major and minor anomalies such as clinodactyly are common.[89] After infancy, patients are taller on average than age-matched controls, owing primarily to a disproportionate length of the legs.[90,91] Other characteristics are delayed emotional development, especially during adolescence, poor motor control, lower verbal I.Q. but normal full scale I.Q.[90,92,93] Increased incidence of speech and learning difficulties is present, but retardation is uncommon. Anti-social characteristics previously attributed to these patients may in fact have been due to ascertainment bias. Many of these patients are indistinguishable from other control males and other hypogonadal males in terms of education, employment, behavior, socioeconomic status, and criminal activity.[94,95]

The testes may be normal at birth or begin to undergo germ cell loss and loss of spermatogonia as early as late gestation.[96,97,98] Baseline gonadotrophin profiles in prepubertal patients are indistinguishable from other prepubertal males, and the response to human chorionic gonadotrophin (HCG) and luteinizing hormone releasing hormone (LHRH) is also normal.[99,100] At puberty, progressive hyalinization of the seminiferous tubules with pseudoadenomatous clumping of the Leydig cells results in loss of testicular function. With loss of Leydig cell function, testosterone levels fail to rise adequately, estradiol levels are normal or increased, and gonadotrophin levels increase. Testosterone production in the postpubertal testes in response to HCG is markedly impaired.[63] Impotence and gynecomastia become significant problems in adulthood. Additionally, adult male patients may experience diminished facial and body hair and poor muscle development. Reports of fertility have not been reliably documented in classical Klinefelter patients (47,XXY), although it is plausible that cases of intra-gonadal mosaicism retain the capacity for spermatogenesis.

In contrast to patients with pure gonadal dysgenesis, Klinefelter patients rarely have thyroid abnormalities. However, approximately 8% of patients will have type II diabetes mellitus.[101,102] Cancer of the breast may occur more commonly than in normal males; 4.2% of men with breast cancer in one series were X-chromatin positive and had seminiferous tubular dysgenesis.[103] Six patients with 47,XXY karyotype and androgen resistance have also been described. It has been hypothesized that in these patients a meiotic error led

to an ovum with two X chromosomes, each carrying a mutation in the gene coding for the androgen receptor.[104]

46,XY/47,XXY mosaicism is the second most common karyotype in patients with Klinefelter's syndrome. In general, patients with sex-chromosome mosaicism are usually less severely affected than those without mosaicism. In patients with this karyotype there may be less gynecomastia, less severe hyalinization of the seminiferous tubules and preservation of testicular function until the fourth to fifth decade of life. As compared to patients with classical 47, XXY karyotype, fertility has been documented in these patients.[105]

48,XXXY, 48,XXYY, or 49XXXYY patients are almost universally mentally handicapped and have tall stature. Peripheral vascular disease and varicose veins are common. As a general rule, the more X chromosomes, the more severe the somatic manifestations of Klinefelter syndrome. Successive paternal or maternal meiotic non-disjunctions are necessary for this phenomenon to occur.

The group of patients with a 49,XXXXY karyotype has more somatic manifestations than other Klinefelter patients. Cardiac anomalies, cleft palate and strabismus are frequently seen. The facies has been described as similar to patients with trisomy 21, that is, epicanthal folds, down slanted eyes, and widened nasal bridge. Degree of mental retardation is very severe, and skeletal abnormalities including radioulnar synostosis and epiphyseal dysgenesis are common. Unlike other forms of Klinefelter syndrome patients, they may have ambiguous genitalia and undescended testes.[106] In adulthood, they do not develop gynecomastia, and androgen deficiency is severe. One theory put forth by Sarto and co-workers is that the extra X chromosomes fail to be inactivated, leading to increased dosage of certain X chromosome genes.[107]

B. Other Sex Chromosome Polyploidies

47,XXX, 48,XXXX, 49,XXXXX, 47,XYY, and 48,XYYY are all associated with increased incidence of mental retardation. The availability of X-linked restriction fragment length polymorphisms (RFLP) has made it possible to identify the parental origin of sex chromosomes. It now seems likely that most sex chromosome tetrasomy (quatrosomy), and pentasomy are due to sequential non-disjunctional events in the same parent. Dose-dependent worsening of phenotype with excess X chromosomes may be due to lack of X-inactivation with subsequent excesses in certain gene expression with negative consequences. There is a slightly higher incidence of congenital malformations in offspring of 47,XXX females as compared to normal females, and amniocentesis and prenatal counseling are recommended.[108] 47,XXX patients may have educational difficulties, particularly in verbal skills. Original descriptions of the 47,XYY karyotype included associations with significant increases in criminal behavior. Recent surveys, however, have disproved this original assumption.[109]

C. Mixed Gonadal Dysgenesis

X chromosomal monosomy occurs in mosaicism with 46,XY cells in approximately 5% of patients with Turner syndrome in large series, although only a minority of 45,X/46,XY (or 45,X/47,XXY) patients manifest TS features. The associated phenotype, ranging from female to male, has been considered in discussions of pseudohermaphroditism, since it has historically been described as ambiguous genitalia and mixed gonadal dysgenesis (streak gonad with contralateral dysgenetic testicular elements, resulting in asymmetry of Wolffian and Müllerian structures).[110] In a retrospective review by Zah and colleagues of 60 patients with mixed gonadal dysgenesis (45,X/46,XY), two-thirds were raised as females.[111] Grumbach and Morishima reported the phenotype in nine patients with either 45,X/46,XY or 45,X/46,XY/47,XYY genotype and found that seven of nine had ambiguous genitalia, one was male and one female. In a large early survey of the 45,X/46,XY literature, Hsu concluded that 25% of cases were phenotypic females with some features of Turner syndrome, 60% presented with ambiguous genitalia and mixed gonadal dysgenesis, and the remaining 15% were phenotypic males with evident undermasculinization.[112] The phenotypic females, who occasionally exhibit mild degrees of virilization such as clitoromegaly, usually have bilateral streak gonads; some of these patients escape diagnosis until evaluated for primary amenorrhea. Infants with the more masculinized phenotype will be evaluated because of small phallus, hypospadias, or undescended gonads. Most of these individuals will have Müllerian structures as well.

Analysis of 45,X/46,XY individuals discovered prenatally has revolutionized our understanding of the phenotypic spectrum. Recent surveys indicate that 85-95% of the fetuses or liveborns with this karyotype are normal phenotypic males, making the earlier ascertainment bias obvious.[113,114,115] In Hsu's survey of prenatally diagnosed cases, of which two-thirds could be confirmed by postnatal karyotype, only 10% exhibited mixed gonadal dysgenesis. In a recent literature review of 92 cases, Chang et al.[115] reported only four exceptions to normal male genitalia, although no follow-up data on reproductive function are available. Furthermore, no Turner characteristics were reported other than a single case of cystic hygroma. Liveborn individuals with 45,X/46,XY karyotype have not been carefully evaluated for features of TS, but these data imply that characteristics of TS are much less common in 45,X/46,XY than in other 45,X mosaicisms. An alternative implication is that 45,X/46,XX is also underreported. No correlations of phenotype with percentages of 45,X versus 46,XY cells have been documented.

Caspersson and co-workers showed that some patients with mixed gonadal dysgenesis had an absence of normal Y chromosome fluorescence.[116] Further work by Magenis and Donlon led to the conclusion that nonfluorescence of structurally abnormal Y chromosomes was due to the formation of an

isodicentric Y.[117] The loss of this structurally abnormal Y chromosome may explain the development of the 45,X/46,XY mosaic pattern.[118,119]

The high incidence of gonadal malignancy in mixed gonadal dysgenesis receives considerable clinical attention. In a series of 30 cases of gonadoblastoma, 10 were associated with 45,X/46,XY mosaicism.[120] Scully and Simpson and others have estimated the risk of developing gonadoblastoma (or dysgerminoma) with this karyotype at 15-20%.[120] Gonadoblastoma is a tumor composed primarily of large germ cells, Sertoli cells, and stromal derivatives. Carcinoma *in situ* has also been found in gonadal biopsy specimens of incompletely virilized patients, and thus gonadal biopsy in patients with male phenotype and mosaicism, and gonadectomy in females is indicated.[122] However, the risk of gonadal malignancy from the 45,X/46,XY karyotype may be exaggerated in light of recognition of the generally normal male phenotype.

IV. X CHROMOSOME MONOSOMY

A. Turner Syndrome

The phenotype resulting from X chromosome monosomy was described separately by Otto Ullrich and Henry Turner in the 1930s.[123,124] Initial elucidation of the chromosomal basis of Turner syndrome came with the report in 1954 that patients were sex chromatin negative.[125] The syndrome was correlated with X chromosome monosomy by Ford et al.[126] in a prepubertal 14 year old with short stature and characteristic features. We now recognize the major features as (1) fetal lethality, often associated with severe lymphedema, (2) short stature, (3) ovarian dysgenesis and failure, and (4) diverse morphologic and functional features of variable expression. In comparison with complete X monosomy, mosaicism generally results in reduced severity of many of the manifestations.

X monosomy is the most commonly occurring sex chromosome aberration, but more than 90% of conceptuses are spontaneously aborted. About 10% of spontaneously aborted fetuses have 45,X karyotypes.[127] Most 45,X fetuses are spontaneously aborted prior to the gestational age at which amniocenteses are performed, and a smaller number of fetuses are aborted in the second trimester, with autopsy findings of massive lymphedema and cystic nuchal hygroma.[128] Because most fetuses die *in utero*, TS has a postnatal incidence of about 1 in 2,500 live female births, compared with approximately 1 in 600 for Klinefelter syndrome or 45,XXX.

It has been postulated that all fetuses with 45,X karyotype are spontaneously aborted and that all survivors with TS exhibit mosaicism, whether detectable or not.[129] The high proportion of 45,X versus mosaic karyotype among TS spontaneous abortions does suggest a fetoprotective effect of mosaicism.

Nevertheless, rigorous techniques to detect small populations of cells with marker chromosomes have documented mosaicism in no more than 80% of children with TS, although that figure is considerably higher than the 50% reported in most series of patients.[130] Theoretically, mosaicism could be present in multiple tissues but undetectable in blood cells, and mosaicism solely in trophoblast or placental tissue might avoid lethality.

Parental origin of the remaining X chromosome has been reported. Through assessment of Xg blood groups, Sanger determined that loss of the paternal X occurred in 77% of cases.[131] X chromosomal RFLP analyses[132] have verified that the intact X in 45,X monosomy is maternal approximately 80% of the time, although the ratio may be close to even in mosaic and isoXq patients. Parental origin of the X does not appear to be a factor in fetal demise.[133,134] Two small studies have failed to discern any other differences in 45,X phenotypes on the basis of parental chromosomal origin.[135,136] Thus, parental origin of the intact X chromosome and hypothetical genomic imprinting do not help explain the great phenotypic diversity in TS.

B. The 45,X Phenotype

The characteristic facial appearance in TS includes epicanthal folds, ptosis, downslanting palpebral fissures, midfacial hypoplasia, small mandible, prominent or anomalous ears, neck webbing, low hairline, and short neck.[137,138] Other head and neck features include high arched palate, which may contribute to feeding difficulties in infancy, and many craniofacial bone anomalies, leading to an abnormal Eustachian tube and a high incidence of otitis media. Hearing loss in adults is common and can be either sensorineural or conductive. The other common bone anomalies are short fourth metacarpal, cubitus valgus, Madelung deformity of the wrist, pectus excavatum, scoliosis, congenital dislocation of the hip, and genu valgum. Patients tend to exhibit a short, square body habitus.[139] Osteopenia has been widely reported and could be secondary to an underlying bone dysplasia or to hormonal deficiencies. Although measurements routinely made by X-ray absorptiometry may artefactually underestimate bone mineral in TS because of their dependence upon body and bone size, osteopenia has also been reported on CT scans in adults with TS. Recent studies have suggested that bone densities in children with TS are normal when corrected for short stature.[140,141] It is not clear whether bone density in adults is still abnormal when estrogen supplementation has been adequate.[142,143]

Echocardiography is routinely performed when TS is diagnosed. Coarctation of the aorta is reported in approximately 20% of patients and is more frequent in association with a 45,X karyotype.[144] Bicuspid aortic valve has been reported in more than one-third of patients,[145] and recent echocardiographic studies have demonstrated isolated aortic root dilation in

about 10% of patients,[146] placing them at risk for aortic dissection and aneurysmal rupture.[147] Mitral valve prolapse is also common, and hypertension may occur without any cardiac anomaly. Heart vessel defects have been noted in association with fetal edema, nuchal cystic hygromas, and lymphatic aberrations at the base of the major vessels, implying a link between lymphedema and cardiac anomalies,[148] but cardiac anatomy has been normal in other fetuses with severe edema. Renal abnormalities occur independently of cardiac anomalies and are seen in approximately one-third of TS patients. The most frequent abnormalities are double collecting systems, horseshoe kidney, rotational abnormalities, ureteropelvic and ureterovesicle junction obstruction, and absent kidney, as shown by Lippe et al.[149] In their study, the incidence of renal dysmorphology in girls with 45,X karyotype was 45%, compared with 18% in other karyotypes combined, but this difference has not been found in other series.

Gonadal dysgenesis is nearly universal in complete X chromosome loss. Oocyte formation and folliculogenesis fail to occur normally in the second trimester of gestation, and connective tissue proliferates. Oocytes undergo a premature atresia that may be completed before birth or not until adolescence or adulthood. Ovarian failure is reflected in reduced steroid feedback on the hypothalamic-pituitary axis and marked elevations in serum gonadotrophins in infancy, and then in adolescence and adulthood.[150] By childhood, the ovaries are usually residual streaks with normal fibrous stroma but greatly diminished numbers of primordial follicles,[151] but the uterus and vagina are anatomically normal and hormonally responsive, allowing for pregnancy by assisted reproductive technologies. Ovarian dysfolliculogenesis constitutes a spectrum from complete absence of pubertal development in the majority of girls, to midpubertal failure, primary amenorrhea, secondary amenorrhea, or early menopause. Unassisted pregnancy occurs occasionally, predominantly in those with mosaicism, and miscarriages and chromosomal anomalies in offspring are common.

Although mild intrauterine growth retardation can be present, growth velocity in the first few years of life is usually normal. Growth velocity begins to decline during childhood and becomes obvious in adolescence when the pubertal growth spurt does not appear.[152] Because of minimal estrogen levels, bone maturation is delayed and epiphyseal fusion may not occur until 18-20 years of age. This delay provides some catchup growth and an opportunity for prolonged growth therapy. Standard growth curves for TS in the absence of hormonal therapy have been developed and serve as the basis for evaluating growth therapy.[153,154] The mean final adult height from most European and U.S. studies is approximately 143 cm (4'8").[155] Etiology of growth failure in TS is essentially unknown, but it may be due to the combined effects of a primary skeletal dysplasia, growth hormone (GH) secretory dysfunction, and estrogen deficiency. Histologic evidence of bone pathology is inconsistent, but

may represent an intrinsic defect in ossification secondary to loss of critical genes on the X chromosome; alternatively, bone pathology might be a manifestation of intrauterine edema.[156] Athough responsive to exogenous growth hormone therapy, most patients with Turner syndrome are not GH deficient as classically defined. However, the normal pubertal increase in GH secretion is absent, probably secondary to estrogen deficiency.[157,158]

Autoimmunity has been observed in TS, including inflammatory bowel disease, rheumatoid arthritis, and thyroid autoimmunity. Apparently, patients with long arm isochromosome are more likely to have autoimmune disorders. Glucose intolerance is widely reported in adults with TS, but frank diabetes, associated with insulin resistance, occurs in only about 5% of adult patients. Thyroid autoantibodies have been documented in 25-60% of patients,[159] in contrast with a 1-2% prevalence of thyroid autoimmunity in the general population. The incidence of clinical hypothyroidism increases with age and develops in 10-20% of patients. There does not appear to be an increase in polyglandular autoimunity associated with thyroid autoimmunity; islet cell and adrenal antibodies are not elevated. Curiously, parents of patients with TS have a higher than expected incidence of thyroid autoimmunity. Investigators have speculated that familial thyroid autoimmunity might be causally related to non-disjunctional events. On the other hand, the increased prevalence of maternal thyroid autoimmunity in liveborn subjects with TS might be attributable to autoimmunity-associated protection against the general fetal lethality of X chromosome monosomy.[160]

Finally, patients with TS are not mentally retarded, but specific impairments in visual-spatial processing and visual memory have been reported.[161,162] These deficits are reflected in mean reductions in performance IQ, whereas verbal IQ is normal. Individuals with mosaicism for a ring X chromosome may be more severely affected.[163] Few studies have examined academic outcome in TS, and social isolation and depression have been reported.[164,165] Life expectancy may be diminished but has been inadequately studied.

C. Phenotype-Genotype Correlations: Evidence from Deletions

In most TS patient series, approximately one-half are 45,X, and the other half is comprised of X structural abnormalities, mosaicism, or both. Patients with mosaicism may have less severe phenotypic manifestations. 45,X/46,XX is a common TS mosaicism, and patients may have less severe growth retardation and a higher incidence of menarche and fertility.[166] Isochromosomes of the long arm, with loss of the short arm, are also common. This structural rearrangement of the X chromosome occurs in both monocentromeric and dicentromeric forms and with or without mosaicism.[167]

The phenotype and karyotype in TS were first compared in the 1960s. Ferguson-Smith hypothesized from review of the literature in 1965 that 45,X

subjects demonstrated a "complete Turner syndrome" due to monosomy of loci on the short arm.[32] In that review, patients with 45,X karyotype had a higher incidence of neck webbing, lymphedema, and cardiac malformations than patients with X structural anomalies or mosaicisms. Spontaneous menses occurred in 8% of subjects with X monosomy, 21% of these with 45,X/46,XX mosaicism, and 25% with deletions of the short arm. Short stature was a universal feature in 45,X and 46,Xi(Xq), but normal stature occurred in 20% of 45,X/46,XX subjects, 50% of 45,X/47,XXX subjects, and in 63% of those with deletions of the long arm. Ferguson-Smith suggested that monoallelic expression of certain loci on the short arm of the X, which normally escape X-inactivation and have presumptive Y-homologues, results in manifestations equivalent to complete X monosomy. In general, these observations and hypotheses have endured.

There is no "complete Turner syndrome" attributable to complete absence of the second chromosome, as shown by the presence of lymphedema and coarctation in only a minority of patients. However, several large patient series over the last three decades have corroborated the relatively greater prevalence of lymphedema, coarctation of the aorta, and neck webbing (or low hairline) in association with the 45,X karyotype. Palmer and Reichman[168] confirmed a low incidence of neck webbing and coarctation of the aorta with long arm isochromosome or ring X. Otto et al.[169] showed that all somatic manifestations other than short stature and gonadal dysgenesis occurred less frequently in 46,Xi(Xq) or i(Xq) mosaicisms than in 45,X. On the other hand, many features such as bony abnormalities appear to occur equally in X monosomy and mosaicisms. Correlation of phenotypic manifestations with recent cytogenetic data in large series of patients has not resolved the imprecision of phenotype-genotype correlations in TS.[170]

In light of newer techniques localizing X chromosome breakpoints, Therman and Susman performed a meta-analysis of long or short arm X deletions in TS adults without mosaicism.[171] In agreement with Ferguson-Smith and Simpson,[172] all of the major features of TS were observed with deletions of either Xp or Xq. Nonetheless, short arm deletions were more strongly associated with short stature, and long arm deletions more frequently resulted in gonadal dysgenesis. Short stature was reported in 88% and 43% of the Xp- and Xq- cases, respectively, whereas ovarian dysgenesis was seen in 65% and 93% of Xp- and Xq- karyotypes, respectively. Turner stigmata other than gonadal dysgenesis and growth failure were less frequently seen with short arm deletions than in complete X monosomy. Lymphedema and its putative consequences were rarely observed from short arm deletion.

Although growth failure is strongly associated with terminal deletion of the short arm, the feature is not universal or as commonly seen as in 45,X patients. Thus, no "short stature" locus has been defined. Fraccaro et al.[173] described short stature as the only feature present in several fertile females with deletion

at X(pter-p21). In five Xp- cases reported by Kalousek et al.,[87] all patients exhibited short stature, but other Turner stigmata were also variable. In familial transmission of distal breakpoints at p22.32, both male and female carriers were short.[174] In contrast, in 11 patients with short arm deletions reported by Fryns et al.,[175] four had normal stature, including a mother and daughter with deletion at pter-p11.6 but no features of TS. Turner stigmata and gonadal function of the seven 46,XdelX(pter-p11.2) karyotypes in this series were highly variable.

Whereas short arm deletions sometimes result in gonadal dysgenesis, long arm deletions and translocations are usually, but not always, associated with some degree of ovarian dysfunction. Skibsted et al.[176] hypothesized that a "critical region" at Xq13-q24 is required for normal gonadal function. Deletions with breakpoints distal to Xq25 result in less severe gonadal dysfunction, such as late secondary amenorrhea or early menopause.[177] Terminal deletion breakpoints at any location on the long arm can result in ovarian dysfunction. However, interstitial deletions at Xq13::Xq26 and Xq13::Xq21.3 have been documented in patients with normal ovarian function.[178]

It is still hypothesized that the phenotype resulting from X chromosomal loss is due to haploinsufficiency of tightly regulated loci with copies on both the X and Y chromosome. "Turner genes" thus might be localizable from Y deletions. XY females with documented Y short arm deletions can manifest features of TS. XYp- females reported by Rosenfeld et al.[47] and Magenis et al.[48] had lymphedema and neck folds, but not short stature. These and other reports[49,50] have suggested that lymphedema may result from deletions of the short arm of the Y, in contrast to the rarity of lymphedema seen with X short arm deletions.

The search for genes whose loss might cause Turner syndrome has concentrated upon loci which are not X-inactivated.[36] Pericentromeric regions and the distal short arm have genes that are not inactivated. Several loci in the pseudoautsosomal region, such as STS, Xg, MIC2, and the gene for the GM-CSF receptor, are transcribed from both X chromosomes. Genes proximal to the pseudoautosomal region known to escape inactivation include *ZFX, RPS4X*, and *KALIG-1. RPS4X* and its homolog *RPS4Y* are ubiquitously transcribed and encode a component of the small ribosomal subunit.[179] While *RPS4Y* maps to the short arm proximal to SRY, RPS4X maps close to the X inactivation center at Xq13.1 in the human. It has been observed that the prenatal lethality and phenotypic abnormality typical of XO humans is not found in XO mice, and Ashworth et al.[180] have demonstrated that *ZFX* and *RPS4* do undergo normal X inactivation in mice. This coincidence suggests the involvement of these genes in normal suppression of the TS phenotype in humans. However, evidence contradicts both RPS4X and ZFX as Turner suppressors. Page et al.[181] observed that an X,t(Y:22) female

lacking *ZFY* did not have features of TS, and Just et al.[182] demonstrated normal *RPS4X* transcription rates in fibroblasts of Turner patients. Thus, no Turner gene has as yet been identified.

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GENETICS OF MALE PSEUDOHERMAPHRODITISM AND TRUE HERMAPHRODITISM

Joe Leigh Simpson

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I. INTRODUCTION

Genetic advances have not only facilitated the delineation of disorders of sexual differentiation, but the converse applies as well. Many of the genes pivotal to sexual differentiation have been localized and sequenced (Table 1). In this chapter we shall discuss only selected disorders of gonadal differentiation that result from mutant genes, namely those forms of male pseudohermaphroditism and true hermaphroditism. Other disorders of sex differentiation have been discussed elsewhere by this author.[1-7] These other publications are inevitably reflected in this review.

Table 1. Some Gonadal and Ductal Differentiation Genes and Their Chromosomal Location

A. Gonadal Genes		
Sex Determining Region Y (SRY) (Testis determining factor (TDF)		Yp22
Anti-Müllerian Hormone (AMH) or Müllerian Inhibiting Substance (MIS)		19p13
Ovarian Maintenance		?multiple on Xq & Xp
?Azoospermia—(AZO) or Spermatogenesis Factor 3(SP3)		Yq11
B. Gonadotropin Genes		
Gonadotropin A subunit (CGA)		6q21.1→12
LH β subunit (CGB)		19q13
FH β subunit (FSHB)		11p13
Kallman (KALIG-1		Xp and other
LH Releasing Hormone (LHRH)		8p21
C. Steroid Biosynthesis and Metabolism		
Cholesterol Side Chain Cleavage	Cytochrome P450scc (CYPSCC) (CYP11A)	15q23-24
3β-o1 dehydrogenase, type II		1p11-13
17α-hydroxylase/ 17,20 desmolase (lyase)	Cytochrome P450c17 (CYP17)	10q24-25
17-ketosteroid reductase		unknown
21-hydroxylase	Cytochrome P450c21 (CPY21)	6p
11β-hydroxylase	Cytochrome P450c11 (CYP11B)	8q22
5α-reductase, type II	SRD5A2	2p23
Aromatase	Cytochrome P450c19 (CYP19)	15q21.1
D. Receptors		
Androgen Receptor (TGM, Testicular Feminization)		Xq11-12
Estrogen Receptor (ESR)		6q24-27
Gonadotropin (LH) Recaeptor (LHCGZR)		2p21
Anti-Müllerian Hormone (AMH) Receptor		unknown

II. REPRODUCTIVE EMBRYOLOGY

Primordial germ cells originate in the endoderm of the yolk sac and migrate to the genital ridge to form the indifferent gonad. 46,XY and 46,XX gonads are initially indistinguishable. Indifferent gonads develop into testes if the embryo, or more specifically the gonadal stroma, is 46,XY (Figure 1). This process begins about 43 days after conception. Testes become morphologically identifiable 7-8 weeks after conception (9-10 weeks gestational or menstrual weeks).

Sertoli cells are the first to become recognizable in testicular differentiation. These cells organize the surrounding cells into tubules. Both Leydig cells[8] and Sertoli cells[9] function in dissociation from testicular morphogenesis, consistent with these cells directing gonadal development rather than the converse.

Fetal Leydig cells soon become differentiated and produce an androgen—testosterone—that stabilizes Wolffian ducts and permits differentiation of vasa deferentia, epididymides, and seminal vesicles (Figure 1). After conversion by

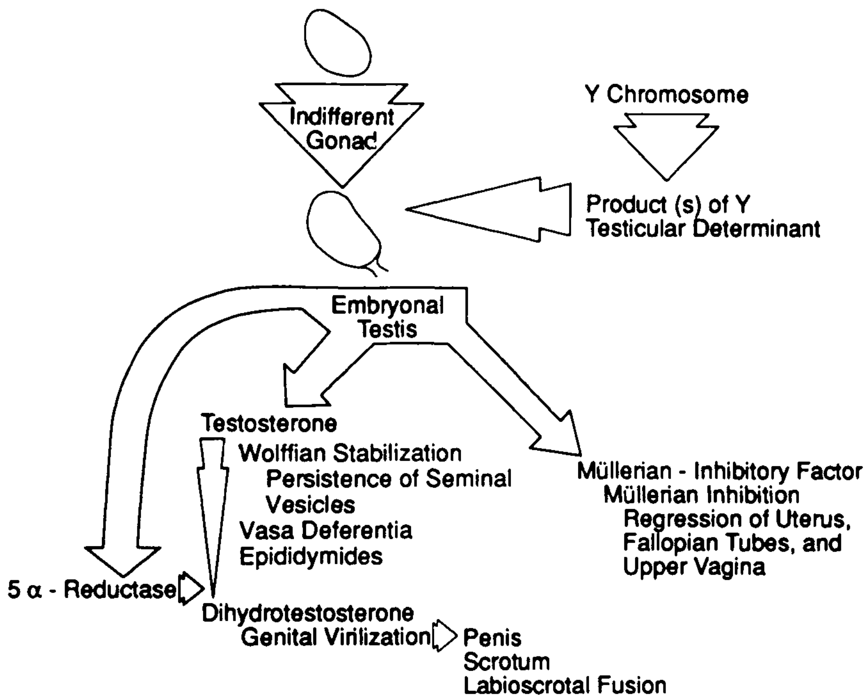

Figure 1. Schematic diagram illustrating embryonic differentiation in the normal male.

5α-reductase to dihydrotestosterone (DHT), the external genitalia become virilized. These actions can be mimicked by the administration of testosterone to female or castrated male embryos, as demonstrated clinically by the existence of teratogenic forms of female pseudohermaphroditism. Fetal Sertoli cells produce anti-Müllerian hormone (AMH) Müllerian inhibitory substance [MIS]), a glycoprotein that diffuses locally to cause regression of Müllerian derivatives (uterus and Fallopian tubes). The AMH may have functions related to gonadal development as well because when AMH is chronically expressed in XX transgenic mice, oocytes fail to persist. Tubule-like structures develop in gonads and Müllerian differentiation is abnormal.[10]

In the absence of a Y chromosome, the indifferent gonad develops into an ovary. Transformation into fetal ovaries begins at 50-55 days of embryonic development. Oocytes differentiate in 45,X embryos,[11] only to undergo atresia at a rate more rapid than that occurring in normal 46,XX embryos. The determinants for ovarian *maintenance* can be localized to specific regions of the X, as we shall discuss later. Certain autosomal genes must also remain intact for successful oogenesis.

Independent of gonadal differentiation is the process of ductal and external genital development. In the absence of testosterone and AMH, external genitalia develop in female fashion. Müllerian ducts form the uterus and fallopian tubes, and Wolffian ducts regress. Such changes occur in normal XX embryos as well as in XY animals which as embryos were castrated before testicular differentiation.

III. GENETIC CONTROL OF TESTICULAR DEVELOPMENT

That 46,X,i(Yq) individuals were female in appearance showed more than 20 years ago that the major testicular determinants (testis-determining factor or TDF) were localized tot he Y short arm (Yp). In the past 15 years a series of candidate genes have been proposed as the testis-determining factor (TDF) and used to localize TDF to distal Yp just below the pseudoautosomal boundary. H-Y antigen (HYA) and later *ZFY* were earlier considered among strong candidates, but consensus now exists that *SRY* (sex-determining region Y) is TDF.[12,13] Identification of *SRY* came as a result of mapping that took advantage of the existence of 46,XX males and sporadic cases of females with XY gonadal dysgenesis, as discused by Sinclair in Chapter 2 of this volume. The etiology of most (80%) 46,XX males involves interchange of not just the pseudoautosomal regions of Xp and Yp, but also the proximal nonpseudoautosomal region that contains TDF. Analysis of XX males revealed *SRY* to be present in the smallest translocated region that is compatible with male differentiation. Moreover, 10% of sporadic XY gonadal dysgenesis show point mutations within *SRY*.[14]

The *SRY* gene is comprised of two open reading frames of 99 and 273 amino acids based on analysis of females with XY gonadal dysgenesis[14-17] the key sequence involves an HMG (high mobility group) box that shares characteristics in common with other DNA binding sequences. When XY gonadal dysgenesis is associated with a point mutation or deletion in *SRY*, the sequence has almost always been located in the HMG box. *SRY* is evolutionarily conserved, being present in all male (XY) mammals. *SRY* is expressed before testicular differentiation is manifested.[12] Finally, transgenic XX mice with *SRY* predictably show testicular differentiation.[18]

Of relevance for this chapter, it is clear that in addition to genes on the Y chromosome, testicular differentiation requires loci on the X and on the autosomes.[6] The importance of genes on the X has long been evidenced by the X-linked recessive form of XY gonadal dysgenesis.[19,20] There is newer evidence that Xp contains a region that can, under certain circumstances, suppress testicular development despite presence of *SRY*. This phenomenon was first recognized by Bernstein et al.,[21] and perhaps a dozen other cases have now been reported.[22-24] The so-called gene responsible for this phenomenon[25] has been called Dose Dependent Sex reversal (DDS). The sex-reversal associated with the presumative locus appears to be the result of duplication and not disruption, given molecular analysis showing break points encompassing the entire region believed to include the gene. The locus is very near that of adrenal hypoplasia (AHC) but the relationship between AHC and DDS is unknown.

In addition to the X, autosomes are clearly important for testicular differentiation. For example, a gene causing campomelic dysplasia and XY gonadal dysgenesis (sex reversal) has been localized to 17q24.3→q25.1.[26] Among the syndromes that affect testicular differentiation and which may be heritable are *agonadia*,[27] *rudimentary testes syndrome*[28], and a syndrome in which *germ cell hypoplasia* occurs in *both males* (*germinal cell aplasia*) and *females* (streak gonads).[29-33]

These disorders point to autosomal influence over testicular differentiation, a concept which can be shown even more definitively in mice. Eicher et al.[34] crossed C57BL females to *Mus posciavinus* males, the Y of the latter being placed on a predominantly C57 autosomal background. The Y did not always prove capable of directing testicular differentiation, demonstrating the capacity of murine autosomes to suppress male differentiation. Redi et al.[35] placed the domestic Y (YDOM) on a C57BL/6J background and reported nine XY true hermaphrodites among 77 offspring. Nagamine et al.[36] and Taketo-Hosotani et al.[37] also found sex reversal. The most frequent explanation is that the *Mus musculus domesticus* Y acts later embryoloically than the *Mus musculus musculus* (C57BL/J)Y.[38] Irrespective, autosomal loci are capable of suppressing the Y.

The Y long arm (Yq) contain several other genes of interest, but these are not immediately relevant to male pseudohermaphroditism or true hermaphroditism.

IV. MALE PSEUDOHERMAPHRODITISM

Male pseudohermaphrodites are defined as individuals with a Y chromosome whose external genitalia fail to develop as expected for normal males. Some authors apply the appellation only to those whose external genitalia are ambiguous enough to produce uncertain sex of rearing; however, applying the term more liberally seems more useful clinically. A few disorders not strictly fulfilling the above definition are usually traditionally considered under the rubric of male pseudohermaphroditism. Cytogenetic forms of male pseudohermaphroditism (45,X/46,XY and variants) are also discussed here because they are important in the differential diagnosis of genetic forms of genetic male pseudohermaphroditism.

A. 45,X/46,XY and Variants

These individuals have both a 45,X cell line and at least one cell line containing a Y chromosome. They manifest a variety of phenotypes, ranging from almost normal males with cryptorchidism or penile hypospadias to females indistinguishable from those with the 45,X Turner syndrome.[39–41] Different phenotypes presumably reflect different tissue distributions of the various cell lines. Not infrequently a structurally abnormal Y chromosomes is present. 45,X/46,XY individuals may be categorized into individuals with either (a) unambiguous female external genitalia; (b) ambiguous external genitalia, that is, the sex of rearing is in doubt; or (c) almost normal male external genitalia.

1. Female External Genitalia

Patients in this category usually have the Turner stigmata and are clinically indistinguishable from 45,X individuals. As in other types of gonadal dysgenesis, the external genitalia, vagina, and Müllerian derivatives remain unstimulated because of the lack of sex steroids. Breasts fail to develop and little pubic or axillary hair develops. Some 45,X/46,XY individuals may be normal in stature and show no somatic anomalies. Fluorescent *in situ* hybridization (FISH) and flow cytometry studies of seemingly nonmosaic 45,X cases only rarely suggest complements (e.g., 46,XY) not readily demonstrable in blood.

Although the streak gonads of 45,X/46,XY individuals seem histologically indistinguishable from the streak gonads of individuals with 45,X gonadal dysgenesis, gonadoblastomas, or dysgerminomas develop in about 15-20% of 45,X/46,XY individuals.[39] Neoplasia may develop in the first or second decade. Lukusa et al.[42] observed that germ cell neoplasia was uncommon in 45,X/

46,XY individuals having a non-fluorescent Y chromosome. Thus, loss of a region (locus) seemed to confer a protective effect, suggesting existence of a cancer-predisposing region (locus) in distal nonfluorescent Yq, a region often lost during formation of a dicentric chromosome.

2. *Ambiguous Genitalia*

The term asymmetrical or mixed gonadal dysgenesis is applied to individuals with one streak gonad and one dysgenetic testis. Such individuals usually have ambiguous external genitalia and a 45,X/46,XY complement. Occasionally only 45,X or 46,XY cells are demonstrable. However, ostensibly nonmosaic cases could merely reflect the inability to sample appropriate tissues. This issue has still not been studied extensively with modern molecular techniques.

An important clinical observation is that 45,X/46,XY individuals with ambiguous external genitalia usually have Müllerian derivatives (e.g., a uterus). Presence of a uterus is diagnostically helpful because that organ is absent in almost all genetic (Mendelian) forms of male pseudohermaphroditism (see below).

3. *Normal or Nearly Normal Male Genitalia*

Occasionally 45,X/46,XY mosaicism is detected in individuals with normal or almost normal male external genitalia. If 45,X/46,XY cases are detected at amniocentesis, most cases prove to have normal male external genitalia.

B. Enzyme Deficiencies in Testosterone Biosynthetic Pathways

An enzyme deficiency should be suspected if testosterone or its metabolites are decreased. Diagnosis is not difficult in older children or adults, but delineation is more difficult in infancy because baseline testosterone levels are normally low. Provocative tests (hCG stimulation) are usually recommended to facilitate diagnosis.

Male pseudohermaphroditism may result from deficiencies of 3β-ol-dehydrogenase, 17α-hydroxylase/17-20 desmolase, 17-ketosteroid reductase, or one of the enzymes required to convert cholesterol to pregnenolone (cholesterol side chain cleavage or congenital adrenal lipoid hyperplasia).

1. *Congenital Adrenal Lipoid Hyperplasia*

In this condition male pseudohermaphrodites show ambiguous or female-like external genitalia, severe salt wasting, and adrenals characterized by

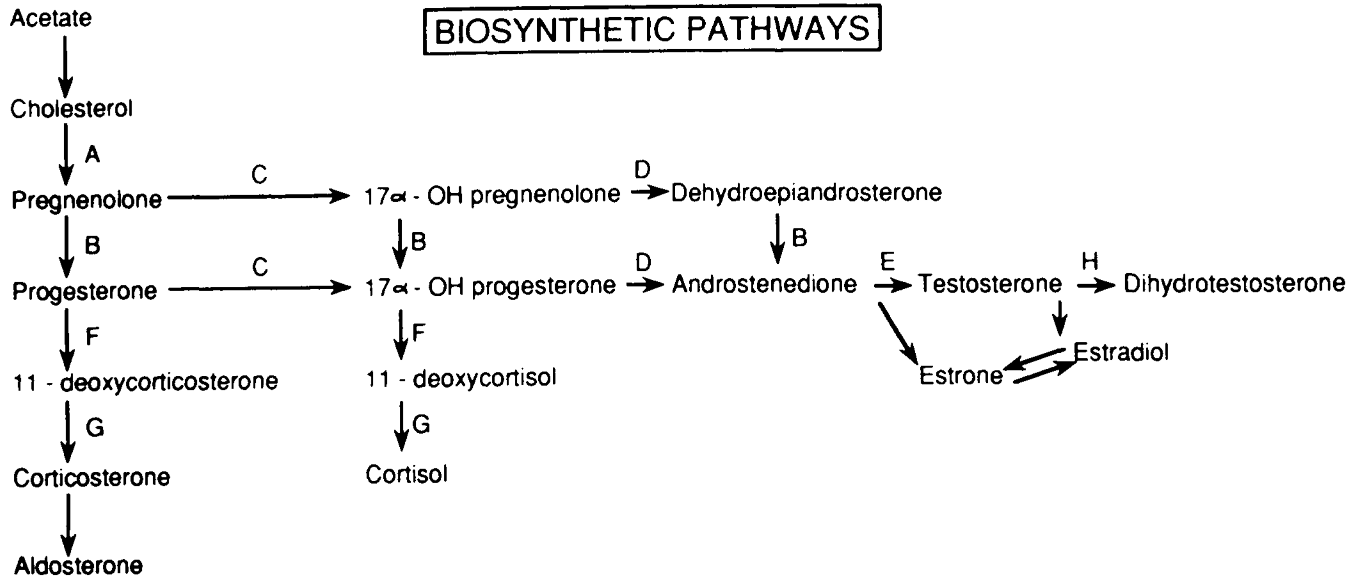

Figure 2. Adrenal and gonadal biosynthetic pathways.

foamy-appearing cells filled with cholesterol.[43,44] Accumulation of cholesterol indicates that this compound cannot be converted to pregnenolone (Figure 2). The enzyme apparently deficient is cytochrome P450 side chain cleavage (P450scc). It converts cholesterol to pregnenolone by 20α-hydroxylase, 20, 22-desmolase, and 22α-hydroxylase. Inheritance is autosomal recessive. This relatively large gene is located on chromosome 15. The length is 20 kb, with 9 exons. However, perturbations of the gene have never been shown in congenital adrenal lipoid hyperplasia (Lin and Gitelman 1991). Instead, various mutations (3 stop codons, 1 splicing error that deleted an exon and caused a frameshift) in the steroidogenic acute regulatory protein (StAR) gene were found [44a] With the mutant StAR cholestoral cannot be converted to cholesterol.

2. *3β-ol-dehydrogenase Deficiency*

Deficiency of 3β-ol-dehydrogenase, is an autosomal recessive disorder in which synthesis of both androgens and oestrogen is decreased (Figure 2).[45] The major androgen produced is dehydroepiandrosterone (DHEA), a weaker androgen than testosterone. Diagnosis is usually made on the basis of elevated 16-hydroxypregnenolone. In addition to genital abnormalities, 3β-ol-dehydrogenase deficiency is often associated with severe salt wasting because of decreased levels of aldosterone and cortisol.

The incompletely developed external genitalia of 3β-ol-dehydrogenase deficiency is clinically similar to the external genitalia of most other male pseudohermaphrodites: small phallus, urethra opening proximally on the penis, and incomplete labiscrotal fusion. Testes and Wolffian ducts differentiate normally. Affected females (46,XX) also show genital ambiguity, 3β-ol-dehydrogenase thus being the only enzyme that, when deficient, produces male pseudohermaphroditism in males and female pseudohermaphroditism in females.

There are two 3BH-SHD genes, both located on chromosome 1 (p11-13) and both consisting of four exons. The length is 7.8 kb. Only Type II is expressed in gonads and adrenals. Point mutations in the Type II gene have been described in a few patients with 3BH-SHD deficiency.[46-49]

3. *17α-hydroxylase Deficiency/17,20-desmolase Deficiency*

A single P450c17 enzyme is located on chromosome 10q24-25 and responsible for both 17α-hydroxylase and 17,20-desmolase activity. The gene consists of eight exons. That a single cytochrome gene is responsible for both these functions was surprising because enzyme studies suggested that were two genetically distinct conditions involving two separate genes. Point mutations have now been reported.[50-53] Males with deficiency of ostensibly only 17α-hydroxylase deficiency have been said to show ambiguous external genitalia,

normal Wolffian duct development, and normal testicular differentiation. Severely affected males may show female external genitalia.[54] Unlike females deficient for 17α-hydroxylase, males display normal blood pressure.

An apparently isolated defect involving 17,20-desmolase was reported in a family in which two maternal cousins had genital ambiguity, bilateral tests, and no Müllerian derivatives.[55] A maternal "aunt" was said to have abnormal external genitalia and bilateral testes. The deficient enzyme was deduced to be 17,20-desmolase on the basis of both cousins showing low plasma testosterone, low plasma dehydroepiandrosterone (DHEA), and normal urinary excretion of pregnanediol, pregnanetril, and 17-hydroxycorticoids. Incubation of testicular tissue demonstrated that testosterone could be synthesized from androstenedione or dehydroepiandrosterone, excluding 17-ketosteroid reductase and thus suggesting deficiency of 17,20-desmolase.

