# Mammalian Gonadal Determination and Gametogenesis

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*Summary.* Although a relationship between the X and Y chromosomes and mammalian sexual development has long been recognized, a detailed understanding of this relation is still lacking. Recent advances in somatic cell genetics and recombinant DNA technology should provide the tools for solving this fundamental problem in developmental genetics.

Mammalian sex determination appears, on first examination, to be genetically one of the simplest developmental processes. Female-male sexual dimorphism parallels XX-XY chromosomal dimorphism; therefore, it must be concluded that structural or regulatory genes governing sex differentiation are on the sex chromosomes. In addition, mutations at these loci have dramatic consequences that are easily recognized as hermaphroditism, pseudohermaphroditism, or infertility. But, although much progress has been made in identifying and localizing the sex-determining genes within the genome, the task is by no means complete. Until this mapping is completed, a full understanding of this biologically important process is not possible.

In this article, we review what is known about the sex chromosomes and major sex-determining loci and their roles in two major aspects of sex differentiation—gonadal determination and gametogenesis (that is, spermatogenesis and oogenesis). Where possible, we attempt to clarify the relation between the major sex-determining genes and the sex chromosomes and to describe efforts to elucidate it through DNA sequence isolation and cloning.

In contrast to most vertebrates, mammalian sex development cannot be modified by manipulating the embryonic environment [except possibly the opossum *Didelphys virginiana* (*1*)]. A particularly instructive example of reversible sex comes from experiments with the fish *Oryzias latipes*. The XY embryos of this organism can be made into fertile females with estrogen (*2*) and can be mated to XY males to produce viable YY male offspring (*3*). The importance of this result lies not only in the demonstration of functional sex reversal; it also shows that the X chromosome is not required for viability in these fish. Clearly, the enzymatic functions coded for by the X are also represented on the Y, a fact strongly suggesting that the sex chromosomes arose from a common ancestral chromosome (*4*). As the X and Y became further specialized, the Y apparently lost most of its structural loci, making the X the predominant repository for these genes. This process has made the X essential for viability, and thus there must be a strong evolutionary pressure that maintains the X intact (*4*). Because of the conservation of X linkage, sex-determining genes on the X of one mammal are likely to be on the X of all others, including man. In contrast, loss of structural loci from the Y has made conservation of this chromosome less important. Data establishing Y linkage in one mammal may not be so easily generalized to others.

## The Y Chromosome

*Gonadal determination.* The obvious differences between the normal male and the normal female karyotypes are that the male has a Y chromosome and only one X, whereas the female lacks a Y and possesses two X's (Fig. 1). For many years, it was not known whether X chromosome dosage or the Y chromosome was the determining factor in gonadal differentiation. The discovery of XO female mice, however (*5*), showed that in this organism the Y determines maleness. Subsequently, XXY male mice were found (*6*), and humans with as many as four X chromosomes and a Y were clearly shown to be males with unequivocally testicular gonadal histology (*7*). It is now accepted that in mammals the Y chromosome carries structural or regulatory loci directing the formation of a testis. Accordingly, much attention has been focused on abnormalities of this chromosome in an effort to define these loci.

Analysis of structural abnormalities of the Y chromosome in man have suggested that the Yp arm of this chromosome carries genes directing testis formation. Several examples of isochromosome for the long (Yq) arm of the Y (iYq) have been observed to result in failure of testicular development (*8*). In addition, simple absence of the short (Yp) arm results in a female phenotype, with streak gonads characteristic of the XO karyotype (*9*). When a portion of the Yq arm is absent and the Yp arm remains intact, testicular tissue is clearly present (*10*). Translocations of the Yp arm to the X chromosome have also been associated with the syndrome of XX maleness. Fergusen-Smith (*11*) has suggested that homology between the X and Y chromosomes may result in X-Y interchange. Madan (*12*) has reported an example of an XX male, one of whose X chromosomes was increased in size by an amount equal to the quinacrine-negative (short arm) of the Y; and Evans *et al.* (*13*) have utilized chromosome banding techniques to demonstrate the presence of Yp material on the X chromosomes of XX males.

These data give support to the proposition that the Yp arm carries the testis-determining genes. However, other studies of abnormal Y chromosomes complicate the situation. One patient has been described (*14, 15*) whose Y chromosomal elements were translocated to chromosome 8 and were exclusively of Yq origin, yet this individual's gonads revealed testicular elements on histologic examination. Another example has been reported of a mosaic whose Y chromosomal material consisted solely of Yq elements but whose gonads contained testicular tissue (*16*). Finally, Siebers *et al.* (*17*) have reported an example of iYp without testes. Thus, the association of

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0036-8075/81/0320-1265$01.75/0 

the Yp arm with testicular determination is not invariable.

