Mechanisms of Gonadal Differentiation

Florence P. Haseltine and Susumo Ohno

Summary. Sex differentiation is the result of the translation of genetic sex into gonadal sex. Without recognizable masculinizing signals the embryonic gonad will undergo ovarian differentiation. The main determinant of gonadal differentiation appears to be the presence or absence of a cell surface antigen, called H-Y antigen. The regulation of H-Y antigen expression is complex and involves the interaction between regulatory sites on the Y chromosome, the X chromosome, and possibly the autosomes.

Sex differentiation in mammals begins at fertilization with the production of two types of embryos differing in chromosomal content. The embryo that will develop into a female has two X chromosomes, becoming the homogametic sex. The male embryo has a single X chromosome and a Y chromosome and becomes the heterogametic sex. The Y chromosome appears to have the dominating regulatory functions for sex differentiation, since any embryo without a Y chromosome develops as a female. The gonads are primarily responsible for the sexual functioning of the adult. In mammals, gonadal sex differentiation is not strongly influenced by environmental or hormonal factors. When the phenotypic sex does not correspond to the karyotypic sex, sex reversal is said to have occurred. In mammals, sex reversal is usually genetically determined. Comparison of normal sex differentiation with that of sex reversal provides some details about the interactions between cells that form gonads.

From the time of fertilization until after blastocyst formation, the male embryo has only one X chromosome functioning, whereas the female has both X chromosomes functioning. The Y chromosome function appears first at the eight-cell stage, as judged by the detection of a cell surface antigen regulated by this chromosome (*1*). The two X chromosomes in the female function until after blastocyst formation. At this time one of the two X chromosomes undergoes a process called inactivation. As the term implies, inactivation results in the gene products being suppressed in the inactive segment of the X chromosome. Inactivation of a large segment of one of the X chromosomes occurs, possibly equalizing the amount of functioning X material present in both sexes so that both sexes express roughly the same amount of genetic material from the X chromosome. In somatic cells X inactivation occurs after blastocyst formation, and before implantation of the blastocyst in the uterine wall (*2*). Two X chromosomes are not needed for early development since XO females in many mammalian species go on to develop as fertile females. In mice, the trophoblastic tissue selectively inactivates the paternal X chromosome, and the cells derived from the inner cell mass randomly inactivate one of the two parental X chromosomes (*3*). In humans, the preferential inactivation of the X chromosome in the trophoblastic tissue does not occur. In the cells destined to become primordial germ cells, the X chromosomes are under a different type of regulatory control (*4*).

Studies with human oocytes before the onset of meiosis but after colonization of the genital ridge have provided evidence for two functioning X chromosomes (*5*). Monk and McLaren have examined the germ cells of the mouse immediately after colonization of the genital ridge and they have presented evidence that there is only one X chromosome active at that time (*6*). These experiments suggest that the X chromosome is inactivated during primordial germ cell migration but that reactivation of the X chromosome occurs soon after population of the genital ridge and before the occurrence of meiosis (*5, 6*). Workers examining sex differentiation in the kangaroo and the tammar wallaby also found evidence for germ cell inactivation during primordial germ cell migration and colonization of the genital ridge, with reactivation of the X chromosome before meiosis (*7*).

If inactivation of the X chromosome does occur in the germ cell line, then reactivation occurs before meiosis. During meiosis both X chromosomes are active in humans (*8*). In males, the X chromosome appears to be inactive after spermatogenesis has begun (*9*). Further, in *Microtus oregonii*, the XY male eliminates the X from the spermatogonium (*10*).

The somatic elements and the germ cells of the mature gonad arise from different embryonic tissues, the former from the mesonephric ridge and the latter from the yolk sac endoderm. The germ cells subsequently migrate through the dorsal mesentery to the genital ridge. The early primordial germ cells have been found to migrate by amoeba-like movement from their site of origin to the developing genital ridge (that is, the undifferentiated embryonic gonad) (*11, 12*). The interaction between the somatic cells and the primordial germ cells leads to the development of the fertile functioning gonad.

The somatic cells and primordial germ cells differentiate under separate, but probably somewhat interdependent, control signals. Differentiation primarily could proceed either because the germ cells direct the differentiation process or because the somatic elements of the gonads contribute to sex differentiation. To decide which is the more likely path, we can examine cases of naturally occurring sex reversal, as well as evidence provided by experimentally produced sex reversal. Several types of tissue can take part in gonadal differentiation, but only one has the function of a primary director. If the destiny of a tissue in an embryonic organ can be reversed, then that tissue is not a critical director of that process although, without the presence of a primary director, the tissue could still undergo the genetically appropriate differentiation. In nonmammalian vertebrates, complete phenotypic sex reversal includes both the germ cells and the somatic cells. Mammals do not have this flexibility.