One informative case involved an individual who during childhood manifested only 17.20-desmolase deficiency but as an adult manifested 17α-hydroxylase deficiency as well.[56] This case is compatible with the idea that a single (gene) enzyme indeed serves both 17α-hydroxylase and 17,20-desmolase functions, temporal differences accounting for differences in enzyme deficiencies manifested.

4. *Deficiency of 17-ketosteroid Reductase*

Inability to convert dehydroepiandrosterone to testosterone is the result of deficiency of 17-ketosteroid reductase (Figure 2).[57] Plasma testosterone is usually decreased; and androstenedione and dehydroepiandrosterone are increased. Affected males show ambiguous external genitalia, bilateral testes, and no Müllerian derivates. Breast development may or may not be present, apparently reflecting the estrogen/testosterone ratio.[58] Pubertal virilization is greater than with some other enzyme deficiencies, and gynecomastia sometimes not even being evident.[59] The disorder is either autosomal recessive or X-linked recessive. This condition is relatively more common in the Gazan Arabs.[60]

The gene has not yet been isolated to my knowledge.

C. Complete Androgen Insensitivity (Complete Testicular Feminization)

In complete androgen insensitivity (complete testicular feminization) 46,XY individuals have bilateral testes, female external genitalia, blindly-ending vagina, and no Müllerian derivatives. Affected individuals undergo breast development and feminization at puberty.

Despite puberal feminization, some individuals with androgen insensitivity show clitoral enlargement and labiscrotal fusion. The term incomplete (partial) androgen insensitivity (incomplete testicular feminization) is applied to these

patients. Both complete and incomplete (partial) androgen insensitivity are inherited in X-linked recessive fashion, but the two disorders are considered distinct because they clearly breed true in a given family. 47,XXY cases have been reported and predictably shown to result from nondisjunction during maternal meiosis II.[61]

Individuals with complete androgen sensitivity may be quite attractive and show excellent breast development, but most are similar in appearance to unaffected females in the general population. Breasts contain normal ductal and glandular tissue, but areola are often pale and underdeveloped. Pubic and axillary hair are usually sparse but scalp hair normal. The vagina terminates blindly. Sometimes vaginal length is shorter than usual, presumably because Müllerian ducts fail to contribute to formation of the vagina. Rarely, the vagina is only 1-2 cm long or represented merely by a dimple.

Neither a uterus nor Fallopian tubes are ordinarily present. Occasionally one detects fibromuscular remnants, rudimentary Fallopian tubes, or rarely even a uterus.[62-64] The absence of Müllerian derivatives is not unexpected because anti-Müllerian hormone (AMH), secreted by the fetal Sertoli cells, is not an androgen; therefore, Müllerian regression would be expected to occur in males with androgen sensitivity, just as in normal males. The only other condition in which a uterus is absent in a phenotypic female is Müllerian aplasia, readily distinguishable on the basis of pubic hair and a 46,XX complement in the latter.

Testes are usually normal in size and located in the abdomen, inguinal canal, or labia, that is, anywhere along the path of embryonic testicular descent. If present in the inguinal canal, tests may produce inguinal hernias. One-half of all individuals with testicular feminization develop inguinal hernias. It may therefore be worthwhile to determine cytogenetic status of prepubertal girls with inguinal hernias, although most will be 46,XX.

Height is slightly increased over that of normal women, but unremarkable compared to 46,XX males. Presumably the increased height reflects influence of the Y chromosome. Likewise, many clinicians have the impression that hands and feet are relatively large.

The frequency of gonadal neoplasia is increased, but less so than often believed. However, the actual risk is probably no greater than 5%.[39,65]

The pathogenesis of complete androgen insensitivity involves end-organ insensitivity to androgens. This has long been deduced on the basis of baseline plasma testosterone being normal, yet patients neither virilizing nor retaining nitrogen after administration of androgen (testosterone or dehydrotesterone). In 60% or 70% of cases, androgen receptors are not present (i.e., the patient is receptor negative). In 30-40%, receptors are present (i.e., receptor positive). The androgen receptor gene has been localized to chromosome Xq11-Xq12. The gene consists of eight exons; exons 2 and 3 are DNA—binding domains and exons 4-8 are androgen-binding domains (Figure 3).

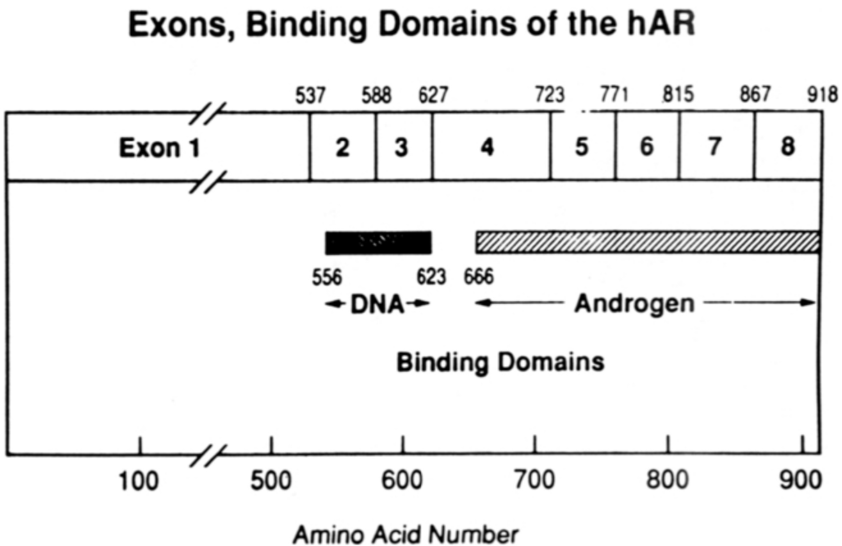

Figure 3. Schematic diagram showing the androgen-binding gene. Exons 2 and 3 relate to DNA binding, whereas exons 4 to 8 confer androgen-binding functions. (Adapted from Dr. Leonard Pinsky, Montreal, Quebec, Canada.)

Molecular heterogeneity has been shown to exist in the androgen receptor gene among affected individuals, but receptor-positive and receptor-negative individuals with complete androgen insensitivity are indistinguishable clinically. Perhaps 70% of cases studied show a mutation in the androgen receptor gene. Deletions are rare[66] as are insertions. More commonly encountered are point mutations involving single nucleotide changes that result in either substitution of an unscheduled amino acid, deletion of three nucleotides with preservation of an open reading frame (ORF), or generation or a stop codon to cause premature termination of the message and production of a nonfunctional protein. Large deletions and mutations that result in premature termination (stop codon) produce no functional receptor and cause complete androgen insensitivity.[67] Point mutation resulting from single nucleotide substitutions may or may not be associated with production of some androgen receptor. Sometimes the receptor may be unstable or characterized by poor binding.[67]

The relationship between phenotype and specific AR gene mutations is still under analysis, with emphasis on understanding consequences in terms of altered androgen receptor kinetics or tertiary structure.[67–70] Especially interesting are those circumstances in which substitution resulting in one new

amino acid produces complete androgen insensitivity, but substitution of another produces only partial androgen-insensitivity. This was shown by Kazemi-Esfarjani et al.[71] with respect to valine-865 being substituted with either methionine or leucine, respectively. Studies like the above are not only of academic interest but clinically important because molecular heterogeneity has left us with no great improvement in diagnostic ability on sporadic cases. Of course, in a given family various strategies (e.g., RFLP analysis) can be devised to detect heterozygotes or hemizygotes once a given mutant sequence is defined.[72]

Finally, a seredipity has been that abnormality of the androgen receptor is found in X-linked spinal and bulbar atrophy.[73]

D. Incomplete (Partial) Androgen Sensitivity (Incomplete Testicular Feminization; Reifenstein Syndrome)

At puberty certain 46,XY individuals feminize (show breast development) despite having external genitalia that are characterized by phallic enlargement and partial labiscrotal fusion. Such individuals are said to have incomplete or partial androgen insensitivity (incomplete testicular feminization). Both incomplete and complete androgen insensitivity share the following features: bilateral testes with similar histological findings, no Müllerian derivatives, pubertal breast development, lack of pubertal virilization, and normal (male) plasma testosterone.

Incomplete (partial) androgen insensitivity is an X-linked recessive condition that encompasses several entities once considered separate: Lubs syndrome, Gilbert-Drefus syndrome, and Reifenstein syndrome. These conditions are now accepted as representing a different spectrum of a single X-linked recessive disorder,[74] preferably called incomplete (partial) androgen insensitivity.

The pathogenesis of incomplete androgen insensitivity appears to involve a decreased number or qualitative defect of androgen receptors (AR).[67,68,75-77] Complete absence of receptors has been observed but this is more likely to be associated with complete androgen insensitivity. There is surprisingly poor correlation between receptor levels (or androgen binding affinity) and the degree of masculinization. No precise correlations are evident between mutation and phenotype. Molecular analysis has revealed several different mutations, predictably in the androgen-binding domains (exons 4-8) but sometimes int he DNA—binding domains (exons 2 and 3) (Figure 3). Complete deletions or point mutations resulting in premature (message) termination are more likely to cause complete androgen insensitivity.[67] Overall, however, fewer cases can be shown to have an AR gene mutation than in complete androgen insensitivity, suggesting that the defect may often involves a more distal step in androgen action.

The clinical significance of incomplete (partial) androgen insensitivity is that this disorder must be excluded before a male sex of rearing is assigned. Presence of androgen receptors and demonstration of a response to exogenous androgen is necessary to exclude androgen insensitivity.

E. Estrogen Receptor Defects

The estrogen receptor gene contains eight exons and is located on chromosome 6(q24-27). Analogous to the androgen receptor gene, there exists DNA-binding (exons 2,3) and estrogen-binding domains (exons 4-8). A case involving an estrogen receptor defect was reported by Lubahn et al.[78] A 28-year old male had normal male sexual development but incomplete epiphyseal closure. Serum gonadotropins and estrogens were elevated, and did not decrease after exogenous estrogen administration. In another case a homozygous transition in exon 2 resulted in a premature stop codon.[79] The rarity of recognized estrogen receptor defects compared to androgen receptor defects is surprising, and has been explained on the assumption of lethality occurring with perturbation of the gene; however, this thesis is belied by the mild phenotype associated with the above case.

F. 5α-reductase Deficiency (Pseudovaginal Perineoscrotal Hypospadias, PPSH)

For more than 20 years it has been recognized that genetic males may show ambiguous external genitalia at birth, yet at puberty virilize like normal males. They undergo phallic enlargement, increased facial hair, muscular hypertrophy, voice depening, and no breast development. Their external genitalia consists of a phallus that resembles a clitoris more than a penis, a perineal urethral orifice, and usually a separate blindly ending perineal orifice that resembles a vagina (pseudovagina). Testes are relatively normal in size and secrete testosterone in normal amounts.

In 1971-1972 my colleagues and I showed conclusively that this trait, then called pseudovaginal perineoscrotal hypospadias (PPSH), was inherited in autosomal recessive fashion.[80,81] Later, the disorder was shown to result from deficiency of 5α-reductase.[82-84] This enzyme converts testosterone to dihydrotestosterone. That intracellular 5α-reductase deficiency results in the PPSH phenotype is thus consistent with observations that virilization of the external genitalia during embryogenesis requires dihydrotestosterone; whereas Wolffian differentiation requires only testosterone. Pubertal virilization can be accomplished by testosterone alone.

The type I SRD5 gene is located on chromosome 5 (SRD5A1) and the type II gene (SRD5A2) is located on chromosome 2p23. There exist five exons in each isoenzyme, but only type II is expressed in gonads. Of the two isoforms

of 5α-reducase, it is type 2 that is deficient in this disorder. The gene contains five exons.[85] The gene has been shown to have undergone deletions[86] or more often point mutations in affected cases.[87] Gene mutations can usually be detected.[87] Different ethnic groups tend to show different mutations (founder effect), scattered among the five exons.

G. Aromatase Deficiency

Conversion of androgens (Δ4-androstenedione) to estrogens (estrone) requires cytochrome p450 aromatase, an enzyme produced by a single 40 kb gene located on chromosome 15q21.1.[88] The gene consists of 10 exons. Ito et al.[89] reported to a mutation in the CYP19 aromatase gene in an 18-year-old 46,XX female with primary amenorrhea. The patient was heterozygous for two different point mutations in exon 10, resulting in a mutant protein having no activity. Shozu et al.[90] reported *placental* aromatase deficiency in a woman who during the third trimester showed progressive virilization. The resulting female infant showed genital abnormalities (female pseudohermaphroditism) but no adrenal enzyme defects. The molecular basis of the mutation was an 87 bp insert in exon 6, altering the splice junction site between exon 6 and intron 6 to produce a new protein with 29 extra amino acids.

H. Agonadia (Testicular Regression Syndrome)

In agonadia the gonads are absent, the external genitalia abnormal, and all but rudimentary Müllerian or Wolffian derivatives absent. External genitalia usually consist of a phallus about the size of a clitoris, underdeveloped labia majora, and nearly complete fusion of labioscrotal folds. A persistent urogenital sinus is often present. By definition, gonads cannot be detected. Neither normal Müllerian nor normal Wolffian derivatives are ordinarily present; however, rudimentary structures may be present along the lateral pelvic wall. About one-half the cases show somatic anomalies—craniofacial and vertebral anomalies, mental retardation.[91]

Any pathogenic explanation for agonadia must take into account not only absence of gonads, but also abnormal external genitalia and lack of internal ducts. At least two explanations seem plausible: (1) fetal testes function sufficiently long to complete male differentiation (hence the appellation "testicular regression syndrome," preferred by some); (2) gonadal, ductal, and genital systems all develop abnormally as a result of either defective anlagen, defective connective tissue, or teratogenic action. Given both the heritable tendencies to be cited below and the frequent coexistence of somatic anomalies, defective connective tissue is an especially plausible hypothesis. Alternatively, cases with and without somatic anomalies could be etiologically distinct.

Among reported cases are several sibships of affected males.[27] Autosomal recessive inheritance seems most likely, but X-linked recessive inheritance cannot be excluded. *SRY* is present, indicating that pathogenesis does not involve perturbation of that system.[14,92]

I. Leydig Cell Agenesis

Several 46,XY patients have shown complete absence of Leydig cells.[93,94] The phenotype predictably consists of female external genitalia, no uterus, and bilateral testes devoid of Leydig cells; epididymides and vasa deferentia are present. LH is elevated.

Affected sibs have been recognized,[95,96] and parental consanguinity reported.[96,97] In addition to the autosomal recessive condition described above, Toledo et al.[98] described a milder phenotype in three affected brothers of consanguineous parents.

Two recent abstracts suggest that the molecular vasis of the disorder involves the LH receptor gene. Themmen et al.[98a] studied two sibs of consanguineous parents, each homozygous for a missense mutation ($Ala^{593} \rightarrow Pro$). Salmeh et al.[98b] detected a deletion in exon 11. Thus, Leydig cells problably fail to develop in this syndrome because of failure of LH to exert its effect during embryogenesis.

V. TRUE HERMAPHRODITISM

True hermaphrodites have both ovarian (follicles) and testicular tissue. They may have a separate ovary and a separate testis, or, more often, one or more ovotestes. The most frequently encountered gonad is an ovotestis.[99] Most true hermaphrodites have a 46,XX chromosomal complement; however, 46,XX/46,XY; 46,XY; 46,XX/47,XXY, and other complements occur.[100]

A. Phenotype

About two-thirds of true hermaphrodites are raised as males; however, external genitalia may be frankly ambiguous or predominantly female. Breast development usually occurs at puberty even with predominantly male external genitalia.

Gonadal tissue may be located in the ovarian, inguinal, or labioscrotal regions. A testis or an ovotestis is more likely to be present on the right than the left. Spermatozoa are rarely present;[101] however, normal oocytes are usually present, even in ovotestes. The greater the proportion of testicular tissue in an ovotestis, the greater the likelihood of gonadal descent. In 80% of ovotestes, testicular and ovarian components are juxtaposed in an end-to-end fashion.[102] An ovotestis can be detected by inspection, or by palpitation given testicular

tissue being softer and darker than ovarian tissue, or by ultrasound or MRI. Accurate identification is essential when extirpating the inappropriate portion of an ovotestis. A uterus is usually present, albeit sometimes bicornuate or unicornuate.

B. Aetiology

46,XX/46,XY cases presumably result from chimaerism (the presence in a single individual of two or more cell lines, each derived from different zygotes). 46,XX/47,XXY cases may result from either chimaerism or mitotic nondisjunction. This author once suggested that many 46,XY cases are unrecognized chimaeras.[100] Braun et al.[103] also reported a postzygotic (gonadal) *SRY* point mutation in a 46,XY true hermaphrodite.

A few 46,XX true hermaphrodites may have undetected chimaerism, but this phenomenon is not considered a common explanation for most 46,XX true hermaphrodites. Explanations for the presence of testes in individuals who ostensibly lack a Y include (1) translocation of testicular determinant(s) from the Y to an autosome, or (2) autosomal sex-reversal genes. Investigators seem to agree that true hermaphroditism is more common in African races than in other ethnic groups and that these cases are usually 46,XX.[99,100,102]

Most (80%) of 46,XX males are sex-reversed as result of *SRY* being interchanged from the paternal Yp to the paternal Xp during meiosis I, but few XX true hermaphrodites show *SRY*. McElreavey et al.[104] detected only three *SRY*-positive cases to 27 *SRY*-negative cases. Still, the few *SRY*-positive cases are informative in showing point mutations within *SRY*.[13, 105,106] Spurdle et al.[107] failed to detect *SRY* in 16 South African Bantu, the ethnic group in which frequency of true hermaphroditism seems highest.[108]

Given the above, Mendelian and more precisely autosomal factors must be presumed to play key roles in the etiology of true hermaphroditism. Sibships of XX true hermaphrodites have long been recognized.[109–115] Familial cases seem more likely to show bilateral ovotestes and no uterus than do nonfamilial cases.[100] Sibships also exist in which both XX males and XX true hermaphrodites occur.[116–122] XX males in the above kindreds differ from the more typical cases in showing genital ambiguity and lacking *SRY*. In aggregate, these data point to autosomal genes that are either inappropriately induced (gain of function in XX individuals or, conversely, not inactivated (repressed).

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PSEUDOAUTOSOMAL REGIONS AT THE TIP OF THE SHORT AND LONG ARMS OF THE HUMAN SEX CHROMOSOMES

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I. INTRODUCTION

The past decade has not only seen remarkable advances in our knowledge of genome organization, function, and variation, but also in the understanding, diagnosis, and treatment of genetic disease. Of all human chromosomes, the sex chromosomes X and Y have always been the focus of intensive gene mapping efforts. For the X, this is due to the unique inheritance of x-linked genes which has allowed the early identification of numerous X-linked recessive traits of medical importance. For the Y, it is due to its small size and its primary role in male development. It seems likely, therefore, that the sex chromosomes will be one of the first chromosomes to be saturated with markers, genes, and sequenced regions. This information is being generated through physical and genetic maps, YAC and cosmid contigs and eventually through the sequence of its approximately 160 (X) and 60 (Y) million base pairs of DNA.[1,2]

For more than 60 years, it has been predicted that the X and Y would retain a segment of persistent homology, referred to as the pseudoautosomal region.[3]. In this review, I will focus on the structure and function of this region (which actually consists of two parts located diametrically at the end of both sex chromosomes, see Figure 1) and discuss some of its unique and fascinating properties.

II. EVOLUTION OF THE SEX CHROMOSOMES IN MAMMALS

Despite being morphologically dissimilar, it is generally accepted that the mammalian sex chromosomes evolved from a pair of autosomes. The textbook model suggests that the acquisition of a sex-determining function by the Y chromosome eventually resulted in the gradual loss of genetic material, with the known consequence of increasingly dissimilar sex chromosomes.[4] This evolutionary process has apparently been accompanied by a series of rearrangements and an accumulation of repetitive DNA known as heterochromatin on the Y. Studies on certain species of reptiles, fish, insects, and birds, which also have developed distinct sex chromosomes, suggest that sex chromosomes have evolved independently many times in vertebrates.

The common past of the human sex chromosomes is emphasized by the observation that different X-Y homologous regions with varying degrees of conservation have been found on both the long and the short arm of the human X and Y (see Workshop Report on the Y chromosome[2]). However, from the analysis of a YAC contig of the human Y chromosome it is clear that possibly only one-third of the Y chromosome reflects the ancient homology between the sex chromosomes and that the majority of non-coding Y sequences is of relatively recent origin.[5]

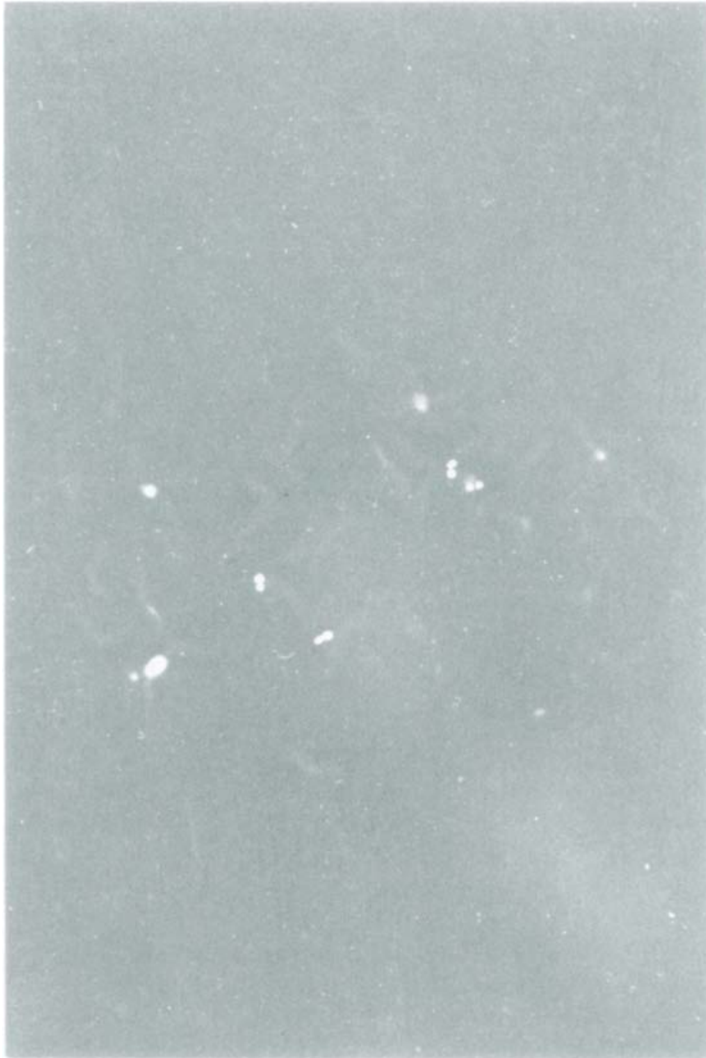

Figure 1. The pseudoautosomal regions PAR1 and PAR2 are located at the ends of the human sex chromosomes. Biotinylated cosmid pools from PAR1 and PAR2 were hybridized to metaphase chromosomes from stimulated lymphocytes of a normal male individual. The hybridized probe was detected via avidin-conjugated FITC and chromosomes were counterstained with DAPI. Images of FITC and DAPI were taken separately by using a cooled charge coupled device camera system (Photometrics, Tucson, AZ) and overlaid electronically using the gene join software.

The gradual process of X and Y chromosome differentiation has been accompanied by the evolution of a corresponding dosage compensation mechanism x-inactivation in the newly unpaired regions of the X chromosome. Most of the human genes known today to escape X-inactivation (with the exception of XIST and UBE1,[6,7] have a homolog on the Y chromosome. This homolog is either an actively expressed gene or a pseudogene. Does escape of X-inactivation therefore reflect a relic remaining from the ancestral homologous chromosome pair?

The observation that practically all human X-Y homologous genes escape X-inactivation and map to Xp (with the single exception of RPS4X) has led to the hypothesis that these genes are of autosomal origin and have been acquired as later additions to the sex chromosomes. This view is supported by the fact that genes on Xp are clustered on two different autosomes in marsupials and monotremes,[8] which evolved from eutherians 150 and 170 million years ago, respectively.[9] The long and short arm of the X chromosome thus seem to have a different history: Whereas the X chromosome is strongly conserved in eutherians (with the exception of the PARs, see below), eutherians and metatherians only share the long arm of the X.[10,11]

III. ORIGIN OF THE PSEUDOAUTOSOMAL REGION

In the textbook model mentioned above, the pseudoautosomal regions are interpreted as remnants of the ancestral autosomal pair. As lack of recombination has been suggested to be the reason for the Y chromosome to decline e.g., by accumulation of deleterious mutations, the converse—stabilization—should be the case for the PARs, where recombination takes place. Do the pseudoautosomal regions therefore represent the most ancient parts on the Y chromosome? It is becoming increasingly evident that the contrary may be the case.

It has been previously noted that steroid sulfatase (STS) and the gene leading to Kallmann Syndrome (KAL) escape X-inactivation and map near, but not within the human PAR1 on Xp22.3.[12,13,14] Both STS and KAL have been found to have pseudogenes, STSP and KALP, in reverse order on the long arm of the Y chromosome in Yq11.2.[12,15,16,17] In mice, the steroid sulphatase gene (Sts) is pseudoautosomal and has functional X and Y linked alleles that also appear to escape X-inactivation.[18] This observation led to the hypothesis that the present day human PAR represents only a part of a previous considerably larger segment of homology that has been disrupted by a pericentric inversion (the model of "shrinking" or "gradual reduction" of the PAR).[19]

However, in contrast to all other genes from the X-specific portion of Xp22.3, a novel gene, PKX1, which resides between XG and STS, has a homolog on Yp rather than on Yq.[20] This is intriguing, as it indicates that the single

pericentric inversion event on the Y chromosome hypothesized to have occured during primate evolution is not sufficient to explain the present X/Y homology pattern of Xp22.3.

Two pseudoautosomal genes, CSF2RA and IL3RA, which map within 100 kbp in the human PAR1 have been shown to map to two different mouse autosomes, MMU19 and MMU14, respectively.[21,22] Comparative *in situ* hybridization in primate and lemur species also have revealed that ANT3 maps to an autosome in two different lemur species, suggesting that the PAR1, too, may be of relatively recent origin.[23]

Sequences from the small PAR2 on Xq/Yq have been transposed from the X onto the Y chromosome even more recently in evolution and are considered to be human-specific.[24] All these results suggest that PAR1 and PAR2 were only recently added to the sex chromosomes with the addition of PAR2 being the most recent event.

The existence of a PAR has so far been shown in man (see above), chimpanzees, mice, and sheep.[18,25,26] Marsupial X and Y chromosomes, however, do not undergo obvious homologous pairing and a chiasma has not been found.[27] Nevertheless, does the association of X and Y chromosomes at one or both of their ends suggest that there is a common region which, however, could be very small? Homologous pairing and recombination have always been considered to be critical for correct segregation of the sex chromosomes and the development of functional sperm, and it is not easy to imagine how marsupials could manage without it.

In summary, the origin and the evolution of the PAR in different mammalian species still remains a major open question, since different species seem to have found their own solution. Due to the features mentioned above it is likely that this region represents a particularly attractive chromosomal target for evolutionary studies by comparative gene mapping.

IV. STRUCTURE OF THE PSEUDOAUTOSOMAL REGIONS

Physical maps of both the PAR1 and PAR2 that are based on pulsed-field gel electrophoresis (PFGE) and yeast artificial chromosomes (YACs) have been established.[28,29,30,5,31,32,33,24,34] The definition accepted for map completion by the U.S. NIH comprises ordered, sequenced-tagged sites (STSs) evenly spaced at 100 kb intervals. Such a "completed map" for the pseudoautosomal regions is not far ahead.

The pseudoautosomal region, PAR1, with its physical length of 2.6 Mbp represents 3-5 % of the human Y chromosome (because of the heterochromatic variation of the Y) and 1.6 % of the X chromosome.

The pseudoautosomal region 1 is located at the distal tip of the short arm of the X chromosome, band Xp22.3, and at the tip of the short arm of the Y chromosome, band Yp11.32. Characterization of DNA within light and dark bands has revealed a series of interesting associations.[35,36,37,38] Chromosomal

band Xp22.3 is a Giemsa-negative band and displays the expected high density of CpG islands that serve as gene markers in all vertebrate species.[39,40] Because of the early replication of this region in the cell cycle,[41] this band was earlier thought not to be subject to random X-inactivation.

Evaluation of the rare cutter restriction cleavage sites within the PAR reveals at least six different CpG islands. Two genes, *MIC2* and *ANT3*, and one pseudogene, MIC2R, have already been shown to be associated with the islands at position 2470, 1300, and 2350 kbp Xptel, respectively.[42,43,44,45] No information is available yet as to whether the cluster of rare cutter restriction cleavage sites at position 470-520 kb from Xptel represents a cluster of potential CpG islands associated with genes. In addition, the very high density of CpG restriction sites in the entire distal 500 kb large subtelomeric region has been previously suggested to possibly mirror an extremely dense area of genes or alternatively a stretch of noncoding, very GC-rich DNA.[28,29,30] A similar accumulation of rare cutter enzyme cleavage sites has been described in several regions of the genome, for example, in the HLA complex in man with five CpG islands within 50 kb[46] or around the L1CAM and G6PD regions on Xq28.[1]

The interface between sex-specific and pseudoautosomal sequences, the pseudoautosomal boundaries (PABY1 and PABX1), defined as the proximal limits of recombination.[47] In man, the pseudoautosomal boundary on Xp/Yp is marked by an Alu repeat, followed by a 220 bp stretch of reduced (78 %) homology.[47] Sequences proximal to this block are not-x-y homologous and sex-specific. Studies on Old World monkeys and great apes have indicated that the Alu repeat did not create the boundary seen in man, but instead was inserted at the pre-existing boundary after the Old World monkey and great ape lineages diverged.[48]

The pseudoautosomal boundary on Xq/Yq (PABY2 and PABX2), on the other hand, is defined by a LINE repeat sequence.[24] Kvaloy et al. have suggested that the boundary PABY2 and PABX2 arose relatively recently as a result of an ectopic recombination event mediated by the LINE sequences which were originally present on non-homologous stretches of X and Y chromosome DNA. The sequence organization of the 320 kbp PAR2 has been analyzed in detail; all of this region is cloned in overlapping lambda and cosmid clones. Several new probes including seven CA repeats have been placed on this contig.[24]

Both pseudoautosomal regions are distally bounded by a telomere. All telomeres consist of a simple repeated sequence $(TTAGGG)_n$ of variable length.[49] In humans, the range is between 2 kb and 20 kb, with the telomeres in germ cells being considerably longer than those in somatic cells.[50] Recently, a link between telomere shortening and aging has been suggested. Proximal to these conserved repeats, a complex mixture of chromosome-specific short repetitive sequences extending over several kb have been described for several

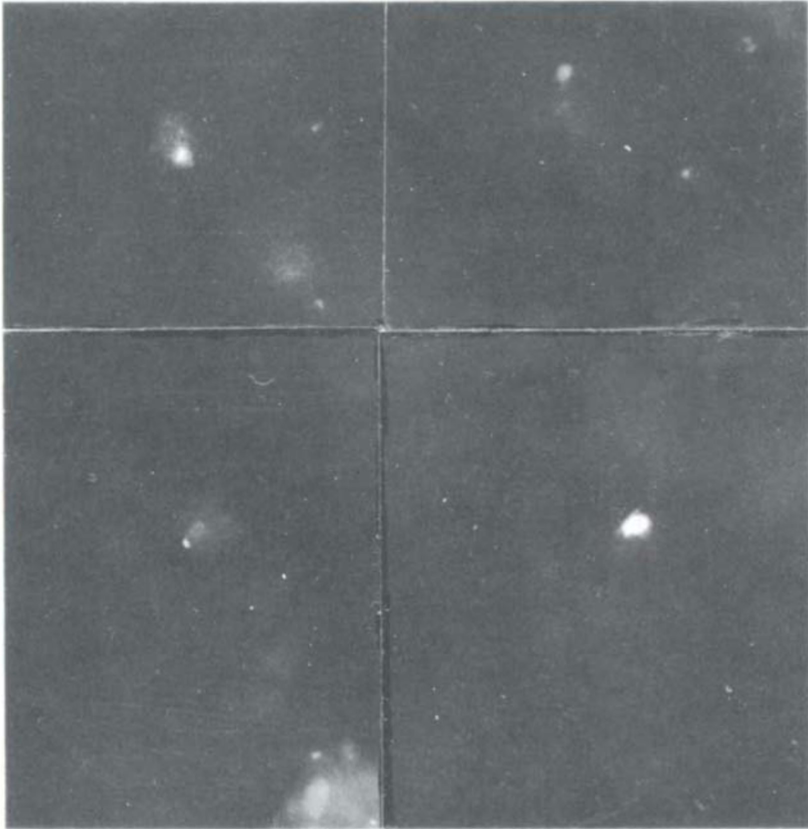

Figure 2. Chromosome painting of the X chromosome (red) and the pseudoautosomal region (PAR1) (yellow) in human testis meiocytes. In spermatocytes, the pseudoautosomal region shows a predominantly peripheral distribution. Courtesy of Dr. Harry Scherthan.

chromosome ends.[51-52] The subtelomeric repeat on Xq/Yq has been characterized in detail (Brown et al., 1990).

Investigations on interphase distribution of the PAR in somatic cells (T. Cremer) and germ cells (H. Scherthan) are currently being carried out. Observations indicate that an association of this region with the nuclear membrane is likely (Figure 2).

Various lines of evidence suggest that the human PAR1 on Xp/Yp contains an especially high density of minisatellite sequences. It has been argued that

minisatellites represent recombination hotspots involved in chromosome pairing, and/or play a role in the initiation of meiotic recombination, which would explain their prevalence in the PAR.[53,54,55,56] Structural studies on several pseudoautosomal tandem-repeats show copy number variations between individuals. Loci DXYS14, DXYS20 and DXYS78, for example, exhibit a 0.3-3 kb, 10-50 kb and 5-30 kb variation range, respectively, between individuals.[57,58,59] It is conceivable that other VNTR (variable number of tandem repeats) loci in the PAR display similar features. Extrapolating from these observations, one may argue for a possible length polymorphism of the PAR as a whole. Whether heterozygosities of pseudoautosomal length differences have an influence on meiotic segregation remains to be shown.

V. GENETICS OF THE PSEUDOAUTOSOMAL REGIONS

A. Pairing and Recombination in Meiosis

During meiotic prophase, physical contact between the homologous chromosomes results in pairing and synapsis, and consequently in the formation of a synaptenomal complex. Chromosome synapsis and genetic recombination are related events, and recent observations suggest that recombination provides the basis for pairing and not vice versa, as generally assumed.[60]

Pairing of human chromosomes usually starts at sites very close to the telomere.[60] On the sex chromosomes, the initiation of pairing and the obligatory crossover event take place within the pseudoautosomal region, PAR1. Based on observations in two cases in the laboratory mouse, a significant pairing failure was, observed however, when pairing and exchange was limited to PAR1. Hunt and Le Maire proposed that sex chromosomes pairing via PAR1 is not a property of the pseudoautosomal region alone, but due to the fact that it resides on an X chromosome.[62]

Crossing over within the PAR1 was first observed cytogenetically[63,64] and was then established with molecular probes by following the segregation of restriction fragment length polymorphism (RFLP) alleles in pedigrees.[65,66,67,68,42,57,69,70,32] The extent of pairing between Xp and Yp is variable, including the PAR1 and most of the short arm of the Y chromosome (see Figure 3), and in some cases extending into the long arm of the Y.[71] It is not known what causes the homologous parts to become precisely aligned; it seems however, that pronounced homology over very long stretches of DNA is not required. Occasionally pairing of the sex chromosomes has been noticed not only between Xp and Yp regions, but also between Xq and Yq[71,72] (see Figure 3), reflecting sequence homologies in the PAR2. Chiasma formation is specific to PAR1 and has only been seen in exceptional cases at the long arm pseudoautosomal region in PAR2.[73]

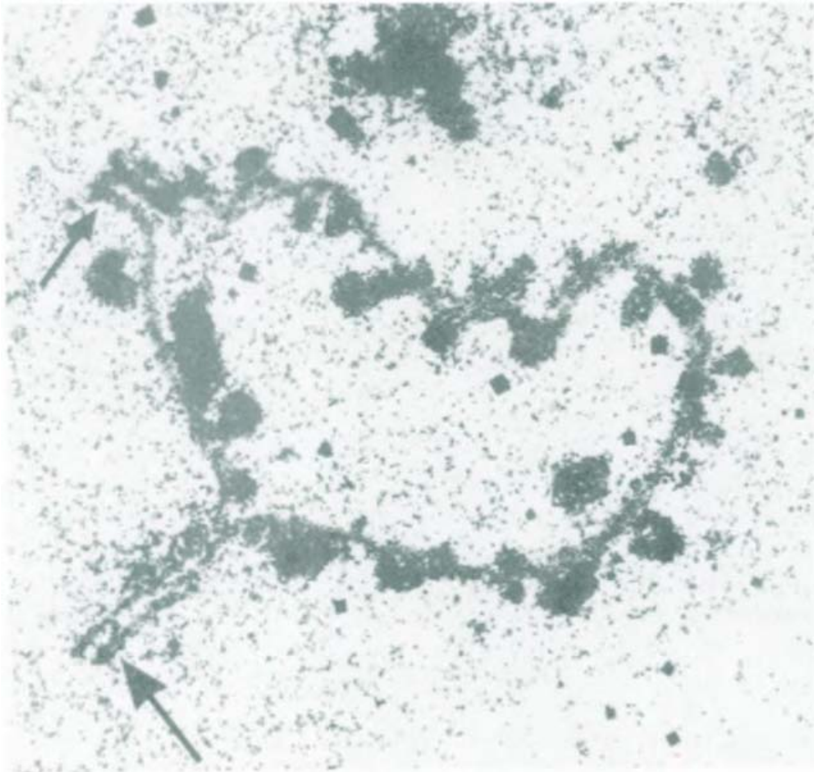

Figure 3. Pairing of the human sex chromosomes X and Y in the early stages of pachytene, examined by electron microscopy.[71] Note the association of Xp and Yp (large arrow), and of Xq and Yq (small arrow). Courtesy of Dr. A. Chandley.

B. Errors in Pairing and Recombination

X-Y pairing has been hypothesized as a prerequisite for the completion of male meiosis.[74] Burgoyne and others have argued that primary spermatocytes containing univalent X and Y chromosomes do not produce functional sperm. Meiotic and segregation data in mice from Sxr-carrier males and XY* males (these are mice which have a rearranged Y and in which the PAR occupies an interstitial position) showed that all functional sperm are derived from spermatocytes with sex chromosomes that had paired and have had an XY chiasma.[65,75,76] Whether X-Y pairing per se or X-Y recombination is a prerequisite for producing functional sperm is still an open question.

X-Y dissociation leads to sterility; in other words, the obligatory crossover is an indispensable condition for the reproduction of the individual. This was recently shown by a particulary striking example that involved crosses between two species of mice. These studies demonstrated a 95 % dissociation rate on the hybrids, which were sterile, compared with a 3-4 % X-Y dissociation rate in the parental strains[77,78]. Genetic divergence of the pairing region can thus contribute to reproductive barriers existing between species and to the process of speciation.

In humans, male individuals lacking X-Y pairing are also known to be sterile. A complete failure of XY pairing was, for example, observed in the primary spermatocytes of a man with a deletion of distal Xp, indicating that this region plays an important role in the initiation of pairing and in the formation of a synaptonemal complex at meiosis.[79] A reduced recombination frequency leads to incorrect segregation, and to 47, XXX or 47,XXY individuals.[80]

Occasionally, the obligatory X-Y crossover occurs outside the PAR1 (illegitimate recombination); in this case, the testis-determining factor, SRY, which is located just 5 kb proximal to the pseudoautosomal boundary on Xp/Yp[81] may be transferred to the X chromosome. Illegitimate crossover events result in 46,XX individuals who display a male phenotype.[82,83] The reciprocal product is a Y chromosome deleted for SRY, as found in some XY females with gonadal dysgenesis.[84]

C. Genetic Map of the Pseudoautosomal Region

Genetic studies on PAR1 and PAR2 have provided clear evidence that recombination in both regions is significantly higher than the average genome rate during male meiosis. This observation was not surprising as genetic map expansions have been observed in many subtelomeric regions of different human chromosomes.[69,85] The PAR, however, exhibits these subtelomeric features in a particularly striking way.

Genetic maps from the PAR1 have been established by family haplotyping[67,68,42,57,32] and by single-sperm haplotyping.[86] Both genetic linkage maps, correspond closely to each other. These studies have established a linear gradient of recombination with frequencies between 50% at the Xp/Yp telomeres and 0% at the pseudoautosomal boundary (PABX1) (see Figure 4). Comparison of the physical and genetic maps of PAR1 has indicated that the rate of recombination is rather uniform throughout the region. High recombination rates which are characteristic for this region are maintained up to the boundary.[42, 86] On average, 53 kpb of DNA correspond to one centimorgan in the PAR1,[32] a rate 10-fold higher than the genome average.

It has been proposed that the recombination frequency could be used to position any locus within this region, implying that there is only one crossover

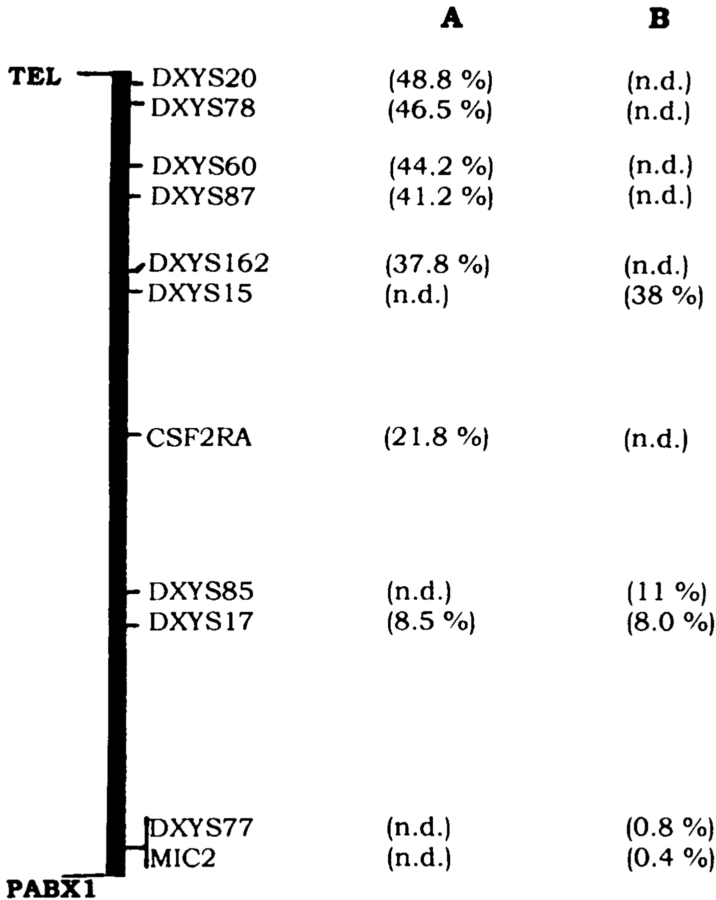

Figure 4. Multipoint genetic map of the PAR1, established by family haplotyping (Column A) and sperm typing (Column B).
Column A: Multipoint analysis of sex-specific recombination rates was performed using a subset of eight markers which were previously typed on more than 600 individuals from 38 CEPH families.[32]
Column B: Multipoint analysis using results obtained on five loci by typing 2,900 sperm from three individuals.[86] Relative order between *DXYS77* and *MIC2* could not be determined on the basis of the results by Schmitt et al.

event and the genetic distances are strictly additive.[67,68] Two recent papers, however, suggest that double crossover may occasionally occur in this region.[86,87] Although it is difficult to definitely rule out a "gene-conversion-like" event mimicking a double crossover, it now seems likely that the occurence of double recombination events are not restricted to the PAR of the mouse, but also occur in the human PAR1.[88,89]

In PAR2, the recombination rate between two specific loci has been estimated to be 2% in male and 0.4% in female meiosis.[70] Extrapolating from these observations and assuming that the probability of exchange is similar in PAR1 and PAR2, the predicted exchange rate is approximately 6%. Clearly, more markers are needed to estimate the relative recombination activity of this region at the Xq/Yq telomere.