It was hoped that the discovery of H-Y antigen, a male-specific cell surface protein (*18*), and the development of a serologic assay for measuring it (*19*) would solve these discrepancies. This antigen has been associated with testicular determination in mammals (*20*) and has been proposed to be the testis-determining substance (*21*). With the testis-determining gene product identified, the problem of mapping the testis-determining locus is reduced to one of mapping a single gene. There are problems, however, with the use of such an assay for mapping. While the assay is more sensitive than histologic analysis, it is also less direct. For H-Y antigen to exert its presumed effect, the gene must be appropriately regulated, and the antigen must be able to interact with the target cells.

Efforts to map the H-Y locus have been based on application of the serologic assay to patients with karyotypic anomalies similar to those described above. The report that XYY patients express more H-Y antigen than XY individuals (*22*) led to the initial proposal that the gene was located on the Y chromosome. H-Y assays on persons carrying translocations of the Y chromosome suggested that the H-Y locus was on the proximal Yp arm (*23*). The difficulty with these latter data was, however, that the presence of Yq material in H-Y positive cases could not be ruled out. In fact, in one H-Y positive individual, Yq material was the only Y chromosomal material identified (*24*). This exception was explained by proposing that the Y chromosome is subject to frequent pericentric inversions, but no data exist as yet to support this assertion. Thus, like their predecessor karyotypic studies, H-Y antigen measurements correlated both arms of the Y with testis determination and did not lead to subchromosomal localization of this genetic information.

These problems were amplified by reports of XY females with apparently normal Y chromosomes but with variable expression of H-Y antigen. In one series (*25*), three such persons were H-Y antigen negative, while nine were H-Y positive. Examples where testes did not develop in the presence of H-Y antigen were explained by postulating a defect in H-Y receptor protein which rendered the target cells unable to respond to the antigen. Other cases of H-Y negativity in the presence of a normal Y chromosome have been explained by postulating abnormal regulation of the H-Y gene, a mutation at the H-Y locus, or by an X-linked gene which suppresses H-Y expression (*26*). The last of these explanations is favored by the identification of human XY females with abnormal X chromosomes—the abnormality consisting of duplication of the portion of the Xp arm (*27*). If this region of the X chromosome contained a gene which suppressed H-Y function, then duplication of the region could lead to oversuppression and failure of H-Y expression. Interestingly, abnormal X chromosomes have been correlated with absence of H-Y expression and the development of XY females in other species (*28*). The problem, of course, is that the existence of this suppressor gene is hypothetical, as are the other defects presumed to be responsible for discordance between H-Y expression and testicular development. This multitude of discrepant results illustrates the problem of mapping a gene by the indirect method of assessing the presence of its protein product. But an important additional factor is that H-Y antigen has not been proved to be the testis-determining gene product. If H-Y antigen were not involved in testis determination, a simple and direct explanation for instances where testes fail to develop in the presence of the antigen would be provided. Therefore, this latter possibility, however, unlikely, must still be considered.

Thus, the H-Y antigen assay has not resolved the apparent contradiction that both arms of the Y chromosome have been associated with testis differentiation; one is still compelled to conclude that testis-determining genes are on both arms. Analysis of abnormal karyotypes involving loss of distal portions of both arms of the Y (*29*) suggests further that the pericentric regions of the chromosome are those involved in gonadal determination. These observations have