Dr. Haseltine is an assistant professor in the Department of Obstetrics and Gynecology and the Department of Pediatrics, Yale University School of Medicine, New Haven, Connecticut 06510. Dr. Ohno is chairman of the Department of Biology, City of Hope Research Institute, Duarte, California 91010.

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Germ cells must be in an appropriate environment to mature and to form functioning gametes. Unless the germinal and somatic sex are the same, a sterile gonad will usually result. The control of the somatic elements is also important. Experimental systems have been created in which normal germ cells of one sex (XY) are found in the presence of genital ridge cells with the opposite genetic sex (that is, XX). These situations were created by producing genetic-mosaic animals. Genetic-mosaic individuals are those that have two cell lines for certain tissues instead of the usual one-cell line, for example, individuals with both XX and XO, instead of one or the other. Techniques of embryo aggregation provide a reliable method for producing XY < --- > XX genetic mosaic mice (often called mouse chimeras) in large numbers (*13*). With the use of this technique, genetically mixed gonads have been produced, and germ cell or somatic cell dominance has been ascertained. This type of study lends itself to evaluating the interaction between two normal cell types that must compete; one genetic component must predominate in regulating sex differences if normal gonads are to result. Mintz (*14*), after examining the offspring of a large number of these animals, observed that there was no evidence that XX cells ever become sperm. She also identified a male chimeric mouse with at least 95 percent of its somatic cells containing XX cells, whereas the germ cells were all XY cells (*14*). The number of competing XX germ cells that might have been present was not examined because histologic data from newborn mosaic mice were not obtained when the XX germ cells would have been still present in a testicular environment. But XX germ cells would be expected to die in a testicular environment and not contribute to the adult (*15*).

Ford *et al.* have described a fertile female chimeric mouse with an ovarian follicle containing 98 percent XY follicular cells (*16*). These experiments show that normal somatic cells can undergo sex reversal. Balanced sex chimeras predominantly become males, and this implies a tremendous competitive advantage for masculinizing properties of male-determining cells in the gonad. The testicular-inducing substance only needs to be produced by a few XY cells to influence a large number of XX cells. Since XX somatic cells can undergo sex reversal, many cells in an XX < --- > XY gonad appear to undergo testicular differentiation, and the small regions of ovarian tissue later degenerate as the testis assumes its endocrine function. Sterile gonads rarely occur in these mosaic animals, and few hermaphrodites are formed.

Germ Cells and Differentiation of the Gonad

Germ cells may also act as gonadal inducers, since, in a male chimera, XY germ cells can be found in a predominantly XX testis, and in a female sex chimeric mouse an oocyte can be surrounded by XY ovarian follicular cells (*16*). We expect to find neither XY germ cells in the ovarian environment nor XX germ cells in the testicular environment, since their usual fate in the hostile, opposite gonadal environment is extermination. For the most part germ cells do not appear to survive or to reach maturity in a sex-reversed environment, although XY cells may start to undergo meiosis in an ovarian environment (*17*). Evans *et al.* (*18*), in expanding upon previous work, described a mosaic gonad in which an early oocyte with an XY karyotype was seen in diakinesis, but there is only one piece of genetic evidence for the Y-containing germ cell becoming a functioning oocyte in XX < --- > XY mice (*18*). Ford *et al.* have reported one instance where a germ cell could possibly have formed a functional egg. In this case, the Y chromosome that was found in an offspring of a female mosaic mouse may have been accompanied by an X in the ovum, since the offspring was an XXY male. Functional germ sex reversal must have occurred if the Y came from the female chimera (*16*).

The question of germ cell dominance has been examined in naturally occurring sex reversal in mice. Studies have focused on an autosomal dominant sex reversal gene (*Sxr*), which causes XX mice to develop as sterile phenotypic males. Testes are formed, but the germ cells are progressively lost so that shortly after birth the testes are completely devoid of germ cells (*18*). Gordon produced chimeric mice that were genetic mosaics, XY < --- > XX/Sxr and XX < --- > XX/Sxr (*19*). Breeding experiments did not provide any evidence that the germ cells from the XX/Sxr line were able to differentiate into either ovum or sperm. Further, his work suggested that these germ cells cannot undergo sex reversal in any systematic way.