The molecular basis of the approximately 10-fold difference in the PAR1 and five-fold difference in PAR2 between the frequencies of recombination in male and female germ cells is unknown. The reason for certain segments of the genome exhibiting highly elevated or low recombination rates remains an enigma. Extreme examples are seen in *Drosophila* males and silkworm females, with a complete absence of recombination. Studies in yeast have shown that chromosome length has a direct effect on the rate of recombination (smaller chromosomes lead to higher recombination rates)[90] and in *Drosophila melanogaster* and *Saccharomyces cerevisiae*, the frequency of meiotic recombination is altered by the proximity of certain chromosomal structures such as centromeres or large blocks of heterochromatin.[91]

VI. GENES IN THE PSEUDOAUTOSOMAL REGION

Genes on the X chromosome are usually only expressed when they reside on the "active X chromosome." From the approximately 100 genes cloned and mapped to the X chromosome,[1] only a few have been shown to escape X-inactivation. Among those genes that are transcribed from both active and inactive X chromosomes are X-linked genes such as STS, KAL, GS1, ZFX, UBE1, SMCX and RPS4X[92,13,14,93,94,6,95,96] and genes from within the pseudoautosomal region (CSF2RA, IL3RA, ANT3, ASMT, XE7, MIC2, XG, see below). It is as yet not clear at what stage these genes escape X-inactivation. It may well be that all were initially inactivated, but subsequently become reactivated. Most of the genes which have been shown to escape X-inactivation, map to the most distal band on the short arm of the X chromosome, Xp22.3 or to the proximal part of the short and long arm of the X. In the human pseudoautosomal region, PAR1, seven genes have been described to date. Their location with respect to the distance from the telomere is depicted in Figure 5. In the following, a brief summary is given on the characteristics of all pseudoautosomal genes.

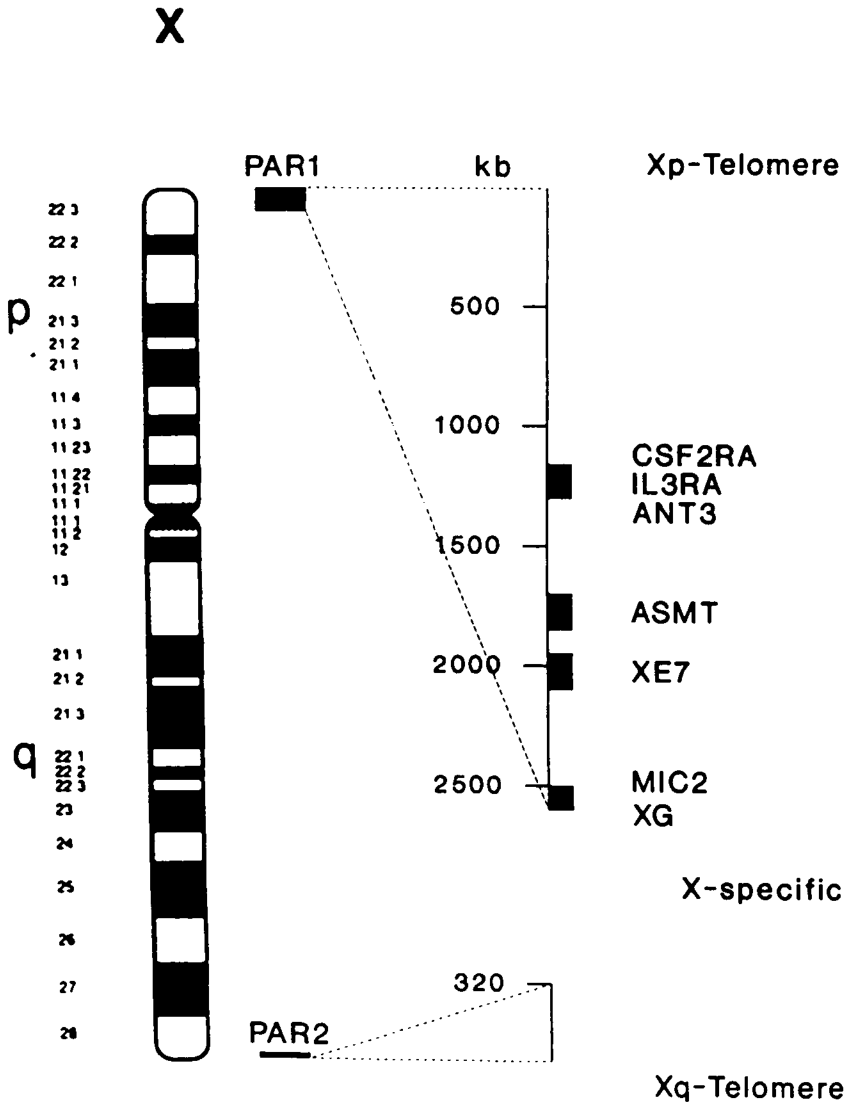

Figure 5. The PAR at the Xp telomere extends for approximately 2.6 Mb as shown by pulsed field gel electrophoresis[28,29,30] and at the Xq telomere for 320 kb as shown by a cosmid contig.[24] Seven different genes have been assigned to the PAR1. All of these are physically mapped; *CSF2RA*, *ASMT*, and *MIC2* are also genetically mapped. Genes in the PAR2 have not been identified to date.

The most distal functional gene is *CSF2RA* (colony stimulating factor receptor (), which encodes the alpha subunit of the GM-CSF receptor.[97] Its physical location from the telomere (1.2-1.3 Mbp) has been determined by PFGE[99]and its genetic location by CEPH family haplotyping.[97,32] GM-CSF (granulocyte-macrophage colony stimulating factor) is a growth and differentiation factor that acts on the monocyte/macrophage lineage.[97,98] Gough et al. have suggested that CSF2RA may be involved in the genesis of acute myeloid leukemia subtype M2, since in about 25% of acute myeloid leukemias, either the X or the Y chromosome is lost.

Another cytokine receptor gene *IL3RA* (interleukin receptor subunit α) resides in close proximity to CSF2RA, between CSF2RA and ANT3.[100,22] Interestingly, both genes IL3RA and CSF2RA share the same B-subunit (which maps to chromosome 22), whereas the -subunits are distinct and locate within 1180 -1280 Xptel in the pseudoautosomal region. This has raised the hypothesis that a cytokine receptor gene cluster containing growth factor receptors in addition to IL3RA and CSF2RA may reside in the PAR1.[100]

ANT3 (adenine nucleotide translocase) represents a highly conserved member of the ADP/ATP translocase family. From all genes of the PAR1 isolated to date, it represents the most highly conserved gene, ideally suited for comparative gene mapping studies. *ANT3* catalyzes the exchange of ADP and ATP across the mitochondrial membrane and thus plays a fundamental role in the energy metabolism of the eukaryotic cell.[101] This pseudoautosomal gene with a CpG island at 1.3 Mbp Xptel was isolated by cross-hybridization with a microdissected clone from the chromosomal subregion Xp22.3.[43,44] Sequencing of the cDNA revealed its identity with a previously characterized member of the translocase family.[101] Expression studies indicated that *ANT3* is a housekeeping gene.[43,44] Interestingly, a homologue of ANT3, viz. ANT2, maps to Xq24-q25 on the long arm of the X chromosome and undergoes X-inactivation.[43,102] ANT3 and ANT2 therefore provide the first evidence of two closely related genes, one on Xp and the other on Xq, that show striking differences in their X-inactivation behavior.

The gene *ASMT* (acetylserotonin methyltransferase or hydroxy-indole-O-methyltransferase, HIOMT) catalyzes the final reaction in the synthesis of the hormone melatonin, which is secreted from the pineal gland. ASMT was cloned by sequence homology to a bovine *ASMT* gene and has been genetically mapped within the PAR1 adjacent to the marker DXYS17.[103] It has been suggested as a candidate for psychiatric disorders because of its tissue-specific expression in pineal glands and retina.[103] No differences have been found, however, in post-mortem *ASMT* levels in the pineal glands from schizophrenics and controls. *ASMT* has been genetically mapped to the PAR1 close to DXYS17[103] and its physical location at 1.8-1.9 Mbp Xptel was established by PFGE and analysis of overlapping YAC clones.[34]

Proximal to *ASMT* maps *XE7*, a gene originally isolated from an inactive X cDNA library.[104] Presently, there is no clue to its biological function.

PFGE studies and analysis of YAC clones have placed XE7 at 2.0-2.1 Mbp Xptel.[34]

MIC2 was the first pseudoautosomal gene described in man.[42,105,106] The physical location of *MIC2* close to the pseudoautosomal boundary (CpG island at 95 kb distal to PABX) has been recently confirmed by analysis of cloned DNA.[107] *MIC2* is a ubiquitously expressed housekeeping gene. It encodes a cell surface antigen (defined by the monoclonal antibody CD99) and although its precise function is unknown, it is involved in cell adhesion processes, for example, in the spontaneous rosette formation of erythrocytes.[108] Elevated levels of expression of *MIC2* have been observed in Ewings sarcoma and primitive neuroectodermal tumors.[109] Further studies on *MIC2* have shown that sequences related to exons 1, 4, and 5 exist and that these form part of a pseudogene called MIC2R. MIC2R has been physically located 120 kb distal to *MIC2* and it has been suggested that the pseudogene represents the result of a duplicating event.[345]

More recently, another gene related to *MIC2, XG,* has been cloned and shown to span the pseudoautosomal boundary of the X chromosome.[110] *XG* represents an unusual gene. Its first three exons reside immediately downstream of *MIC2* in the pseudoautosomal region, whereas the other seven exons reside in the X-specific region. Hence, the 5'end of *XG* is pseudoautosomally inherited and its 3'end is X-linked. The amino acid sequence of *XG* is 48 % homologous to *MIC2* (including conservative amino acid substitutions), suggesting that all three *MIC2, XG,* and MIC2R form members of a small gene family, originating from an ancestral duplication event. Genetic evidence for a cis-acting regulation near or in MIC2 has been obtained some time ago.[111,112] The recent cloning of the *XG* blood group gene which is close to and coregulated with *MIC2* has very nicely confirmed those studies.[110,113]

No genes have been reported yet in the PAR2, but a possible locus implicated in the determination of sexual preference[114] has been suggested to reside there.[1]

A summary of the available information on the seven so far known human pseudoautosomal genes is given in Table 1. Are genes residing in an area of exceptionally high male recombination rates (possibly the highest in the entire human genome) put under special constraint? Does the genomic organization of a pseudoautosomal gene, for example, reflect those unusually high recombination rates in male meiosis? Data on physical gene size, exons, and introns suggest that this is not very likely.

Are pseudoautosomal genes then preferentially genes for which a certain degree of genetic divergence between individuals would have a selective advantage? Is the degree of polymorphism in pseudoautosomal genes between different individuals, for example, higher than of genes in low rate recombination areas? There are no data yet on the genetic divergence of pseudoautosomal genes.

Table 1. Genes in the PAR1

Gene	Physical location	Orientation	Genomic size [kb]	No. exons	cDNA	Expression
XG	2530	Tel-Cen	65	10	0.82	Bone marrow fibroblast
MIC2	2470	Tel-Cen	52	10	1.24	Ubiquitous
XE7	2000-2100	?	11	6	3.25	Ubiquitous
ASMT	1700-1850	?	>6	?	1.25	Pineal gland, retina
ANT3	1300	Cen-Tel	5.9	4	1.3	Ubiquitous
IL3RA	1180-1300	Tel-Cen	?	>4	1.5	Haemopoietic cells
CSF2RA	1180-1300	?	>45	>5	1.8	Haemopoietic cells

Notes: References to gene isolation and characterizations are given in the text. Physical mapping data was derived by PFGE and by YAC analysis.[34] Physical locations are based on a physical map length of the PAR of 2560 kpb (as determined for individual AH). 5'-3'orientation of the gene loci is given with respect to the telomere and centromere.

Are gene duplications due to unequal crossover more frequent within this region of high recombination rates? It may well be that the duplication event as seen in the proximal part of the PAR1, involving the loci *MIC2,* MIC2R, and *XG* exemplifies a more general phenomen of this region. Further molecular analysis on the structure of the pseudoautosomal region should clarify this question.

The unusual mode of inheritance of pseudoautosomal genes with varying degrees of partial sex-linkage stimulated very early the proposal of a wide range of hypotheses concerning different diseases.[115] All of these hypotheses could not be confirmed, illustrating the difficulty in distinguishing between pseudoautosomal inheritance from autosomal and X-linked inheritance with variable penetrance. Nowadays, genes predisposing to schizophrenia in PAR1[116,117,118,119] or homosexuality in PAR2[114] are a matter of discussion.

Besides these more controversial issues, there is evidence that a locus (or loci) controlling height maps (map) to the pseudoautosomal region.

VII. SHORT STATURE AND THE PAR1

Genotype-phenotype correlations in patients with sex chromosome abnormalities have argued for the presence of growth-related genes in the proximal part of Yq and in the distal part of Yq. Short stature is also consistently found in individuals with terminal deletions of Xp[120]. This

association between short stature and terminal deletions of both Xp and Yp pointed to a pseudoautosomal location of one of the Y-located growth genes.

CSF2RA appeared to be a promising candidate for a gene controlling height, because of both its pseudoautosomal location and its role in stimulating skeletal and gonadal tissue growth. Haploinsufficiency of this gene could have conceivably produced a sub-normal dosis of the gene product, leading to abnormal growth and development. However, genotype-phenotype correlations in patients with small terminal deletions of the sex chromosomes have excluded this gene as a candidate.[121,122] Recently, the candidate region has been narrowed down to a critical interval of less than 700 kb, covering the most distal quarter of the PAR1.[121,122]

VIII. TURNER AND KLINEFELTER SYNDROME AND THE INVOLVEMENT OF THE PAR1

Gene pairs which are active on the active and inactive X and Y chromosome were suggested early on to be candidates for numerical X chromosome disorders such as Klinefelter syndrome (47,XXY) and Turner syndrome (45,XXY).[123,124] Furthermore, X-Y homologous gene pairs which escape X-inactivation in man, as they undergo X-inactivation in mice as they may be the candidates for Turner syndrome in man, as 39,XO mice have a near-normal phenyotype with only slightly reduced fertility and growth and a slightly increased rate of fetal loss).[125]

Since all the pseudoautosomal region PAR1 escapes X-inactivation, some genes it contains are thought to lead, in a dosage-dependent way, to some of the features seen in Turner syndrome. However, with the exception of the gene causing short stature, possibly all genes for Turner stigmata seem to reside on the specific portion of the Y and the X-specific portion of Xp.[84,126] A goal of current studies is the identification of these Turner genes.

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TEMPERATURE DEPENDENT SEX DETERMINATION:

EVALUATION AND HYPOTHESES

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I. THE PHENOMENON

Temperature dependent sex determination (TSD) is most frequently observed in species which lack heteromorphic sex chromosomes. The phenomenon of TSD was first recorded by Charnier in 1966[1]when he observed that the lizard *Agama agama* had a skewed sex ratio which was dependent upon temperature of egg incubation. Subsequently, TSD has been described for many turtles[2,3,4], some lizards,[2,5] and all crocodilians studied.[6] This phenomenon of TSD has been shown to exist in the wild as well as in the laboratory; studies of the American alligator have shown that the sex ratio in the wild is approximately five females to every male.[7]

For animals with TSD sex is not determined at conception, but rather some time during embryonic development, by the temperature of egg incubation. A single sex is commonly seen to develop at the extreme temperatures with a mixture of sexes developing at intermediate temperatures.

II. TSD IN LIZARDS

Two species of gecko lizard have been documented as having TSD, *Eublepharis macularius* and *Gecko japonicus*. *Eublepharis*, the leopard gecko, a native of Western Asia, has all female offspring at incubation temperatures of 26°C, mostly female at 34°C, and mostly male (94%) at 32°C. Varying sex ratios, with no observable intersexes, are found at intermediate temperatures.[8] The period of incubation during which the sex of the embryo is affected by changes in incubation temperature is known as the temperature sensitive period (TSP) and has been defined for the leopard gecko as between stages 32-37, during the first half of development.[9,10] TSD is not the only sex-determining mechanism described for gecko lizards as some species have been reported to have heterogametic sex determination with XX/XY chromosomes, such as *Gekko gecko*.[11] *Gecko japonicus*, the Japanese gecko has been shown to have TSD producing mostly females at 24°C and 32°C and males at the middle temperature of 28°C.[12] Varying sex ratios are produced at intermediate temperatures and again no evidence of intersexes exists.[13] It was thought at one time that *G. japonicus* was a unique example of a species in the process of evolving sex-determining mechanisms. Separate studies had reported both heterogametic sex chromosomes and TSD for this lizard.[14] Further work has revealed that the identification of sex chromosomes may have been attributed wrongly to *G. japonicus* and that the lizard in question could in fact either be a close relative, *Gekko hokouensis* or an as yet unidentified species. Further taxonomic studies are required to conclusively comment about the evolutionary status of the sex determining mechanisms of *G. japonicus*.

III. TSD IN TURTLES

TSD is the predominant sex determining mechanism in turtles, the only documented occurrence of sex chromosomes is one of male heterogamety in the mud turtle, family *Kinosternidae*.[2] Temperature dependent sex determination appears to have been selectively maintained in the majority of turtles.[15] Why chromosomal sex determination has evolved in the *Kinosternidae* is unknown. Studies on TSD in turtles began when *Emys oblicularis*, the European pond turtle, was found to produce all males at 24-26°C.[16] In contrast to other TSD species discussed so far Pieau[16] found intersexes developing at intermediate temperatures. TSD has subsequently been described for many fresh water and marine turtles and has been verified in the wild.[17] It has also been suggested that other environmental factors such as hydration may also affect sex ratios. Chronic dehydration has been suggested to affect the sex ratios of the painted turtle.[18,19] However, this effect does not show a consistent bias and may reflect the premature condition of stressed hatchlings rather than a true effect on sex.

All sea turtles studied so far have TSD, but as sample availability is limited, and most species are threatened or endangered, the data obtained have been obtained from only a few clutches. The loggerhead turtle *Caretta caretta* produces all males at 26-28°C and all females at 32°C or above.[20,21] Similarly the green turtle *Chelonia myda* produces all males at 28°C and below with 90% females being produced at temperatures of 30.3°C and above.[22] The Olive Ridley sea turtle also has low temperature males (28°C) and high temperature females (32°C).[23,24]

IV. TSD IN CROCODILIA

All crocodilians studied have been documented as having TSD, including the Australian salt and fresh water crocodiles, *Crocodylus johnstoni* and *Crocodylus porosus*, respectively,[25,26] the Nile crocodile, *Crocodylus niloticus*,[27] and the Mugger crocodile, *Crocodylus palustris*.[28] TSD has been most extensively studied in the American alligator, *Alligator mississippiensis*, which was first described as having low temperature females (30°C) and high temperature males.(33°C)[7] Subsequently to this pattern of sex determination has been revised to describe a bimodal pattern of sex determination with respect to temperature and includes the production of mostly females at 34°C as well.[30]

Temperature not only affects sex determination but also influences other developmental processes, including growth rate and pigmentation patterns.[6] The sex determining effect of temperature is not due to differential mortality and has been verified both in the wild and the laboratory.[32]

The effect of temperature on sex ratios is well documented but it is not known how temperature exerts its effect on an undifferentiated embryonic gonad to direct development toward the male or the female pathway. The following section outlines the progress made to date in elucidating the mechanism of TSD.

V. THE PROBLEM

Since its discovery a great deal has been written about TSD but to date nothing is known about its molecular control. Despite the recent advances made in identifying the primary sex determinant in mammals, the genetic mechanism by which temperature exerts its effect has proven elusive. Unlike mammals, where the Y chromosome is known to be sex determining, reptiles with TSD have no structural distinctions in their chromosomes which can be used as the starting point for the study of sex determination.
Before we can hope to understand the mechanisms controlling TSD we must address the following fundamental questions.

1. Is the basic mechanism of TSD the same as chromosomal sex determination?
2. How is it possible to produce two sexes at the same temperature without any genetic differences (sex chromosomes)?
3. If the basic mechanism of TSD is conserved, how is it possible to produce different sexes in different species at a single temperature?

Based on the little experimental data that has emerged over the last few years there are currently no satisfactory answers to any of these questions. The aim of this review is to evaluate the latest molecular data and place it in context by suggesting possible hypotheses. The differences in phenomenology between different species with TSD are ignored for the purposes of this review, since the premise for this discussion is that the common underlying molecular mechanism will be the same for all reptiles with TSD.

VI. DEFINING THE TEMPERATURE SENSITIVE PERIOD (TSP)

For the American alligator temperatures of 30 °C result in all female offspring with temperatures of 34 °C giving mostly females.[30] Incubation at 33 °C gives 100% males while intermediate temperatures gives rise to mixed ratios of sexes with no intersexes observed.
During the 60-72 day gestation period of *Alligator mississipiensis* there is a defined window of development during which a change in incubation temperature can induce sex reversal in the hatchling. Experiments involving

the shift of eggs from one temperature to another at various times during development have defined the temperature sensitive period (TSP) of sex determination as between stages 21-24 corresponding to days 28-42 of incubation at 32°C.[30]

VII. SEX DETERMINATION GENES IN REPTILES

Closely related species of turtle or lizard have been documented as having either heterogametic sex determination or temperature dependent sex determination. It may, therefore, be postulated that similar genes could be involved in at least the early control of both mechanisms. Alternatively the mechanism of sexual differentiation could be the same in closely related species with TSD versus GSD and would be dependent on an "evolutionary" change in the mode of control of the hierarchy.

VIII. H-Y

The search for genes involved in TSD has largely centered around the cloning of homologues of candidate genes for mammalian sex determination. In the 1980s the male histocompatibility antigen, H-Y, was briefly thought to play a role in mammalian sex determination. Although H-Y is now known to be associated with the heterogametic chromosome, studies in species with TSD were initiated. The mud turtle, a species known to be ZZ/ZW, was found to express H-Y[33] and the European pond turtle, a species with TSD was found to express H-Y in association with testicular cells and sex reversed ovarian cells.[34] As H-Y usually associates with the heterogametic sex it was proposed that the European pond turtle was in fact a species with genetic sex determination at the intermediate incubation temperature and a temperature dependent sex determination at extremes of temperature.[35] The existence of sex chromosomes has never been proven but studies of estrogen sex reversal in TSD species have shown H-Y antigen to be associated with estrogen levels and not chromosomal composition.[36] The H-Y status in the ZZ/ZW salamander, *Pleurodeles waltii,* which can be sex reversed, was also found to be dependent upon the development of ovarian tissue and not the primary sex determining signal.[37] Although this data refuted the hypothesis that H-Y was the primary sex determinant, the evolutionary conservation of this molecule has been shown to be so extensive that it supported the hypothesis that other genes including ancestral sex determining genes may also have been conserved through evolution.[38,39]

IX. BKm

Bkm satellite DNA, originally isolated from the W chromosome of the snake, the banded krait, is characterized by repetitive sequences and has been found throughout the genomes of many species, often associated with the W or Y chromosome.[40] Bkm sequences were, for a short time, thought to be involved in primary sex determination in mammals and their expression in reptiles with TSD was studied. Male and female specific sequences were found in the sea turtle, *Chelonia mydas,* and female specific sequences in *Lepidochelys kempi* species which do not have identifiable sex chromosomes.[41] It was proposed that temperature controlled excision of Bkm fragments altered the expression of adjacent sex determining genes in these species.[42] Additional studies of Bkm in reptiles did not provide further evidence for a role in sex determination but showed by a fingerprinting technique that Bkm satellites could be used to study the genetic profiles of reptiles.[43] The phylogeny of turtles and the parental genetic status of wild and captive populations of endangered species of crocodiles is now assessed by such fingerprinting.[44,43] The function of Bkm satellites is not known but the observed evolutionary conservation of sequences is another example of homology in evolutionarily diverged species.

X. ZFY

The search for sex determining genes in TSD reptiles began in earnest with the discovery of *ZFY* in 1987 by David Page and his group in Boston.[45] At the time the discovery of *ZFY* generated much excitement since it was the first gene to be identified in the region of the human Y chromosome known to be sex determining. Moreover, since *ZFY* encoded a zinc finger protein it was capable of acting as a transcriptional regulator. This provided a plausible explanation for its suggested role in the control of a developmental cascade leading to testis development. *ZFY* encodes a protein with a classic structure of a transcriptional regulator; an acidic activation domain, a short nuclear localization signal, and a 13 cys-cys, his-his zinc finger DNA binding domain. All eutherian mammals have been found to have at least two *ZFY* genes, one on the Y chromosome [*ZFY*] and one on the X chromosome [*ZFX*]. The existence of *ZFX* was explained in terms of a dosage phenomenon with males having two functional genes, *ZFY*/*ZFX*, and females, through X inactivation, having only one, *ZFX*. *ZFY* was subsequently shown not to be sex determining in mammals but may still have an, as yet, unidentified role in gonad differentiation or spermatogenesis.[46,47]

Two genes highly related to *ZFY*/*ZFX* have been cloned from the American alligator [*Alligator mississippiensis*].[48] One of these genes, *Zfc,* is a homologue of *ZFY*/*ZFX* showing 91% identity at the amino acid level with *ZFY* in the

395 amino acid zinc finger region. Not surprisingly, in light of the evolutionary relationships between birds and reptiles, *Zfc* is most highly related to the chicken *ZFY* homologue, *Zfb*. PCR analysis, genomic library, and Southern blot screening indicate that the alligator has only one gene equivalent to *ZFY/ZFX*. The other alligator zinc finger isolated, *Znc6*, is homologous to *ZNF6*, a gene related to *ZFY* and *ZFX* found on the human X chromosome. The zinc finger exon of *Znc6* is composed a of a two-finger repeat of only 12 complete zinc fingers. The region which comprises the third finger in the *Znc6* gene has been altered such that a finger cannot form. Moreover a Phe for His substitution at position 215 may also disrupt the structure of the fifth finger. The amino acid sequence of the "third finger" region is highly conserved in different species and has been identified in mice, fish, marsupials, and several other reptile species.[49] This would imply that the " missing-finger" at position three in this protein is not simply acting as a spacer region in the protein but has some important function.

Embryonic expression of *Zfc* and *Znc6* has been studied using Northern blots and *in situ* hybridization. The Northern analysis showed that both genes are expressed throughout development with no differences in embryos developing at different temperatures. Although transcripts of both genes can be detected in RNA from genital ridge tissue (urogenital system), *in situ* hybridization of whole embryos shows that expression of both *Zfc* and *Znc6* appears to be widespread (Figure 1). It seems unlikely therefore that either of these two genes have specific roles in TSD or gonadal development.

Since *Zfc* and *Znc6* are homologous with genes that are present on mammalian sex chromosomes they can provide useful initial markers in alligator chromosome mapping studies. The alligator genome is about the same size as the human genome and is distributed on 16 pairs of chromosomes. The haploid karyotype is distinguished by five large chromosomes and 11 small chromosomes. No heteromorphic chromosomes are detectable in either sex although one chromosome of chromosome pair 13 possesses a secondary constriction on the short arm which is possibly caused by variation in the copy number of rRNA genes.[50]

Using metaphase chromosome spreads prepared from alligator embryos we have produced a detailed karyotype based on G-band staining and mapped the location of *Zfc* using *in situ* hybridization.[50] *Zfc* maps to chromosome 3 and since a position on either arm could not be determined statistically we assume *Zfc* is located close to the centromere. Preliminary mapping studies with *Znc6* also suggest this to be located on chromosome 3 although insufficient spreads have been counted to confirm this. *ZFX* and *ZNF6* are on different arms of the human X chromosome and mapping studies of marsupial and monotreme X-linked genes have shown that the two arms of human X chromosome have different evolutionary origins. Genes on the human long arm are also found on marsupial X chromosomes whereas genes on the human

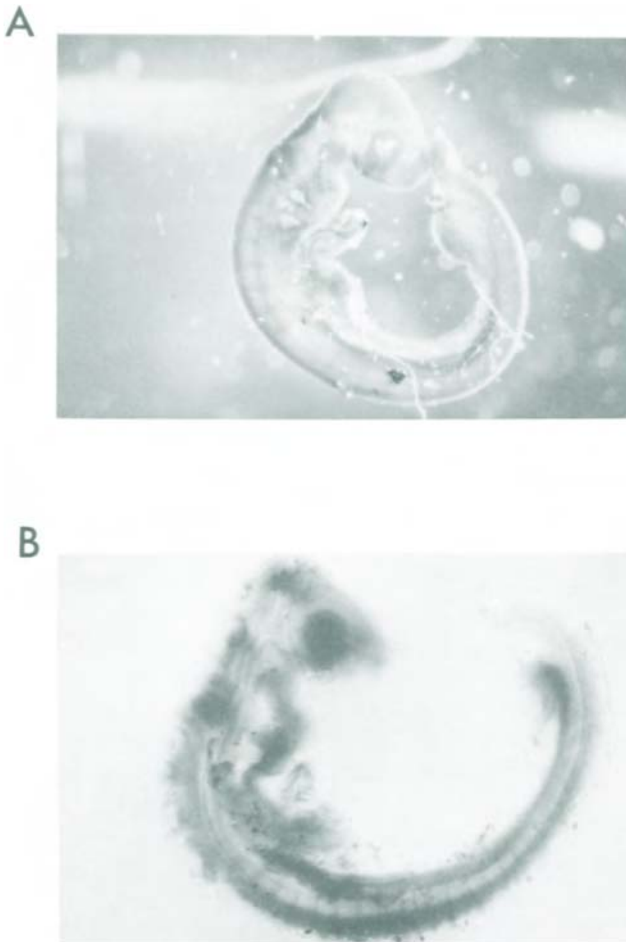

Figure 1. Expression of *Zfc* and *Znc6* genes in stage 7 alligator embryos revealed by whole embryo *in situ* hybridization. A. *Zfc*.; B. *Znc6*.

short arm are found on marsupial autosomes. It would be interesting, therefore, if human long and short arm genes are located on the same reptile chromosome.

XI. SRY

With the discovery of *SRY* as the mammalian sex determining gene (testis determining factor) it became important to determine whether reptiles with

1
TCC CCC AAG ATG CAC AAC TCG GAG ATC AGC AAG CGG CTC GGG
S P K M H N S E I S K R L G

43
CGC GAG TGG AAA CTT TTA TCC GAG GCG GAG AAG AGA CCC TTC
A E W K L L S E A E K R P F

85
ATC GAT GAA GCC AAG CGC CTC CGC GTC CTG CAC ATG AAG GAG
I D E A K R L R A L H M K E

127
CAC CCG GAT TAT AAA TAC CGA CCC CGG CGA AAA ACC AAG ACC
H P D Y K Y R P R R K T K T

169
CTG ATG AAG AAG GAT AAG TAC ACG TTG CCA GGG GGC TTG CTG
L M K K D K Y T L P G G L L

211
GCG CCG GGC ACC AAT ACC ATG ACC ACC GGG GTC GGG GTT GGG
A P G T N T M T T G V G V G

253
GCC ACC TTG GGA GCG GGG GTG AAC CAG AGG ATG GAC AGT TAT
A T L G A G V N Q R M D S Y

295
GCT CAC ATG GGC TGG ACC AAC GGA GGG TAT GGG ATG ATG CAA
A H M N G W T N G G Y G M Q

337
GAG CAG CTC GGC
E Q L G

Figure 2. Nucleotide and translated amino acid sequence for a partial cDNA clone of ASox2.

TSD had an homologous gene. If an *SRY* gene could be found in TSD species this would represent a considerable leap in our understanding of TSD since it is likely that the function would be evolutionarily conserved. Unlike the cloning of *ZFX/Y* however, definitive identification of an *SRY* homologue has proved very difficult. Low stringency screening of alligator genomic and embryonic cDNA libraries has repeatedly failed to isolate any sequences identifiable as *SRY*. PCR with degenerate primers based on the conserved DNA binding domain of *SRY* (SRY-box) and genomic DNA resulted in the cloning of a number of sequences with a recognisable SRY-box.[51] These

sequences ranged in amino acid homology with SRY between 45 and 63%. Greatest homology seen with these PCR generated sequences however was with SRY-boxes from Sox genes (*S*ry b*ox*) . Because the PCR primers were only designed to amplify the conserved SRY-box, it was not therefore possible to obtain sequences outside the box to confirm the identity of the products. Although *SRY* genes have now been cloned from a number of mammalian species PCR primers cannot be designed to *SRY*-specific sequences outside the conserved SRY-box because no conservation of sequence exists between any of these species (see Chapter 2 of the volume).

A number of alligator embryonic cDNAs have been isolated through cDNA library screening with SRY-box sequences generated by PCR from genomic DNA (above). The predominant cDNA clone isolated from a screen of a mixed sex/stage cDNA library was identified as the homologue of Sox-2. Partial sequence of three cDNAs shown in Figure 2 show 100% amino acid identity with each other with nucleotide differences only occurring at third base positions. These three sequences show 100% amino acid identity with bird and mammalian *Sox*-2 and probably represent different allelic forms since the

```
Alligator SPKM  HNSEI SKRLG AEWKL LSEAE KRPFI DEAKR
Chick     N---  ----- ----- ----- ----- ----- -----
Mouse     N---  ----- ----- ----- ----- ----- -----

Alligator RLALH MKEHP DYKYR PRRKT KTLMK KDKYT LPGGL
Chick     ----- ---Y- ----- ----- ----- ----- -----
Mouse     ----- ----- ----- ----- ----- ----- -----

Alligator LAPGT NTMTT GVGVG ATLGA GVNQR MDSYA HMNGW
Chick     ----- ----- ----- ----- ----- ----- -----
Mouse     ----G ----- ----- ----- ----- ----- -----

Alligator TNGGY GMMQE QLG
Chick     ----- ----- ---
Mouse     ---S- ----- ---
```

Figure 3. Translated amino acid sequence for ASox2 compared with chick and mouse protein sequences respectively. All amino acid differences are conservative. Dashed lines represent identity with respect to ASox2. The difference between sequences seen at 5′ end of the above sequence could be due to a cloning artefact.

Figure 4. Expression of *Asox2* in a stage 10 alligator embryo showing expression predominantly in the developing brain and central nervous system.

library was made from RNA from embryos from several different clutches of eggs. Further sequencing of the alligator *Sox-2* cDNA (*Asox-2*) confirmed its homology with *Sox-2* with amino acid identity between *ASox-2, cSox2*, and *Sox-2* being 93-98% over 117 amino acids (Figure 3).

Since *Sox-2* expression had been detected in developing murine gonads (Lovell-Badge personal communication) the expression of *ASox-2* was studied in alligator embryos using *in situ* hybridization. Both whole embryo and section *in situs* showed the main site of *Asox-2* expression as the developing nervous system, consistent with the expression of bird *cSox2*.[52] No obvious expression was detected in the developing urogenital system of either male or female embryos (Figure 4). It is possible that *Asox-2* expressed at a low level in the urogenital system and may not have been detected by *in situ* hybridization. More sensitive techniques such as RNase protection and RTPCR need to be used to confirm this.

Comparison of all SRY-box sequences published to date reveals that *Sox-3* is most closely related to *SRY/Sry* within the DNA-binding region. The complete lack of conservation between different *SRY* genes from mammals outside the SRY-box means that a full comparison of amino acid sequence between *Sox-3* is not informative. However the fact that mammalian *Sox-3* genes map to the X chromosome and are expressed in developing mouse gonads supports the idea that, in non-mammalian species *Sox-3* might be a candidate for an early evolutionary ancestor of *SRY*.[53] If this is so then alligators would be expected to have a recognizable *Sox-3* gene which could be predicted to be expressed in the developing urogenital system. Using degenerate PCR primers against a highly conserved region of *Sox-3* outside the SRY-box found

in bird, murine, and human sequences, a sequence from alligator genomic DNA has been amplified that shares 85% nucleotide homology with bird *cSox3* in the SRY-box confirming that alligators do indeed have a *Sox-3* gene (Asox-3). The embryonic expression of *ASox3* is currently under investigation.

The identification of a reptile equivalent of *SRY/Sry* using standard cloning approaches has proved difficult. The possibility remains that *Sox* gene expression in reptile gonads may be important in TSD and so a direct functional assay for detecting *SRY*-like activity in gonads was developed. SRY-box-containing proteins bind to a consensus DNA sequence of A/T A/T CAAAG.[54] We have explored the possibility that binding of proteins present in embryo tissue extracts to this sequence *in vitro* may provide a useful assay for detecting the presence of SRY-box-containing proteins in developing reptile gonads. Protein extracts were prepared from dissected urogenital systems from alligator embryos at equivalent developmental stages during the temperature sensitive period at 33°C and 30°C. Extracts were also prepared from the brain tissue of the same embryos to provide controls as a tissue where *Sox* genes are known to be expressed and where activity would be unlikely to be influenced by temperature. Extracts were incubated in standard gel shift reactions with 32P-labeled double stranded oligonucleotides containing the DNA-binding consensus sequence. Figure 5 shows the gel shift pattern obtained with urogenital tissues from stages 20 and 32 male (33°C) and female (30°C) embryos. A surprisingly simple band pattern was obtained with two faint bands visible in the male extracts that were barely detectable in the female extracts. Extracts from brain tissue of the same embryos produced more bands on the gel shift but no differences were detectable at different temperatures. The majority of bands were competed with excess of unlabeled oligonucleotide but not with unrelated sequences. These results indicate the presence of SRY-box proteins in extracts from alligator urogenital systems developing as male, that are not present at the same stage in female embryos.

These results thus provide the first evidence for a possible role for SRY-box proteins in TSD. In both cases the gel shift band patterns were remarkably simple suggesting that, at least with the conditions used, there are few SRY-box proteins expressed during the temperature sensitive stages of reptilian gonad development. SRY-like DNA binding activity was present in urogenital tissues developing at all male producing temperatures but not at all female producing temperatures at two stages within the temperature sensitive period. The start of the temperature sensitive period thus corresponds to the first detectable difference in protein activity and the end of the period of commitment to a sexual differentiation pathway (Figure 6). Significantly although bands were detected in brain extracts there was no difference in the band patterns at male or female temperatures. Thus there appears to be a male gonad-specific *SRY*-like activity. The lack of any *SRY*-like activity in female gonads at two different stages further implies that if these proteins have a role

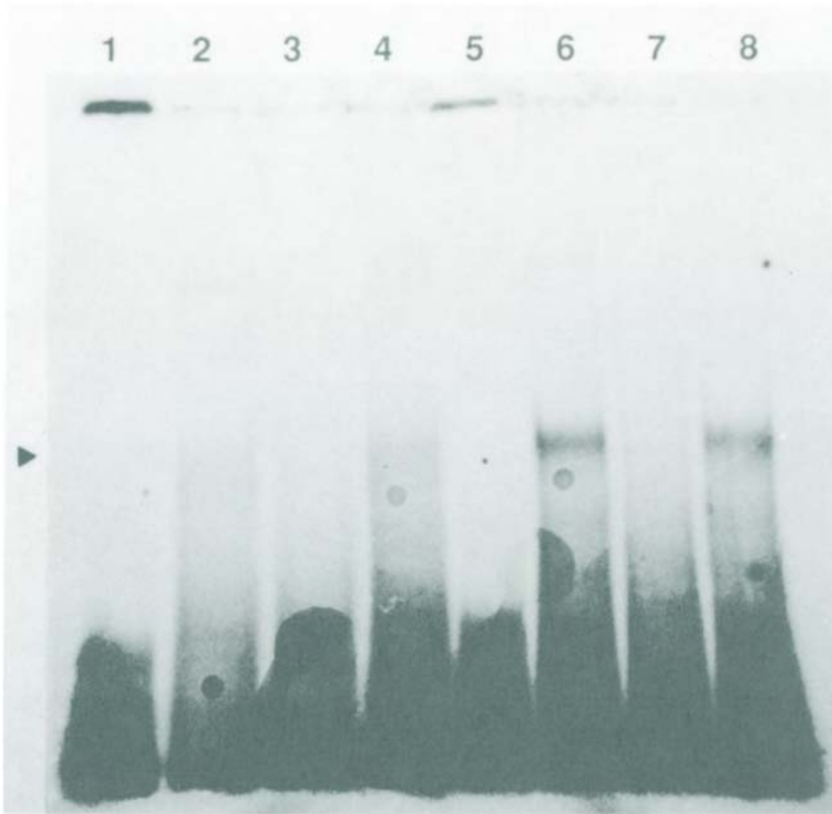

Figure 5. Gel retention assay showing binding of proteins extracted from alligator embryo urogenital tissues at stage 20 developing at 33 °C and 30 °C to a double stranded oligonculeotide containing an SRY-binding sequence.
1. Free probe; 2. 30 °C extracts; 3. 30 °C extracts plus 200 fold excess of unlabeled *SRY* oligo (specific competition); 4. 30 °C extracts plus 200 fold excess of unrelated oligonucleotide (non-specific competition); 5. free probe; 6. 33 °C extracts; 7. 33 °C extracts, specific competition; 8. 33 °C extracts, non-specific competition. Arrowheads indicates the position of the retarded band with 33 °C extracts.

in TSD, their mechanism of action may not be dosage or time related but an "all or nothing" switch (below). This clearly also has implications for considering how two sexes might be produced at intermediate temperatures.

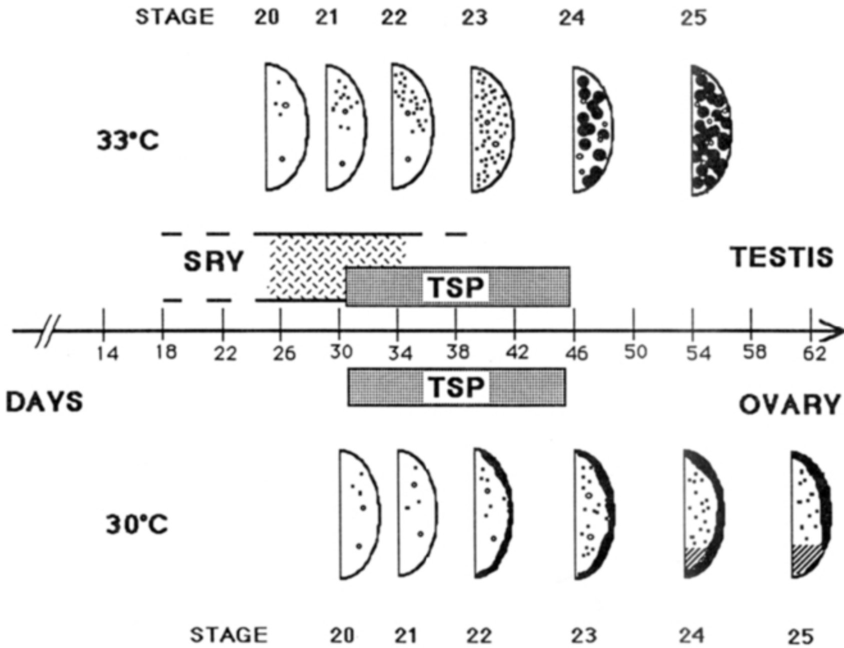

Figure 6. Diagrammatic representation of gonad development in *Alligator mississippiensis.* TSP = temperature sensitive period as defined by Lang and Andrews (1994); *SRY* = the activity of an *SRY*-like protein; o = germ cells; ● = enlarged medullary cord cells; ■ = medullary rest; //// = small, irregular-shaped medullary cord cells. White background is germinal epithelium. Ovarian cortex including germ cells shown as black shading. Not to scale. Dark interstitial cells have been omitted. Adapted from Smith and Joss (1994).