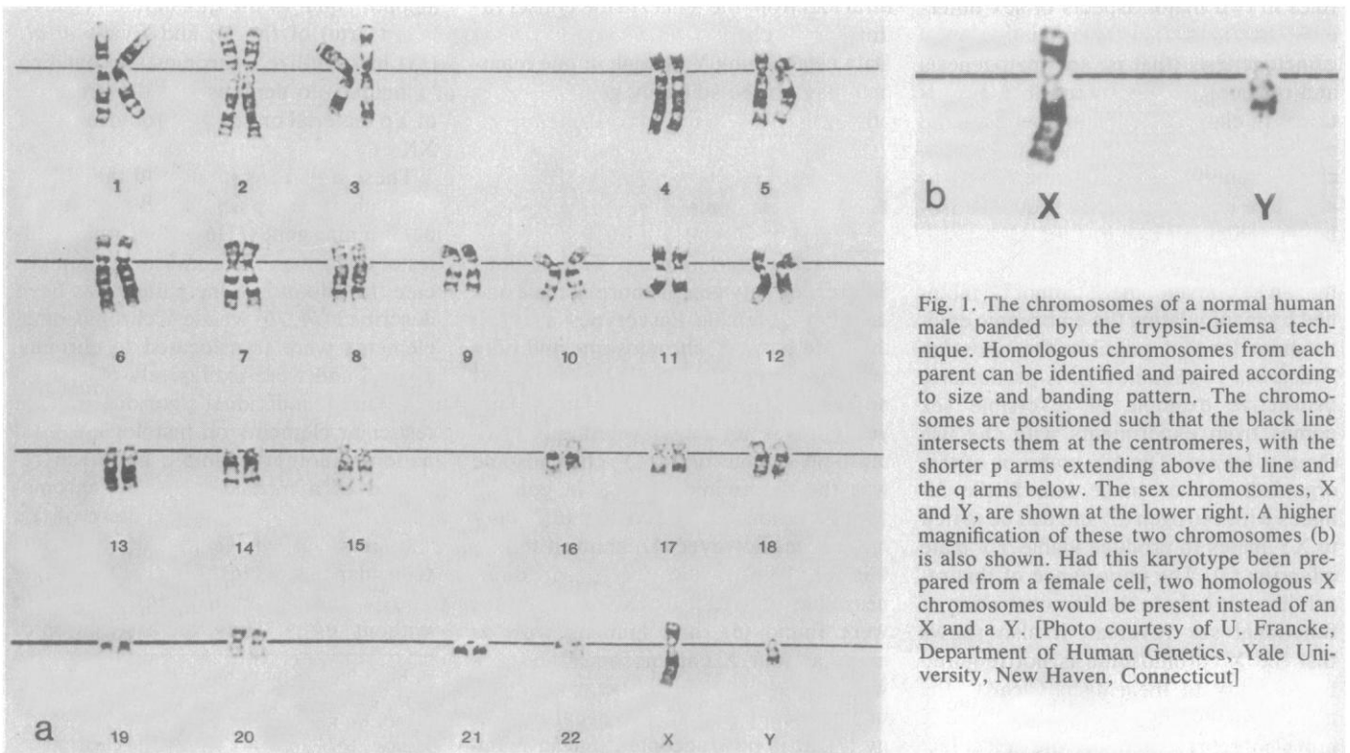


Fig. 1. The chromosomes of a normal human male banded by the trypsin-Giemsa technique. Homologous chromosomes from each parent can be identified and paired according to size and banding pattern. The chromosomes are positioned such that the black line intersects them at the centromeres, with the shorter p arms extending above the line and the q arms below. The sex chromosomes, X and Y, are shown at the lower right. A higher magnification of these two chromosomes (b) is also shown. Had this karyotype been prepared from a female cell, two homologous X chromosomes would be present instead of an X and a Y. [Photo courtesy of U. Francke, Department of Human Genetics, Yale University, New Haven, Connecticut]

led to the postulate that testis-determining genes are duplicated many times and are clustered in the region of the centromere (*15*). Interestingly, independent investigations of H-Y antigen in animals with autosomal inheritance of male-determining genes has led to the proposal that the H-Y gene is present in multiple copies, and that recessive patterns of autosomal male inheritance are due to translocation of a fraction of these copies to an autosome (*4, 30*). Thus, independent lines of investigation have led to similar conclusions regarding the genetic basis for testis determination.

Thus under normal circumstances, the Y chromosome contains genes that act in a dominant fashion to cause testicular determination. Studies correlating histologic evidence of testicular differentiation with karyotypes have indicated that both arms of the Y contribute to this process. Independent correlative studies with the serologic assay for H-Y antigen, the presumed testis-determining substance, have been consistent with the idea that both arms of the Y exert an effect on gonadal determination, although the evidence from these studies is more difficult to interpret because of the complex sequence of events required for H-Y antigen expression and function. Other evidence suggests that the proximal portions of both arms of the Y are those that influence gonadal determination. A viable working hypothesis has evolved which asserts that the testis-determining gene, perhaps H-Y antigen, is present in many copies and is situated in the centromeric region of the Y chromosome. Conclusive evidence is still lacking to show that H-Y antigen is the testis-determining substance, or which shows that Y-linked loci directing testis formation act as structural rather than regulatory entities.

*Spermatogenesis.* In addition to fostering spermatogenesis through induction of testicular differentiation, the Y chromosome probably plays a direct genetic role in this process. Indirect evidence for this comes from several sources. In the marsupials *Isoodon* and *Parmeles* some or all of the somatic tissues may be XO, but spermatogonia are always XY (*31*). It appears in these organisms that selective mitotic nondisjunction occurs, which eliminates the Y chromosome from somatic tissues. That the germ line specifically fails to undergo such nondisjunction suggests that spermatogonia which lose the Y chromosome are inviable and thus are never found when the gonads are examined.