XII. TSD—MOLECULAR HYPOTHESES

Any model (hypothesis) of the molecular basis of TSD has to include an explanation of three important features; (1) how do small differences in temperature function at the molecular level, (2) how can the same sex be produced at different temperatures, and (3) how can different sexes be produced at the same temperature?

XIII. DOSAGE AND THRESHOLDS

A popular idea that has been proposed in several different forms is that TSD involves a "threshold" phenomenon.[31] Deeming and Ferguson proposed that TSD might involve dosage of a gene product(s) which accumulate in gonadal

cells faster at 33°C than at 30°C (in alligators), and that sex is determined by the concentration of this product(s) at a particular threshold point in development. If sufficient product has accumulated during this period then cells are directed to follow the male pathway. If insufficient product has accumulated, gonadal cells follow a "default" pathway of female development. 33°C is assumed in this model to be the optimum temperature for gene transcription or translation and temperatures higher or lower are sub-optimal. The production of both sexes at the same temperature is explained in this hypothesis by natural variation in the population of embryos. Thus in any one clutch of eggs variation will exist both in the length of the time or rate of accumulation and the dose of product required by individual embryos. The generation of the different sexes at the same temperature in different species is difficult to explain by this hypothesis

XIV. "ALL OR NOTHING"—PROTEIN CONFORMATION

The concept that the molecular basis of TSD might involve an "all or nothing" phenomenon as suggested by the gel shift data (above) is difficult to reconcile with any of the three important features of TSD (above). If TSD is controlled by a switch, that is, the presence or absence of a protein, this must be exquisitely sensitive to small temperature changes. The "switch" must operate only in certain embryos at intermediate temperatures and must have different consequences in different species.

One attractive possibility is that the *SRY*-like DNA binding activity detected in alligator gonads at male temperatures may reflect a temperature dependent conformational change in the protein, allowing it to bind to DNA. At female temperatures this change may not occur and thus although the *SRY*-like gene is transcribed and translated no DNA binding activity is detected. Attempts to alter the binding activity in male extracts by reducing the incubation of protein extracts and DNA unfortunately resulted in abolition of all binding *in vivo* so this could not be tested directly.

If we assume the same *SRY*-like protein is involved in TSD in different reptiles then the same temperature would be expected to produce a similar conformation and hence activity. Since different sexes are produced at the same temperatures in different species, for example, *A. mississippienesis* produces 100% males at 33°C, whereas *E. macularis* (Leopard gecko) produces 60% females at 33°C, the same protein conformation would be predominantly having opposite effects—female rather than male.[56] This might be easily explained by the fact that *SRY* is a very rapidly evolving gene: for example, the sequences outside the DNA binding domain are completely different between mouse and human. The sequence outside the DNA binding region appears to be important for the activity of *SRY*/*Sry* since human and mouse

SRY proteins differ in their ability to bend DNA. This has profound effects on the activity of the proteins, such that human *SRY* cannot functionally replace mouse *Sry*.[57] Thus in two closely related mammals, the process of testis determination appears to be controlled in the same way, by activity of an *SRY*/*Sry* DNA binding protein, but the structure and properties of the two proteins are quite different. It seems plasuible therefore that reptile *SRY*-like proteins involved in TSD will exhibit similar properties and so conformation of the proteins could be very different in different species and result in different activities (male vs. female) at different temperatures.

The protein conformation hypothesis proposes that *SRY*-like genes are transcribed at the same level at male and female producing temperatures and the effects of temperature is on the activity (DNA bending or binding) of the protein. The production of both sexes at the same temperature cannot be easily explained by this hypothesis as it stands. It is possible that at intermediate temperatures that produce both sexes, the *SRY*-like protein adopts a partially active conformational change that in some embryos is sufficient to activate male differentiation but in others is not sufficient. This clearly implies that there has to be a process independent of the *SRY*-like protein structure that could enable a partially active protein to function in one embryo but not another. However, the production of two sexes at the same temperature could be a result of variation in the timing of development of individual embryos. In order to get male differentiation at intermediate temperatures the reduced activity of the *SRY*-like protein will be sufficient only in embryos whose gonadal development has advanced to a certain stage. If this stage has not been reached then the *SRY*-like protein does not function and the female pathway is activated. Thus there must be synchronization between *SRY*-like protein activity (conformation) and gonad development to switch-on male gonad differentiation.

XV. GONAD ASYNCHRONY

The concept that the mechanism of TSD involves a temperature-dependent asynchrony during gonad differentiation has been proposed by Smith and Joss[59] based on modifications of ideas originally proposed by Joss and Haig (1991).[60,61] They proposed that TSD may involve a temperature dependent mismatch between the timing of pre-Sertoli/Sertoli cell differentiation and ovary determination. Pre-Sertoli/Sertoli cell differentiation appears to be temperature dependent: at 33°C (in alligators) proliferation is greater than at 30°C. Thus, at any developmental stage there are always more pre-Sertoli/Sertoli cells in developing gonads at 33°C than at 30°C (Figure 6). Based on this observation, Smith and Joss propose that the basic pathway of sex determination in alligators is female and hypothesize that if a threshold number

of pre-Sertoli/Sertoli cells is reached before the stage of ovary determination then a male gonad develops. If the threshold number is not reached then a female gonad will develop. This hypothesis is a morphological manifestation of the threshold and dosage mechanism outlined earlier where accumulation of a product, in this case pre-Sertoli/Sertoli cells, by a particular developmental stage leads to a particular developmental pathway. The accumulation of pre-Sertoli cells might be linked to accumulation of an *SRY*-like protein at 33°C. This hypothesis also supports the "all or nothing" concept whereby activity of an *SRY*-like protein would be synchronized with pre-Sertoli/Sertoli cell number for male development.

At present no one theory can adequately explain TSD but now hypotheses have been put forward which can be tested experimentally. It is likely that the future for this field lies in the identification of molecular markers of early gonad development in reptiles and that the most useful markers will be reptile homologues of genes involved in mammalian sex determination .

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MAMMALIAN SPERMATOGENESIS

David W. Hale

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I. INTRODUCTION

In this chapter, I present an overview of the cellular, cytogenetic, genetic, and, to some extent, evolutionary aspects of mammalian spermatogenesis, and highlight some of the salient differences between spermatogenesis and oogenesis in mammals. The first section, which describes the cellular progression from the primordial germ cell to the mature spermatozoon, is intended to provide the reader with the sequential and terminological perspective necessary to fully appreciate the remainder of the chapter. As an extension to this opening discussion of the cell biology of spermatogenesis, the section on intranuclear structures presents and discusses those structures which are particularly relevant to the progression (i.e., the synaptonemal complex and the dense body) or the microscopic study (i.e., the synaptonemal complex and the nucleoli) of spermatogenesis. The cytogenetics section, which chronicles the chromosomal events of normal spermatogenesis, necessarily and appropriately concentrates on the events and phenomena of the meiotic stages (particularly prophase I). This rather lengthy section also examines chromosomal modification (telomere elongation, X-chromosome inactivation, and genomic imprinting), the pseudoautosomal region, obligate X-Y recombination, chromosomally based male-hybrid sterility, nondisjunction, and premeiotic chromosomal rearrangements. The section on the genetics of spermatogenesis focuses on genes that affect or play some role in the spermatogenic process. The endocrinological aspects of the initiation and maintenance of spermatogenesis are very complex, and consequently a complete discussion of this topic is beyond the scope and intent of the this chapter. For an appropriately detailed treatment of this subject, the reader is referred to a more comprehensive review.[1] Because my technical and conceptual training is in cytogenetics and evolutionary biology, it is unavoidable (and not wholly unintended) that discussion in these areas will

comprise much of this chapter. I hope that, by departing somewhat from the more traditional emphases on the cellular, endocrinological, biochemical, and genetic aspects of spermatogenesis, this chapter will provide a rather different perspective on this fascinating and highly complex biological process. While some of the content of this chapter will undoubtedly overlap with that of other chapters in this volume, I hopefully have succeeded in highlighting those aspects, events, and structures of spermatogenesis that are not usually considered in reviews on this subject.

II. SEXUAL REPRODUCTION, MEIOSIS, AND SPERMATOGENESIS

The vast majority of eukaryotic organisms exploit a sexual mode of reproduction in which haploid gametes are produced from diploid cells, and diploid zygotes are subsequently formed by fusion of these haploid gametes (syngamy). Although there are some disadvantages associated with sexual reproduction (e.g., inefficiency in terms of energy expended on meiosis and syngamy, necessity of finding and perhaps competing for a mating partner, inability to fix certain advantageous genotypes, parasitism by transposable elements, susceptibility to sexually transmitted diseases),[2] this reproductive mode exhibits considerable advantages relative to asexual reproduction. Perhaps most importantly in a population-genetic and evolutionary context, a sexually reproducing organism can produce progeny exhibiting a diversity of genotypes. This genetic reassortment promotes genetic variation within populations, thereby allowing more rapid response to selection (clearly a distinct advantage in an unpredictably variable and changing environment).[3,4] Genetic variation and the resultant developmental, anatomical, and physiological variability pave the way for evolutionary innovation and the exploitation of a wide variety of habitats and ecological niches; indeed, the advent of sexual reproduction might have facilitated the accelerated evolution and diversification that occurred in planktonic algae approximately 1 billion years ago.[5] Sexual reproduction also facilitates the incorporation of favorable mutations at different loci into one individual by means of recombination during meiosis and at syngamy, and, through the same mechanisms, confers the ability to eliminate deleterious mutations from a population through the death or sterility of individuals carrying large numbers of disadvantageous mutations.[2] It has been hypothesized that sexual reproduction and recombination originally evolved as DNA-repair system.[6] According to this hypothesis, the diploid condition and chromosomal synapsis during prophase I provide a juxtaposed undamaged chromosome for double-stranded DNA repair in the damaged homolog. Because recombinational repair effects crossing over, genetic recombination is viewed as a secondary effect of DNA repair.

Meiosis is a highly specialized type of nuclear division that occurs during gametogenesis in sexually reproducing organisms. The meiotic process, which is apparently derived from mitosis (both utilize the spindle apparatus, microtubules, and kinetochores for segregating chromatids and chromosomes prior to cytokinesis), is basically a single chromosomal duplication (preleptotene S phase) followed by two successive nuclear divisions. Through the two divisions of meiosis, haploid gametes are produced from diploid germ cells. The first meiotic division is a reductional division that exactly halves the chromosome number (i.e., from the diploid number of chromosomes to the haploid number of chromosomes), thereby averting the doubling of the diploid chromosome number that would otherwise occur at syngamy. Like mitosis in diploid somatic cells, the second meiotic division is an equational division involving the separation of sister chromatids and their subsequent incorporation into different daughter nuclei. However, unlike mitosis, the second meiotic division involves haploid cells and is not immediately preceded by a period of DNA synthesis (S phase).

Spermatogenesis is the inclusive process by which diploid cells undergo two meiotic divisions to produce the highly differentiated and specialized haploid gametes in males. Spermatogenesis includes: (1) spermatogonial renewal and proliferation, (2) meiosis, which reduces the chromosome number to the haploid condition (and, during which genetic recombination occurs), and (3) spermiogenesis, which is the process by which the immediate haploid products of meiosis (spermatids) undergo further differentiation to form mature haploid spermatozoa.[1,7] Although the basic sequence of events in female and male meiosis is essentially the same, the overall gametogenic processes are fundamentally different in male and female mammals (Table 1).[8,9] In both male and female embryos, the diploid primordial germ cells migrate from the endoderm-derived yolk sac to the mesoderm-derived genital ridges, which will ultimately form ovaries in females and testes in males. The mitotic oogonia develop from the primordial germ cells in females, and proliferate only during the fetal stages of the female individual.[8] In contrast, the mitotic spermatogonia proliferate throughout the postpubertal life of the male individual.[9] Oogonia ultimately enter meiosis as they differentiate into primary oocytes in the fetal female. The primary oocytes then arrest at an extended diplotene stage (dictyonema), which may last up to 50 years in human females. These "arrested" primary oocytes increase in size and perform various biosynthetic activities, but do not continue through meiosis until hormonal stimulation and maturation at sexual maturity; from this time, the oocytes are ovulated at intervals throughout the fertile life of the female mammal. The development of the oocyte is once again arrested at metaphase II, and it remains at this stage until fertilization stimulates resumption of the second meiotic division towards completion of oogenesis.[8] In males, spermatogonial proliferation and gametogenesis are continuous processes with the onset of puberty.[9] The cellular

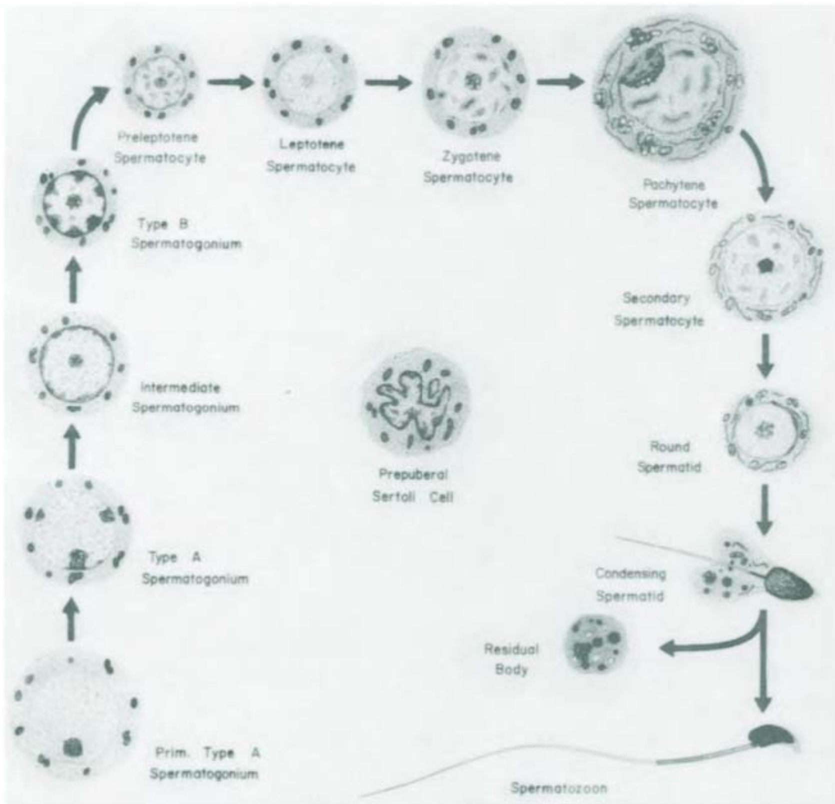
Table 1. Comparison of Oogenesis and Spermatogenesis in Mammals

	Oogenesis	*Spermatogenesis*
Location of meiotic cells	Ovarian cortex	Seminiferous tubules of testis
Premeiotic proliferation	During fetal stage	Continuous at sexual maturity
Time of onset of meiosis	Prenatal in most species[a]	Puberty
X-chromosome activity	Both active in XX females	Single X inactivated in XY males
Meiocyte Population	Finite[b]	Essentially unlimited
Meiotic arrests	Diplonema (dictyonema), metaphase II	None; continuous process from onset of meiosis
Postmeiotic differentiation	None	Spermiogenesis
Meiotic products from 1 primary meiocyte	1 ovum + 2-3 polar bodies	4 spermatozoa + 4 residual bodies
Fertile life of gamete	6-24 hours post-ovulation[c]	6-144 hours post-ejaculation[d]

Notes: [a] 2 months fetal development in humans; 12.5 days fetal development in laboratory mouse.
[b] 400-500 oocytes ovulated in humans.
[c] $\leq$ 24 hours in humans; 8-12 hours in laboratory mouse.
[d] 28-48 hours in humans; 6 hours in laboratory mouse.

outcomes of oogenesis and spermatogenesis are also different. Four haploid spermatozoa are ultimately formed from each primary spermatocyte during spermatogenesis. In contrast, only one haploid ovum is generated from each primary oocyte that completes oogenesis. Each meiotic division during oogenesis involves an asymmetrical cytoplasmic division, which yields a small haploid polar body (which may or may divide at meiosis II) and a larger haploid cell (which continues through meiosis and becomes the ovum following the second meiotic division).[8] This unequal apportionment of cytoplasm through both meiotic divisions ensures a large size and an abundant supply of nutrients and organelles for the developing ovum.

These sex-related differences in gametogenesis impart unique genetic, evolutionary, and clinical attributes and consequences to spermatogenesis. The rapid and continuous proliferation of the spermatogonia throughout the reproductive life of male mammals provides considerable opportunities for genetic mutations (from errors during DNA replication), chromosomal rearrangements, and aneuploidy-generating nondisjunctions. The amount of premeiotic germ-cell proliferation is considerably less in females, and consequently the male germline is expected to be the primary source of mutation and, ultimately, of genetic variation within populations and species.



Source: This figure was originally published in ref. 7, and was kindly provided by Dr. Anthony R. Bellvé.

Figure 1. Diagram of representative cellular stages of spermatogenesis in the laboratory mouse illustrating the relative size and characteristic morphology of each cell type. A Sertoli cell is shown at the center of the diagram. The ascending portion of this figure (primitive type A spermatogonium through the type B spermatogonium) represents the mitotic proliferation of spermatogonia in the juvenile and adult testis. The horizontal segment shows primary spermatocytes at representative meiotic stages preceding the first meiotic division in the adult testis; diplonema, diakinesis, and metaphase I cells are not shown (see description of these three stages in text). The descending part of the figure

Indeed, molecular data from primates indicate that the mutation rate in males is higher than that in females, thus supporting the hypothesis that DNA-sequence evolution is male-driven.[10] Additionally, genetic mutations or chromosomal rearrangements and aneuploidies in a spermatogonium can lead to a cluster of cells exhibiting the same mutant genotype or karyotype.[11] The effect would be particularly pronounced in males, as 512 sperm can be derived from a single spermatogenic cycle in some mammals.[12] Because of these proliferative characteristics of male germinal cells, genetic mutations, chromosomal rearrangements, and chromosomal aneuploidies occurring in primordial germ cells or spermatogonia can potentially enter the gametic pool as a cluster, and thereby enter the population at a higher initial frequency than would a unique mutation or rearrangement in a single sperm.[11] Because genetic mutations and chromosomal anomalies within premeiotic cells can produce germline mosaicism, these events can generate elevated aneuploidy levels or increased genetic or chromosomal mutation, and have considerable clinical implications in humans (see discussion below) and evolutionary implications in natural populations.[11]

III. CELL BIOLOGY OF SPERMATOGENESIS

The formation of male gametes and the secretion of various androgens, including testosterone, take place in the various compartments within the testis.[1,9] Among different mammals, the paired ovoid testes are known to occupy a variety of positions within or just outside the abdominal cavity.[9] When located outside the abdominal cavity, the testes reside in a pouch-like "external" scrotum in many mammalian species. These sexual organs are permanently confined to the scrotum in some mammalian species (e.g., cattle, canids, and higher primates), whereas in others (e.g., various rodents) the seasonal descent of the testes into the scrotum is coincident with the onset of breeding

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illustrates those stages of the second meiotic division and spermiogenesis (i.e., the secondary-spermatocyte stage through the ultimate formation of a spermatozoon and anucleate residual body. During spermatogenesis, a diploid primary spermatocyte progresses through the first meiotic division (reductional division) to produce two haploid secondary spermatocytes. The two secondary spermatocytes subsequently pass through the second meiotic division (equational division) of spermatogenesis, each producing two spermatids (the final haploid products of male meiosis). The four haploid spermatids derived from the "original" diploid primary spermatocyte further develop and differentiate during spermiogenesis, and ultimately become mature flagellated spermatozoa.

behavior. For those mammals with scrotal testes, the "evolutionary logic" of deploying an animal's reproductive capacity in a seemingly vulnerable location becomes evident in the temperature differences between the scrotum and the abdominal cavity. The temperature of a scrotal testis in these mammals is typically 4-7° C cooler than that of the body core; the spermatogenic process can be disrupted if the testicular temperature approaches that of the body.[9] This temperature sensitivity in mammals with scrotal testes is in part due to the lower temperature optimum of meiosis-specific recombinase activity.[13] In contrast, the testes are permanently located within the body cavities of a variety of mammals, including elephants, hyraxes, armadillos, bats, whales, and dolphins. For volant and aquatic mammals, in which minimizing drag is a consideration for efficient locomotion, the disadvantages that would be associated with scrotal testes are obvious.

A. Nongerminal Cells of the Testis

The internal structure of the mammalian testis is dominated by the highly coiled seminiferous tubules in which spermatogenesis actually occurs. Within and between these tubules are two nongerminal cell types, Leydig cells and Sertoli cells, which are particularly relevant to the stimulation, maintenance, and progression of spermatogenesis. The androgen-producing Leydig cells, which are distributed in the vascular connective tissue outside and among the seminiferous tubules, respond to luteinizing hormone (secreted by the anterior pituitary under stimulation by gonadotropin-releasing hormone from the hypothalamus) by producing and secreting the male gonadocorticoid, testosterone.[1,9] The nonmitotic Sertoli cells (also called sustentacular cells) are large, tall, narrow cells that rest upon the basement membrane of the avascular seminiferous tubule; their extensive cytoplasm ramifies throughout the germinal epithelium, thereby enveloping the spermatogenic cells from the basal lamina to the lumen of the seminiferous tubule.[9,14] The irregular shape of the Sertoli cells is very dynamic, as it constantly changes as the developing spermatogenic cells progressively move towards the lumen of the seminiferous tubule. The large Sertoli cells provide structural and metabolic support for the proliferating and developing spermatogenic cells, and phagocytize the cytoplasm shed from spermatids as they develop into spermatozoa. With the onset of puberty, the Sertoli cells participate in the formation of the blood-testis barrier, which biochemically isolates the contents of the seminiferous tubules from the remainder of the body. The cellular basis of this barrier is the continuous layer of Sertoli cells, which are bound to one another by tight junctions. This layer partitions the seminiferous tubule into the basal compartment (bounded by the tubule basal lamina and the first "set" of Sertoli-cell tight junctions), the intermediate compartment (a transitional compartment bounded on both sides by Sertoli-cell tight junctions), and the

adluminal compartment (the lumen proper, internal to tight junctions).[9,14,15] As spermatocytes move from the basal to the luminal portions of the seminiferous tubule, the tight junctions temporarily dissociate to allow passage of these developing cells. The intermediate compartment allows meiotic cells to migrate from the basal compartment to the adluminal compartment without compromising the integrity of the blood-testis barrier.[14] The blood-testis barrier excludes various hormones and antibodies from the internal microenvironment of the seminiferous tubules, and prevents the unique "foreign" proteins produced by the spermatogenic cells from provoking an autoimmune reaction.[9,15] In contrast to other hormones, testosterone traverses the blood-testis barrier easily, and apparently acts upon the Sertoli cells.[9] Follicle-stimulating hormone (also secreted by the anterior pituitary) stimulates the Sertoli cells to secrete androgen-binding protein, which facilitates binding and concentration of testosterone by spermatogenic cells.[15] Thus, testosterone and the gonadotropins (luteinizing hormone and follicle-stimulating hormone) are involved in the stimulation and maintenance of spermatogenesis in mammals.[1,9,15] Recently, it has been determined that the Sertoli cells produce a "steroidogenesis-stimulating protein" that, together with luteinizing hormone, regulates the production of androgens by the Leydig cells.[16]

B. Spermatogonia

The following discussion is a simplified chronology of the cell types and sequential progression from primordial germ cell of embryonic stages to primary spermatocyte at the onset of meiosis. Various workers and authors further subdivide the general spermatogonial cell types described herein, and the interested reader is referred to these more detailed treatments of the distinctions among these specific cell types (refs. 9, 17-19).

The germ-cell lineage produces progeny that ultimately form mature gametes. The initial events of this process occur early in the ontogeny of the male mammal. During embryogenesis, the primordial germ cells migrate to and colonize the genital ridges, which will ultimately differentiate into the testes.[17] Upon arrival at the genital ridges, these cells cease dividing and enter a period of proliferative quiescence, at which time they are known as gonocytes.[9] As puberty approaches, the gonocytes initiate proliferation, and differentiate into a population of spermatogonia that continuously divide in the adult mammal. Because the proliferating spermatogonia are located below the Sertoli cells (in direct contact with the basal lamina, and external to the tight junctions of the Sertoli cells), they are considered to reside within the basal compartment of the seminiferous tubule.

At the onset of puberty, there are several distinct types of spermatogonia present within the seminiferous tubule. Type A spermatogonia undergo repeated mitoses to form more spermatogonia; thus, these constitute the

renewing stem cells of germ-cell lineage. In addition to generating more type A spermatogonia, the divisions of the type A spermatogonia also give rise differentiating spermatogonia known as intermediate spermatogonia and type B spermatogonia. The type B spermatogonia are the cells that will further differentiate and enter meiosis as primary spermatocytes.

C. Meiosis

The various stages of meiosis are primarily defined by the morphology and behavior of the chromosomes. Accordingly, discussion of these stages is included in the cytogenetics section of this chapter, and they will not be considered further here.

The last division of type B spermatogonia provides preleptotene primary spermatocytes that enter the premeiotic S phase. From this point, the primary spermatocyte enters the relatively prolonged prophase stage in preparation for the first meiotic division. As the cells enter meiosis, they migrate away from the basal lamina and progress through a set of Sertoli-cell tight junctions to enter the intermediate compartment.[9,14] As meiosis progresses, the spermatocytes pass through a final array of tight junctions to enter the adluminal compartment, where meiosis is completed and spermiogenesis takes place.

The haploid spermatogenic cells undergoing the second meiotic division are known as secondary spermatocytes. These small cells are relatively scarce within the seminiferous tubule, as this meiotic phase is short in duration. The immediate endproducts of the second meiotic division are the haploid spermatids.

D. Spermiogenesis[9,15]

Spermiogenesis is the postmeiotic spermatogenic process by which the round haploid products of meiosis differentiate into the morphologically specialized mature spermatozoa. This process involves considerable morphological differentiation, including restructuring of the nucleus, ultracompaction of the haploid genome by protamines, formation of the protease-containing acrosome, and elaboration and elongation of a flagellum. These morphological modifications ultimately produce a streamlined cell designed for efficient delivery of the paternal genome to the egg.

Following the second meiotic division, the spermatid nucleus enters an interphase, and embarks upon its spectacular course of spermiogenic metamorphosis. The histones are displaced from the chromatin and replaced by the tightly binding protamines, and the nucleus and the paternal genome become highly compacted. The membrane-bound acrosome, which is derived from the Golgi apparatus, assumes a position at the anterior surface of the

nucleus. This cap-like structure contains hyaluronidase, proteases, and various other enzymes that will enable the spermatozoan to penetrate the zona pellucida of the ovum at fertilization. The two centrioles migrate to the posterior side of the nucleus, and one begins to elaborate the microtubular axoneme of the growing flagellum. The cytoplasm moves to the posterior portion of developing spermatid, thereby concentrating the mitochondria in a helical array at the base of the forming flagellum. This displacement of the cytoplasm and its organelles serves to concentrate the energy-producing mitochondria in the vicinity of the energy-consuming flagellum. The excess cytoplasm, which contains those organelles and materials not critical to sperm function, is shed as a residual body by the developing spermatozoon. The fully formed spermatozoa are then released into the lumen of the seminiferous tubule, from which they pass into the coiled epididymis. There, they are stored and undergo further maturation to become the highly motile transport vehicles for the paternal genome.

At fertilization, the spermatozoan contributes its nucleus and a centriole to the new zygote. All other cellular structures and organelles are provided by the ovum. Thus, it has been assumed that mitochondria, like other cellular organelles, are maternally inherited (cytoplasmic inheritance). However, by amplification of mitochondrial-DNA by the polymerase chain reaction (PCR), a small paternal contribution to the mitochondrial pool of the zygote has been demonstrated in laboratory mice.[20] This indicates that a few of the approximately 50 mitochondrial-DNA molecules contained within the sperm midpiece may be transferred to the zygote in each generation. Although the "leakage" of paternal mitochondrial-DNA molecules appears to be small (less than 4×10^{-5} per generation), it is nonetheless a possible mechanism for the generation of heteroplasmy, and its occurrence may explain those mitochondrial disorders exhibiting biparental inheritance.[20]

E. Intercellular Bridges of Spermatogenic Cells

During spermatogenesis, germ cells do not undergo complete cytokinesis during spermatogonial mitosis and meiotic divisions. Consequently, the progeny derived from the same spermatogonium are connected by cytoplasmic bridges, forming a syncytium.[12] Cells derived from same spermatogonium remain connected by cytoplasmic bridges, which persist until the latter stages of spermiogenesis. Extravesicular mRNAs and/or proteins are freely exchanged through these intercellular bridges, such that genetically haploid spermatids are phenotypically diploid.[21] This physical connection among haploid spermatogenic cells effectively allows differentiation to be directed and coordinated by a diploid genome. Because of the cytoplasmic connections and the sharing of gene products through them, postmeiotic gene expression does not result in genetic differences among haploid spermatids.

IV. INTRANUCLEAR STRUCTURES IN SPERMATOCYTES AND SPERMATIDS

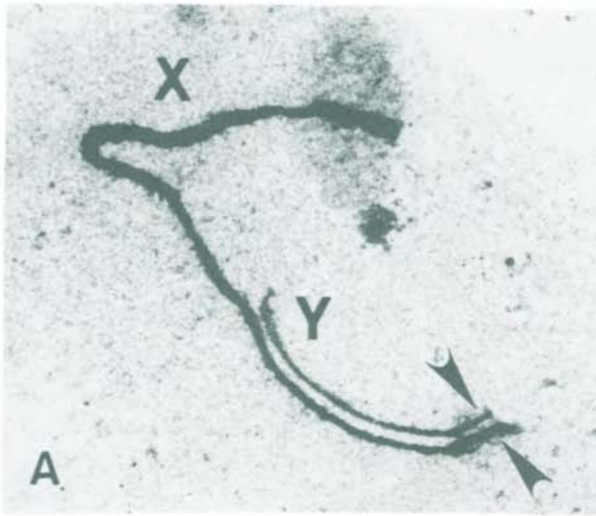
Spermatogenic cells exhibit several conspicuous intranuclear structures which are present at different stages of meiosis and spermiogenesis. The inclusion and discussion of these structures in this section (and chapter) are not intended to imply that they are unique to spermatogenesis; the nucleolus, for example, is present in most nucleated somatic and meiotic cells in both females and males. The presence of this readily discernible structure provides an obvious and convenient cytological marker for assessing meiotic and postmeiotic expression of rRNA genes during spermatogenesis. Additionally, the progressive morphological changes in the nucleoli have served as a useful means for substaging zygonema and pachynema in both spermatocytes[22–25] and oocytes.[26,27] Because these substage-specific physical changes in the development and ultimate dispersal of the nucleolus are directly comparable during prophase I of oogenesis and spermatogenesis, they also facilitate identification of equivalent zygotene and pachytene substages for comparisons of synaptic events between oocytes and spermatocytes.[26–28] Although the synaptonemal complex is present during prophase I of both oogenesis and spermatogenesis, its fundamental role in the meiotic process and its unparalleled utility in analyses of X-Y behavior clearly warrant its inclusion and discussion in this chapter. Recombination nodules are intimately associated with the synaptonemal complex, and their occurrence in phylogenetically diverse species and the intriguing possibility that they represent the cytological manifestation of a zygotene-pachytene "recombination apparatus" merit discussion herein. Of the intranuclear structures discussed in this section, only the sex body and the dense body are unique to male meiosis. Although a structurally (and functionally?) abnormal dense body is present in pachytene oocytes from sex-reversed XY females (Hale, unpublished observations), comparable intranuclear structures have not been observed in the oogenesis of normal XX females.

A. The Synaptonemal Complex

The synaptonemal complex is a ribbon-like proteinaceous structure that forms to the longitudinal axis of pachytene bivalents.[29,30] This structure is universally present in primary meiocytes of sexually reproducing eukaryotes, and is thought to stabilize chromosomal pairing and mediate genetic recombination.[29,31] Staining with silver or tungsten preferentially differentiates the proteinaceous chromosomal axes (= two lateral elements of the synaptonemal complex) from their associated chromatin and surrounding nucleoplasm, thereby providing simple linear representations of chromosomal behavior and orientation during zygonema and pachynema.[32,33] Transmission

electron microscopy of the synaptonemal complex provides for exquisite, high-resolution visualization of meiotic pairing configurations, and, accordingly, this analytical approach has gained considerable popularity among cytogeneticists over the last two decades.[22,32,34-40] Within a complete pachytene complement, a specific synaptonemal complex can often be identified (i.e., the identity of the corresponding autosomal bivalent can be ascertained) by its characteristic relative length, p/q arm-length ratios (if the kinetochores are visualized in appropriately stained preparations), "attached" nucleoli, or structural heteromorphism (due to heterozygosity for a chromosomal rearrangement or to differences in homolog length).

Synaptonemal-complex analyses have yielded considerable insight into the details and flexibility of the synaptic process, particularly in regards to the pairing behavior of structurally heterozygous autosomal bivalents and heteromorphic X-Y bivalents (Figs. 2A, 3, 4). For example, pachytene analyses of synapsis in laboratory mice heterozygous for paracentric inversions[35] and duplications[34] revealed that heterozygous pairing configurations (e.g., inversion loops, duplication "buckles") undergo "synaptic adjustment" to become fully synapsed, straight-paired bivalents with nonhomologous pairing within and adjacent to the rearranged or duplicated chromosomal segments. In deer mice heterozygous for naturally occurring pericentric inversions, however, the sizeable inverted segments proceed directly to nonhomologous synapsis (heterosynapsis) without an intervening phase of homologous pairing and reverse-loop formation during zygonema and pachynema.[36,42] Synaptonemal-complex analyses of sex-chromosome behavior have revealed that the pattern of synapsis and desynapsis of the heteromorphic X and Y chromosomes is asynchronous relative to that of the autosomes.[25,26,41] In the majority of mammalian species examined, the X and Y chromosomes initiate synapsis at late zygonema or early pachynema, and form a synaptonemal complex of variable length (e.g., Figs. 2A, 3, 4).[22-26,33,40,41,43-45] In contrast, the X and Y chromosomes of didelphid and dasyurid marsupials[46-48] and various cricetid rodents[38,46,49,50] remain univalent throughout pachynema (i.e., a synaptonemal complex is not formed between the X and Y chromosomes; however, the X and Y axes are typically associated with one another within the sex body). Synaptonemal-complex analyses have also provided new insight on the potential origin of aneuploidy in mammals (see discussion in the cytogenetics section). Examination of the large numbers of analyzable zygotene and pachytene nuclei typically generated in synaptonemal-complex preparations have revealed low but appreciable levels of numerical chromosomal abnormalities in karyotypically normal individuals. These observations indicate that aneuploid primary spermatocytes, and perhaps aneuploid offspring, can result from premeiotic nondisjunction events in spermatogonial lineages.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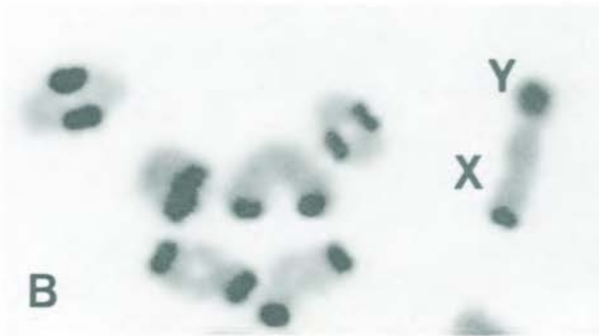
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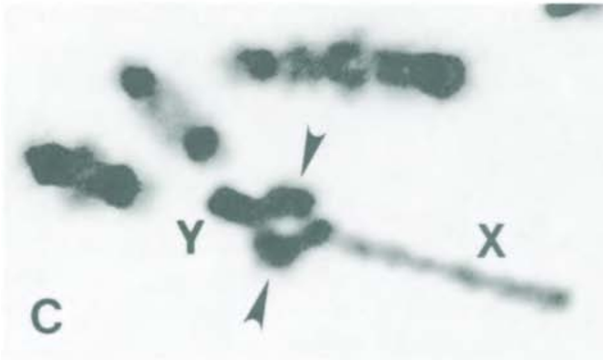
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Analyses of synaptonemal complexes have also provided insight into various enigmatic cytogenetic/genetic phenomena for which convincing explanations were previously unavailable. For example, mensural data from synaptonemal-complex analyses suggest an underlying cytogenetic basis for the gender-related length differences in the genetic maps of mammals. In humans,[51,52] laboratory mice,[53] and various marsupials,[54] recombination frequencies are higher in oogenesis than in spermatogenesis; consequently, the overall genetic map is longer in females than in males of these species. This sex-specific difference is especially striking in humans, as the overall genetic map of the autosomes is approximately 90% longer females than in males.[51] These recombination-frequency differences between females and males have been attributed to

Figure 2. **A.** Electron micrograph of a silver-stained X-Y bivalent in a surface-spread early-pachytene nucleus from a laboratory mouse (LT/Sv inbred strain). Surface spreading and silver staining of testicular material allow for visualization of the chromosomal axes and synaptonemal complexes. The densely stained X and Y axes form a segment of synaptonemal complex that includes 40% and 100% of the lengths of the X and Y axes, respectively. Although 80-100% of the Y chromosome is typically paired with the X chromosome at this substage of pachynema, homologous X-Y synapsis is restricted to the pseudoautosomal regions at the distal regions of the X and Y chromosomes (*arrowheads*) in laboratory mice. The darkly stained "cloud" surrounding the unsynapsed end of the X axis is the condensed centromeric heterochromatin of the X chromosome. **B.** Light micrograph of a C-banded X-Y bivalent and six autosomal bivalents at metaphase I from a laboratory mouse (C57BL/6J inbred strain). The long arms of the X and Y chromosomes exhibit a characteristic end-to-end association reflecting the terminal position and very small size of the X and Y pseudoautosomal regions. The chiasma formed between the distal (noncentromeric) ends of the X and Y chromosomes is too small to be resolved by light microscopy. An interstitially or terminally positioned chiasma is present within each of the autosomal bivalents. The Y chromosome and the centromeric heterochromatin of the X chromosome and of six autosomal pairs are darkly stained. **C.** Light micrograph of a C-banded X-Y bivalent and three autosomal bivalents at metaphase I from the deer mouse, *Peromyscus keeni* (Family Cricetidae). The X-Y chiasma (*arrowheads*) is clearly discernible at this stage, as the X and Y pseudoautosomal regions of this species are very large. The autosomal bivalents each exhibit an interstitially or terminally positioned chiasma. The heterochromatic short arm of the X chromosome (which comprises the pseudoautosomal region of the X chromosome), the totally heterochromatic Y chromosome, and the centromeric heterochromatin of three autosomal bivalents are darkly stained.

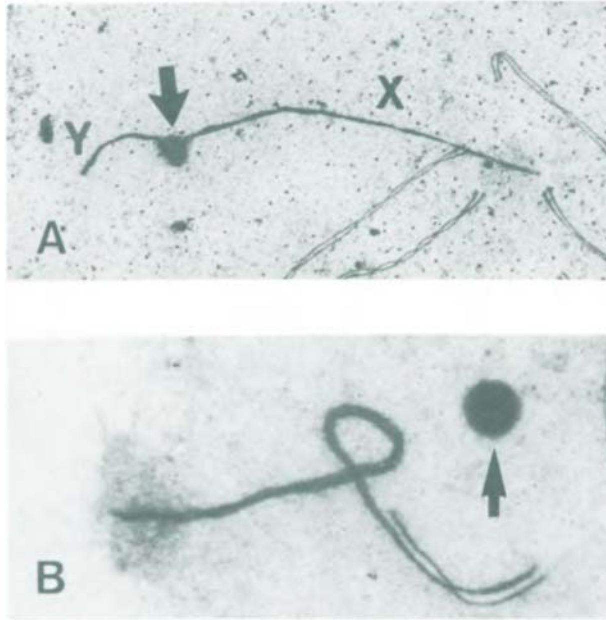

Figure 3. Electron micrographs of X-Y bivalents and associated dense bodies in silver-stained, surface-spread nuclei from male laboratory mice. **A.** X and Y chromosomes at synaptic initiation in a late-zygotene nucleus from a LT/Sv male. The darkly stained, ellipsoid dense body (*arrow*) is associated with the X and Y axes at their point of pairing initiation. In the laboratory mouse, X-Y synapsis commences at the distal (noncentromeric) ends of the acrocentric X and Y chromosomes (i.e., at their respective pseudoautosomal regions), and then progresses interstitially towards the proximal (centromeric) end of the Y chromosome.[45] **B.** X-Y bivalent in an early-pachytene nucleus from a C57BL/6J male. The Y axis is completely synapsed with the distal half of the X axis. The round, darkly stained dense body (*arrow*) is closely associated with the X-Y bivalent. Once X-Y synapsis is complete, the dense body disengages from the X and Y chromosomes, and typically remains proximate to the sex bivalent throughout the remainder of pachynema.[23,45]

homogamety/heterogamety,[55] to differential interference with or predisposition to crossing over due to DNA methylation differences in oogenesis and spermatogenesis,[56] or to physiological factors associated with the occurrence of female and male meiosis in completely different tissues.[51] Sex-related differences in chromosomal size at pachynema (when genetic recombination

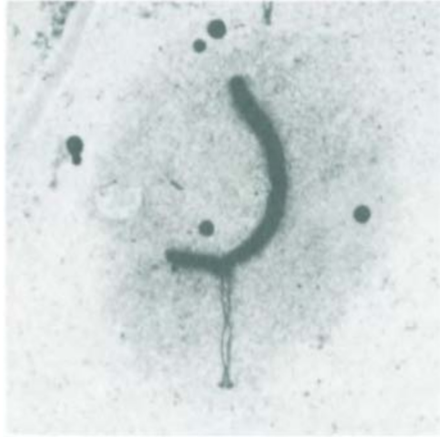

Figure 4. Electron micrograph of the sex body in a silver-stained pachytene nucleus from the deer mouse *Peromyscus keeni.* This densely stained structure is composed of the X and Y axes and their surrounding condensed chromatin.

between homologous chromosomes occurs) suggest an alternative and more convincing explanation for the expanded genetic map derived from female humans and laboratory mice. Measurements of synaptonemal complexes in human males and females reveal that the total-complement length in pachytene oocytes is nearly twice that in pachytene spermatocytes;[57] thus, more chromatin is in direct contact with the synaptonemal complex in female meiosis than in male meiosis. This gender-related length difference must result from differential chromatin condensation and distribution along the synaptonemal complex, as the small difference in female and male genome size would have a negligible effect on the respective total-complement lengths. If the synaptonemal complex does actually mediate genetic recombination between homologous chromosomes,[29] then the greatly increased "pairing surface" available for recombination in pachytene oocytes could account for the higher incidence of recombination observed in female meiosis relative to male meiosis in humans.[57] Similarly, the higher frequency of female recombination in laboratory mice[53] may be related to the longer total-complement length observed in pachytene oocytes (Hale, unpublished observations). Another gender-related meiotic phenomenon "explained" by the synaptonemal-complex approach is the male-biased F_1 sterility associated with Haldane's rule;[27,55] the cytogenetic basis for this biological generalization will be discussed in the section on obligate X-Y recombination and male-hybrid sterility.

Despite the intensive efforts to isolate and identify the components of the synaptonemal complex, the molecular composition of this fundamental structure and the details of its assembly remain relatively unknown. Monoclonal antibodies against isolated synaptonemal complexes from the rat identify various proteins as major or unique proteins of the chromosomal axes and lateral elements.[58-60] Another protein has been localized to the central region of the synaptonemal complex.[61] The localization of a microtubule-associated protein to the nuclear matrix and the lateral elements suggests that the synaptonemal complex may in part be organized from preexisting structures within the meiotic nucleus.[62]

B. Recombination Nodules

Recombination nodules are small, dense, spheroid or bar-shaped structures situated between the lateral elements of the synaptonemal complex in a variety of eukaryotic organisms. Each of these localized aggregations of proteinaceous material is thought to represent a multienzyme "recombination apparatus" within the central region of the synaptonemal complex.[29,31,63] There are two types of recombination nodules, which differ in their numbers per nucleus, the timing of their appearance during prophase I, and their distribution along zygotene and pachytene bivalents. "Early" recombination nodules appear upon the forming synaptonemal complexes at zygonema, whereas "late" recombination nodules are located upon fully formed synaptonemal complexes at mid pachynema. The early recombination nodules are randomly distributed among bivalents in approximately twice the numbers as the late recombination nodules.[29] While early recombination nodules are thought to mediate gene conversion,[63] only the late form of recombination nodule is considered to catalyze reciprocal genetic recombination across the 100 nm separating the synapsed homologous chromosomes.

The considerable indirect evidence that recombination nodules play a role in homologous genetic recombination is primarily based upon their shared attributes with chiasmata.[29,31,63] The total number of recombination nodules along synaptonemal complexes in pachytene nuclei closely corresponds to total number of chiasmata observed among bivalents in diakinesis/metaphase I cells. Additionally, the number of recombination nodules per chromosome, and their distribution along chromosomes, closely parallel the numbers and distribution of chiasmata. In mammals whose sex chromosomes exhibit a typical end-to-end association at metaphase I (e.g., humans and laboratory mice), the telomeric location of the recombination nodules within the X-Y synaptonemal complex is consistent with the terminal position and small size of the pseudoautosomal region.[64,65] The X-Y recombination nodule appears at more interstitial positions along the X-Y synaptonemal complex in those mammalian species exhibiting large pseudoautosomal regions and chiasmatic X-Y bivalents

(e.g., the Armenian hamster *Cricetulus migratorius*).[65] Recombination nodules and chiasmata both show positive interference, and they are absent from those synaptonemal-complex or chromosomal segments, respectively, that correspond to heterochromatic regions. Additionally, the distribution and numbers of recombination nodules mirror the abnormal distribution of crossovers and overall reduced recombination observed in various recombination-defective mutants of *Drosophila*.[66] Perhaps the best evidence for a recombinational role of recombination nodules is the demonstration of small amounts of pachytene DNA synthesis (the expected concomitant of the resolution of a crossover) in association with these structures.[67] While the combined cytogenetic and genetic data strongly implicate recombination nodules in effecting genetic recombination, the biochemical nature and actual function of these interesting structures remain to be elucidated.

C. The Dense Body

A darkly staining "dense body" has been observed in zygotene and pachytene spermatocytes from a variety of mammalian species, including representatives of the orders Rodentia, Primates, Artiodactyla, and Marsupialia.[23,24,33,41,45] In prophase I nuclei stained with silver nitrate or phosphotungstic acid, dense bodies appear as densely stained ellipsoid or spherical structures associated with the X and Y axes (Fig. 3). The dense body may be single, as in the laboratory mouse (Fig. 3), or double ("the double dense body"), as in the Chinese hamster *Cricetulus griseus*.[23,33,68] Cytochemical studies with various lipases and proteases suggest the presence of lipoproteins within the dense body,[68] and its argentophilic properties, stainability with acridine orange, and positive reactivity with "anti-nucleolus" antibodies indicate a nucleolar origin for this structure.[23,33] Since the early 1980s, the dense body has failed to capture or maintain the interest of biochemists, and consequently the cytochemical nature of this intriguing structure remains largely unknown.

Electron-microscopic analyses of surface-spread primary spermatocytes reveal that the dense body of the laboratory mouse and other mammalian species is associated with or proximate to the X and Y chromosomes during zygonema and pachynema (Fig. 3). In pachytene nuclei from the Eurasian harvest mouse (*Micromys minutus*), the dense body is typically associated with the centromeric regions of the axes of the submetacentric X and Y chromosomes. Because the synaptic-initiation sites of the harvest-mouse sex chromosomes are located in the vicinity of the centromeres, the characteristic association of the dense body with this region suggests a role in X-Y synaptic initiation.[41] In the laboratory mouse, pairing-initiation sites are located at the distal (noncentromeric) telomeres of the X and Y chromosomes, and electron-microscopic analysis similarly implicates the dense body in the initiation of X-Y synapsis (Fig. 3).[45] This hypothesis is indirectly supported by the apparent

association between irregular sex-chromosome behavior and dense-body morphology in primary oocytes from sex-reversed XY laboratory mice; the high incidence of zygotene and pachytene X-Y pairing failure is coincident with a structurally abnormal dense body (Hale, unpublished observations). Furthermore, dense bodies have not been reported in those mammalian species in which the X and Y chromosomes normally exhibit telosynaptic ("end-to-end") or asynaptic associations at pachynema. The occurrence of a sex chromosome-associated dense body in primary spermatocytes from such a wide variety of mammalian species suggests some fundamental role in male meiosis. The exact role of this conspicuous structure in X-Y synapsis, however, remains to be determined.

D. The Sex Body

The densely staining sex body (also called the "XY body" or "sex vesicle") is a nearly ubiquitous feature of pachytene spermatocyte nuclei in eutherian mammals.[65] The original designation "sex vesicle" is actually somewhat of a misnomer, as this intranuclear structure is not membrane-bound. The unique chromatin structure of the sex body is formed by condensation of the X and Y chromatin, which reflects the functional inactivation of the X and Y chromosomes during male meiosis.[69,70] The sex body typically occupies a position at the periphery of the pachytene nucleus, where it often appears as a densely stained protrusion or "bleb" on the surface of the nuclear envelope. In conventional Giemsa-stained testicular preparations, the sex body appears as a amorphous mass of homogeneously stained chromatin in which the individual X and Y chromosomes cannot be resolved. The higher resolution afforded by transmission electron microscopy of surface-spread, silver-stained preparations clearly reveals that the X and Y axes and their surrounding condensed chromatin comprise the densely stained sex body (Fig. 4). The majority of the mass of the sex body is contributed by the condensed chromatin of the larger X chromosome, and the interspecific variability in the size of the this intranuclear structure is attributable to variation in the sex-chromosome size.[65] In those mammals in which a synaptonemal complex is not formed between the X and Y chromosomes, the sex chromosomes remain separate, with their association being limited to variable telosynapsis or asynaptic coinclusion within the sex body.[46-48] The absence of a sex body in some sternodermatine bats[71] is not consistently associated with naturally occurring sex-chromosome-autosome translocations, as other studies of multiple sex-chromosome systems in mammals reveal the chromatin condensation consistent with sex-body formation.[44,65,72] The timing of the peripheral positioning and dense staining of the sex body is consistent among pachytene spermatocytes in a particular species, and, accordingly, these cytogenetic events serve as useful criteria for the substaging of pachynema.[22,25]

E. Nucleoli and Nucleolus-Organizer Activity

The nucleolus is the site of transcription and processing of rRNA and assembly of ribosomal subunits (proteins and rRNAs) within the nucleus.[73,74] Although nucleoli are not membrane-bound structures, they each appear as a fairly coherent, darkly stained "cloud" when silver-stained and viewed by light or electron microscopy.[22,33,74,75] The nucleoli of somatic and meiotic cells are characteristically associated with two or more chromosomes at the nucleolus-organizer regions (NORs), which contain clusters of tandemly repeated rRNA genes (each encoding a 45S rRNA primary transcript).[73] At each NOR, the DNA containing the rRNA genes extends into the nucleolus in large loops. Because the size of the nucleoli is related to the transcriptional and translational activity of the cell,[73] they tend to be large in interphase-prophase spermatogonia and prediplotene spermatocytes and small in the relatively quiescent spermatids.[76] The nonmitotic Sertoli cells typically display one or two large nucleoli.[76]

As mentioned in the introduction to this section, the characteristic changes in NOR activity and nucleolar morphology during spermatogenesis have served as useful cytological markers for discerning and delimiting the various substages of zygonema and pachynema in a variety of mammalian species.[22-25] Because of their dense staining and frequent large size in silver-stained testicular material, the nucleoli are the most readily visualized intranuclear structures at these stages of prophase I. At the onset of zygonema, the nucleoli appear as small, extremely dense clouds in contact with the axes of the NOR-bearing chromosomes. The nucleoli enlarge throughout zygonema, and ultimately assume a "comet-shape" with their dense "heads" in contact with the chromosomal axes. As zygonema progresses into pachynema, the number of nucleoli appears to decrease due to homologous synapsis, which brings active NORs at homologous chromosomal positions into apposition,[76] and to nucleolar associations among NOR-bearing bivalents, which result in coalescence of the nucleoli from two or more bivalents.[33] In the laboratory mouse, synapsis is often delayed or disrupted in the vicinity of the NORs;[23] the relative decondensation of the NOR chromatin may preclude proper alignment and synapsis of this chromosomal segment. The NORs remain active through early pachynema, at which time the transcription of rDNA genes ceases and the nucleoli "detach" from the axes (= lateral elements of the synaptonemal complexes) of the NOR-bearing chromosomes. The nucleolar material becomes progressively dispersed throughout the remainder of pachynema, and imparts an increased granularity to the nucleoplasm in late-pachytene and diplotene spermatocytes).[22,75]

With the cessation of rRNA transcription during pachynema, the NORs remain inactive throughout the remainder of meiosis. Postmeiotic reactivation of the NORs briefly occurs at the early round spermatid stage. There is no

detectable NOR activity in the nuclei of elongation-phase spermatids or in the nuclei of mature spermatozoa.[76]

Nucleolus-organizer regions located on the sex chromosomes often do not conform to the general activity patterns exhibited by nonpseudoautosomal genes in somatic cells and during spermatogenesis. For example, the X chromosome of the gray short-tailed opossum (*Monodelphis domestica*) exhibits a NOR, and, as expected, the NOR of the single X chromosome is transcriptionally active in all somatic cells examined for males. However, this X-linked NOR apparently escapes somatic X-inactivation and dosage compensation, as it is transcriptionally active in 98.3% of the fibroblasts analyzed from females.[48] During spermatogenesis in this species, the transcriptional quiescence of the X-linked NOR throughout prophase I[48] is consistent with the inactive state of the single X chromosome.[69,70] In contrast to X-chromosome NORs, Y-linked NORs are transcriptionally active despite the general inactivity and condensation of the Y chromosome during male meiosis.[69,70] The Y-linked NORs of the domestic dog (*Canis familiaris*)[75] and the Mediterranean mole rat (*Spalax ehrenbergi*) (Hale, unpublished observations) clearly remain transcriptionally active into prophase I, as the axes of their Y chromosomes each display a large "attached" nucleolus in silver-stained pachytene nuclei (Fig. 5). Male mole voles (*Ellobius talpinus*) also possess an active NOR on one of their sex chromosomes. However, it is not cytologically apparent which of the sex chromosomes bears the NOR in this

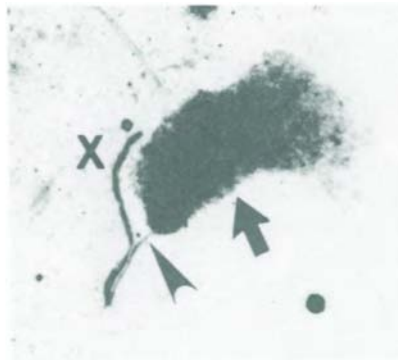

Figure 5. Electron micrograph of a X-Y bivalent in an early-pachytene nucleus from a Mediterranean mole rat, *Spalax ehrenbergi* (Family Spalacidae). A large, densely stained nucleolus (*arrow*) is associated with the unpaired end of the Y-chromosome axis. The Y-associated nucleolus-organizer region is located within the small segment of the Y chromosome (*arrowhead*) that does not synapse with the X chromosome during pachynema.

rodent, as the X and Y chromosomes exhibit equal lengths and similar G-banding patterns in metaphase preparations.[77] In silver-stained pachytene nuclei from male mole voles, a densely stained nucleolus is clearly associated with one of the equal-sized axes of the X-Y bivalent.[43] Presumably, this nucleolus is associated with the Y chromosome of the mole vole, as X-linked NORs appear to be subject to X-inactivation during male meiosis.[48] The presence of sex chromosome-associated NORs and their differential expression in primary spermatocytes and female somatic cells poses some interesting questions relative to sex-related dosage and transcriptional regulation of nucleolar components in mammals.

V. CYTOGENETICS OF SPERMATOGENESIS

Because of the remarkable behavior of chromosomes during meiosis, the cytogenetic analysis of the events of spermatogenesis is particularly rewarding and aesthetically pleasing endeavor. Anyone who has had the pleasure of microscopically examining the intricacies of chromosomal pairing can appreciate the beauty of heterozygous synaptic configurations and delicate chiasmata in meiotic cells. The fidelity of homologous chromosomal synapsis provides the striking reciprocal-translocation quadrivalents and graceful inversion loops in some chromosomally heterozygous mammals,[34,40,46,78-80] while the flexibility of the pairing and recombination mechanisms averts the adverse fertility effects of such configurations in others.[28,36,42] Through the specialized reductional division, the meiotic mechanism is impressively efficient at equally and precisely distributing a haploid chromosomal complement to each of two daughter cells. While the subsequent second meiotic division exhibits various mechanistic similarities to mitotic divisions, this process is nonetheless unique among the equational divisions occurring among mammalian cells.

A. Sertoli Cells

Because Sertoli cells are nonmitotic *in vivo*, their cytogenetics is rather mundane compared with that of the other cells residing within the testes. However, within the adult testis of humans and laboratory mice, the Sertoli cells and zygotene spermatocytes are unique in exhibiting decondensed Y-chromosome segments.[81,82] In contrast, the Y chromosome of prepubertal Sertoli cells and nonzygotene meiotic cells is highly condensed. The Y-chromosome decondensation observed in human zygotene nuclei may be related to X-Y synapsis (and subsequent recombination at pachynema), which typically commences at this stage (see discussion below). In Sertoli cells, the decondensed state of portions of the Y chromosome suggests that these regions

are transcriptionally active in the adult testis. Such genetic activity would implicate these nongerminal cells in controlling some aspects of spermatogenesis.[82]

B. Chromosomal Behavior during Spermatogenesis[15,22,30,83]

From a cytogenetic perspective, the most critical stage of spermatogenesis is prophase I, in which synapsis and recombination occur. By ensuring the physical connection (i.e., chiasmata) between homologous chromosomes at metaphase I, synapsis and recombination during prophase I facilitate the reductional division and the ultimate production of haploid gametes. Additionally, genetic recombination between homologous chromosomes at pachynema and independent assortment of maternal and paternal chromosomes at anaphase I allows for production of an astonishing diversity of genotypes among offspring.[3,4] The following discussion of the cytogenetics of spermatogenesis will necessarily and appropriately concentrate on this stage of meiosis. Description of the second meiotic division will primarily serve to highlight the differences between this division and mitotic divisions of somatic and spermatogonial cells.

As the a spermatogenic cell makes the transition from spermatogonium to preleptotene primary spermatocyte, it enters a prolonged premeiotic interphase.[30] This stage involves checkpoints at which the cell evaluates its readiness for meiosis. The regulatory factor responsible for the initiation of meiosis is unknown.[18,30]

The first stage of meiosis, prophase I, is traditionally divided into five consecutive substages defined by various progressive morphological changes in the chromosomes (i.e., condensation, degree of synapsis or desynapsis, chiasma formation and terminalization).[22,25] Throughout most of prophase I, the ends of the meiotic chromosomes remain attached to the inner surface of the nuclear envelope. At leptonema, the chromosomes condense and become visible as thin threads; although the DNA has been replicated, the sister chromatids are tightly bound to one another, and consequently the duplicated chromosomes appear as single threads. When appropriately stained with silver or tungsten, one can see that each chromosome (i.e., sister-chromatid pair) condenses around a proteinaceous axial element.[22,29] The initiation of homologous chromosomal pairing defines the onset of zygonema. Autosomal synapsis is typically initiated at one set of telomeres, and then progresses in a unidirectional ("zipperlike") fashion towards the opposite end of the forming bivalent. Synapsis of the heteromorphic sex chromosomes is characteristically delayed relative to that of the autosomes).[23,25,41,45] Although the mechanism of homologous pairing is unknown, the synaptic process is apparently mediated and stabilized by the proteinaceous synaptonemal complex[29] (see previous discussion under intranuclear structures). The completion of autosomal

synapsis and bivalent (synaptonemal complex) formation defines the beginning of pachynema; in some mammals, the X and Y chromosomes do not commence pairing until pachynema,[26] and in many species the sex chromosomes begin to desynapse during this substage.[23,45,64] In conventionally stained preparations, it is now evident that each chromosome is composed of two chromatids. If homologous pairing is complete, structural [chromosomal] heterozygosity will be apparent as heterozygous synaptic configurations such as inversion loops, fusion-fission trivalents, translocation quadrivalents, and duplication buckles.[34,40,43,78-80,84] Because genetic recombination occurs when the chromosomes are homologously aligned, this is perhaps the most critical stage of meiosis. At diplonema, the homologous chromosomes initiate desynapsis. As the chromosomes separate along most of their lengths, they remain attached at one or more chiasmata (= the cytological manifestations of crossing over). The final substage of prophase I is diakinesis. During this substage, the chromosomes further condense, and detach from the inner surface of the nuclear envelope. The chiasmata continue to maintain the association between homologous chromosomes. The disintegration of the nuclear membrane defines the end of diakinesis and prophase I.

At metaphase I, the bivalents align at the equatorial region of the dividing primary spermatocyte. Here, one function of chiasmata becomes apparent: they maintain the bivalent associations until this stage, and thereby ensure proper poleward orientation of the chromosomes for the first meiotic division.[85] Univalent chromosomes (i.e., those not chiasmatically associated with a homolog) are prone to nondisjunction at anaphase I, as they will segregate randomly relative to their similarly univalent homolog (if present). It is interesting to speculate that recombination and chiasma formation first evolved as a means of maintaining bivalents through metaphase I, and that the promoting of genetic diversity[4] was a fortuitous secondary effect of recombination.

The homologous chromosomes separate at anaphase I, and move towards opposite poles of the dividing cell. Completed poleward migration and chromosomal decondensation characterizes the telophase I, which is the final stage of the first meiotic division.

The cytogenetic events of the second meiotic division superficially resemble those of the mitotic divisions of somatic cells and spermatogonia. In both types of equational divisions, condensed chromosomes composed of two chromatids ("dyads") congress at the equatorial region of the cell, centromeres divide, and sister chromatids segregate and become incorporated into different daughter nuclei. However, various cytogenetic and genetic aspects of this equational meiotic division depart from those typical of mitotic divisions. Of course, haploid cells enter the second meiotic division and diploid cells undergo mitosis. An S phase precedes the onset of mitosis, while spermatocytes essentially progress directly from telophase I to prophase II; thus, there is no duplication

of chromosomal material prior to the second meiotic division. Unless mutation has occurred during DNA replication, the sister chromatids in post-S-phase mitotic cells are genetically identical, and the daughter cells ultimately produced are likewise genetically identical (to one another and to the original parental cell). In secondary spermatocytes, the "sister" chromatids (i.e., chromatids attached at the same centromere) are genetically dissimilar as a result of recombination at pachynema. Thus, the daughter cells of the second meiotic division are not genetically identical.

The names of the major stages of the relatively short second meiotic division parallel those of the first meiotic division. However, the respective behavior of the chromosomes in each of these changes is considerably different. During the short prophase II, the chromosomes typically recondense, and the nuclear membrane breaks down. The single chromosomes (each composed of two chromatids) align at the equator at metaphase II. At anaphase II, the centromeres divide and the "sister" chromatids migrate to opposite poles. The chromosomes reach the poles at telophase II, and the nuclear membrane reforms around the decondensing chromosomes.

Postmeiotic chromosomal phenomena are described in the following section.

C. Chromosomal Modification during Spermatogenesis

As spermatogonia, spermatocytes, and spermatids pass through spermatogenesis, their chromosomal DNA may be structurally and chemically modified. Consequently, the chromosomes present in mature spermatozoa are not structurally and biochemically identical to the chromosomes present in somatic cells of the same individual. In addition to minor heritable alterations in chromosomal structure (telomere elongation), these modifications affect the transcriptional activity of the sex chromosomes during spermatogenesis (X inactivation) and of specific paternally inherited genetic loci in offspring (genomic imprinting).

Elongation of Telomeres

Telomeres define the ends of the linear chromosomes and are essential for normal chromosome function in eukaryotic organisms. Because DNA polymerase cannot replicate the 3′ ends of each DNA strand, the repetitive sequences comprising each telomere undergo progressive shortening with increasing age and/or number of cell divisions during the life of the individual.[86] Counteracting this progressive loss of telomeric sequences requires the activity of the enzyme telomerase (a ribonucleoprotein reverse transcriptase), which catalyzes the 5′-3′ addition of TTAGGG repeats to the telomeres in mammals and other vertebrates.[86] Telomerase is apparently synthesized and biochemically active during mammalian spermatogenesis, as the chromosomes

of human spermatozoa have considerably longer telomeres than the chromosomes of somatic cells from the same individual.[86–88] Germline-limited telomerase activity (which has yet to be demonstrated in gonadal cells from mammals) thus serves to provide the sperm chromosomes with "full-length" telomeres for the next generation. Presumably, telomeres are similarly elongated during oogenesis so that, at syngamy, the zygote can begin development with a diploid complement of chromosomes possessing telomeres of sufficient length.

X-inactivation in Spermatogenic Cells

In therian mammals, inactivation of one X chromosome in somatic cells of normal XX females is a well-documented phenomenon. The transcriptionally inactive X chromosome typically appears heterochromatic or positively heteropycnotic in metaphase preparations from somatic cells and mitotic oogonia. At the onset of prophase I of oogenesis, the inactive X chromosome is reactivated, and transcriptional activity of the formerly inactive X chromosome resumes (Table 1). In normal XY males, the single X chromosome is transcriptionally active in all somatic, Sertoli, and spermatogonial cells. However, the single X chromosome is apparently inactivated as a spermatogenic cell enters meiosis, as this chromosome (and the Y chromosome) are transcriptionally quiescent by the time a spermatocyte reaches pachynema.[70,89,90] The inactivation of the X chromosome in primary spermatocytes is considered necessary for the normal progression of spermatogenesis in heterogametic males;[91] interference with X-chromosome inactivation during this process is thought to result in primary-spermatocyte loss and infertility in mammals.[27,28,84,92,93] Inactivation of the X chromosome in spermatogenic cells is apparently mediated by the *Xist* gene (*XIST* in humans),[94,95] which exhibits testis-specific expression in adult males. The expression of some spermatogenesis-specific genes, such as *A1s9Y-1* and *Pgk-2*, is thought to biochemically compensate for the transcriptional inactivity of their respective X-linked counterparts.[96–99]

Genomic Imprinting

Genomic imprinting refers to the phenomenon in which the expression of alleles at the same gene is dependent on whether they were maternally or paternally inherited.[56] The chromatin is evidently modified in germline cells, resulting in differential genetic expression in subsequent offspring. Often, expression of the gene inherited from one parent is completely repressed, while the gene derived from the other parent is transcriptionally active. The molecular imprint placed upon a specific chromosomal region during gametogenesis is not permanent; this genomic modification can be erased and reestablished

within the gametogenic lineage of the next generation.[56] Although DNA methylation seems to play a role in genomic imprinting,[100] the exact mechanism for parental-germline modification and differential expression of maternally and paternally inherited genes in offspring remains unknown. The specific spermatogenic stage at which the imprinting occurs is also unknown.

Histone Replacement by Protamines

In mammalian spermatozoa, the histone component of the chromatin is replaced by highly positively charged protamines, which tightly bind and compact the DNA.[9,101,102] These small testis-specific proteins are synthesized during spermiogenesis, as the histones are being displaced during nuclear condensation. The high compaction of the protamine-bound genome contributes to the overall streamlined nature of the mature spermatozoan.

D. The Pseudoautosomal Region of Mammalian Sex Chromosomes

During zygonema and pachynema, homologous chromosomes synapse and recombine with one another. Cytologically, the crossovers which occur between homologous chromosomes are manifested as chiasmata within bivalents at diplonema, diakinesis, and metaphase I. Thus, analysis of chiasma position at these post-pachytene stages (usually, at diakinesis and metaphase I) allows assessment of the extent and distribution of crossing over that occurred during zygonema and pachynema. In the absence of structural heterozygosity or heterochromatic blocks, which would suppress recombination within certain chromosomal segments,[36,42] autosomal bivalents typically exhibit one or more chiasmata along their lengths (e.g., Fig. 2B, C). Among diakinesis and metaphase I cells, the chiasmata within any particular autosomal bivalent usually appear to be randomly distributed (i.e., these chiasmata may be interstitially or terminally positioned within the bivalent). The X and Y chromosomes of many mammals (including the human and the laboratory mouse), however, often exhibit a characteristic end-to-end association at diakinesis and metaphase I (e.g., Fig. 2B).[44,45] This seemingly "achiasmatic" association reflects the small size and terminal position of homologous region (the "pseudoautosomal region") of the X and Y chromosomes.

The pseudoautosomal region is the segment of genetic homology and recombination between the X and Y chromosomes. The designation "pseudoautosomal" reflects the autosomal (rather than strictly sex-linked) inheritance pattern exhibited by sequences and loci within this unique sex-chromosomal segment.[103] The pseudoautosomal region actually constitutes an additional linkage group (i.e., a 22nd linkage group in the laboratory mouse, and a 25th linkage group in humans) in the chromosomal complement, as its genes are technically not X- or Y-linked. Because all X-Y recombination is

restricted to the typically small pseudoautosomal region, this region is a recombinational hotspot in male meiosis. [In female meiosis, recombination can potentially occur over the entire length of the X-X bivalent; presumably, the frequency of pseudoautosomal recombination in females is proportional to the relative length of the X pseudoautosomal region within the overall length of the X chromosome.] In humans, the frequency of pseudoautosomal recombination is 10-to-20 times higher in male meiosis than in female meiosis.[104,105] Similarly, genetic and molecular analyses in laboratory mice indicate that the frequency of pseudoautosomal recombination is seven times higher in males than in females.[106] The pseudoautosomal region in humans is very small; physical mapping of large pseudoautosomal restriction fragments by pulse-field gel electrophoresis indicates that this chromosomal segment is only 2.6 Mbp in size.[105] The pseudoautosomal region of the laboratory mouse is presumably somewhat larger, as the ratio of pseudoautosomal recombination frequency in male meiosis to that in female meiosis (~7) is less striking than that observed in humans (10-20).[104,106] Additionally, the occurrence two-strand double crossovers in the pseudoautosomal region of the laboratory mouse[106] and only single crossovers in that of humans suggests that the former is larger than the latter. Because normal X-Y recombination is restricted to these small pseudoautosomal regions during spermatogenesis, these chromosomal segments constitute the most highly recombinogenic regions in the genomes of these two species.

The pseudoautosomal regions of mammals exhibit considerable variability in overall structure (i.e., size, euchromatin/heterochromatin), location (p arm or q arm, or both, of the sex chromosomes), and genetic content. The small pseudoautosomal region of the laboratory mouse is terminally positioned on the long arm of the acrocentric X and Y chromosomes.[45] In humans, there are apparently two distinct pseudoautosomal regions positioned at opposite ends of the submetacentric X and Y chromosomes[107] (the Xp-Yp pseudoautosomal region is the one for which the size of 2.6 Mbp was determined[105]). The sex chromosomes of the mole vole may also possess two separate pseudoautosomal regions (one at each end of the X and Y chromosomes), as a small segment of synaptonemal complex is typically formed at each end of the X-Y bivalent during pachynema.[43] The only genetic locus mapped to the pseudoautosomal region of the laboratory mouse is steroid sulfatase (*Sts*);[108] in humans, however, this locus (*STS*) is clearly X-linked.[109] A variety of genetic loci, including *MIC2* (encoding a cell-surface antigen defined by the monoclonal antibody 12E,[110,111] *CSF2RA* (encoding the a chain of the granulocyte-macrophage colony-stimulating factor receptor),[112,113] and *ANT3* (encoding an ADP/ATP translocase),[114] have been mapped to the human pseudoautosomal region. The genetic homologs of some of these human pseudoautosomal genes (*CSF2RA* and *ANT3*) are autosomal in the laboratory mouse.[115] Interestingly, pseudoautosomal genes and some X-linked

genes proximate to the pseudoautosomal region apparently escape X inactivation in somatic cells of normal X-X females.[109,114,116] Sex-chromosome evolution in the hare wallaby *Lagorchestes conspicillatus*,[47] various gerbils,[117] and some species of stenodermatine bats[71] has involved the recruitment of new or additional pseudoautosomal chromatin onto the X and Y chromosomes by means of translocations involving the X and Y chromosomes and both members of an autosomal pair. Following these sex chromosome-autosome translocation events, the formerly autosomal segments (and all of their resident genes) of such "neo-XY" sex-chromosome systems technically comprise the pseudoautosomal regions and exhibit pseudoautosomal inheritance. Replication banding of somatic chromosomes from females of these gerbil and bat species indicates that the pseudoautosomal segment of the allocyclic "neo-X" is not subject to X-inactivation.[117,118] In deer mice[26,28] and European harvest mice,[41] the respective pseudoautosomal regions consist of the heterochromatic short arm of the submetacentric X chromosome and a corresponding arm of the totally heterochromatic Y chromosome; presumably, these heterochromatic (and length-variable) pseudoautosomal segments are devoid of functional genes in these species. Although molecular and cytological studies have documented X-Y homology and recombination in a wide variety of mammalian species,[26,41,44,45] it should be noted that the presence of a pseudoautosomal region is not a ubiquitous feature of mammalian sex chromosomes. The X and Y chromosomes of most marsupials[46-48] and various cricetid rodents[38,46,49,50] depart from the more typical mammalian pattern, as these sex chromosomes do not possess pseudoautosomal segments; the cytogenetic mechanism ensuring proper anaphase I segregation of the asynaptic and achiasmatic X and Y chromosomes in these species is not known.

In addition to serving as the genomic residence of various genetic loci, the pseudoautosomal region plays an important role in the cytogenetic behavior of the X and Y chromosomes during prophase I, metaphase I, and anaphase I. At zygonema and pachynema, the presence of structurally homozygous X and Y pseudoautosomal regions is critical for the proper initiation and subsequent progression of X-Y synapsis. Deletion of the pseudoautosomal region of the X chromosome is associated with sterility and the complete failure of X-Y pairing in humans;[119,120] presumably, loss of the Y pseudoautosomal region (or both the X *and* Y pseudoautosomal regions) would have a similar adverse effect on male fertility and sex-chromosome synapsis. Additionally, structural heterozygosity within the pseudoautosomal regions (due to Y-chromosome rearrangements or to length differences in the pseudoautosomal segments) can disrupt proper pairing alignment and preclude normal synaptic initiation, thereby generating substantial X-Y univalency at pachynema.[28,45,121] Because of the relatively small size of the pseudoautosomal region in laboratory mice and their wild relatives (i.e., *Mus musculus, M. domesticus, M. castaneus, M. spretus*), genetic heterozygosity within this segment (generated by

interstrain and interspecific crosses) can hinder X-Y synapsis in F_1 males;[27,93] when the X and Y chromosomes do manage to pair in such hybrid individuals, pseudoautosomal heterozygosity effectively suppresses X-Y recombination in a large proportion of pachytene cells.[27,122] Normally, crossing over and chiasma formation within homologously aligned pseudoautosomal regions maintain the X-Y bivalent throughout the remainder of prophase I and metaphase I, thereby ensuring the proper poleward orientation of the sex chromosomes during metaphase I and their proper reductional segregation at anaphase I.[85] The failure of pseudoautosomal recombination results in the premature dissociation of the sex bivalent during diplonema and the consequent production of X and Y univalents at diakinesis and metaphase I. While nonrecombinant X and Y univalents would seemingly be prone to missegregation at anaphase I,[28,85] their presence is often associated with spermatocyte loss prior to or soon after metaphase I (see the following discussion of obligate X-Y recombination).

Compared to its critical cytogenetic role in male meiosis, the pseudoautosomal region would seem to be of no special consequence to the events and progression of female meiosis. The normal fertility of human females lacking one Xp pseudoautosomal region[119] suggests that X-X pairing is not adversely affected by structural heterozygosity within the telomeric region of the X short arm. Furthermore, the initiation of X-X synapsis can presumably occur at either end of the X chromosomes, so the X pseudoautosomal region would not necessarily be involved in homolog recognition and presynaptic alignment in all mammalian species. However, the two X chromosomes of female deer mice (*Peromyscus maniculatus*) exhibit a pattern of asynchronous synapsis (relative to the autosomes) and interstitial synaptic initiation that is very similar to that of the X and Y chromosomes during male meiosis.[26] Moreover, the presence of a medium-sized late-synapsing bivalent in zygotene and pachytene oocytes from other mammals[57,123] suggests that asynchronous sex-chromosome pairing may be a common feature of both male and female meiosis).[26] Because X-X synapsis may be initiated at the nonpseudoautosomal telomeres in many mammals, it is not apparent whether the meiotic pairing asynchrony [if this is indeed an attribute of the X-X bivalent] is related to the pseudoautosomal regions of the X chromosomes or to some other property of the sex chromosomes in female meiosis. The pseudoautosomal region is not necessarily involved in X-X recombination and chiasma formation, as crossing over can occur anywhere along the length of the X-X bivalent. Indeed, the greatly expanded genetic distances of the pseudoautosomal region in male meiosis relative to female meiosis[104-106] indicate that pseudoautosomal recombination seldom occurs within the X-X bivalent. Recombination and chiasma formation at any point along the length of the X-X bivalent fulfill the function of maintaining the bivalent and ensuring its proper orientation within the spindle apparatus at metaphase I. Thus, pseudoautosomal recombination is not necessary for proper anaphase I segregation or oocyte survival.

E. The Pseudoautosomal Region, X-Y Synapsis, and Obligate X-Y Recombination

In addition to the cytogenetic role of the pseudoautosomal region in X-Y synapsis, crossing over, chiasma formation, and proper anaphase I segregation during spermatogenesis, the meiotic behavior of this sex-chromosomal segment during prophase I has important implications for the survival of primary spermatocytes during meiosis. Homologous synapsis and recombination between the X and Y chromosomes during pachynema typically yield a chiasmatic X-Y association that is maintained until the onset of anaphase I. The failure of X-Y pairing and/or recombination, which results in sex-chromosome univalency in pachytene and diakinesis/metaphase I cells,[27,45,124] is often coincident with primary-spermatocyte loss, reduced testis weight, low sperm count, and infertility in a variety of mammals.[27,28,45,120,125–128]

The relationship between X-Y univalency and primary-spermatocyte death has been considered in the context of two partially conflicting hypotheses for the sex chromosome-related requirement for a spermatocyte to successfully complete meiosis and generate functional spermatozoa. The "pairing hypothesis"[128–130] states that homologous synapsis of the X and Y pseudoautosomal regions is sufficient for the normal progression of male meiosis. In contrast, the "obligate recombination hypothesis"[45,124] predicts that homologous X-Y synapsis *and* pseudoautosomal recombination are necessary for a spermatocyte to successfully progress beyond the first meiotic division. In practice, it can be difficult to distinguish between these two alternative hypotheses. Because homologous synapsis necessarily precedes recombination, much of the data supporting the obligate X-Y recombination hypothesis are also consistent with X-Y pairing hypothesis. However, chromosomal synapsis does not necessarily commit the homologous chromosomes to recombination. An elevated incidence of X-Y univalency at diakinesis/metaphase I relative to pachynema is typical of normal male meiosis.

The available cytogenetic data from humans and laboratory mice are most consistent with the operation of two meiotic "quality-control" mechanisms that monitor different events during prophase I during spermatogenesis. Thus, elements of both the pairing and obligate recombination hypotheses are relevant to the progression of male meiosis. The first mechanism ensures that proper X-Y synapsis has occurred, and thereby acts as a first level of quality control to identify and eliminate pachytene spermatocytes containing X and Y univalents. The existence of this cellular selection mechanism is consistent with the apparent prediplotene loss of primary spermatocytes with sex-chromosome univalents in humans[119,120] and laboratory mice.[27,45,131] While proper X-Y synapsis allows a spermatocyte to progress to metaphase I, pairing alone is apparently not sufficient to ensure successful progression through meiosis. According to the obligate X-Y recombination hypothesis,[45,124] homologous X-

Y synapsis and crossing over are necessary for spermatocyte survival and the normal progression of male meiosis. This hypothesis is derived from and supported by cytogenetic and inheritance data from male laboratory mice carrying the *Sxr* (*sex-reversed*)[126,127] and Y* rearrangements[45,132] of the Y chromosome. The Sxr rearrangement involves the duplication and subsequent terminal transposition of the pericentromeric portion of the Y short arm (including the testis-determining region) to the distal end of the pseudoautosomal region.[127,133,134] The Y* rearrangement involves the translocation of a functional centromere from an unidentified chromosome (the X chromosome?) to a terminal position adjacent to the Y pseudoautosomal region).[132] Thus, the pseudoautosomal region of the rearranged Y* chromosome occupies an interstitial position adjacent to the translocated functional centromere. The consequent structural heterozygosity within the pseudoautosomal segments of the Y^{Sxr}[121] and Y* chromosomes[45] results in considerable synaptic failure of the sex chromosomes in XY^{Sxr} and XY* males. In these male mice, pairing and recombinational failure of the sex chromosomes at pachynema resulted in very high levels of sex-chromosome univalency at diakinesis and metaphase I. Additionally, the stainability of Y-derived chromatin (in G- or C-banded testicular preparations) and the pronounced chromatid asymmetry generated by crossing over within X-Y^{Sxr} and X-Y* bivalents provided unambiguous cytological markers for assessing the recombinational status of sex chromosomes at diakinesis, metaphase I, and metaphase II. In both XY^{Sxr}[45,127,135] and XY* males[45] cytogenetic analyses conclusively revealed that: (1) all sex-chromosome bivalents at diakinesis and metaphase I were chiasmatic; (2) all sex-chromosome univalents at diakinesis and metaphase I were nonrecombinant (i.e., sex-chromosome univalents were the consequence of recombinational failure at pachynema); and (3) recombinant sex chromosomes were present in all metaphase II complements. Clearly, those metaphase I spermatocytes containing nonrecombinant sex-chromosome univalents failed to progress to the secondary-spermatocyte stage in these male mice. Furthermore, the presence of equal numbers of recombinant and nonrecombinant paternally derived sex chromosomes among offspring of XY^{Sxr}[126] and XY* males[132] indicated that functional spermatozoa originated only from those primary spermatocytes in which the sex chromosomes had recombined. These cytogenetic and inheritance data are therefore consistent with the obligate X-Y recombinant hypothesis, as they indicate that only those spermatocytes in which the sex chromosomes have synapsed and recombined can successfully complete meiosis and ultimately give rise to functional spermatozoa. Thus, while X-Y synapsis alone may assure spermatocyte viability to metaphase I, recombination between the X and Y chromosomes is a prerequisite for the normal progression of spermatogenesis beyond the first-meiotic division. The physiological/biochemical basis for the pre-meiosis II loss of spermatocytes with asynapsed and/or nonrecombinant sex chromosomes is not known.

The X and Y chromosomes of metatherian mammals (marsupials) do not conform to either the pairing hypothesis or the obligate recombination hypothesis, as these species display a characteristic lack of X-Y synapsis and synaptonemal-complex formation during prophase I.[46–48] Comparison of the morphology and genetic structure of the sex chromosomes in monotremes, marsupials, and placental mammals indicates that the fully differentiated metatherian X and Y chromosomes represent an evolutionarily derived condition for mammals.[136] For marsupials, therefore, the characteristic pattern of X-Y univalency at prophase I and metaphase I is apparently the consequence of reduction or absence of a functional pairing (i.e., pseudoautosomal) region on the sex chromosomes.[47] Males of these species do not exhibit any of the adverse fertility effects associated with total sex-chromosome univalency in laboratory mice[125,128] and humans.[119,120] Similarly, some cricetid rodents typically exhibit X-Y univalency at pachynema and diakinesis/metaphase I without experiencing diminished fertility.[38,46,49,50] The exceptional asynaptic patterns displayed by the X and Y chromosomes of these rodents presumably involve the secondary loss of pairing initiation sites and pseudoautosomal segments on one or both of the sex chromosomes [in human males, deletion of the X-chromosome pseudoautosomal region results in complete X-Y synaptic failure at pachynema[119,120]]. The marsupials and aforementioned cricetid rodents have evidently evolved a means of averting the spermatocyte loss and reduced fertility usually associated with the failure of X-Y pairing and recombination in eutherian mammals, perhaps by elimination or relaxation of the meiotic quality-control mechanisms monitoring sex-chromosome pairing[129] and recombination.[124] Although autologous synapsis ("self pairing") of sex-chromosome univalents apparently fulfills pairing requirements in female meiosis,[86] this type of X and Y synapsis has not been reported for marsupials or those placental mammals that normally exhibit X-Y univalency at pachynema.

F. X-Y Recombination, XY-Autosome Associations, and Male-Hybrid Sterility

"Haldane's rule" is the well-documented biological generalization that the heterogametic sex is sterile, rare, or absent among the F_1 offspring of hybridizing animal taxa.[55] Among mammals, the male-specific hybrid sterility consistent with Haldane's rule[27,84,122,137] has been ascribed to a variety of physiological and genetic factors. Several putative hybrid-sterility genes have been mapped in laboratory mice,[92,138] including one locus (*Hst-3*) localized within the distal half of the X chromosome.[139] Observations from F_1 hybrids between BALB/c laboratory mice and inbred wild-derived mice (*Mus domesticus*) suggest that interaction of hybrid-sterility genes reduces the steroidogenic capability of Leydig cells, thereby causing sterility by hindering

the progression of spermatocytes beyond pachynema.[140] While degeneration of prophase I spermatocytes and absence of spermatids and spermatozoa seem to comprise a common pathology in hybrid sterility in many crosses,[27,122,140-142] abnormal sperm morphology has been implicated in others.[140] Despite the numerous examples of male-hybrid sterility in mammalian crosses, the underlying genetic basis or bases for this phenomenon are not known.