Strong but still indirect evidence for involvement of the Y chromosomes in spermatogenesis comes from examination of a 39,X/41,XYY mosaic mouse (*31*). This animal was found to have spermatogonia exclusively derived from its XYY cells. Such a single anomalous individual could not have evolved a mechanism for eliminating XO cells from the germ line. Thus, the XO cells must have been deficient in their ability to give rise to spermatogonia. Still more evidence supporting a direct role for the Y chromosome in spermatogenesis comes from the analysis of a human male with a small deletion of the nonfluorescent portion of the Yq arm of the Y. This deletion was sufficiently distal to the centromere such that the individual's testicular development appeared normal. He was, however, azoospermic (*32*). This observation suggests that a gene or genes on the long arm of the Y influences spermatogenesis. But these data are also indirect. Normal spermatogenesis depends so greatly on close interactions between the germ cells and gonadal soma, that any subtle defect in the somatic component of the testis could result in azoospermia. Thus, it is not possible to state with conviction that this region of Yq affects the germ cells directly. Until DNA sequences from this region are isolated and characterized, their exact relationship to the process of spermatogenesis will not be known.

## The X Chromosome

*X chromosome inactivation.* Because ovarian development and gametogenesis in both sexes are vitally dependent on X chromosome activity, these subjects cannot be discussed cogently without first briefly describing one of the most remarkable phenomena yet observed in mammalian developmental genetics—that of X chromosome inactivation. This topic has been extensively reviewed (*33, 34*) and will therefore be described only briefly here.

In 1949 Barr and Bertram (*35*) reported that the neurons of female cats contained an additional piece of heterochromatin adjacent to the nuclear membrane. It was later proposed (*36*) that this heterochromatin represented the second X chromosome of females, and that inactivation of this chromosome equalized the dosage of X chromosomal genes between the sexes. This inactivated X was shown to be late replicating (*37*), a finding which subsequently allowed its rapid identification. Evidence supporting the idea that this chromatin is genetically inactive was produced by several experiments. This chromatin was shown not to incorporate labeled messenger RNA precursors (*38*). Translocation of an autosomal gene affecting coat color in mice to the inactivated chromosome resulted in inactivation of the autosomal gene and the development of pigment mosaicism (*39*). When cells from either pigmented or unpigmented patches from such mosaics were examined cytologically, expression of the dominant coat color marker was correlated with the presence of an active X chromosome which was larger than normal and contained translocated material; unpigmented regions were associated with absence of the large X chromosome (*40*). This study provided strong evidence that the inactivated chromosome was in fact the second X. Biochemical evidence of genetic inactivity of the heterochromatic X came first from the demonstration that clones of cells grown from humans heterozygous for allelic variants of glucose-6-phosphate dehydrogenase (G6PD) (an X-linked enzyme) expressed only one of the G6PD alleles (*41*). In females of the horse-donkey hybrid, the mule, the X chromosomes differ both in morphology and electrophoretic mobility of enzymes coded within them. These differences were exploited to conclusively show that the heterochromatic X does not produce enzyme products (*42*). Thus, the mammalian X chromosome is subject to a special form of genetic regulation whereby the second X chromosome of females is turned off as a unit. This mechanism explains why levels of activity of X-linked enzymes are the same in both sexes.

While the generalization that the supernumerary X chromosome is genetically inactive still holds true, some exceptions have been discovered. The cell surface antigen $Xg^a$, which is coded for on the X chromosome, appears to be such an exception. Chronic myelocytic leukemia (CML) is a tumor presumed to be derived from a single cell. When CML cells from females heterozygous for $Xg^a$ were examined for expression of the antigen, all of 11 cell lines were found to be $Xg^a$ positive (*43*). Since 50 percent of such tumors would be expected to be derived from $Xg^a$ negative cells, this result suggested that $Xg^a$ was expressed in cells in which the $Xg^a$ positive X was inactivated. The possibility that the antigen could be passively transferred between cells was ruled out by examination of a twin chimera who was mosaic both for ABO blood group antigens and for $Xg^a$. This individual's O cells were always $Xg^a$ positive, while the others were $Xg^a$ negative (*44*). In addition, hematopoietic stem cells have been shown to be

capable of synthesizing $Xg^a$ (*45*). Finally, Fellous *et al.* (*46*) showed that cells cloned from $Xg^a$ heterozygotes all expressed the antigen, again consistent with expression of the $Xg^a$ gene from the inactivated X. Another X-linked gene that appears to escape inactivation is that coding for steroid sulfatase (SS). Cells cloned from double heterozygotes for G6PD and SS deficiency have been shown to produce SS regardless of their G6PD expression (*47*). Thus, SS is also produced from the inactivated X chromosome.

Preliminary evidence had suggested that $Xg^a$ was located on the Xq arm, close to the gene coding for α galactosidase (*48*). However, later analysis of iXq individuals showed that Xg was on the Xp arm (*49*). In addition, the Xg locus was known to be closely linked (10 centimorgans) from the SS locus (*48*). Somatic cell hybridization studies subsequently demonstrated that SS was on the distal portion of Xp (*50*). Thus, both loci that escape inactivation are linked and are on the short arm of the X chromosome.