In the context of obligate X-Y recombination, the elevated incidence of metaphase I X-Y univalency apparent in male-hybrid meioses[27,28,93,122,142-144] suggests that the failure of X-Y synapsis and recombination may play a role in the male-limited sterility consistent with Haldane's rule. High levels of X-Y univalency are correlated with the sterility of F_1 and first-backcross mice of the C57BL/6 X *Mus spretus* intercross; in these infertile individuals, meiosis typically does not progress beyond the first meiotic division.[27,122] Subspecific and specific hybrids of deer mice (genus *Peromyscus*) also exhibit relatively high levels of X-Y univalency (~40%), yet these males still generate sufficient quantities of sperm for normal fertility. Interestingly, despite frequencies of 40% X-Y univalency at metaphase I, all metaphase II cells from these hybrids contained a single sex chromosome.[28] Only the loss of those primary spermatocytes with univalent [nonrecombinant] sex chromosomes can satisfactorily explain these consistently euploid metaphase II complements. Similarly, cytogenetic observations from other mammals reveal that metaphase I cells with X and Y univalents do not progress beyond the first meiotic division.[120,145,146] Low levels of X-Y univalency (5-20%) are typical of diakinesis and metaphase I stages in nonhybrid males;[27,28,45,146] presumably, this univalency is the consequence of pairing and recombinational failure between the small pseudoautosomal regions of various mammalian species.[45] In hybrid individuals, however, the failure of synapsis and recombination between the sex chromosomes and the consequent high incidence of X-Y univalency are correlated with genetic and/or structural differences between pseudoautosomal regions derived from different subspecies or species.[27,28]

The nonrandom contact or association of unpaired autosomal segments with the normally asynapsed portion of the X chromosome is frequently observed in pachytene cells in which normal autosomal synapsis has been disrupted by heterozygous pairing configurations)[40,84,90] or hybridization.[27,28,39,93] As discussed previously, inactivation of the X chromosome in primary spermatocytes is considered to be essential for the normal progression of spermatogenesis in heterogametic males.[91] The pachytene association of unpaired autosomal segments with the X chromosome is thought to reactivate the inactive X chromosome or interfere with the process of X inactivation, thereby disrupting the unfortunate cell's progression through spermatogenesis.[27,92] A reactivation of the genetically quiescent X chromosome during pachynema is consistent with the observation of high levels of X-linked gene products in primary spermatocytes of sterile laboratory mice exhibiting

pachytene X-autosome associations.[147] It has been suggested that the products of X-linked genes are somehow inhibitory or nonpermissive for spermatogenesis,[92] and, accordingly, the spermatocyte loss and spermatogenic impairment in mammals with chromosomal anomalies have been attributed to X-autosome associations and the consequent disruption of normal X inactivation.[27,92] Alternatively, the contact of autosomal segments with the inactive X chromosome could result in the inactivation of the autosomal genes, thereby causing metabolic problems and spermatogenic breakdown.[90] Despite numerous examples of autosomal asynapsis and X-autosome associations in mammals, there is no conclusive evidence for reactivation of the inactive chromosome.[89,90] However, there is some evidence suggesting that genetic inactivation does spread into autosomal segments associated with the sex chromosomes.[89,90] Although the underlying physiological or genetic basis for this type of spermatogenic disruption is unknown, pachytene X-autosome associations are clearly correlated with male infertility in humans and laboratory mice.[27,89,90,93]

While fixed differences for euchromatic autosomal rearrangements are often thought to facilitate the divergence leading to speciation,[148] the potential role of the X and Y chromosomes in generating hybrid sterility and effecting reproductive isolation between mammalian populations is generally not considered.[27] Clearly, the spermatocyte loss associated with the failure of X-Y pairing and recombination can reduce the reproductive fitness of F_1 males, and thereby effect or at least reinforce reproductive isolation between hybridizing mammalian taxa. Because the fertility-reducing cytogenetic irregularities associated with X and Y chromosomes are necessarily limited to hybrid males, these sex-chromosome phenomena can generate the gender-specific F_1 sterility consistent with Haldane's rule in mammals. The manifestation of Haldane's rule represents the initial stage in the evolution of postzygotic reproduction isolation,[149] and consequently the genetic or cytogenetic components underlying this biological phenomenon can facilitate the divergence leading to speciation. The regular occurrence of X-autosome associations and the failure of X-Y synapsis and recombination in a variety of mammalian hybrids[27,28,93,122,143,144] suggests that sex chromosome-related phenomena have a significant impact on the dynamics and ultimate outcomes of many mammalian hybridizations.

High levels of pachytene and diakinesis/metaphase I X-Y univalency are not consistently associated with male-hybrid sterility in mammals, and thus the failure of X-Y pairing and recombination is not a ubiquitous feature of male-biased F_1 sterility in mammalian crosses. In F_1 males of the white-tailed deer (*Odocoileus virginianus*) X mule deer (*O. hemionus*) cross, normal X-Y bivalents are present in pachytene and diakinesis/metaphase I cells;[150] the gender-biased sterility observed in this cross is apparently attributable to the production of morphologically abnormal spermatozoa.[137] Additionally, the

possible role of genetic incompatibilities other than the X-Y-related irregularities in F_1 and first-backcross male sterility cannot be excluded in the C57BL16 *Mus spretus* cross described earlier in this section. The existence of sterile first-backcross males with small testes and a low incidence of prophase I and metaphase I X-Y univalency[27,122] suggests additional genetic components to hybrid sterility in this interspecific cross. Perhaps autosomal and X-linked loci involved in spermatogenesis, when derived from C57BL16, function improperly in conjunction with a Y chromosome derived from phylogenetically distant species *Mus spretus*.

G. Generation of Aneuploid Gametes during Spermatogenesis

The majority of human aneuploidies is attributed to nondisjunction of homologous chromosomes at anaphase I or of sister chromatids at anaphase II. The combined application of cytogenetic and molecular approaches to studies of monosomy and trisomy indicate that most human aneuploidies are of maternal origin, and that most of these maternal nondisjunction events occur at the first meiotic division.[151-154] With the exception of X-Y nondisjunctions[155,156] (occurring at the first meiotic division to produce hyperhaploid sperm containing a X and Y chromosome) and Y-chromatid nondisjunctions (occurring at the second meiotic division to produce hyperhaploid sperm with two Y chromosomes), only a small proportion of trisomic offspring are the consequence of nondisjunctions during paternal meiosis. The paternal origin of a substantial proportion of sex-chromosome aneuploidies, as indicated by analysis of cytogenetic and molecular markers in XXY and XYY offspring[155,156] is corroborated by karyotypic analyses of haploid complements from human spermatozoa (chromosomes obtained from the male pronucleus following human sperm-hamster egg cross-species fertilizations) and secondary oocytes. These analyses reveal that sex-chromosome aneuploidy frequently occurs in spermatozoa, but that X-chromosome aneuploidy is relatively rare in oocytes.[157] In addition to meiotic segregational errors, postzygotic nondisjunction (at early zygotic stages) is a recognized source of aneuploid offspring.[153-155] However, the possibility that premeiotic nondisjunctions in can produce aneuploid meiocytes, gametes, and offspring is generally not considered.

The high resolution provided by electron microscopy of synaptonemal-complex preparations has allowed for the detection of small numbers of trisomic primary spermatocytes in a variety of mammals, including laboratory mice,[80] deer mice (Fig. 6),[26,158] domestic cattle,[39,159] domestic sheep,[40] domestic horses,[159] domestic goats,[159] blue foxes (*Alopex lagopus*),[79] and the common vole (*Microtus arvalis*).[38] These aneuploidies were clearly the result of nondisjunctions in spermatogonia, as the extra chromosomes observed in primary spermatocytes were not present in the somatic karyotypes of

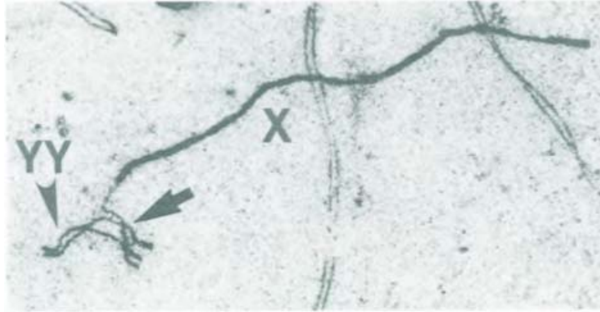

Figure 6. Electron micrograph of a X-Y-Y trivalent in a silver-stained, surface-spread early-pachytene nucleus from a XY brown deer mouse, *Peromyscus megalops* (Family Cricetidae). The axes of the two Y chromosomes have synapsed to form a short segment of synaptonemal complex (*arrowhead*). Pairing-partner exchange among the X axis and the two Y axes has occurred within the segments that would normally synapse in X-Y bivalents from this species (*arrow*). Although both Y axes participate in pairing with the X axis, synapsis is limited to two axes within any particular segment of this trivalent configuration. As in normal X-Y bivalents, the unpaired (differential) portion of the X axis is densely stained. The occurrence of hyperploid primary spermatocytes in a karyotypically normal individual indicates that these cells are the consequence of a mitotic nondisjunction event in a spermatogonium.

individuals reported in these studies. The occurrence of these trisomies is potentially of some consequence, as the incidence of aneuploid primary spermatocytes exceeds 0.5% in some of these species);[26,38,40,79,159] in humans, the incidence of potentially viable trisomies among clinically recognized pregnancies ranges from 0.04% (for XYY) to 0.45% (for trisomy 21).[151] While first- and second-division segregational errors are clearly implicated in the generation of human aneuploidy (and probably account for the majority of human aneuploidies), the occurrence of aneuploid primary spermatocytes in mammals suggests the possibility that some aneuploid offspring may be the consequence of premeiotic nondisjunction events.

The production of trisomic spermatogonia from segregational errors during mitotic proliferation would generate variable levels of euploid/aneuploid mosaicism within the germinal epithelium of the testis. In males, the descendants of these aneuploid spermatogonia would continually enter meiosis as trisomic primary spermatocytes, which could ultimately join the gametic pool as groups of trisomic spermatozoa.[11] The successful completion of spermatogenesis by trisomic cells derived from euploid/aneuploid mosaicism

in the germline perhaps accounts for reports[160,161] of recurrent trisomic offspring originating from a single individual.

While the trisomic cellular products of spermatogonial nondisjunctions are clearly capable of surviving within the testis and entering meiosis, the fate of the reciprocal monosomic products is unknown. Presumably, the adverse genetic effects of autosomal monosomy would result in the eventual loss of a monosomic spermatogonium from the spermatogenic cell lineage. Although XO and XYY spermatogonia are the expected reciprocal products of premeiotic Y-chromosome nondisjunction, XO primary spermatocytes were not been detected in conjunction with the XYY primary spermatocytes (Fig. 6) observed in two species of deer mice.[26,158] XO spermatogonia are generally absent from the testes of XO/XYY[162] and XO/XY/XYY[131,163] mosaic laboratory mice, and XO primary spermatocytes are not produced. Such XO cells are apparently incompatible with the testicular environment,[162,163] and consequently they do not thrive or survive within the testis. Not surprisingly, XO oogonia are clearly not similarly constrained in XX females, as a XO primary oocyte has been recovered from a fetal laboratory mouse.[164]

H. Spontaneous Chromosomal Rearrangements

In addition to premeiotic nondisjunction events, various chromosomal rearrangements also occur in spermatogonia. These spontaneous rearrangements are manifested as heterozygous synaptic configurations (i.e., translocation quadrivalents, inversion loops, Robertsonian trivalents) in zygotene and pachytene spermatocytes. Spontaneous reciprocal translocations have been detected in nearly 1% of zygotene and pachytene spermatocytes from two species of deer mice.[78] In various cattle, heterozygous pairing configurations consistent with spontaneous inversions were evident in nearly 0.3% of the zygotene and pachytene nuclei examined.[39] Spontaneous translocations have been detected in humans,[37] and centric fusions and X-autosome translocations have been recovered from laboratory mice (Hale, unpublished observations). It is interesting to note that the spontaneous centric fusions observed in pachytene spermatocytes of laboratory mice are the very type of chromosomal rearrangements that appear spontaneously in laboratory stocks and that characterize chromosomal variability and evolution in wild house mice (*Mus musculus*).[84] The genetic implications and potential of these premeiotic rearrangements are similar to those of viable spermatogonial nondisjunctions.[11] Lineages of chromosomally heterozygous spermatogonia will be generated, and, if their descendants enter meiosis, these cells ultimately have the potential to affect the chromosomal constitution of spermatozoa and subsequent offspring.

VI. GENETICS OF SPERMATOGENESIS

The genetics of spermatogenesis has been the subject of several excellent and comprehensive reviews.[19,101,102,165] While many genes undoubtedly encode proteins or RNAs specific to spermatogenic stages (e.g., genes encoding the protein components of the synaptonemal complex), the expression of other genes has pleiotropic effects on both somatic and spermatogenic cells (e.g., *sks*[166] and *Paf*[167]). Indeed, mutations of the majority of these pleiotropic genes have primary phenotypic effects other than sterility.[101,165] In this section, I present a temporal classification scheme for genes expressed during the different stages of spermatogenesis,[99] and then list and briefly describe various structural and catalytic proteins and several recently reported genes (or mutations) that affect spermatogenesis or that effect spermatogenesis-specific processes. This discussion includes enzymes specific and/or important to spermatogenic stages and processes (e.g., telomerase), even though the genes encoding their protein or RNA components have yet to be identified. The reader is referred to previous reviews of the genetics of spermatogenesis[19,101,102,165] for more comprehensive treatment of this topic and for more extensive listings of loci affecting the spermatogenic process.

Genes that are differentially expressed during spermatogenesis genes have been divided into three general classes on the basis of stage of expression.[99] The first class includes genes that are already active or that commence activity in spermatogonia (and, often, are also expressed in somatic cells). An example of this class is the X-linked gene encoding phosphoglycerate kinase-1 (*Pgk-1*), which is expressed in spermatogonia as well as somatic cells. Transcriptional cessation of this locus appears to be a concomitant of X-chromosome inactivation with the onset of meiosis.[96,99,168] The second class of differentially expressed spermatogenesis genes includes those loci transcribed in meiotic cells, but not premeiotic spermatogonia. Such genes are not expressed in somatic or oogenic cells. The autosomal gene *Pgk-2* (phosphoglycerate kinase-2) is typical of this class. Transcription of this gene commences with the onset of meiosis, and continues through the spermatid stage.[96,99,] *Pgk-2* expression was not detected in spermatogonia. Genes initiating transcription during spermiogenic stages comprise the third class of differentially expressed spermatogenesis genes. Various protamines are examples of this class.[102] Because of their "on-and-off" pattern of transcription during spermatogenesis, the rRNA genes residing within the NORs conform to the expression criteria defining all three of these gene classes.

A. Proteins of Spermatogenesis

Numerous proteins are variously synthesized and deployed throughout the spermatogenic stages (including mature functional spermatozoa). These

proteins (which are not necessarily spermatogenesis-specific) fulfill a variety of structural, cytogenetic, and catalytic functions, such as structurally modifying the chromosomes, mediating chromosomal behavior (i.e., synapsis, congression, segregation), effecting genetic recombination, and facilitating sperm-egg fusion and fertilization.

A variety of proteins are known to be unique to spermatogenic cells. Isozymes, isotypes, or splice variants of many somatically expressed proteins, such as lactate dehydrogenase,[19] actin,[102] angiotensin-converting enzyme,[169] cytochrome c,[170] and the cystic fibrosis transmembrane regulator[171] are specific to various spermatogenic cell types. In many mammals, prophase I cells possess recombinases exhibiting different temperature optima than recombinases present in somatic cells.[13] Other proteins, such as skeletal proteins involved in the nuclear architecture of spermatocytes[172] and the DNA-binding protamines that displace the histones during spermiogenesis,[19] are completely unique to spermatogenesis (i.e., they have no isozymic or isotypic counterparts in somatic cells). Telomerase is apparently synthesized and biochemically active only during spermatogenesis, as the chromosomes of human spermatozoa exhibit considerably longer telomeres than the chromosomes of somatic cells from the same individual.[86-88] The genes encoding the protein and RNA components of this enzyme have not been identified or mapped in any mammal. Other proteins, by virtue of their activity upon chromatin, effect critical functions in both somatic and meiotic cells. An example of such a protein is the DNA-binding enzyme topoisomerase II, which introduces transient double-strand DNA breaks and apparently functions in chromosome condensation and segregation. Topoisomerase II is associated with the chromatin and synaptonemal complexes (lateral elements) of pachytene and diplotene chromosomes.[173] In addition to topoisomerase II, a variety of novel synaptonemal-complex proteins have been identified using polyclonal antibodies from patients with autoimmune diseases[174] or monoclonal antibodies from isolated synaptonemal complexes. Immunofluorescence microscopy reveals that these proteins are localized to the central element or lateral elements of the synaptonemal complex.[58-61] While the majority of proteins comprising the central element and lateral elements of the synaptonemal complex are assembled from components synthesized during leptonema and zygonema,[58-61] others are apparently derived from preexisting nuclear matrix components present in both somatic and meiotic cells.[62]

B. Genes Expressed during Spermatogenesis

In this section, I have attempted to limit the discussion to those loci not included in these previous publications and to those for which new insights or information is available. Many of these loci have been characterized at the molecular level (e.g., *PGK-2*), while others (e.g., *sks* and *Paf*) are defined on

the basis of mutations affecting male fertility. Not listed in this section are unnamed genes whose existence is inferred from spermatogenic abnormalities in laboratory mice lacking portions of the Y chromosome. An example is a "spermiogenesis gene" mapping to the long arm of the mouse Y chromosome; this gene (or genes) is apparently required for normal development of the sperm head.[128] The locus designations in upper-case letters refer to human genes, while those with lower-case letters denote loci or mutations in the laboratory mouse.

Spy (Spermatogenesis gene, Y chromosome)[175,176]

This uncharacterized locus, which maps to short arm of mouse Y chromosome, is apparently required for proliferation of type A spermatogonia.

A1s9Y-1 [97] (= Sby [98])

This testis-specific gene maps to the short arm of the laboratory mouse Y chromosome. Its chromosomal location, testis-specific expression, homology to X-linked ubiquitin-activating enzyme E1, and loss in the deletion mutant *Sxr*^{b} suggest this gene as a candidate for the spermatogenesis gene *Spy*. Expression of *A1s9Y-1* (*Sby*) during spermatogenesis may compensate for the cessation of the X-linked *A1s9X* (ubiquitin-activating enzyme E1) expression at prophase I.

AZF (azoospermia factor)[177]

This Y-linked gene controls spermatogenic development. Molecular analysis of candidate *AZF* sequences suggests a protein product containing a RNA recognition motif; thus, *AZF* may function in RNA processing or translational regulation. Expression of the candidate gene is restricted to adult testis. Cytogenetic data indicate the possibility of expression in Sertoli cells, and suggest that this gene might play a role in the contraction and expansion of the Y chromosome of zygotene nuclei, thereby affecting synapsis and recombination.[82] Southern-blot analysis using the human clone as a probe reveals hybridization patterns consistent with a single-copy Y-linked gene in several mammalian species.

sks (skeletal fusions with sterility)[166]

In addition to various skeletal defects, this autosomal (Chr 4) recessive mutation in the laboratory mouse causes spermatogenic arrest at pachynema. It is also associated with disruption of autosomal pairing and lack of X-Y pairing throughout pachynema; these synaptic anomalies may represent a secondary defect of the mutation. For the laboratory mouse, *sks* is the first

identified single-gene mutation that causes meiotic arrest during spermatogenesis (other mutations affect postmeiotic stages). This mutation also causes female sterility when homozygous.

Paf (patchy fur)[167]

This semi-dominant X-linked mutation causes an abnormal coat in laboratory mice. Additionally, this mutation, or a closely linked chromosomal abnormality (e.g., a small inversion), interferes with normal disjunction of the X and Y chromosomes at the first meiotic division. Hemizygous male exhibit high levels of X-Y nondisjunction at anaphase I, and consequently they produce a high frequency of XO and XXY offspring. *Paf* has no apparent meiotic effects in female mice. *Paf* maps to the distal end of the X chromosome (near the pseudoautosomal region).

Xist/XIST (X-inactive specific transcripts)[94,95,178]

This X-linked gene is a strong candidate for the regulator of X-chromosome inactivation in both XX females and XY males.[179] In XX females, this gene is transcribed from the inactive X chromosome in somatic cells. Transcription of this gene in XX females is discontinued at the time of X-chromosome reactivation (i.e., meiotic reactivation of the X chromosome that is inactive in oogonia) during oogenesis. In adult XY males, *Xist/XIST* expression is limited to spermatogonia, primary spermatocytes, and spermatids in testes exhibiting normal spermatogenic profiles. *Xist/XIST* transcripts are not detectable in cultured Sertoli cells or various male somatic tissues, or in testicular tissue lacking germ cells. The cessation of *Xist/XIST* transcription in postmeiotic spermatids is correlated with the general transcriptional quiescence of spermiogenic cells.[94] The available data are consistent with the role of *Xist/XIST* in X-chromosome inactivation in male meiosis.

Pgk-2/PGK-2 (phosphoglycerate kinase-2)[96,99,168]

As mentioned previously, *Pgk-2* transcripts are first detected in preleptotene spermatocytes, indicating that initiation of transcription coincides with the onset of meiosis and with the cessation of X-linked *Pgk-1* expression. The level of *Pgk-2/PGK-2* transcription increases as spermatogenesis progresses, and continues throughout the meiotic and spermiogenic stages; transcripts are detectable in spermatids and residual bodies. In humans, the autosomal *PGK-2* locus lacks introns and exhibits the characteristics of a processed gene. This locus evidently originated from reverse transcription of processed phosphoglycerate kinase mRNA and the subsequent integration of double-stranded DNA product at an autosomal location. Spermatogenesis-specific

expression of this autosomal *Pgk* locus may have evolved to compensate for the inactivation of the X-linked *PGK-1* locus at prophase I (phosphoglycerate kinase is necessary for enabling mature spermatozoa to utilize the fructose encountered in secretions of the male and female reproductive tracts[96]).

Zfp-35 (zinc -finger protein 35)[180]

This gene, which is selectively expressed in pachytene spermatocytes, encodes a zinc-finger protein in the laboratory mouse. The zinc-finger motif is known to have a variety of regulatory functions in development and differentiation, and thus *Zfp-35* presumably regulates genetic activity during pachynema. Its specific expression at this meiotic stage suggests potential roles in synaptonemal-complex formation, recombination, or the reductional division.

Sxa (sex-chromosome association)[181]

The high levels of X-Y univalency observed in F_1 hybrids between wild-derived *Mus musculus* subspecies and standard inbred strains of laboratory mice, together with the observation that the frequency of X-Y univalency decreased with repeated backcrossing, led to the hypothesis that autosomal genes were controlling X-Y synapsis.[181,182] This putative gene, *Sxa*, was subsequently mapped to the distal region of the mouse X chromosome.[181] However, the segregation data[27,122] obtained from backcrossing within the C57BL/6 X *Mus spretus* cross indicate that *Sxa* actually represents the entire pseudoautosomal region, rather than being a distinct genetic locus).[27] As discussed in the section on obligate X-Y recombination, the "effect" of pairing and recombinational failure within the X and Y pseudoautosomal is apparent at or prior to the first meiotic division.

VII. SUMMARY AND PROSPECTS

From this decidedly brief overview, it is immediately apparent that mammalian spermatogenesis is an exquisite example of cellular differentiation and specialization. This complex and fascinating process involves the progression of diploid cells through two unique nuclear divisions to form genetically diverse haploid cells, which then undergo further differentiation to become the only flagellated cells within the body of an adult mammal. Spermatogenesis involves unique cellular divisions, proteins, chromosomal behavior, quality-control mechanisms, and morphological specializations. Through this chapter, I hope to have provided the interested reader with sufficient background information and citations to pursue and explore this subject in greater detail.

Many questions pertaining to mammalian spermatogenesis remain to be addressed and answered. What are the genetic bases and mechanisms for the regulation of the spermatogenic process? Is the genetic totipotency of the germ cell line maintained from the primordial germ-cell stage, or is it reestablished during spermatogenesis?[17] What is the meiotic inducer[18,30] that directs cells to enter meiosis? What is the physiological/biochemical basis for the loss of primary spermatocytes with asynapsed and/or nonrecombinant sex chromosomes? Perhaps one of the most fundamental biological questions for sexually reproducing organisms pertains to the mechanism of homologous recognition and pairing of homologous chromosomes during zygonema and pachynema.

A variety of tools and techniques are available for ongoing investigations on spermatogenesis. The availability of additional mutations that disrupt the normal progression of spermatogenesis in laboratory mice will facilitate genetic dissection of the spermatogenic process. Laboratory mice comprising the *Mus spretus* cross may prove to be a valuable system for elucidating the genetics, physiology, and regulation of the spermatogenic process in mammals. Cytogenetic data from sterile F_1 and first-backcross males of this intercross suggest the existence of a cellular feedback mechanism operating during spermatogenesis; the diminished number or complete lack of postmeiotic cells is associated with an apparent breakdown in the control of spermatogonial multiplication (Hale, unpublished observations). Recently developed techniques for injecting and repopulating sterile testes with viable spermatogonia from donors may provide new approaches for the study of spermatogenesis, infertility, and self-renewing stem cells.[18,183,184] The characterization of the recombination-monitoring mechanism and the elucidation of its mode of action should provide valuable insight into the physiology and regulation of spermatogenesis. At the molecular level, the characterization of testis-derived cDNA clones provides useful markers for analyses of transcriptional patterns during spermatogenesis.[185] The molecular characterization of spermatogenesis genes and the biochemical characterization of their products will facilitate methods of correcting spermatogenic problems in infertile men who want to become fathers—and of disrupting spermatogenesis in fertile men who do not (see discussion below). Because spermatogenesis may be influenced by many of the same regulatory mechanisms as differentiating somatic cells, further investigations into the underlying genetic and developmental mechanisms of spermatogenesis should yield insight on the differentiation of gametogenic cells as well as somatic cells.[19]

A. Nonsurgical Contraceptives for Men?

The recognition of potential health risks associated with long-term use of the oral contraceptive pill by women and the growing willingness among men

for equal responsibility and participation in non-barrier methods of birth control provide social and financial incentives for the development of nonsurgical contraceptive alternatives for men. The multiple steps in the spermatogenic process provide a spectrum of potential targets for chemically or hormonally precluding the ultimate production of viable and fertilization-competent sperm. The present level of knowledge of the cellular and endocrinological aspects of spermatogenesis and the biochemistry of sperm capacitation and fertilization indicates a variety of promising approaches for male contraception. Potential targets and approaches for male contraceptives include hormonal modulation of spermatogenesis and sperm production, chemical (i.e., drug) disruption of normal sperm function and physiology, "immunization" with antibodies to spermatogenesis-specific proteins, prevention of sperm-egg fusion (by blocking the deployment of membrane-bound cell-recognition molecules), and interference with the acrosomal reaction at fertilization.[186,187] Perhaps our growing understanding of the genetic and biochemical events controlling the specific stages of spermatogenesis and the function of spermatozoa may soon provide the necessary insight for the timely development of a male "pill."

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MECHANISM OF SEX DETERMINATION IN MAMMALS

Ken McElreavey

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I. INTRODUCTION

The purpose of this review is to outline current knowledge of the genetic and molecular mechanisms whereby sex is determined in mammals. Although the mammalian sex determining gene *SRY* was isolated in 1990, and some of its biochemical properties have since been characterized, our knowledge of the other components of the pathway and the mechanism of mammalian sex determination is rudimentary. Insights into the principles underlying the process of mammalian sex determination may be gained from comparison with sex determination mechanisms in other taxonomic groups. For this reason the first half of this review discusses the genetic and molecular strategies of sex determination in several well-studied species. The sex determination pathways of *Drosophila melanogaster* and *Caenorhabditis elegans* are two of the best understood developmental regulatory pathways in eukaryotes. The mechanisms of regulation within the pathway have been revealed by a combination of sophisticated genetic, molecular, and biochemical investigations. Aspects of the molecular and genetic strategies used to determine sex in these two model organisms will be discussed. The genetics of sex determination in the order *Hymenoptera* (ants, bees, wasps) has also been subject to considerable study. Several genetic models have been proposed to account for sex determination in *Hymenoptera*. Each of these will be reviewed.

The second half of this paper will summarize recent data concerning the process of sex determination in mammals, concentrating on aspects of human sex determination. *SRY* is the primary signal which determines sex in mammals. The genetics and biochemistry of the *SRY* gene are discussed. The genetic and molecular mechanisms that control sex determination in *Drosophila* and *C. elegans* were identified by studying mutants that cause sex reversal. Similarly the study of sex reversed conditions in humans and mice were essential for the identification of *SRY*. The analysis of these altered sexual phenotypes will continue to be vital for our understanding of the mechanism of mammalian sex determination. The relationship between *SRY* and the various classes of human sex determination pathologies is complex. Mutations

involving the *SRY* gene account for the altered sexual phenotype in only a minority of patients with sex reversed or intersex conditions. Other non-Y linked sex determining genes must be involved. Although these other components of the pathway have yet to be identified, several genes other than *SRY* are implicated in human sex determination. These include Wilms' tumor gene, *WT1*, the orphan nuclear receptor, *SF1*, and Müllerian inhibiting substance. In addition a number of loci (on the X chromosome and autosomal) are associated with a failure of testicular development. These are the dosage sensitive sex reversal locus (DSS) located at Xp21.3, the sex reversing autosomal (SXR-A) locus maping to chromosome 17q24.3-q25.1, the long arm of chromosome 10, and the short arm of chromosome 9. The relationship between *SRY* and each of these genes/loci will be discussed.

The final sections of this review will discuss pedigrees of human sex reversal and how they may be used to propose a model which can adequately explain the genetic mechanism of mammalian sex determination. Parallels between this model and genetic sex determining mechanisms in other organisms will be outlined.

II. SEX DETERMINATION IN THE FRUITFLY AND THE NEMATODE

Two sexes are present in *Drosophila* and nematodes[1,2]. Sex determination is an important aspect of development in both organisms. The two sexes differ in their biochemistry, morphology, and behavioral traits. Extensive anatomical differences exist between the two sexes. In *C. elegans* about 30% of the cells in the adult hermaphrodite and about 40% of the cells in the adult male are sexually specialized, and all tissues appear to be sexually dimorphic. Adults of *Drosophila* also exhibit considerable sexual dimorphism. Differences can be observed in pigmentation, number of abdominal segments, and the presence of bristle structures (the sex comb) on the forelegs of the male. In nematodes XX animals are normally self-fertile hermaphrodites and XO animals are male. Hermaphrodites are essentially somatic females that first produce sperm (about 200) and then oocytes. In *Drosophila* XX flies are female and XY flies are male, however, the Y chromosome of *Drosophila* has no role in sex determination. In both species somatic sex determination is based on the ratio of X chromosomes to autosomes (X:A ratio).[3] The primary chromosomal signal of the X:A ratio governs all aspects of sexual development[1,2]: somatic sex determination, dosage compensation, to ensure that levels of X-specific transcripts are equal in XX and XY or XO cells, and germ line sex determination, that is, whether spermatogenesis or oogenesis is initiated. The elements which control sex determination in these two model systems have been identified by the isolation of mutants that transform one sex phenotype to the other. The comparative analysis of these genes has revealed no sequence

similarity between the two species, although the overall strategy is broadly similar. In response to the primary signal (X:A ratio), a master regulator gene controls the activity of an ordered regulatory hierarchy of sex determining genes which govern the activity of sex differentiation genes. The molecular mechanisms which have evolved to achieve sexual dimorphy differ widely between the two species. Sex determination in *Drosophila* involves a cascade of positive regulatory interactions generally mediated by the use of alternative RNA splicing while in *C. elegans* the cascade is characterized by negative regulatory interactions[1,2].

A. Somatic Sex Determination in *Drosophila* and *C. elegans*

In *Drosophila* the X:A ratio dictates the activity of the binary switch gene *Sxl*.[4-7]. *Sxl* regulates the three major aspects of sexual cell fate in *Drosophila*, somatic sexual differentiation, germ-line sex determination, and X chromosome dosage compensation. A simplified overview of the somatic sex determination cascade is shown in Figure 1. In female fruitflies (2X:2A), the *Sxl* gene product is functional, while in males (1X:2A) the gene product is non-functional. However, because of its dual role in dosage compensation, mutations in the gene are lethal and do not result in sex reversal: loss-of-function mutations result in female-specific lethality and gain-of-function mutations result in male-specific lethality. *Sxl* is transcribed in both sexes.[7] Functional sex specific activity in female fruitflies is achieved by RNA splicing which results in a functional *Sxl* polypeptide. In males the mRNA contains an additional exon with an in-frame termination codon. Male-specific transcripts therefore give rise to an inactive truncated protein.[7] Female-specific expression of *Sxl* is initially regulated by the positive-acting does-sensitive "X:A numerator" genes (*sisterless-a* (*sis-a*), *scute or sisterless-b* (*sis-b*) and *runt*).[8-11]. The products of these genes act in concert with the maternal *daughterless* (*da*) product[12,13], to generate a functionally active *Sxl* gene product through the use of a female-specific "early" promoter.[14] Synthesis of this polypeptide occurs only in females. This active product catalyzes the productive splicing of transcripts produced by a "late" non-sex-specific promoter. *Sxl* activity, once established in the female, is maintained throughout development and into adulthood by an autoregulatory feedback loop which controls its expression.[15] The observation that certain flies with two X chromosomes and three sets of autosomes will develop as intersexes indicates the existence of denominator genes. These genes are predicted to negatively regulate the functional activity of *Sxl*. Only the denominator gene *deadpan* has been cloned and encodes a negatively acting protein, that inhibits *Sxl* activity in males.[16]

How do the nominator and denominator gene products interact to select the *Sxl* early promoter in females? One possibility[1] is that heterodimers of the products encoded by the numerator and denominator genes bind to DNA and

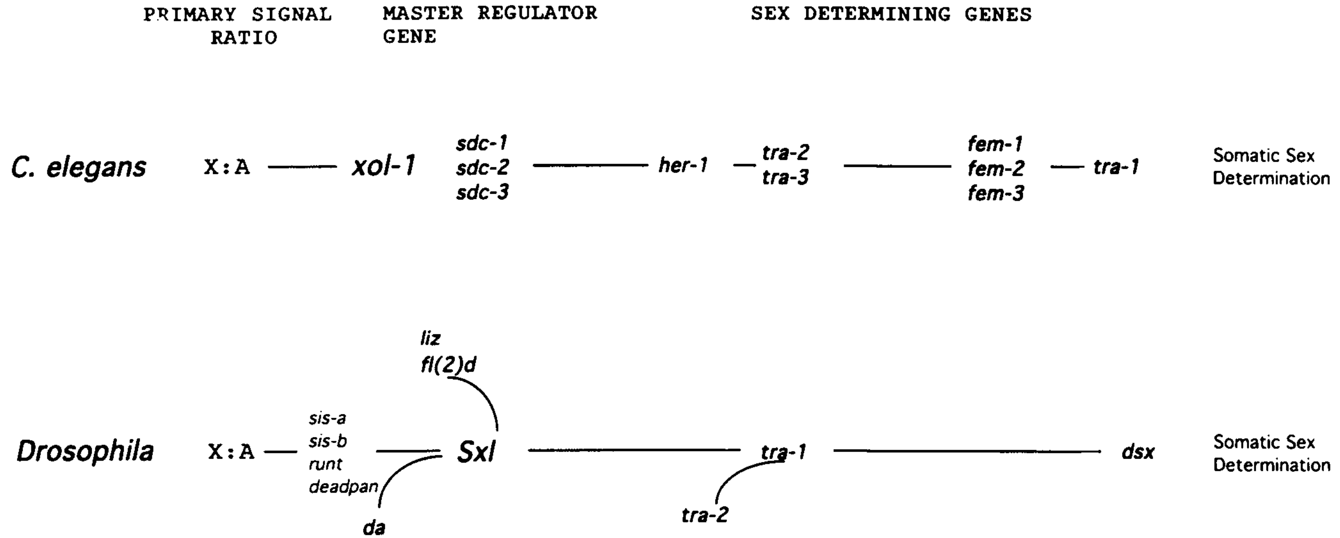

Figure 1. Simplified Schematic diagram of the somatic sex-determining pathways in *Drosophila melanogaster* and *Caenorhabditis elegans*. In both species the primary signal is the X:A ratio. This controls the activity of a key gene, which in turn controls the activity of a few subordinate sex-determining genes. Only the major components are shown. Other genes involved in the process have been recently reviewed.[1,2]

activate the early *Sxl* promoter. In a male with one X chromosome there may be insufficient numerator gene products to achieve this. Other more complex hypotheses are possible. The choice of early *Sxl* promoter may be achieved by a combination of competition and cooperation between denominator, nominator, and maternal proteins. Nominator and denominator gene products may interact directly with each other. Heterodimers formed between the *da* gene product and numerator gene products may be necessary to activate transcription from the *Sxl* early promoter. However heterodimers may also form between denominator and nominator gene products thereby inhibiting this activation. Since an XX fly has an excess of nominator gene products, heterodimers between *da* gene product and nominator gene products will be favored. As a consequence transcription is initiated from the *Sxl* early promoter in an XX fly.

What benefit does the organism receive from having a switch gene that is autoregulated? As suggested by Hogkin[2] there may be two reasons for this. Somatic sex determination and dosage compensation in *Drosophila* are governed by the activity state of *Sxl*. In an XY male (*Sxl* inactive) the lack of X chromosome gene transcripts compared to an XX female is compensated by increased transcription of the single X chromosome. Since nominator genes are X-linked their activity may also increase, leading to an apparent increase in the X:A ratio and activation of *Sxl*. In turn an active *Sxl* product will cause a decrease in the transcription rate and therefore lead to an apparent decrease in the X:A ratio, and so the cycle continues. An autoregulatory mechanism stabilizes the system. Another possibility may be that this type of autoregulated switch may increase the fidelity of transducing the X:A ratio signal to other genes in the cascade.

Subsequent steps in the sex determining pathway of *Drosophila* are characterized by a series of positive regulatory interactions mediated by alternative RNA splicing of *tra* mRNA culminating in the productive splicing of the RNA encoding the terminal regulator *doublesex* (*dsx*).[17-20] Production of the male-specific dsx protein leads to repression of female differentiation genes while production of the female-specific dxs protein represses male differentiation.

The sexual phenotype in *C. elegans* is also controlled by a regulated hierarchy of sex-determining genes (see Figure 2). Again the primary signal is the X:A ratio which controls the activity of a series of sex-determining genes. In response to a low X:A ratio the gene *xol-1* negatively regulates the sex determination and dosage compensation genes (*sdc-1, sdc-2, sdc-3*).[21-25] Precisely how the X:A signal is transduced to downstream genes is not clear. Numerator genes analogous to those identified in *Drosophila* have not yet been found in *C. elegans*. Although the *xol* gene negatively regulates the activity of the *sdc* genes, it may itself not be a true master regulator gene. Both the sex determination and dosage compensation pathways are controlled by the

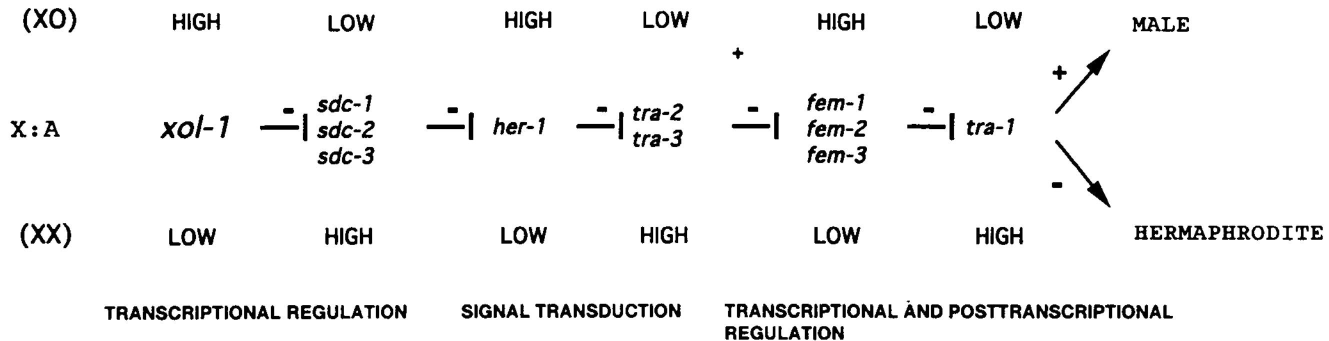

Figure 2. A simplified model of the regulatory network controlling somatic sex determination in *C. elegans*. The activity state (either high or low) of each component of the pathway is indicated. The pathway is characterized by a series of negative regulatory interactions. The postulated mechanisms whereby the sex-determining signal is transduced from the X:A ratio to the sex differentiation genes are shown. *Tra*-1 may also positively regulate the activity of *tra*-2.

xol and *sdc* genes. Genetic studies indicate that the *sdc* genes negatively regulate the next gene in the sex-determining cascade *her-1*.[26] A high level of *her-1* is found in males whereas little or none can be detected in hermaphrodites. *Sdc-1* is predicted to encode a protein containing several zinc finger domains and therefore *sdc-1* may negatively regulate *her-1* by controlling the expression of *her-1*.[27] Genetic epistasis analysis indicate that *her-1* also negatively regulates the activity of *tra-2* the next gene in the somatic sex-determining pathway, which is required for female development. Since sequence analysis predicts that *her-1* encodes a secreted protein[28] and that *tra-2* encodes a membrane protein,[29] the *her-1* gene product probably regulates *tra-2* activity by binding to the extracellular domain of a *tra-2* encoded receptor.[30] The interaction between *her-1* and *tra-2* gene products allows the *fem* gene products to inhibit the activity of *tra-1,* the final step in the sex-determining cascade.[31] *Tra-2* may negatively regulate the *fem* gene products via its intracellular, carboxy-terminal domain. Therefore in *Caenorhabditis* a range of molecular mechanisms including transcriptional, posttranscriptional, and signal transduction are used in the somatic sex-determining pathway. Intercellular signaling ensures that surrounding cells are coordinated to adopt a similar sexual developmental fate, even if their cell-autonomous induction is disrupted.

The liability of both sex-determining systems is surprising. By using different mutations it is possible to entirely alter the sex-determining mechanism of these organisms in the laboratory. For example, complete sexual transformation in *C. elegans* can be achieved by appropriate mutations in *tra-1*. Null *tra-1* alleles (*tra-1*(*O*) result in the development of both XX and XO individuals into males.[32] *Tra-1* gain-of-function (*tra-1*(*gf*) mutations result in XX and XO animals developing into females. As *tra-1*(*gf*) is dominant to *tra-1*(*O*), heterozygous *tra-1*(*gf*)/*tra-1*(O) animals are also females. These can be crossed fertilized by *tra-1*(*O*) males to give equal numbers of *tra-1*(*gf*)/*tra-1*(*O*) females and *tra-1*(*O*) males. The result of this is the establishment of a stable male/female population. The system now behaves like a mammalian XY system and no longer responds to the X:A ratio. Similarly in *Drosophila* the genetic control of sex determination can be changed to one that is dependent on temperature. Chromosomal females flies (XX) homozygous for temperature sensitive *tra-2* mutants (*tra-2*^{ts}) will develop as males if the larvae are incubated at 29^{0}C, whereas at 16^{0}C they will develop as females.[33] These examples also illustrate an important difference between mammalian sex determination and sex determination in *Drosophila* and *Caenorhabditis.* In these species sex is continuously determined during development whereas in mammals sex is determined at a precise moment during embryonic development.

B. Dosage Compensation and Germ Line Sex Determination

Dosage compensation is controlled in both species by the X:A ratio, but again the molecular mechanisms used to accomplish this are different. Dosage

compensation is *Drosophila* is regulated by transcribing the single male X chromosome at twice the rate of each of the two X chromosomes of the female (hypertranscription). In contrast the nematode uses a different strategy to control the levels of X-specific transcripts by downregulating the expression of each set of X-linked genes in XX hermaphrodites.[34,35] Germ line sex determination refers to the developmental choice of germ cells to enter oogenesis or spermatogenesis. Germ line sex determination in both species requires both inductive signals from the stroma and cell autonomous components.[36,37]

In contrast to germ cell sex determination in the fruitfly and nematode, in mammals the choice of germ cells to enter spermatogenesis or oogenesis depends only on whether or not a germ cell is exposed to a testicular environment and therefore is not directly dependent on a signal from the Y chromosome.[38] Mammalian germ cell sex determination is not cell-autonomous. For example some XY germ cells fail to enter the genital ridge and can end up at the adrenals.[39] These cells will enter meiosis. XY germ cells therefore have the potential to develop as oocytes when separated from testicular tissue. Dosage compensation in mammals is not linked to sex determination (although see discussion in section VII). Equalizing the levels of X-linked transcripts is achieved by the independent process of inactivation of one of the two X chromosomes during early female development. Since both germ cell sex determination and dosage compensation occur independently of sex determination in mammals they will not be considered further in this review.

The genetic hierarchy which has evolved to generate two sex phenotypes is fundamentally different between the fruitfly and the nematode. It involves a complex interplay of positive, negative, and autoregulatory signals, some of which are cell-autonomous and others intercellular. This lack of molecular and mechanistic conservation is in striking contrast to other development processes, such as body pattern formation which is controlled by homeobox gene clusters. Although at a mechanistic or sequence level there is a lack of similarity, the general organization of sex-determining pathways is similar. There is a regulatory hierarchy for cell specialization crowned by a master regulatory gene whose activity depends on a primary signal. The pathway consists of a series of selector genes whose state of activity specifies one of two alternative developmental pathways. At the end of this chapter we will return to this concept of sex determination as a genetic hierarchy of binary switches controlled by a key gene which responds to a primary signal.

III. SEX DETERMINATION IN THE ORDER HYMENOPTERA

The order *Hymenoptera* comprises the ants, bees, and wasps. The most frequent mechanism of reproduction in *Hymenoptera* is arrhenotoky.[40] Males

develop from unfertilized eggs and are haploid, whereas females develop from fertilized eggs and are diploid. The males contain only a single series of maternal chromosomes and the females contain both maternal and paternal chromosomes. No morphologically distinct heterosomes can be recognized. At first sight it would appear that sex is determined simply by the level of ploidy, however, in strongly inbred crosses males can develop from fertilized eggs, which excludes ploidy as the sex-determining mechanism.[41] This observation lead to a one locus multi-allele hypothesis. The one locus multi-allele model proposes that sex is determined by a series of complementary alleles of one locus.[41,42] In this system homozygotes as well as haploid hemizygotes are male and heterozygotes are female. This hypothesis was based on the observation that in strongly inbred crosses of the parasitoid *Bracon hebetor,* diploid males could develop from fertilized eggs. Experimental data are consistent with a single-locus sex-determining mechanism in some other species (for example *Diadromus pulchellus*[43]; *Augochlorella straita*;[44] *Athalia rosae ruficornis*[45]). However, in other species data from inbreeding experiments is inconsistent with a single sex-determining locus. The single-locus model predicts that inbreeding should produce diploid males, yet some species of *Hymenoptera* habitually inbreed, without producing diploid males. This observation lead to the proposal of a multi-loci multi allele hypothesis.[46,47] In this model heterozygotes at one or more loci develop as females, homozygous or hemizygous at all loci are male. The number of diploid males is maintained at a low level by occasional outcrossing between inbreeding lines. This model is difficult to test experimentally since in principle there is no limit on the number of possible loci involved. This is illustrated by inbreeding experiments of the sib-mating species *Goniozus nephantidis* (Chrysidoidea: Bethyliade), which over 22 generations failed to introduce changes in the sex ratio.[48] This indicates that at least 15 loci are involved or that the multi-loci model is incorrect, at least in this species.

A third model, the genic-balance model[49,51] proposes that sex is determined by a series of compensated maleness genes (m) and a series of dose-dependent femaleness genes (f). The maleness genes are non-cumulative, hence the effects of m genes (M) are the same in haploids and diploids. The femaleness genes are cumulative hence the effects of f genes are F in haploids and 2F in diploids. Sex is determined by the effects of m and f genes, $2F<M<F$, diploids are therefore female and haploids male. Again it is difficult to experimentally prove or disprove this hypothesis, but to date no evidence has been found for maleness and femaleness genes with additive effects.

Other apparently more diverse mechanisms that determine sex may exist in some *Hymenoptera* species. Thelytoky (female-to-female parthenogenesis) and spanandrous arrhenotoky (arrhenotoky in which only a few males are produced) occur in some species.[52–54]. Thelytoky can be distinguished from arrhenotoky because virgin thelytokous females produce female offspring

whereas virgin arrhenotous females produce only male offspring. Thelytoky occurs in some *Trichogramma* species. Backcrossing experiments by Stouthamer and colleagues[55] of *Trichogramma* parasitic wasps indicated that thelytoky was not inherited as a simple chromosomal trait. In a series of experiments eggs were exposed to either high temperatures or newly emerged wasps were permitted to feed on honey mixed with antibiotics. In some lines this lead to a reversion to arrhenotoky. These observations strongly inferred that symbiotic microbes are responsible for maintaining thelyotoky. The use of PCR technology has allowed the identification of some of these microbes, which cannot be cultured outside their insect hosts.[56,57] These bacteria all appear to be closely related to each other and have been assigned to the species *Wolbachia pipientis*. Since antibiotics can cause a change from thelytoky to arrhenotoky, it suggests that the symbiotic microbes influence in some way the "normal" sex-determining mechanism of arrhenotoky. In spite of apparent diversity of sex-determining systems in *Hymenoptera* (arrhenotoky and thelytoky) the underlying mechanism may be similar.

All the genetic models proposed to account for the determination of sex in *Hymenoptera* are compatible with many molecular mechanisms. The increasing use of molecular biology coupled with genetic studies should shed light on the molecular mechanisms underlying these systems.

IV. MAMMALIAN SEX DETERMINATION, THE CHOICE OF A TESTIS OR OVARY

To clarify the terminology used in this section and to put it into the context of this review I will define the term mammalian sex determination. Mammalian sex determination is the ensemble of genetic events that give rise to the choice of male or female-specific gonad formation. Sex differentiation is the series of events which occurs after this choice; the formation of the gonad and the development of secondary sexual characteristics leading to the complete sex phenotype. This boundary is somewhat artifical since there is a continuum of events leading from gonad determination to a sex phenotype. In vertebrates sex specific differentiation depends largely on circulating sex hormones. This contrasts with the situation in *Drosophila* in which each somatic cell determines and maintains its sex independently.

Until the late 1950s mammalian sex determination was thought to have a similar genetic control mechanism to that discovered in *Drosophila*. Sex determination was considered to be controlled by the X:A ratio. The first indication that sex determination in mammals was dependent on the presence or absence of a Y chromosome came from observations made in the late 1950s and early 1960s, when both XO mice and XO humans were found to develop

ovaries.[58-60] Subsequently XXY humans and XXY mice were discovered who developed as phenotypic males with testes.[61,62] The conclusion drawn from this observation was that the mammalian Y chromosome contains one or more genes involved in the induction of testicular tissue.

A classic series of experiments conducted in the early 1940s by Alfred Jost and colleagues[63] defined the basic principles of mammalian sex determination. Jost castrated fetal rabbits just before the onset of gonad differentiation. He found that all embryos (both chromosomal males and chromosomal females) developed female internal genitalia with no development of the Wolffian ducts. From these observations he concluded that (1) the testis products substances that cause both the development of Wolffian structures and regression of Müllerian ducts in a male foetus, and (2) that the presence of an ovary was not necessary to induce Müllerian duct differentiation. Therefore in mammals the female ovarian pathway is the default pathway; the Y chromosome in some way must directly intervene to initiate testis formation. Since the testes produce all the hormones necessary for the complete male phenotype, sex determination in mammals can be equated with testes determination.

The formation of the mammalian gonad and differentiation of the external genitalia is relatively well understood both in terms of morphology and endocrinology, although molecular details have not yet been worked out. Up to the eighth week of gestation the reproductive tract is similar in male and female human fetuses.[64] Both testes and ovaries are derived from the genital ridge which develops along the ventral cranial part of the mesonephros. The formation of the testis from the indifferent gonad is associated with two events. Germ cells and Sertoli cells become enclosed in testicular cords, followed shortly by the differentiation of Leydig cells. Subsequent differentiation of male genitalia is the result of products secreted by Sertoli and Leydig cells. Sertoli cells produce Müllerian inhibiting substance (MIS) which causes regression of the Müllerian ducts.[64] In humans Müllerian duct regression begins at about eight weeks gestation and ducts have almost disappeared by 10 weeks. Leydig cells produce testosterone which influences the development of both internal and external genitalia. Testosterone induces Wolffian duct development leading to the formation of epididymides, vasa deferentia, and seminal vesicles. Dihydrotestosterone, derived from testosterone by the action of the enzyme 5α-reductase, influences the external bipotential primordia to develop into penis shaft, scrotum, and glans penis. In human females the differentiation of Müllerian ducts (into fallopian tubes, uterus, and upper vagina) and degeneration of Wolffian ducts begins at about 10 weeks.

V. THE MAMMALIAN TESTIS DETERMINING GENE, *SRY*

A full description of the *SRY* gene is given elsewhere in this volume. I will restrict this discussion of *SRY* to those aspects which are directly relevant to our understanding of the mechanism of mammalian sex determination. The human *SRY* gene consists of a single exon encoding a 223 amino acid proten[65-69]. The central one-third of the gene encodes a conserved motif of 79 amino acids that is present in a number of proteins that are associated with DNA. These include the Mc protein encoded by the *mat3-M* locus of the fission yeast *Schizosaccharmoyces pombe*[70] and the nuclear non-histone high mobility group (HMG) proteins.[71] The *SRY* conserved motif has been termed an HMG-box. The gene lies 5 kb proximal to the Y pseudoautosomal boundary with transcription of the gene proceeding in the direction of centromere to the telomere.[65]

The conserved HMG-domain is related to the DNA-binding motifs of several other biochemically-defined regulators of transcription and genetically-defined regulators of cell specification[72-74]. Hybridization and PCR studies suggest that there are a large number of as yet unidentified genes which encode this motif.[75,76] The most common members of the family are HMG-1 and its homologue, HMG-2. These are closely related proteins which show intraspecies sequence identities of approximately 80%. HMG-1/-2 are highly abundant proteins that bind to linker DNA between nucleosomes in chromatin, and although the data are far from conclusive, experimental evidence suggests a role for these proteins in a wide variety of activities including transcription, replication, chromatin assembly and stabilization of chromatin structure. HMG-box containing proteins have several physical properties in common. The box contains approximately 80 amino acids, has a net positive charge, and has a large number of conserved aromatic and basic amino acid residues. Although sequence conservation between HMG-boxes is low (20-30% identity), NMR spectroscopy studies[77] indicate that they probably have a common tertiary structure consisting of three alpha helices forming an L-shape with an angle between the two arms of 80^0. How such a structure may recognize and interact with DNA is unclear.

The family of HMG-domain proteins is somewhat unusual in that its members recognize DNA with substantially different DNA-binding specificities. Proteins with multiple HMG-binding domains have typically no or low sequence specificity of DNA-binding, whereas proteins with a single HMG-domain recognize specific DNA sequences[78-80]. The *SRY* HMG-box is both necessary and sufficient to bind DNA, however, little is known concerning possible target sequence(s) for SRY-binding although candidate motifs have been described.[81,82] Like other proteins containing a single HMG-box, SRY will bind to DNA in a sequence-specific manner, although there is a fairly high non-specific binding affinity for linear DNA irrespective of the sequence.[81,82]

Human SRY, as a monomer in solution will bind specifically to double-stranded DNA with the sequence CTTTGTT, whereas murine Sry will bind with higher affinity to the sequence CATTGTT.[81-83] Differences in binding affinity may reflect distinct differences in the interactions of the two proteins with DNA. This view is supported by *in vitro* studies which indicate that human SRY interacts with DNA primarily through minor groove contacts, murine Sry mainly recognizes the major groove.[84] This may explain in part the observation that both human and murine SRY will bend linear DNA to different degrees.[83,85] The low DNA-binding specificity exhibited by hSRY suggests that interaction with another protein may be necessary to aid recognition of specific target sites.

Although SRY will bind to linear DNA with sequence specificity, it can also bind to four-way junctions.[85] These are DNA structures that can be generated by recombination events and by intrastrand base pairing of inverted repeat sequences. Binding to irregular DNA structures such as four-way junctions, kinked DNA, and supercoiled DNA may be a general property of HMG proteins. HMG-1 and the HMG-proteins SSRP, LEF-1, and UBF will bind to non-linear DNA.[86-89]. The binding affinity of SRY for four-way junctions is similar to that observed in binding to linear DNA. SRY binding is independent of the sequence that makes up the junction. How can one reconcile the apparently contradictory observations that SRY will bind to sequence-specific linear DNA and also to four-way junctions independent of the sequence? One simple mechanistic model proposed by Bianchi and coworkers[85] suggests that HMG-1 proteins, which are highly abundant non-specific DNA-binding proteins will occupy available four-way junctions forming a stable protein-DNA complex. SRY also has the capacity to bind to bend DNA without sequence specificity. However, since HMG-1/-2 proteins are much more abundant they could compete for and inhibit non-specific binding by SRY. This would have the effect of limiting the interaction of SRY with DNA to specific sites in the genome.

The HMG domain of SRY not only has the ability to bind to DNA but also to induce DNA-bending.[83,85] Other HMG-domain proteins share this property.[83] Circular permutation assays, which detect protein-induced DNA-specific bending indicate that human and murine SRY will induce sharp bends in linear DNA of approximately 83^0 and 85^0, respectively.[83,85] This feature of SRY proteins may be functionally important. It might permit regulation of gene expression by juxtaposing distant sites in the DNA helix, thereby allowing interaction with other transcription factors. This may be achieved by the SRY protein acting alone to augment protein-protein contacts facilitating the assembly of a multiprotein complex, or there may be direct physical interactions between the SRY protein and other regulatory proteins to create a "scaffold" structure. Support for the latter model can be inferred from experimental data of the transcriptional activities of the HMG-domain protein

LEF-1.[89] LEF-1 regulates gene expression from the minimal T cell receptor alpha enhancer motif which contains multiple binding sites for distinct regulatory proteins. LEF-1 will not alter basal promoter activity from multimerised LEF-1 binding sites. Likewise, the replacement of the LEF-1 HMG-domain with other DNA-binding domains such as Gal4 result in a sharp reduction of TCRα enhancer activity, suggesting the importance of specific protein-protein contacts.

VI. RELATIONSHIP BETWEEN *SRY* AND PATHOLOGIES OF HUMAN SEX DETERMINATION

A. Classification of Human Pathologies of Sex Determination

Completely sex reversed individuals can be generally classified as either 46,XX males or 46,XY females. Incomplete or partial sex reversed syndromes are comprised of 46,XY and 46,XX true hermaphrodites.

About one in 20,000 new-born males have a 46,XX karyotype and yet develop testes. These individuals have a male habitus, are sterile with small and azoospermic testes.[90] 46,XX true hermaphroditism is a rare disorder, defined by the presence of both ovarian and testicular tissue present in the same individual.[91] The gonads usually consist of an ovary on one side and a testis on the other, or an ovotestis on one side and an ovary on the other. In all cases histological examination of the gonads should show distinct tubule structures in the testicular-like tissue and the presence of follicles in the ovarian-like tissue before a case is classified as a true hermaphrodite. The external genitalia of 46,XX true hermaphrodites is usually ambiguous or feminine. The degree of masculinization broadly correlates with the amount of testicular tissue present.

A much rarer condition is 46,XY gonadal dysgenesis. The term 46,XY gonadal dysgenesis refers to all conditions characterized by abnormalities of testis determination in subjects with an apparently normal XY karyotype.[92,93] This group can be further subdivided into complete (or pure) gonadal dysgenesis and partial gonadal dysgenesis. Complete gonadal dysgenesis is characterized by completely female external genitalia and well-developed Müllerian structures. The gonad consists of a streak of fibrous tissue with no evidence of testicular differentiation. 46,XY partial gonadal dysgenesis is characterized by partial testis determination and a normal 46,XY karyotype without mosaicism. Internal ducts usually consist of a mixture of Wolffian (epididymis, vas deferens, and seminal vesicle) and Müllerian ducts (fallopian tube, uterus, and upper third of the vagina). Affected individuals exhibit varying degrees of masculinization of the external genitalia. The degree of virilization generally correlates with the extent of testicular differentiation.

Embryonic testicular regression sequence can also be regarded as part of the clinical spectrum of 46,XY gonadal dysgenesis.[94] Affected individuals have a 46,XY karyotype and usually present with ambiguous external and internal genitalia. Gonad tissue is absent on one or both sides. Patients with this disorder are considered to have incomplete testicular determination and loss of gonad material early in gestation before testis differentiation was complete.

Mosaicism (defined as the presence of two or more cell lines with different karyotypes in the same individual, derived from a single zygote) can give rise to all the above phenotypes. For example individuals with 45,X/46,XY karyotype usually present with partial gonadal dysgenesis, and occasionally true hermaphroditism.

There are difficulties in attempting to classify pathologies of human sex determination comprehensively. For example an XX male who presents with ambiguous genitalia may be a true hermaphrodite. Classification can only be ascertained by means of a gonad biopsy, which is not always feasible. Similarly, detailed histological analysis of the streak gonads of patients with 46,XY pure gonadal dysgenesis often reveals the presence, to a lesser or greater extent, of tubule-like structures indicating induction of testicular determination. Therefore we are not dealing with distinct categories of sex reversed conditions but rather a continuum of sex reversed and intersex phenotypes.

B. Relationship Between *SRY* and 46,XY Complete Gonadal Dysgenesis

46,XY complete gonadal dysgenesis is characterized by a total failure of testis development and a female phenotype without ambiguities. Thus, affected subjects are ideal candidates for harboring mutations in the primary steps of the sex-determination pathway. Since the cloning of the *SRY* gene in 1990 we have screened[95] more than 50 XY females with complete gonadal dysgenesis for mutations in both *SRY* coding sequences and by Southern blotting for mutations in the testis-determining region (the testis-determining region is 35 kb of Y-specific DNA which is the minimum portion of the Y chromosome known to confer a male phenotype in certain XX males[65]). Mutations in *SRY* coding sequences are present in approximately 20% of patients with this disorder. Figure 3 shows mutations which have been published in the *SRY* HMG DNA-binding domain and in the testis-determining region. Careful analysis of the streak gonad of patients carrying a mutation has revealed[96] that without exception there has been a failure of the *SRY* to initiate testicular formation (as indicated by the absence of testicular cords in the entire gonad).

In addition to nonsense and missense mutations in *SRY* coding sequences, deletions outside the *SRY* gene and microduplications of *SRY* sequences may also be associated with abnormal gonad determination. These can be easily identified by Southern blotting (Figure 4). One patient was identified[95] with a small *de novo* interstitial deletion 5' to the *SRY* gene. This deletion begins

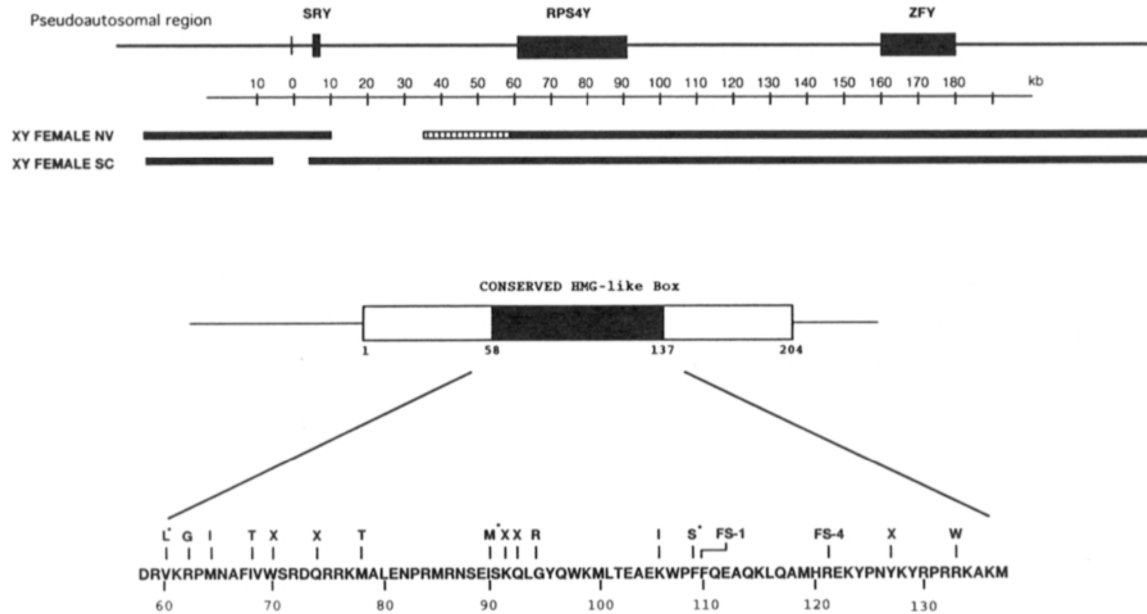

Figure 3. Mutations in the testis determining region associated with complete and partial forms of 46,XY gonadal dysgenesis. Top: Schematic representation of the distal short arm of the Y chromosome is shown. Distances from the Pseudoautosomal boundary are shown in kilobases. Genes identified in this region are indicated. Transcription of *SRY* proceeds from the contromere to the telemere. Black arms indicate Y-chromosomal material present in XY females with gonadal dysgenesis who harbor small interstitial deletions flanking the *SRY* gene, XY female NV has complete gonadal dysgenesis and has a *de novo* deletion which begins 0.8 kb 5′ to the *SRY* transcription initiation site and extends for a further 25-52 kb. XY female SC has partial gonadal dysgenesis and harbors a de novo deletion 3′ to the *SRY* gene.

approximately 0.8 kb 5' to the *SRY* transcription initiation site and extends for a further 25-50 kb toward the next known gene on the Y chromosome, *RPS4Y*. The patient presented with complete gonadal dysgenesis. Histological analysis of her streak gonads demonstrated a complete absence of tubule-like structures, indistinguishable from the gonads of patients carrying mutations in *SRY* coding sequences.[96] The deletion may define *SRY* regulatory elements (see discussion of patients with partial gonadal dysgenesis).

Data from other studies also indicate a low frequency of mutations in the *SRY* gene in this group of patients.[97–104] Some of these individuals may harbor mutations in other downstream sex-determining genes, but there may also be other reasons for this low figure. The most obvious is the criteria used for patient selection. It is not always possible to have detailed clinical data concerning a patient. Patients with other conditions which can also result in a sex-reversed phenotype, such as testicular feminization (caused in some cases

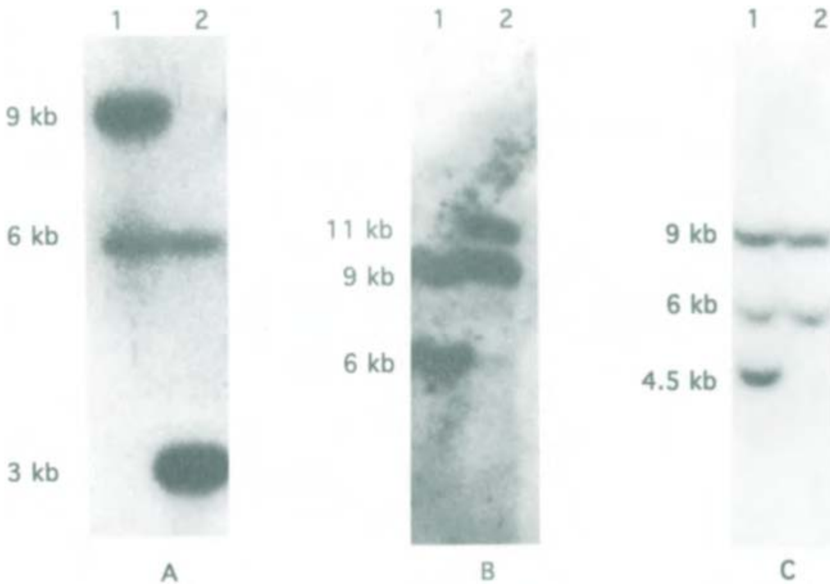

Figure 4. Hybridization of pY53.3 probe which contains the *SRY* gene to Southern blots of DNA from XY females with complete or partial 46,XY gonadal dysgenesis. Digestion of DNA by StuI followed by hybraidization with pY53.3 gives a 9 kb and a 6 kb Y-specific fragment in a normal male. (A) Lane 1 normal male, lane 2 XY female with a deletion 5′ to the SRY gene. (B) Lane 1 normal male, lane 2 XY female with a deletion 3′ to the SRY gene.

by mutations of the X-linked androgen receptor gene) may be inadvertently included. A chromosomal mosaicism limited to the gonad may account for gonadal dysgenesis in a proportion of cases. In addition some workers have screened only the HMG DNA—binding domain of the gene for mutations. Mutations may be present in flanking coding sequences.[104] Mutations in regulatory elements outside the *SRY* gene may also be associated with complete or partial gonadal dysgenesis. For example point mutations in Sp1 and ATF regulatory motifs in the promoter region of the human retinoblastoma gene have been described that cause hereditary retinoblastoma.[105]

Sequence changes in the *SRY* gene do not always cause sex reversal. Three familial cases of 46,XY sex reversal have been described where the affected individuals share the same amino acid change as their father.[106,107] In each case a single base-pair change has resulted in an amino acid substitution in the HMG DNA-binding domain. The amino acid substitutions were Val60-Leu(V60L), Ile90-Met (I90M), and Phe109-Ser(F109S). Each of the patients had 46,XY complete gonadal dysgenesis. In the familial case of SRY^{V60L} analysis of the streak gonad from affected XY females revealed only wavy ovarian-like tissue. Tubule-like structures were not observed, indicating a complete absence of SRY activity. There are several possible hypotheses that might explain sex reversal in these families. It is possible that the sequence variants may be trivial polymorphisms not associated with the sex reversed phenotype. A mutation in autosomal gene may be responsible for the phenotype. This hypothesis is unlikely since studies by ourselves and others have not found any polymorphism in *SRY* coding sequences in more than 200 normal XY males. An alternative hypothesis is interaction between the variant SRY protein and another genetic determinant. A third hypothesis si that in the *in vivo* activity of the variant SRY protein is altered such that it is around a critical threshold level. The ability of recombinant SRY proteins containing these mutations has been tested for binding activity to a potential 7-bp recognition motif TTGTTTTC. No difference in binding activity was detected in in vitro assays between wild-type SRY and SRY^{F109S}. In contrast binding was not observed with SRY^{V60L} to the same target site. Binding of the protein SRY^{I90M} was reduced.

These *in vitro* observations must be interpreted with caution. The binding motif used in the experiments may not be the true *in vivo* recognition target site for the SRY protein. Assay conditions *in vitro* may not reflect *in vivo* conditions, accessory factors for example may be required to aid SRY to recognize its target site. Also SRY is known to bend DNA. This could give rise to a situation in which modified SRY proteins may exhibit a wild-type binding affinity, yet bend the DNA to varying degrees.

C. Relationship Between *SRY* and Partial Gonadal Dysgenesis

The relationship between SRY activity and partial testicular formation in an XY individual is not clear. This phenotype could result from a mutation involving the *SRY* gene, which may alter SRY activity or change the timing/tissue specificity of *SRY* expression. Incomplete testicular formation could also be due to mutations in downstream responder genes in the sex-determining cascade, or to mutations in other genes which result in an inhibition of normal testicular development. Mosaicism may also give rise to this phenotype. Mutations involving the *SRY* gene in this phenotype are very rare[99] with only one complex case of an XY individual with gonadal dysgenesis and two postzygotic mutations in the SRY open reading frame published.[108] The individual had intersex genitalia and was described as a true hermaphrodite. The *SRY* gene sequence in DNA extracted from perpheral blood lymphocytes was normal. However DNA from the gonad was found to be of two types, either normal or harboring two point mutations (one silent, the other resulting in a leucine to histidine substitution). The cause of the two postzygotic mutations is unknown. Dysgenesis presumably occured in gonad cells containing a Y chromosome with the *SRY* mutation, while normal testicular development occurred in those cells with a normal *SRY* gene.

In a joint study with Gary Berkovitz and colleagues at The Johns Hopkins University, we have systematically screened 21 individuals presenting with 46,XY partial gonadal dysgenesis and embryonic testicular regression sequence for mutations in the testis-determining region. No mutations were detected in *SRY* coding sequencing but one individual was found to harbor a *de novo* deletion 3' to *SRY*. This deletion begins approximately 2 kb 3' to the *SRY* polyadenylation site. The Y pseudoautosomal boundary is deleted. The distal breakpoint is located between about 1 kb and 4 kb distal to the Y boundary. How a deletion 3' to the *SRY* gene may influence its expression is unclear. There are several possible explanations. The deletion may remove regulatory elements normally required for the proper expression of *SRY* or a position effect may partially silence *SRY* expression. In the former case the loss of regulatory elements may reduce the level of transcripts, change the timing or tissue specificity of expression. Sequences 3' to many genes are important for regulation of gene expression, for example an enhancer motif 3' to the Wilms' Tumor gene *WT1* appears to increase basal transcription levels and confer tissue specificity.[109] Alternatively the deletion may alter the timing of *SRY* expression during development. The precise moment when the testis determinant acts during fetal development is important. Murine studies suggest that testis determination depends on the activity of Sry at a critical moment of fetal development. Murine *Sry* is expressed in the fetal genital ridge of the developing embryo during a limited period from days 10.5 to 12.5 post coitum. Studies by Palmer and Burgogyne,[110] of crosses between

different mouse strains, in which a proportion of the progeny are XY females, indicated that the sex reversed phenotype may be due to a mismatch of developmental timing. Subtle differences in the exact timing of Tdy activity in the genital ridge relative to the developmental rate may result in male to female sex reversal.

Another explanation is that the phenotype is due to a position effect. Studies in the mouse suggested that *Sry* expression is sensitive to position effects.[111] Deletions of the Y chromosome Sx1 repeat sequences located outside the minimal testis-determining region resulted in XY sex reversal, even though the *Sry* gene itself was intact.

Other cytogenetically undetectable interstitial deletions in this region have been described including a deletion that led to the gene *ZFY* being proposed as a candidate for TDF.[112] This suggests that microdeletions in the testis-determining region may be more prevalent than previously supposed. The Y chromosome is rich in highly repetitive DNA.[113] These include tandem clustered repeats, short interspersed repetitive elements (Alu repeats are particularly frequent[114]), and long interspersed repetitive elements.[115] Simple illegitimate recombination events involving repetitive elements are the most likely mechanisms responsible for these deletions, however, insights into the origins of these rearrangements must await detailed molecular analysis of their junctions.

D. Relationship Between *SRY* and XX Sex Reversal

In principle the development of testicular tissue in an XX subject could result from (1) the presence of Y DNA sequences including *SRY* translocated from the paternal Y to the X chromosome during male meiosis, (2) a mutation of an autosomal or X-linked gene permitting testis determination in the absence of *SRY*, and (3) an undetected mosaicism of a Y chromosome bearing cell line. Examples of conditions 1 and 3 are well documented; mutations in other genes leading to testicular development is inferred from pedigrees of *SRY*-negative XX sex reversal (see Section IX).

The incidence of Y chromosome DNA sequences in 46,XX males and 46,XX true hermaphrodites is indicated in Table 1. In general the presence of Y chromosome sequences in an XX individual is associated with a more masculinized phenotype. Most XX males without genital ambiguities harbor Y-chromosome material including the *SRY* gene. A minority of XX males with genital ambiguities carry *SRY* and a small proportion of XX true hermaphrodites are positive for Y material. In rare cases a complete male phenotype can occur in the absence of *SRY*. In one of these cases mosaicism was excluded as Y chromosome sequences were not detected in DNA extracted from testicular tissue.[116]

Table 1. Presence or Absence of *SRY* in XX Males or XX True Hermaphrodites

	SRY Present	*SRY Absent*
XX male without ambiguities	36	3
XX male with Ambiguities	4	39
XX true hermaphrodite	6	32

In situ hybridization studies have indicated that Y-chromosome positive XX males and XX true hermaphrodites carry the *SRY* gene on the distal tip of the short arm of one of their X chromosomes.[117,118] This observation is consistent with an unequal exchange of material between the X and Y chromosomes during male meiosis, however, it does not explain the spectrum of phenotypes observed in Y-positive XX individuals. A close relationship between XX maleness and XX true hermaphroditism in this group of patients is suggested by familial cases of sex reversal where XX males and XX true hermaphrodites coexist.[118] One such pedigree is shown in Figure 5. Southern analysis has shown that the XX true hermaphrodite and the XX male carry exactly the same amount of Y-specific DNA (35 kb), localized by *in situ* hybridization to one of their X chromosomes at position Xp22. The analysis of DNA from their father indicated that he also carried *SRY* on his X chromosome as well as on his Y-chromosome. He is a normal healthy fertile male. This indicates that the crossing-over event occurred in a previous generation. Since the father of the two sibs received his *SRY*-bearing X chromosome from his mother, she may have been a fertile true hermaphrodite. It cannot be formally excluded that a complex genetic event occurred in the early development of the father's zygote, generating an *SRY*-bearing X chromosome. Since both sibs inherited the same Y-positive X chromosome from their father the phenotypic variability is most simply explained by X inactivation silencing *SRY* expression to a lesser or greater extent. Other studies have also demonstrated that the Y-bearing X chromosome in 46,XX individuals is subject to nonrandom inactivation.[117]

E. *SRY* and Human Sex Reversal: Conclusions

Several conclusions concerning the mammalian sex-determining pathway can be reached from these observations.

1. Other non-Y genes are necessary for mammalian sex determination. Mutations involving *SRY* only explain a minority of cases of sex reversal. The majority of XY females with gonadal dysgenesis do not carry mutations

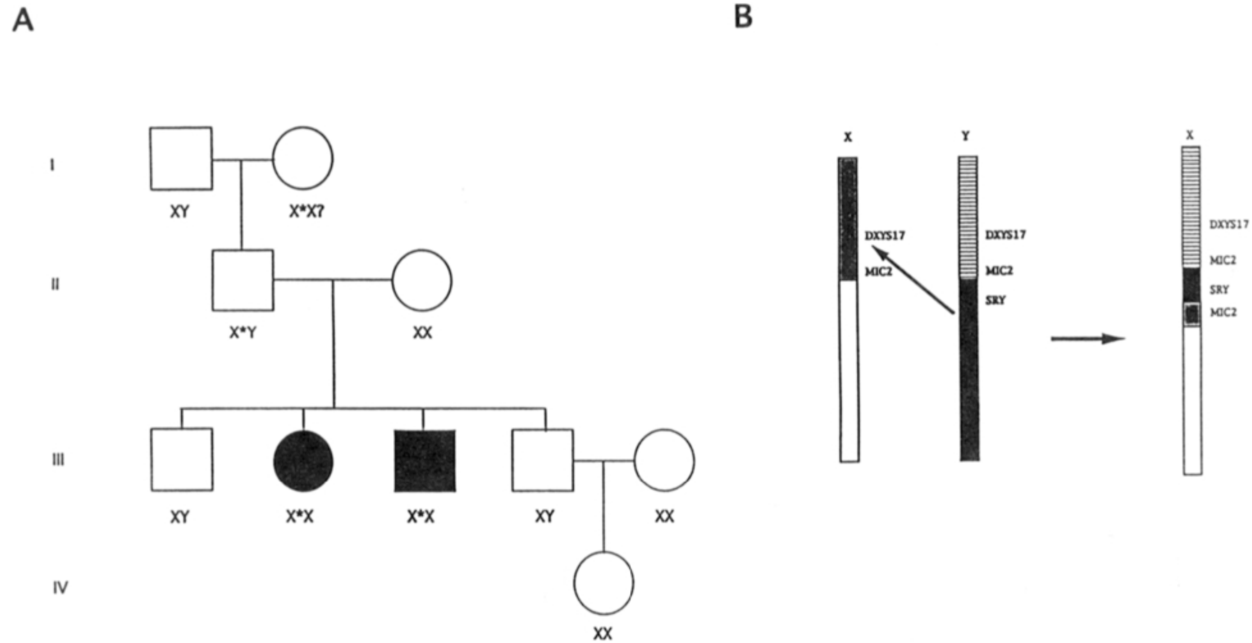

Figure 5. (A) Pedigree of family L. Solid symbols indicate the XX true hermaphraodite (circle) and the XX male (square). The *SRY*-bearing X chromosome is indicated by an asterisk. The X* chromosome can be detected in DNA from the peripheral blood of the father suggesting that he inherited X* from his mother. (B) Model of the formation of the abnormal X* chromosome. The breakpoint on the original X chromosome was between DXS17 and MIC2 in the pseudoautosomal region and on the Y chromosome 35 kb proximal to the Y-boundary. This unequal crossing-over event generated the X* chromosome which contains 35 kb of Y-specific DNA and two copies of the pseudoautosomal gene MIC2. A single copy of DXS17 is present.

involving the *SRY* gene. Likewise some XX males without genital ambiguities and the majority of XX males with genital ambiguities and XX true hermaphrodites do not carry the *SRY* gene. Complete XX sex reversal can occur in the absence of *SRY*. This indicates that there must be other genes, either on the X chromosome or on the autosomes that are required for sex determination. Evidence for the existance of these genes will be discussed in the next section.

2. DNA-binding and DNA-bending by the SRY protein may be functionally important. Mutations in HMG-domain of the SRY protein in some XY females alters the *in vitro* DNA-bending property of the SRY protein.

3. *SRY* expression and activity may be sensitive to position and threshold effects. Observations which favor this hypothesis come from studies of human and murine sex reversal. Position effects can be inferred from deletions of the Y chromosome outside the *SRY* gene, described above and long range deletions of the murine Y chromosome that lead to either partial or complete failure of testis determination.[111] The presence of variable amounts of Y-chromosome sequences on the X chromosome of certain XX males and XX true hermaphrodites also suggests that *SRY* is sensitive to position effects. The importance of thresholds in sex determination is suggested by human pedigrees with sequence variants of the *SRY* gene which alter the biochemical properties of the gene product.

VII. AUTOSOMAL AND X-LINKED LOCI ASSOCIATED WITH ABNORMAL GONAD DETERMINATION

A. Syndromes Associated with Abnormal Testis Development

Several human syndromes are known which are associated in some cases with abnormal testicular determination. These include (1) the Smith-Lemli-Opitz syndrome (SLOS)[119,120] is an autosomal recessive trait, characterized by multiple congenital abnormalities, including microcephaly, mental retardation, hyponia, and short nose with anteverted nostrils. Ambiguous genitalia are frequently observed in male patients with SLOS. Differentiation of the gonads ranges from apparently complete testis (XY male pseudohermaphrodite) to dysgenetic streak gonads (XY female with pure gonadal dysgenesis). (2) Campomelic dysplasia (CD)[121] is a semi-lethal autosomal recessive disorder characterized by multiple shortening and bowing of the lower limbs, hypoplastic scapulae, cleft palate, tracheomalacia, and other defects. In a minority of cases with an XY karyotype there is a failure of the testis to develop and affected individuals have a female phenotype. The *SRY* gene is apparently normal. The chromosomal location of campomelic

is not precisely known, although chromosome 17 abnormalities are occasionally associated with the phenotype.[123-125] The analysis[126] of three individuals with apparently balanced *de novo* reciprocal translocations (two associated with sex reversal), suggests that the disease maps to 17q24.3-q25.1, distal to the growth hormone locus and proximal to thymidine kinase. Multiple abnormalities may be due to pleiotropic effects of a single gene or a contiguous gene syndrome.

B. Autosomal Abnormalities Associated with a Failure of Testicular Development

Sex reversal has been reported in a number of XY subjects with an apparently normal Y chromosome and rearrangements of the autosomes. There are six reported cases of females with an XY karyotype and a terminal deletion of chromosome 9p.[127] Patients have a female phenotype with development of the gonad ranging from partial to complete gonadal dysgenesis. The most distal breaks reported are at 9p24 and the most proximal at 9p21. This suggests that a gene located in the 9p24 region is involved. The most likely explanations are that the deletion has lead to the expression of a recessive allele on the other normal chromosome 9 or that the phenotype is caused by haploinsufficiency of a gene at 9p24-pter.

Abnormal sexual development has been described in 10 XY individuals with terminal deletions of the long arm of chromosome 10.[128] Again there is a range of sexual phenotypes from XY female with gonadal dysgenesis to male pseudohermaphrodite. The unmasking of a recessive allele on the other chromosome 10 is suspected in these cases.

C. An X-linked Gene Involved in Human Sex Determination?

Several observations in humans and other mammals suggest the presence of a gene on the X chromosome which when mutated interfers with normal testicular determination.

1. 46,XY gonad dysgenesis has been described in several human kindreds where there is an apparent X-linked recessive (or sex-limited autosomal dominant) mode of transmission.[129-131] An example of a human pedigree of partial gonadal dysgenesis with a mode of transmission compatible with X-linkage is illustrated in Figure 6. Pedigrees of XY females have also been described in the horse,[132] where the transmission of the sex reversed phenotype is compatible with an X-linked recessive mode of inheritance. Until linkage studies are performed pedigrees of XY sex reversal are a weak argument for the existence of an X-linked gene since the transmission of the sex reversed

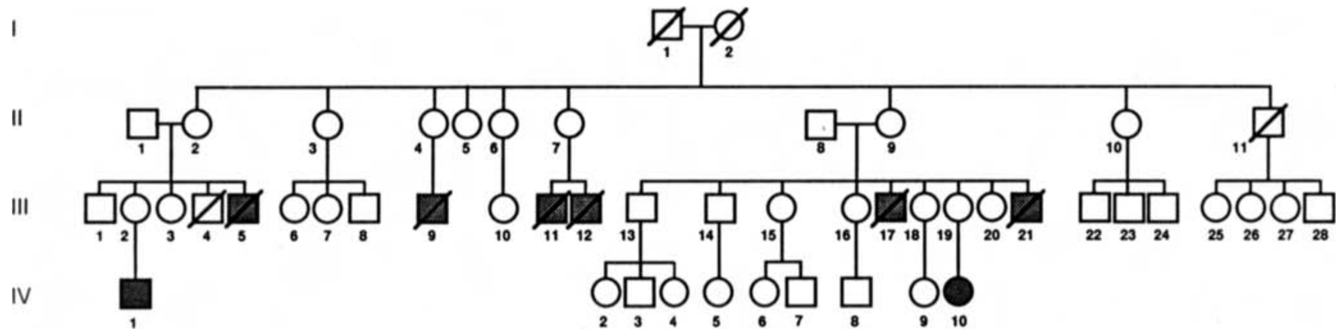

Figure 6. Pedigree of family C with a complex syndrome of mental retardation, partial gonadal dysgenesis, diffuse retinal pigmentation, and facial abnormalities. Affected males are indicated by solid squares. Patient IV10 is raised as a female. She presented with 46,XY partial gonadal dysgenesis. Expression is variable, with mental retardation and gonadal dygenesis the most common features.

phenotype is compatible with an autosomal sex-limited dominant mode of transmission.