*The X chromosome; ovarian development and oogenesis.* The knowledge that the second X chromosome of females is inactivated would lead us to predict that females with a single X chromosome would be normal. However, this is not the case; XO human females are infertile and have streak gonads which are devoid of germ cells [rare exceptions have been described (*51*)]. Analysis of abnormal X chromosome karyotypes have shown that when portions of the Xp arm are missing from the second X chromosome, gonadal dysgenesis typical of the XO phenotype is again observed. For example, fusion of Xp to Xp with loss of a part of Xp results in gonadal dysgenesis (*52*). Simple deletion of Xp from p11 to the terminus also results in gonadal dysgenesis (*53, 54*). When portions of Xp distal to p21 are lost, however, fertility is maintained (*55*). These observations are fully consistent with the proposal made by Fergusen-Smith (*56*) that a portion of the Xp arm is not subject to X inactivation, and that loss of this material results in monosomy and subsequent infertility. These data correlate nicely with the biochemical data indicating that two loci near this region, $Xg^a$ and SS, escape inactivation. In fact, in several examples mentioned above in which loss of Xp material was associated with gonadal dysgenesis, the Xg locus was apparently deleted (*52, 54*). But abnormal function of the Xq arm resulting either from deletion (*57*) or translocation (*58*) can also lead to gonadal dysgenesis. These observations could be explained if genes on the Xq arm were found which, like $Xg^a$ and SS, escaped inactivation. However, it is clear from studies of X chromosome inactivation alluded to earlier that most of the second X is certainly genetically inactive. Thus, we are left with the contradiction that an inactive and apparently nonfunctional piece of chromatin, the Xq arm of the inactivated X, is required by female cells for maintenance of fertility.

A resolution to this problem may reside in an examination of the genetic requirements of the female germ cells. Oocytes and their associated follicle cells make up such a large component of normal ovarian structure that they have been considered a requirement for ovarian differentiation (*59*). Studies of mammalian oocytes have led to the observation that these cells are the only ones of the adult in which both X chromosomes appear to be active. Comparing X-linked enzyme activity between oocytes from XO mice and XX mice [XO mice are fertile (*60*), although their fertile life-span is reduced (*61*)], Epstein (*62*) and Kozak *et al.* (*63*) showed that XO oocytes had half the activity of these enzymes as normal XX eggs. Later, studies of human females carrying allelic variants of the dimeric enzyme G6PD showed that the hybrid isozyme was present in oocytes (*64*). This result provided definitive evidence that both X chromosomes are active in this cell. Moreover, in XO mice, even those oocytes that survive to the point of fertilization manifest impaired development during subsequent cleavage (*65*). It is clear, therefore, that oocytes require genetic activity from both X chromosomes for their normal development and differentiation. Since the differentiation of these cells is in turn required for normal ovarian development, absence of any portion of an X chromosome could result in gonadal dysgenesis secondary to abortive oogenesis. We therefore offer this tentative explanation for the correlation of Xq deletions with gonadal dysgenesis.

Thus, despite the fact that the second X chromosome of females is inactivated, both X chromosomes are required for female fertility. Deletions of portions of either arm of this chromosome result in gonadal dysgenesis. In the case of the Xp arm, gonadal dysgenesis may relate to the fact that portions of this arm remain active on the inactivated X chromosome. Deletions of the Xq arm may result in gonadal dysgenesis because of as yet undiscovered loci that escape inactivation, but may also cause ovarian developmental failure through disruption of oocyte differentiation, which in turn is required for normal ovarian development.

*The X chromosome and spermatogenesis.* While the mammalian oocyte is unusual in that it possesses two active X chromosomes, the primary spermatocyte is perhaps even more unusual—in this cell, the single X is inactivated. In the primary spermatocyte, heteropycnosis typical of that seen in female cells with an inactivated X is observed (*66*), and this heteropycnosis is correlated with transcriptional inactivity (*67*). Thus, the X chromosome of the premiotic spermatocyte shows all of the characteristics of the inactivated female X.

To what function is X inactivation in the male germ line designed? The answer apparently lies in the observation that the retention of active X chromosomal material in these cells is strongly correlated with meiotic failure. The autosomal dominant gene in mice, *Sxr*, reverses the sex of females to males (*68*) (Fig. 2). The XX *Sxr* mice develop testes, but no postmeiotic germ cells are found. When the gene is passed to XO mice through XY *Sxr* males, however, meiosis can occur (*68*). Thus, the presence of a second X chromosome in XX *Sxr* mice prevents meiosis. Similar observations have been made in XXY humans who, although they are males and develop testes, are invariably aspermic (*69*). On the basis of their observations in *Drosophila*, Lifschytz and Lindsley (*70*) suggested that X-to-autosome translocations which cause male sterility do so by interfering with X chromosome inactivation. These translocations are also associated with sterility in mice (*71*) and in man (*72*). An interesting adaptation to the hazards of an active X chromosome in the male germ line has been made by the creeping vole *Microtus oregoni* (*73*) whereby the X is eliminated entirely from the germ cells by nondisjunction so that only O and Y sperm are formed. Such examples emphasize the importance of a silent X to spermatogenesis.

It is clear that the X chromosome contains many loci that affect gonadal development and gametogenesis profoundly. Exactly which genes must be present in two copies in the ooctye or turned off completely in the spermatocyte is not known. It is likewise unclear how each region of this chromosome relates to the development of the ovary. The specific genes involved have yet to be identified, their positions on the chromosome precisely mapped, and their physiologic roles in these developmental processes elucidated.

## Isolation of Sex Chromosomal DNA Sequences

Even though various regions of both the X and Y chromosomes have been associated with certain aspects of gonadal development and gametogenesis, the precise mechanism of action of the genes located within these regions is not understood. Moreover, karyotypic analysis is not sensitive enough to distinguish the presence or absence of an individual gene sequence. Since the chromosome is a complex package of the DNA double helix, absence of a given band on a karyotype could indicate deletion of sequences that are tens of thousands of base bairs apart on the DNA strand. In order for the major sex-determining genes to be identified and their individual contributions to sex differentiation defined, the specific coding sequences must be isolated and analyzed directly. Efforts in this direction have been made, and the results, although still preliminary, illustrate the potential of this approach to these problems.

In 1976 Kunkel *et al.* (*74*) used liquid hybridization to purify sequences specific to the Y chromosome of humans. Because the Y is small and composed in large part of heterochromatin, the approach taken by these workers was to isolate reiterated sequences which were presumed to comprise a significant proportion of the Y chromosomal material. This was accomplished by exhaustive hybridization of radioactively labeled reiterated DNA from cells with supernumerary Y chromosomes against a large excess of DNA from female cells. Those sequences that did not hybridize to XX cellular DNA were again exposed to that DNA and the unhybridized sequences were again recovered. At the end of this procedure, sequences were isolated which (i) hybridized extensively to XY DNA and (ii) hybridized to XX DNA and *Escherichia coli* DNA with identical kinetics. Moreover, the extent of hybridization to DNA from cells containing Y chromosomes increased linearly with the number of Y chromosomes (for example, XYY) present in those cells. Though these Y-specific sequences were not further characterized in this initial report, the results demonstrated that sequences from this very small chromosome could be purified.

The discovery of restriction endonucleases shortly led to the observation that DNA from human XY cells, when cut with Hae III, subjected to electrophoresis on Agarose, and stained with ethidium bromide, showed two distinct bands not present in DNA from female cells (*75*). The molecular size of these fragments were $2.24 \times 10^6$ (3.4 kilobase pairs) and $1.6 \times 10^6$ (1.9 kilobase pairs). That such sequences were visible after ethidium bromide staining suggested that they represented reiterated DNA, and this was further supported by the observation that the larger sequence was not digestible with several other restriction enzymes. Densitometric analysis indicated that the two reiterated bands constitute as much as 70 percent of the Y chromosomal DNA. The RNA nick-translated from the larger fragment was exposed to DNA from XX cells and did not hybridize; in addition, this probe did not hybridize with the smaller reiterated Y-specific fragment. Lack of cross-hybridization between the two fragments indicated that each isolate came from a distinct portion of the Y chromosome.

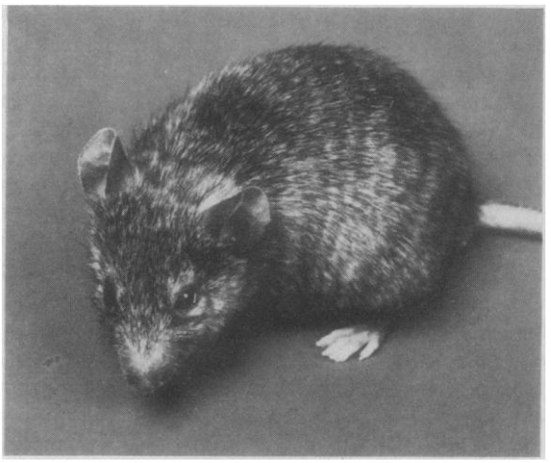

Fig. 2. An XX sex-reversed (*Sxr*) male mouse. The banded coat color pattern indicates the presence of two X chromosomes, which normally produce a female phenotype. XX *Sxr* mice are thus easily identified as banded males and selected from breeding populations for further study. [Photo by B. Sacco]