2. Duplications of portions of the X chromosomes are associated with human XY sex reversal. A total of 15 XY individuals have been reported who presented with ambiguous or female external genitalia and carried partial duplications of the short arm of the X chromosome.[133-143] Where examined the *SRY* gene was apparently intact. In five cases histological examination of the testis revealed gonadal dysgenesis. Duplicated Xp material is present either on the X chromosome or translocated to the long arm of the X chromosome. The phenotype of these patients is variable. The severity of the phenotype loosely correlates with the size of the duplicated fragment. Some subjects present with severe pyschomotor retardation and facial dysmorphia as well as abnormal genital development, while others have sex reversal as the only phenotypic abnormality. The development of the gonad varies from complete to partial gonadal dysgenesis. Initial studies suggested that duplications involving the region Xp21.3 were associated with abnormal testis formation, while duplications outside this region did not affect testis development. How may a duplication of a portion of the X chromosome impair testis determination? Duplications of Xp leading to sex reversal in an XY individual contrasts with 47,XXY and 48,XXXY individuals who in spite of the presence of more than one copy of Xp, develop a male phenotype. To explain why XY individuals with Xp duplications are sex reversed and individuals with supernumary X chromosomes and a single Y are not, Ogata and colleagues[136] proposed that sex reversal is caused by a double dosage of an X-linked gene(s) which is normally subject to X-inactivation. Thus XY Xp+ subjects have two active copies of the gene and are sex reversed, and XXY subjects have only one active copy as only one X chromosome is active. Although the gene *ZFX* (the X homolog of *ZFY* a previous candidate gene for the testis-determining factor) maps to Xp21.3 it is unlikely to be the cause of sex reversal since it escapes X-inactivation.

In an attempt to precisely map the dosage sensitive region, Camerino and colleagues[142] defined the duplicated portion of the X in (a) four patients with cytogenetically visible duplications who presented with ambiguous or female external genitalia (partial gonadal dysgenesis was confirmed in three of these patients by histological examination of the internal genitalia) and compared this data with four other individuals who had duplicated regions but exhibited normal male development. The extent of the duplicated portion of the X in each of the four sex reversed patients was found to be different, each had a unique breakpoint. This excluded gene disruption generating the phenotype. Correlation between the portion of Xp duplicated and the phenotypic sex defined the minimal region to a 20 Mb region located between DXS418 and DXS 274, and between the Duchenne muscular dystrophy locus (DMD) and DXS319.

The majority of XY females do not harbor cytogenetically visible duplications of Xp. However, some may carry submicroscopic duplications of the sex reversing locus (designated DSS for dosage sensitive sex reversal) that might be detected by Southern analysis. To test this hypothesis, Camerino and colleagues screened XY females having apparently grossly intact sex chromosomes with probes from the Xp region. An analysis of 27 individuals with a female phenotype and an XY karyotype revealed a single patient (BI) who carried a duplicated fragment of less than 1 Mb distal to the DMD locus, in the proximal portion of the DSS critical region of 20 Mb. The minimum DSS region was further defined by determining the extent of overlap of the duplications of BI and the other cytogenetically detected duplications. Thus a common region of 160 kb was identified which defines the DSS critical interval. This region is immediately adjacent to the locus for adrenal hypoplasia congenita (AHC) and hypogonadotrophic hypogonadism. The AHC locus was mapped by deletion analysis of XY patients. Some of these patients are deleted for the entire region and have a complex AHC-GK-DMD syndrome and yet develop testis and have male external genitalia. It is possible that DSS and AHC correspond to the same locus and duplications of the same gene lead to abnormal gonad development, while deletions result in abnormal adrenal development.

3. XY sex reversal has been described in a number of rodent species.[144] Of particular relevance to this discussion is sex reversal in the wood lemming (*Myopus schisticolor*). In the wood lemming three sex chromosome types of females exist XX, X*X, and X*Y.[145] The asterisk designates an X-linked mutation that influences the sex-determining pathway, resulting in X*Y females. Males are XY. Occasionally X*XY animals occur. These may develop as males if the X* chromosome is inactivated or as females if the normal X chromosome is inactivated. The X* chromosome leads to a skewed sex ratio in population of these species. The two types of X chromosome in Myopus can be distinguished cytogenetically by G-banding. The short arm of the X* chromosome is about 7% shorter than the normal X chromosome. The X* chromosome probably arose from complex rearrangements of the short arm of the normal X chromosome. The molecular basis of X*Y reversal in the wood lemming is unknown, although one study[146] has localized the wood lemming homolog of the human *ZFX* gene to the rearranged portion of the X* by *in situ* hybridization and identified a variant *Zfx* gene (designated *Zfx**) on the X* chromosome by RFLP analysis. However, since the pattern of expression and transcript size of *Zfx* and *Zfx** are apparently identical, a causative role for *Zfx* in sex reversal in the wood lemming remains spectulative.

VIII. MAMMALIAN GENES ASSOCIATED WITH ABNORMAL GONAD DEVELOPMENT

A. Müllerian Inhibiting Substance

Müllerian inhibiting substance (MIS) is a glycoprotein dimer normally secreted by Sertoli cells of the fetal and adult testis.[147] It is the first substance known to be secreted by fetal Sertoli cells. It is also produced by the granulosa cells of the postnatal ovary. Production of MIS in the male fetus causes regression of the Müllerian ducts. The role of MIS in the adult ovary is unclear. Purified MIS induces the formation of seminiferous cord-like structures in fetal rat ovaries *in vitro*.[148] This observation suggests that MIS may have two roles: to repress formation of female genitalia and influence testicular formation. Transgenic mice chronically expressing *MIS*[149] exhibit a range of sexual ambiguities, which suggests that MIS may well have pleiotropic effects. Because of its biological activities in the developing fetal gonad MIS has been considered a candidate target gene for SRY in the testis-determining pathway. Genetic, molecular, and biological evidence suggests that this is not the case. An indirect relationship between SRY and MIS is suggested by the expression profiles of both molecules. *MIS* transcripts can be detected[150] by *in situ* hybridization in murine Sertoli cells at 12.5 d.p.c. *MIS* expression can be detected in Sertoli cells until after birth. However expression of *Sry* can be detected 48 h prior to the appearance of *MIS* transcripts.[151] This suggests that *SRY* does not directly control the expression of *MIS* (with the caveat that transcript levels do not necessarily reflect functionally active protein). Phenotype-genotype comparisons of individuals with mutations in the *MIS* gene also suggest that *MIS* is not a key gene in the testis-determining pathway. Certain individuals have been identified with defective regression of the Müllerian ducts (termed Persistent Müllerian Duct Syndrome [PMDS]). They have an XY karyotype with normal male external genitalia, though they often present with undescended testes. Their internal genitalia are feminine. They have bilateral fallopian tubes, a uterus, and an upper vagina. Some of these individuals carry mutations in the *MIS* gene.[152] The gonad is composed of testicular tissue. The *MIS* mutant phenotype (male pseudohermaphrodite) indicates that *MIS* cannot be a major gene in the sex-determining pathway although it does not formally exclude a regulatory function for *SRY* in *MIS* expression.

B. SF1 the Missing Link?

Recently, a murine factor (Steroidogenic factor 1 [SF1]) has been identified that may be the link between the sex determining and sex differentiation pathways. SF1 was originally identified as a transcriptional regulatory protein involved the expression of cytochrome P450 steroid hydroxylases in murine

adrenocortical cells.[153] The cytochrome P450 steroid hydroxlyases convert cholesterol to various steroid products. Murine SF1 closely resembles an orphan nuclear receptor isolated from *Drosophila,* designated fushi tarazu factor 1 or FTZ-F1.[154] FTZ-F1 protein is a positive regulator of transcription of the *ftz* gene, a well-studied *Drosophila* zygotic segmentation gene. Like other members of this family the SF1 protein has distinct ligand-binding and DNA-binding domains. *SF1* and another cDNA designated embryonal long terminal repeat-binding protein (*ELP*) arise from the same structural gene by the use of alternative promoters and splicing.[155] The mouse gene giving rise to both isoforms has been designated *Ftz-F1.*[156]

As well as its regulatory function in adrenocortical cells several observations suggest that SF1 controls gene expression in gonadal tissues.[157] Rat *SF1* gene transcripts were detected in ovarian tissue and in a rat Leydig tumor cell line. SF1 also appears necessary for the expression of P450 aromatase, an enzyme that catalyzes the conversion of androgens to estrogens including estradiol 17β. Aromatase is expressed primarily in the adult ovary. In adult mice *SF1* transcripts can be detected in all primary steroidogenic tissues including the adrenal cortex, testicular Leydig cells, Sertoli cells, and theca cells of the ovary.[156,158] The timing of *ELP* and *SF1* expression is different. *ELP* transcripts cannot be detected in murine tissues from embryonic day 8 to adult, whereas *SF1* transcripts can be detected in the urogenital ridge as early as embryonic day 9-9.5. Expression can be detected in fetal Sertoli cells. This suggests that the protein may have a role in the sex-determining process. Targeted disruption of the *Ftz-F1* gene to produce mice homozygously deficient in both isoforms, *SF-1* and *ELP,* also favors this hypothesis.[159] All *Ftz-F1* null animals lacked adrenal glands and gonads and died by postnatal day 8, probably from adrenocortical deficiency. This demonstrates that *SF1* is essential for the embryonic development of the adrenal glands and gonads.

What is the relationship between *SF1* and *MIS*? Expression of the mouse *MIS* gene in transfected Sertoli cells requires a conserved CCAAGGTCA element which matches exactly the recognition sequence of the nuclear receptor *SF1.*[160] This element is bound by a single Sertoli cell nuclear protein that is recognized by a specific anti-SF1 antibody. *In situ* hybridization and RNase protection methods demonstrate that *SF1* and *MIS* are co-expressed in Sertoli cells during embryonic development. Both *SF-1* and *MIS* transcripts rise simultaneously in the male in the period just following testis formation, during Müllerian duct regression. In the female the level of *SF1* transcripts sharply declines during this period. These observations imply that *SF1* is a major positive regulator of *MIS* gene expression during the period of Müllerian duct regression.

C. The Wilms' Tumor Gene, *WT1*

Wilms' tumor or nephroblastoma is a pediatric cancer of stem cells of the developing kidney.[161] Some patients with Wilms' tumor also present with genitourinary abnormalities such as cryptorchidism, hypospadias, and complete or partial gonadal dysgenesis. Cytogenetic analysis indicates that in a proportion of cases Wilms' tumor is associated with a loss of gene function at chromosome 11q13.[162,163] A gene from this region was isolated and termed *WT1*.[164,165] The *WT1* gene spans approximately 50 kb and is comprised of 10 exons, encoding a protein with a proline/glutamine rich amino-terminal domain, and four contiguous $(Cys)_2$-$(His)_2$ zinc-fingers.[166] The zinc finger domains share homology with those of the early growth response (ERG) family of transcription factors. *WT1* is alternatively spliced giving rise to four different transcripts.[166] The alternative splices have been conserved between human and mouse genes. Expression of the gene is limited to the developing genitourinary system. Transcripts can be detected in the developing kidney, genital ridge, fetal gonads, and mesothelium.[167-169] WT1 has the capacity to function as both a positive and negative transcriptional regulator. In cotransfection assays, WT1 transcriptionally repressed several growth-related genes including EGR-1, insulin-like growth factor II, and the platelet-derived growth factor (PDGF) A-chain gene.[170-173] WT1 can also act as a positive regulator by activating transcription through a separate activation domain.[174,175] This suggests that WT1 has a complex role in regulating cellular differentiation and proliferation. Two lines of evidence define *WT1* as a tumor suppressor gene. First, heterozygous deletions or point mutations in the *WT1* gene are associated with a predisposition to Wilms' tumor, with a loss of heterozygosity occuring at the *WT1* locus in tumor tissue.[165] Second, wild-type WT1 protein will suppress the growth of a Wilms' tumor cell line expressing an endogenous WT1 variant.[176]

Of particular interest to this discussion are a group of patients who present with Denys-Drash syndrome. This is a complex phenotype of nephropathy and abnormal development of the gonads.[177,178] Patients have a high incidence of developing Wilms' tumor. The invariable feature of this syndrome is a complex nephropathy which results in proteinuria and usually leads to nephrotic syndrome. Initially this syndrome included only XY males with ambiguous genitalia, but was broadened to include XX individuals. The most common genital abnormality described is XY male pseudohermaphroditism, although true hermaphroditism has been described. In patients with an XY karyotype malformations of the external genitalia vary considerably, from penoscrotal hypospadias with bilateral cryptorchidism to a completely female phenotype with a streak of fibrous tissue instead of a testis. XX patients usually have normal female external genitialia, although the ovaries may occasionally be dysgenetic.

The vast majority of Denys-Drash patients harbor a constitutional point mutation in one of the *WT1* alleles,[179–183] frequently there is a missense mutation in the region encoding the zinc-finger binding domains although mutations which alter the balance between alternatively spliced forms of WT1 have been documented[184] (unpublished data). In particular, more than 80% of the mutations occur in exons 8 and 9 encoding zinc fingers 2 and 3. Of these more than one-half occur at a hot-spot residue Arg^{394} in exon 9.[179,180,182] It is predicted that these mutations will alter the physiological function of the WT1 protein. To account for the severe phenotype observed in these patients with a constitutional mutation in only one allele a dominant negative mechanism has been proposed,[185] whereby the abnormal product from one allele functionally antagonizes the product from the other allele.

If mutations in *WT1* can interfere with normal gonad determination, what is the relationship between *SRY* and *WT1*? Several observations suggest a close relationship between *WT1* and *SRY*. In the murine genital ridge *wt1* transcripts can be detected during the same period as *Sry* expression.[167–169] Mutations in the *WT1* gene can be associated with abnormal gonad development. Further evidence for a role of *WT1* in gonad development was demonstrated in transgenic mice with constitutional homozygous mutations in the murine *wt1* gene.[186] Heterozygous mice developed normally and did not develop tumors. However mice homozygous for the *wt1* mutation died *in utero* at 13-15 d.p.c., probably because of a failure of the heart to develop properly. In the homozygous mice kidneys failed to develop because of the absence of the ureteric bud. The gonads also failed to develop. This suggests that *WT1* may not be sex determining per se but be necessary for the commitment of gonad tissue from the mesonephros. Since it is also expressed in fetal and adult Sertoli cells of both humans and mice, it may be necessary for maintenance of gonadal tissue.

IX. A SIMPLE GENETIC MODEL OF MAMMALIAN SEX DETERMINATION

Many questions remain unanswered about mammalian sex determination. What controls the expression of *SRY*? Does *SRY* have a positive or negative regulatory function? Does it control the activity of one or more genes? How many genes are in the sex-determining cascade? Many mutants altered in sexual phenotype permitted the identification of key regulatory genes in *Drosophila* and *C. elegans*. Extensive genetic analysis of these mutants led to the construction of models of sex determination. Likewise the analysis of mammalian sex reversed phenotypes may provide a response to at least some of these questions.

A. SRY Negatively Regulates an Inhibitor of Male Development

Familial cases of sex reversal where sex reversal is either complete (XX males) or partial (XX true hermaphrodites) and affected individuals lack *SRY* are informative. In Figure 7 two pedigrees of human XX sex reversal are indicated. In family J one affected sib is an XX male with ambiguities while his sister (a phenotypic female with ambiguities) is an XX true hermaphrodite. In family P the two affected sibs are XX males with ambiguities.

From observations of *SRY*-negative familial cases of XX sex reversal one can draw the following conclusions:

1. Male sex determination can occur in the absence of *SRY*.
2. Affected sibs exhibit a spectrum of phenotypes ranging from XX male to XX true hermaphrodite. This indicates that these clinically distinct phenotypes may be attributed to a single genetic event.
3. Since the parents and other ancestors did not exhibit sex reversed phenotypes it is simplest to interprete these pedigrees in terms of a single autosomal or X-linked locus *Z*, whose mutant recessive allele (Z^-) confers a male phenotype. XX individuals who are Z^-/Z^- homozygotes will express a male phenotype (see Figure 8). Therefore XX sex reversal can be regarded as a loss-of-function at the *Z* locus rather than a gain-of-function mutation in the testis-determining pathway.

What can an autosomal recessive mode of inheritance of XX maleness in the absence of SRY tell us about the nature of the sex-determining pathway? The SRY protein is formally a positive regulator of male development. However, the genetic observation that *SRY* is not needed for sex reversal in the absence of *Z* (Z^-/Z^- homozygotes), suggests that the function of SRY is to repress the synthesis of *Z* or antagonize the activity of Z.[187] In this model the wild-type function of Z is to negatively regulate the expression of male-specific genes (Figure 9). In normal XX females Z negatively regulates the expression of male-specific genes. In XY males SRY may repress the synthesis of *Z* or inhibit Z activity. This would allow the expression of male-specific genes. XX patients who lack *Z* due to mutation will develop testis as inhibition of male-specific genes will not occur.

This model makes certain predictions. Mutations may occur in *Z* termed Z^i such that *Z* is insensitive to SRY action. Since SRY is a DNA-binding molecule, Z^i mutations may be insensitive to SRY repression and thus may be considered to be constitutive mutations. An XY individual harboring a Z^i mutation would be predicted to exhibit a female phenotype. The *SRY* gene is apparently normal in about 80% of XY females with complete gonadal dysgenesis. Some of these individuals may carry a Z^i mutation.

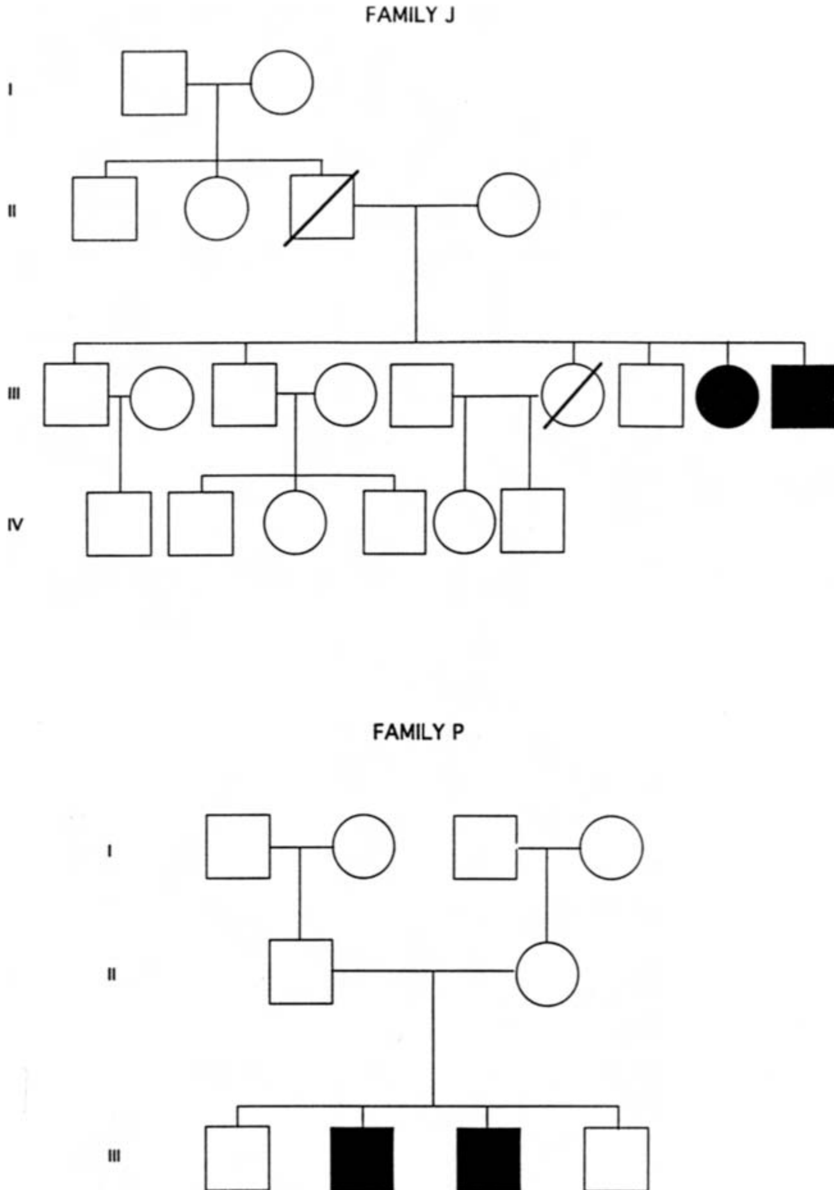

Figure 7. Human pedigrees of XX-*SRY* negative sex reversal. Symbols are as described in the legend of Figure 5. In family J one case is an XX male with ambiguities, the other case is an XX true hermaphrodite. Family P. consists of two cases of 46,XX true hermaphroditism.

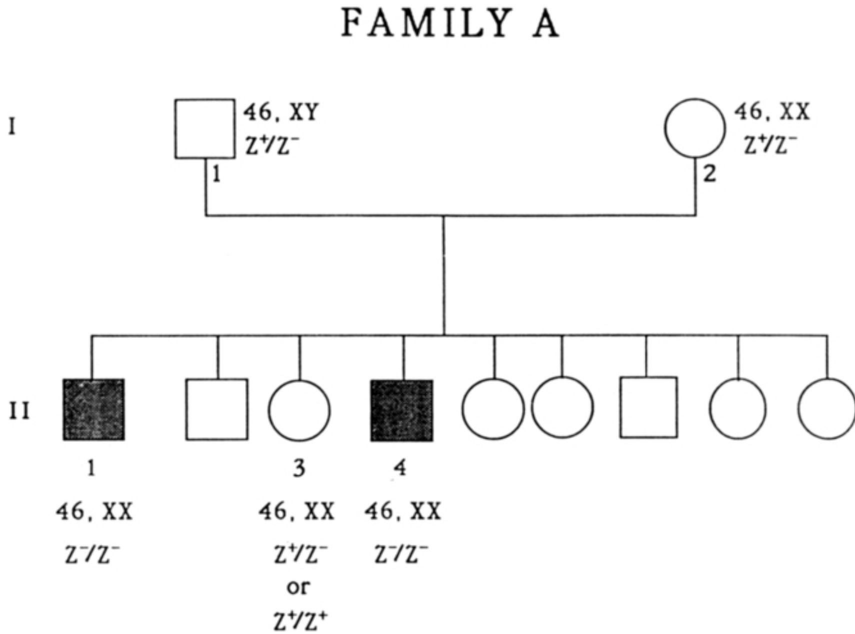

Figure 8. Pedigree of family A. Two cases of SRY-negative 46,XX males with genital ambiguities are indicated by filled squares. The inferred genotypes Z+ and Z- are indicated

This model proposes that the major function of SRY is to control the activity of a single gene *Z*. It is possible that SRY may have several target genes, however, this is unlikely since the range of phenotypes in XXZ^- individuals is considerable, from XX male to XX true hermaphrodite. The phenotypic variability can be explained by proposing that the Z^- mutations are leaky to different extents. This indicates that the major action of SRY is to inhibit *Z*.

This genetic model makes few predictions about the molecular nature of the interactions between SRY and Z. SRY may control the synthesis of Z. However, other more complex hypotheses are possible. *SRY* may control the activity rather than the synthesis of Z protein. For example Z protein might inhibit the transcription of male-specific genes and SRY protein might compete with Z for its binding site. Ultimately the answers to these questions await the cloning of the *Z* gene. If SRY represses the transcription of Z, then the *Z* gene may be cloned as a female-specific cDNA.

The model may also be applicable to other mammals. XX sex reveral has been described in dogs, pigs, and goats where in some cases, it has been

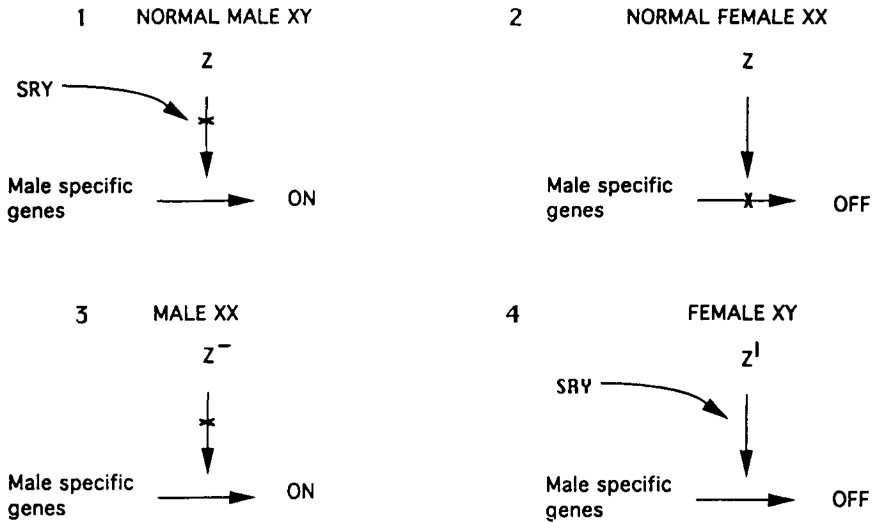

Figure 9. A regulatory cascade hypothesis for mammalian sex determination: *SRY* antagonizes a negative regulator of male development. Normal and sex reversed conditions are indicated. ON and OFF represent the activity state of male-specific genes. (1) In wild-type males, SRY protein is present and antagonizes Z activity (as drawn) or inhibits Z by acting as a repressor of Z synthesis (not drawn). In the absence of Z activity, male-specific genes are ON and a male phenotype develops. (2) In wild-type XX females *SRY* is absent. Z is active and inhibits male-specific genes. As a consequence female differentiation occurs. (3) XX sex reversed patients who lack *SRY* and yet develop a male phenotype are proposed to be defective in the Z gene due to being homozygous for a *Z*- mutation (indicated by a X). In the absence of functional Z activity, male development occurs. (4) XY sex-reversed females with a wild-type *SRY* gene are predicted to harbor a mutation at the Z locus (Z^i) which renders them insensitive to the antagonizing effect of SRY, Consequently Z protein inhibits male sex determination, permitting female sex determination.

attributed to a monofactorial mode of inheritance.[188-191] One such recessive mutation leading to sex reversal is the *polled* mutation of goats.[192] The *polled* mutation is characterized by the suppression of horn formation and in XX individuals the formation of variable amounts of testicular tissue. The absence of horns is due to a dominant gene, whereas the mutation associated with genital abnormalities is recessive. XX intersex pigs and goats homozygous for the *polled* mutation lack the *SRY* gene (C. Cotinot, personal communication).

The proposal that a positive regulator, SRY may function mechanistically by inhibiting an inhibitor has precedents from other organisms. In yeast, the regulatory protein a1-α2 triggers the initiation of meiosis by repressing transcription of an inhibitor of meiosis. As discussed earlier many of the steps in somatic sex determination in *C. elegans* involve inhibitors of inhibitors. For example *her-1* negatively regulates *tra-2* activity, thus permitting functional activity of the *fem* gene products.

B. Abnormalities of Xp and Sex Reversal

Data consistent with this model of mammalian sex determination have come from the study of XX females with Xp duplications. As discussed earlier duplications of distal Xp sequences have been described in XY sex reversed females with either complete or partial testicular dysgenesis. An important observation is that deletions of the *DSS* region in an XY individual do not effect testis determination. This results in the apparently paradoxical situation that the gene is not required in the presence of *SRY* for normal testis development and yet a double dosage disrupts testis formation, however, these observations are entirely consistent with the genetic predictions of the model (Figure 10). *DSS* may be an inhibitor of male development. In a normal XY male *SRY* represses or functionally antagonizes the activity of the *DSS* gene thereby permitting testis formation. Individuals who are XY and are deleted for *DSS*, lack *DSS* inhibitory activity and male specific genes will be expressed leading to testis formation. The sexual phenotype of these individuals is predicted to be identical to that of a normal male. XY individuals who are duplicated for Xp and carry two doses of *DSS* may have impaired testicular development due to incomplete repression of the *DSS* gene by SRY.

If *DSS* corresponds to the "*Z*" gene one would predict that certain XX true hermaphrodites or XX males should harbor loss-of-function mutations at this locus. We have recently identified[193] an XX true hermaphrodite with a terminal deletion of the short arm of one of her X chromosomes (Xp11.3-pter). The deleted X chromosome is inactivated in all cells analyzed. The *SRY* gene was not detected in DNA samples extracted from peripheral blood or from ovotestes, excluding a mosaicism. In addition to sex reversal the patient also has other somatic abnormalities: profound psychomotor retardation, strabismus, absence of the vermis of cerebellum, and unusual skin pigmentation. Deletions of Xp21.1-pter in XX individuals are usually only associated with short stature in otherwise phenotypically normal females, since the deleted X chromosme is subject to inactivation.[194] The phenotype of the true hermaphrodite can be interpreted in two ways. Either it is an indirect consequence of the deletion or it is an independent event. The deletion may have unmasked a recessive allele(s) on the apparent normal X chromosome. This constellation of phenotypic abnormalities is unusual. Mental retardation,

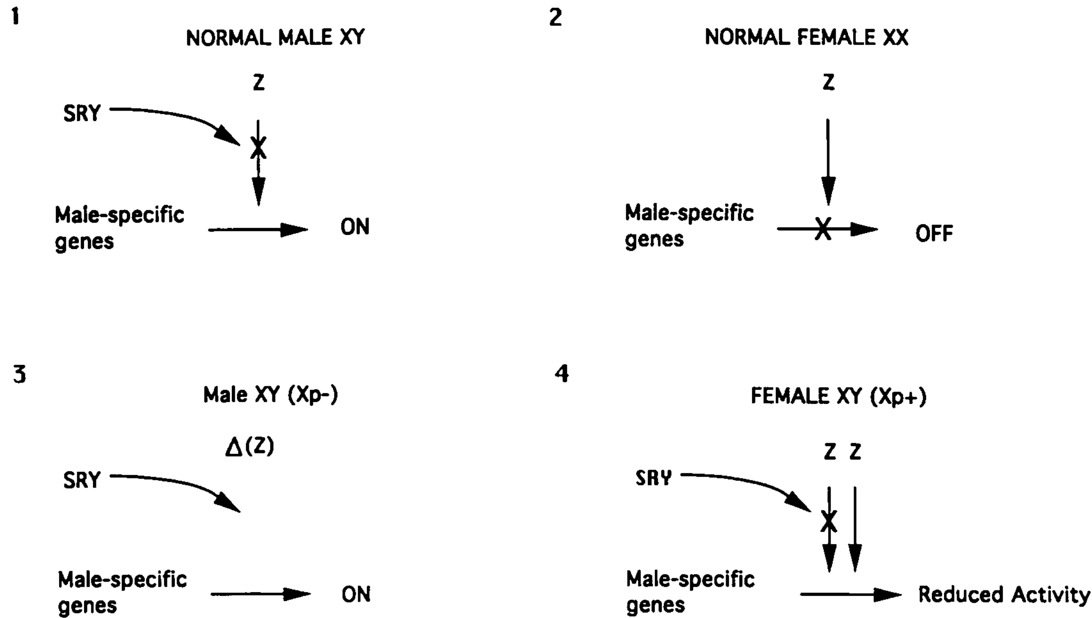

Figure 10. Schematic representation of interaction between *SRY* and *Z* gene located on the X chromosome. (1) and (2) are as described in the legend for Figure 9. (3) In XY individuals deleted for *Z* there is no inhibition of male-specific genes, male-specific genes are *ON* a male phenotype occurs. (4) When the *Z* gene is present in two copies as described for some XY females *SRY* is unable to completely repress the synthesis of *Z* activity. As a consequence there is partial *Z* activity and male-specific genes are inhibited to a lesser or greater extent.

strabismus, and anomalies of skin pigmentation have been mapped to a number of regions of the short arm of the X chromosome. Absence of the vermis of the cerebellum is characteristic of a rare disorder, the Dandy-Walker syndrome. The etologie of this syndrome is obscure, however, Cowles et al.,[195] described a rare familial case with this condition. The transmission of the phenotype was compatible with an X-linked recessive mode of inheritance.

X. CONCLUSIONS

It is evident from the analysis of the sex determination pathway in non-mammals that there is a lack of mechanical or molecular conservation. Sex determination is one of the most diverse developmental pathways. One cannot therefore extrapolate molecular data from these organisms for example to clone mammalian sex-determining genes, or make predictions about the molecular interactions between the components of the sex-determining pathway. This situation contrasts with other developmental processes such as body pattern formation which is controlled by homeobox gene clusters, where similarities in sequence, function, and structure are conserved between vertebrate and invertebrate embryos.[196] However, at a superficial level there are broad similarities between these sex-determining systems.[2] The basic scheme consists of a signal, a key gene, and a few subordinate control genes. The purpose of the cascade is to achieve differential activity of the last gene in the cascade.

In 1987 Nöthiger and Steinmann-Zwicky[195] proposed a common principle for sex determination in insects. A primary signal is read by a key gene. The activity of this gene, either *ON* or *OFF*, controls the activity of a double switch gene. The activity of the double switch gene has mutually exclusive functions resulting in the expression of either male or female differentiation genes. The primary signal inhibits or represses the activity of the key gene. In this system the key gene must be *ON* to determine the female pathway and *OFF* to determine the male pathway. *ON* and *OFF* refer to the activity states of the key gene. Although Nöthiger and Steinmann-Zwicky used *Drosophila* as a paradigm, parallels can be drawn with genetic model of mammalian sex determination described earlier. *SRY* is the primary signal of mammalian sex determination. It is formally a positive regulator of testis formation and consequently male development, however, it may function mechanistically by repressing the synthesis or antagonising the activity of a key gene (*Z*). This postulated gene *Z* must be *ON* to determine the female pathway and *OFF* to determine the male pathway. Does this mean that there is a unifying principle underlying sex determination in different taxomonic groups including mammals? Ultimately the answer to this and other questions relies on the identification of other mammalian sex-determining genes.

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COMMENTARY:
GENES, CHROMOSOMES, AND SEX

RAM S. VERMA

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I. INTRODUCTION

Sex determination is a biological process which leads to sexual differentiation. The presence of testes in males and ovaries in females are the events that ultimately lead to sex determination.[1] One of the most perplexing questions which remains elusive is the developmental process which governs the fate of

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a cell during differentiation. Whether it is a whole chromosome or a single gene which determines sex is being currently investigated using various species.[2] Nevertheless, it is a long held assumption that sex differences in many species, including humans, are due to the heteromorphic sex chromosomes that segregate to offspring in an orderly fashion; males having XY and females having XX. It provided some clues about the initiation of mammalian sex differentiation. However, some intriguing exceptions have been found: Humans with a single X chromosome are female whereas individuals with multiple X and a single Y develop as males,[3] while in *Drosophila melanogaster* and some other invertebrates, sex determination is triggered by the X:autosome ratio. In marsupials, the phenotypic sex differences take place before the development of gonads. Therefore, sex determination and sex differentiation are two related but distinct fields of development that are currently being examined.[4] Deviations from the normal sex chromosomal constitution in humans have provided new insight into the role of sex chromosomes in sex determination. One such extreme example the discovery of XX males and XY females which has resulted in the volumenous literature concerning the role of gene(s) in sex determination.[5] The discovery of the sex-determining gene for males termed testis-determining factor, *TDF*, in humans and *Tdy* in the mouse have resulted in numerous accounts of speculation and it is a widely accepted scenario that the sex-determining region of Y (SRY) is the *TDF*.[6] Numerous reviews are available on this subject.[7-10] Therefore, I shall provide a brief commentary concerning the role of genes and chromosomes concerning sex determination.

II. X CHROMOSOME INACTIVATION

The developmental process of X chromosome inactivation is a means of regulating gene dosage in females where only one X chromosome is genetically active which in turn results in dosage compensation for X-linked genes.[11] Nevertheless, the mechanism controlling X-chromosome inactivation in mammals remains unknown.[12] The general rule is that both X-chromosomes are active during oogenesis and in the cleavage stage of female embryogenesis the inactive X chromosome undergoes reactivation prior to entering meiosis. The inactivation process is random in somatic cells. The inactivated X chromosome is late replicating and transcriptionally inactive. The inactivation is initiated by an inactivation center called X1C.[13] However, at present there are eight genes which escape inactivation in humans.[14,15,16] Nevertheless, seven of them are active in both chromosomes while the X1ST gene is exclusively found on the X-chromosome (for review see reference #16). Genes which escape inactivation in humans are an intriguing phenomenon.[17] Inactivation is random but a fixed process and is maintained during somatic cell division. This process is maintained by the process of DNA methylation. It is remarkable to note

that only one X-chromosome remains active in somatic cells.[18] The inactivation phenomenon plays a very important role concerning human diseases. For example, there are more than 200 diseases which are X-linked whose severity depends upon the nature of inactivation.

Ongoing research pertaining to the X-inactivation center is being performed at a rapid pace and its role in the initiation of inactivation has begun to unfold.

III. THE HUMAN Y CHROMOSOME

The mammalian Y chromosome carries the male sex determinant genes. The human Y chromosome is unique and has been divided into two portions: the euchromatic portion comprises the entire short arm and one third of the proximal long arm. The remaining portion is heterochromatic. The combination of molecular genetics and deletion mapping have provided a wealth of knowledge pertaining to the human Y chromosome. The pseudoautosomal regions of the Y chromosome is involved in homologous recombination with X-auromosapeia while the Y-specific region is not. This has been the fundamental basis for proper segregation of the X and Y chromosomes. There are not hot spots of recombination in the pseudoautosomal region. Males with XX and females with XY genotypes have led to the localization of *TDF* where Y-specific sequences have been interchanged. The incidence of sex reversal in the general population is approximately one in 20,000 births. At least two genes have been isolated from the pseudoautosomal region; granulocyte macrophage colony stimulating factor (*GM-R*) and 12E7 (*MIC2*). *MIC2* escapes X-inactivation. There are at least two genes in the Y-specific region and we recommend reading the excellent reviews which are available on this subject. 19-24.

IV. EVOLUTION OF SEX CHROMOSOMES

The gamete dimorphism which involves distinctly different sex chromosomes among males and females has been universal among eukaryotes. In some circumstances the male is heterogametic having X and Y chromosomes while the female is homogametic with only XX chromosomes. The reverse situation is noted among amphibia.[25] The variables size of sex chromosomes in many species suggests that highly repeated DNA sequences have no functional significance.[26-28] The evolutionary history of sex chromosomes cannot be traced. However, there are a number of models which explain the mutational events leading toward the origin of dioecy.[29] Whether the allelic differences of sex chromosomes are due to recombination or mutation, the heteromorphy between the sexes remains a matter of debate. The environmental sex determination based upon temperature has begun to shed some light on the

evolutionary process.[30] The evolutionary process of the Y chromosome and the conservation of two genes in the Y-specific region in eutherians is interesting, but autosomal sex-determining gene in marsupials has intrigued us all.[31] Conservation of the pseudoautosomal region on Xp and Yp in humans and the absence of a pairing region between the marsupial X and Y chromosomes raises a serious question concerning the origin of this region. Convergence and divergence of certain regions on sex chromosomes have provided an armamentarium to molecular biologists to further explore the evolutionary concept of mammalian sex chromosomes.

V. GENETIC DISORDERS OF SEX DIFFERENTIATION

Sex chromosomal abnormalities have more than their fair share which can be either structural or numerical with an overall frequency of about one in 500 births, but the clinical consequences are far less severe than those associated with autosomal disorders. The most common genetic disorder of sex chromosomes is the trisomic type (XXY, XXX, and XYY) while monosomy is rare in live births as the majority of cases spontaneously abort. Structural abnormalities are rare except for the Xq which may result in an isochromosome while mosaicism is far more common. There are at least four well characterized syndromes associated with sex chromosome aneuploidy: Klinefelter syndrome (47, XXY), trisomy X (47, XXX), double Y (XYY), and Turner syndrome (45, X and variant forms). The karyotype of a true hermaphrodite is 46, XX while about 10% of the cases could be 45, XY. Male pseudohermaphrodites are 46, XY while females are 46, XX. Individuals with androgen insensitivity syndrome, formerly known as testicular feminization, are 46, XY. Currently, we are searching for genes which have been postulated to be implicated in the development of abnormal phenotypes with so-called normal karyotypes.[33,34]

VI. SEX CHROMOSOME IMPRINTING

The functional differences between the maternal and paternal chromosomes is a most perplexing phenomenon resulting in the differential expression of genes during development which is termed imprinting. The imprinting phenomenon has been further vocabulized as chromosomal, parental, genomic, genetic, gamete, or gene imprinting.[35] One of the most common problems in humans is sex-chromosomal aneuploidy. Currently X chromosome imprinting is a heated subject owing to X chromosome inactivation. It is suggested that the paternal X chromosome is preferentially inactivated in certain tissues of female mammals.[36] Furthermore, gamete-specific imprinting reflects the randomness of X chromosome inactivation.[37,38] The preferential expansion of triplet repeats on the X chromosome in patients with fragile-X syndrome needs

to be addressed. Though the exact nature of X chromosome imprinting is unknown, methylation has been proposed as a possible mechanism for imprinting.

VII. SEX REVERSAL

In most mammals, the sex-determining signals are on Y chromosomes. Embryos bearing Y chromosome irrespective of the number of Xs (i.e., XY, XXY, XXXY, and XXXXY) develop testes while its absence (i.e., X, XX, XXX, and XXXX) results in the development of ovaries.[39] Nevertheless, in humans, XX males and XY females are the best known examples where the Y-borne gene is referred to as the testis-determining factor or TDF which is presently the focus for the zeroing in on the sex reversal mechanism.[40] The Y-specific sequences has been isolated where the *SRY* gene has been implicated for sex reversal due to aberrant X-Y interchanges.[41] The phenotype of XX males and XY females depends upon the amount of the chromosomal material exchanged from Yp.[42] There are a number of reviews available on this subject and readers are advised to refer to them.[43-51]

VIII. TEMPERATURE-DEPENDENT SEX DETERMINATION

The genetic basis of sex reversal in humans and temperature-dependent sex determination in lizards, turtles, and all crocodilians do not imply that chromosomal sex determination (CSD) and temperature dependent sex determination (TSD) are inclusive events. While the TSD phenomenon is well established[52-55] the genetic basis still remains to be worked out.[56] The ontogenetic patterns of sexual development remain the same despite the differences in causation. It is a process in terms of an initial gene trigger leading to differentiation related to regulatory gene hierarchies.[57] The genetic cascade leading to sex differentiation in vertebrates is not yet known but is evident in *Drosophila*.[58-63] It is the thermosensitive locus which will determine which one (CSD and/or TSD) determines the underlying pathways in temperature-dependent sex determination for its maintenance and evolution of dimorphism.

IX. SUMMARY

The origin of differential tissues from pluripotent cells in the embryo during the developmental process has intrigued geneticists, biologists, and evolutionists alike. The testis and the ovary both develop from the gonodal ridge resulting in two sexual forms with a few exceptions. Sex determination leading to sex differentiation depends upon a number of factors, such as the

X:autosome ratio, temperature, hormones, and dominant male or female genes. The fundamental theory behind dimorphisms is that the Y chromosome acts in some way as a dominant male-determining factors while the presence of two X chromosomes leads to normal development of a female. Cloning of a gene on the Y chromosome has been carried out. A number of views regarding mammalian chromosome inactivation are reviewed here. The evolutionary concept concerning pseudoautosomal regions and pairing or mispairing of them has dire consequences. The diversity of sex-determination systems in various taxa is quite intriguing while similarities must have arisen through a convergent path. The most recent investigations using molecular techniques in mapping sex-linked genes have been reviewed and the sex reversal phenomenon in mammals have opened new vistas concerning *SRY* variant, but other various mutated genes which affect the sex phenotypes remain to be explored.

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