Isolation of such Y-specific fragments subsequently permitted a detailed analysis of structural anomalies of the Y chromosome in man (*76*). Samples of DNA's from normal human males with Y chromosomes whose Yq arms were of varying length were digested with Hae III and exposed to a radioactively labeled probe that was nick-translated from the larger male-specific fragment (3.4 kilobase pairs). This experiment showed that the extent of hybridization was proportional to the length of the Yq arm. Thus, the large Hae III fragment was tentatively mapped to the Yq arm. This is not surprising, since this highly fluorescent, heterochromatic portion of the Y undoubtedly contains repeated sequences, and this fragment was isolated on the basis of its presence in multiple copies in the genome. The fragment was also shown to have some homology with the X chromosome, which again indicated that it was composed of reiterated DNA. Since all of the donor DNA's used for this study, regardless of their extent of hybridization to the probe, were obtained from phenotypically normal males, the purified fragment was considered unlikely to have been derived from sequences involved in testis determination.

This latter expectation was supported in a subsequent study utilizing a male-specific Bsu fragment (Bsu is a restriction endonuclease with the same specificity as Hae III) (*77*). In this study, normal males lacking the fluorescent portion of Yq were examined for the presence of the Bsu fragment and found not to possess it. Thus, the fragment was localized to Yq and shown not to be involved in primary sex determination. The Y-specific fragments obtained by exhaustive liquid hybridization were similarly applied to analysis of human beings with abnormal Y chromosomes (*78*), and these fragments were likewise shown not to represent testis-determining genes. More recently, probes constructed from such Y-specific fragments have been mapped by in situ hybridization techniques. Bostock *et al.* (*79*) purified an Hae III fragment from satellite III DNA which did not hybridize with DNA from XX cells. A probe constructed from this fragment was localized by in situ hybridization to the long arm of the Y. One individual examined in this study had a deletion from Yq12 to the terminus of the long arm; DNA from this person did not show the Hae III reiterated band after ethidium bromide staining, but it was detected in small amounts after filter hybridization. Thus, this sequence was localized to the q12 region of Yq. As pointed out by Buhler (*15*) this result is intriguing, because it is precisely this region on the Y whose absence was previously correlated (*33*) with azoospermia. Thus, this probe may represent the first purified sex chromosomal sequence

which is involved in sex differentiation. Further studies are obviously required to determine whether this is indeed the case.

Szabo *et al.* (*80*) have utilized these Hae III fragments for further in situ hybridization studies. Whereas the 3.4-kilobase band was localized over most of the Yq arm, the 1.9-kilobase fragment was found only on the distal long arm. Interestingly, exposure of gorilla chromosomes to these probes showed that the gorilla Y did not hybridize at a level significantly above background, although grains were found over many autosomes. This result is consistent with the notion that Y chromosomal sequences were not highly conserved as a linkage group.

Liquid hybridization has also been applied to the purification of specific DNA sequences of the X chromosome. Schmeckpeper *et al.* (*81*) annealed DNA isolated from a mouse-human somatic cell hybrid containing only the human X with mouse DNA. The unhybridized sequences which remained were presumed to be derived from the human X chromosome. This expectation was confirmed by three criteria. First, these remaining DNA sequences hybridized strongly to human DNA, which indicated that they were of human origin. Second, a single copy component of this material hybridized to human DNA in a manner proportional to the number of X chromosomes in the target cells. Third, a reiterated component also hybridized proportionally with the number of X chromosomes in the target. The total amount of X chromosome–specific DNA isolated in this experiment accounted for 50 percent of the human X chromosome. Presumably, the other 50 percent could not be isolated by this technique owing to sequence homology between the human and mouse X chromosomes. These workers pointed out that when sequences specific to a human autosome are isolated in this manner, only 30 percent of the autosomal sequences are characteristically homologous with mouse DNA. This observation is consistent with Ohno's proposal (*4*) that the X chromosome has been highly conserved. Taken together with the results from purification of Y-specific sequences, these data suggest that the liquid hybridization approach is more likely to succeed with the Y than with the X chromosome, which has extensive sequence homology with DNA from other mammalian species.

Recently, Wolf *et al.* (*82*) have taken an approach which has greater potential for purification of X chromosomal DNA. They isolated genomic DNA from human cells with supernumerary X chromosomes, digested it with the restriction enzyme Bam HI, and cloned the resultant fragments in the bacterial plasmid pBR322. Probes constructed from the cloned fragment were tested (by filter hybridization) against DNA from a mouse-human hybrid containing the human X chromosomes, and the clones were judged to be X-specific by three criteria. (i) When DNA from the original hybrid line was digested with Bam HI (the same enzyme used for the cloning procedure) and hybridized against probes derived from the cloned sequence, only a single band was found after autoradiography. (ii) The single band was a sequence of the same size as the original cloned sequence. (iii) The probe hybridized more strongly to human DNA from XXX cells than from XY cells. Thus, compelling evidence was presented that human X chromosomal sequence had been cloned in pure form.

Although this approach is particularly applicable to the X chromosome, it might also be used for isolating additional sequences specific to both the X and Y chromosomes. Since cloning of the entire human genome is effected by such a procedure, it can be assumed that all sequences specific to both sex chromosomes are contained somewhere in the cloned DNA. Isolation of a cloned sequence derived from the X or Y chromosome may be difficult, since these chromosomes comprise only a small percentage of the total genomic DNA. In addition, some sex chromosomal sequences are undoubtedly composed of reiterated DNA that is not unique to the sex chromosomes, but is found on most or all of the autosomes as well. It is evident, however, that the sex chromosomes do contain genetic information that is important to sex differentiation. Searching a cloned library from these sequences may be tedious, but it is difficult to imagine that such a search would not eventually be productive.

Along with probes constructed from such sequences, filter hybridization can be used to evaluate DNA from individuals with abnormal sex chromosome karyotypes. These methods are very sensitive, and therefore they should add a new dimension to the study of sex differentiation. For example, probes constructed from sequences encoding the H-Y antigen could be applied to DNA from individuals that have testicular tissue but lack identifiable Y chromosomal material. Positive hybridization can be used in such cases to map the position of the antigen. With this approach, expression or function of the gene product is not required. If the DNA sequences are present, they can be located by such a probe. This technology has already been coordinated with somatic cell hybridization to map genes such as human insulin gene (*83*) and the mouse immunoglobulin heavy chains (*84*).

A problem with cloning the entire genome, however, is the determination of the protein product coded for by randomly cloned sequences. Unless the amino acid sequence of a gene product is known, it is not yet possible to identify the DNA sequence coding for that product. A potential solution to this problem lies in the transfer of cloned sequences into recipient cells and examination of the effect of the donor sequences on the host cells' function. Of course, such analysis depends on the genetic expression of the donor sequence in the recipient cell, and the ability of the investigator to detect such expression. Gene transfer experiments have already succeeded in correcting cellular deficiencies of the enzyme thymidine kinase (*85*). Recently, our laboratory has succeeded in transferring gene sequences into intact mice by microinjection into fertilized eggs (*86*), although expression of the transferred genes has not yet been demonstrated. These results are encouraging, however, and may eventually permit an analysis of the effect of cloned sex chromosomal sequences on embryonic sexual development and gametogenesis.

## Conclusions

The initial realization that the sex chromosomes are specialized for the direction of primary sex determination and gametogenesis has been extended by more sophisticated analyses. The pericentric region of the Y chromosome has been implicated as the source of genes directing testis determination, whereas regions of the Yq arm have been associated with spermatogenesis. The Xp arm contains genes that escape inactivation, and deletions within these same regions are associated with gonadal dysgenesis and female infertility. Deletions of the long arm of the X also result in infertility, even though all evidence suggests that even in the absence of such deletions, only one Xq arm is genetically active. However, the female germ cell requires two active X chromosomes, and germ cell atresia may itself result in gonadal dysgenesis. Thus, deletions of the Xq arm may cause gonadal dysgenesis via germ cell atresia. The X chromosome is also important to spermatogenesis in that

it must be inactivated prior to meiosis. The precise regions of the X where the genes must be turned off have not been identified.

Although some subchromosomal localization of sex-determining functions has been achieved, specific sequences and their gene products have not been isolated. New advances in gene cloning technology now make possible such isolation, although genes encoding these functions have not as yet been purified. Initial analysis of the sequences thus far obtained has supported, however, the notion that the X chromosome is highly conserved in evolution, and that such conservation has not been characteristic of the Y chromosome. We might expect from initial efforts in this area that sex-determining genes located on the X chromosome in one organism will likewise be X-linked in many or all others, while functions related to the Y chromosome may be distributed about the genomes of mammals. The XO male vole, *Ellobius lutescens*, is perhaps an extreme case of this distribution of male-determining functions, since this organism has no identifiable Y chromosome at all (*87*). In the more common cases where the Y chromosome is present, it may perform a regulatory role, governing the expression of autosomal genes whose map positions vary substantially between species. As more sequences are cloned and used as probes for mapping, these aspects of Y chromosomal function will be further clarified. Finally, the advent of gene transfer technology may also permit the examination of the physiologic roles of cloned sequences in host cells and intact animals, advances which should allow for a more detailed understanding of mammalian sex differentiation.

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88. Dr. Ruddle's laboratory is supported by National Institutes of Health grant GM09966-19 (to F.H.R.) and a Hudson Brown fellowship administered through the Department of Obstetrics-Gynecology, Yale University School of Medicine (to J.W.G.) and NIH grant 1F32GM07959-01 (to J.W.G.).