One of the possible reasons for the relative irreversibility of germ cell sex may have to do with either the number of X chromosomes or the presence of a Y chromosome. The Sxr mice with XX karyotype form testes without germ cells; the XO/Sxr mice also form testes, and these mice have active spermatogenesis although no functional sperm form (*20*). Genetic sex reversal of a germ cell demonstrates that, in the presence of only one X, a masculinizing factor could determine germ cell sex. However, germ cells with two X chromosomes cannot undergo spermatogenesis; they appear to attempt spermatogenesis, but the process stops at the first meiotic division. Two X chromosomes may inhibit spermatogenesis because these germ cells do not proceed as far as the XO/Sxr cells. These XX/Sxr germ cells might lack the ability to direct ovarian differentiation, and so the genital ridge undergoes masculine differentiation. Whether differentiation is guided by the genital ridge cells, or is influenced by the XX/Sxr primordial germ cells, is still not known. However, if XX/Sxr germ cells direct masculinization, they become surrounded by an environment hostile to their further growth, and will die. The XX/Sxr testis found in mice appears to be analogous to that found in XXY humans. These testes form, promote early masculinization, and secrete androgens; but their germ cells disappear before puberty. In the mouse, there is an autosomal dominant gene called W that, in the homozygous condition, prevents the proliferation of primordial germ cells. Thus, while thousands of primordial germ cells reach the genital ridges in normal embryos, fewer than 50 do so in WW mice (*11*). Nevertheless WW males, rescued from lethal anemia (another pleiotropic effect of W) by syngeneic bone marrow transplants, develop sterile testes. In these mice the germ cells do not have a major role in gonad formation.

Female germ cells in mammals have an XX chromosomal constitution. However, two active X chromosomes are not needed to complete oocyte development because XO mice become fertile females with a reduced reproductive life-span (*21*). Humans with XO karyotypes are usually sterile, and degeneration of the primary oocytes near the time of birth leads to follicular degeneration and streak gonads. The process of follicular atresia in humans normally occurs throughout the life-span of the individual, resulting in loss of the hormone-producing Graafian follicles and, eventually, in menopause. A viable oocyte is required for development, survival, and function of follicles and for the successful formation of tightly condensed follicular cells in fetal life (*22*).

Germ cell sex reversal is not common in mammals. But there is one mammal,

the wood lemming (*Myopus schisticolor*), in which XY germ cell sex reversal is common. There are fertile XY females that produce mainly females. This trait is X-linked and the X chromosome has a readily identifiable X-linked morphological marker that is found when the cells from the XY females are karyotyped. Most of the XY germ cells undergo a nondisjunctional event, first producing two X chromosomes and then X-bearing oocytes. However, breeding studies have produced evidence that Y-bearing oocytes are formed, leading to the birth of XY and XX males; here the Y chromosome could have been derived from the egg and the X chromosome from the sperm, unless both X and Y chromosomes were derived from the sperm (*23*).

The observed abilities of the testis to develop, survive, and secrete androgen in the absence of germ cells is in sharp contrast to the inability of the ovary to mature, form follicles, and secrete estrogens. Possible factors contributing to this may be found in the functions that the ovary and testis must perform early in embryologic development. The ovaries produce estrogens in the fetus and postnatally. However, the fetal ovary has no known major function in directing sex character development, and the female ductal, internal, and external genitalia form without any known organizing factors (*24*).

The Role of the Testis in Sex Differentiation

The burden of sex differentiation falls on the testis. The testis must be formed early and masculinizing hormones must be produced very early in development. Masculine extragonadal differentiation is induced by testicular products, and both genetic males and females are responsive to the masculinizing effects of the testis. The XX extragonadal cells are as responsive as the XY counterparts, and their sex chromosome constitution plays no role in secondary sex-determining mechanisms. A group of marsupials is distinguished by the ability to eliminate a sex chromosome from most of the extragonadal cells, one of the X chromosomes from the XX cells or the Y from the XY cells. Their extragonadal cells are XO (*25*). Clearly here the sex chromosomal content of extragonadal somatic cells is irrelevant to the development of secondary sex characteristics.

The fetal testis performs two early functions. The first is to cause regression of the Müllerian duct system, which it does with the production of a Müllerian inhibiting substance produced by the fetal Sertoli cells. The second function is to produce testosterone, which is excreted by the developing testicular Leydig cells and binds to an androgen receptor found in target cells. Only one form of androgen receptor is necessary to perform this function. Androgens may not have a direct role in sperm cell differentiation, but function by stimulating the Sertoli and Leydig cells to provide a supporting environment for sperm cell differentiation. The X-linked gene for testicular feminization (Tfm) results in the inability of cytosol receptors to bind androgens in target tissues (*26*). The $X^{Tfm}Y$ animals are male pseudohermaphrodites and, although they have testes, they are phenotypic females externally. With X inactivation occurring during spermatogenesis, resulting in inactivation of the X-linked Tfm locus, spermatogonia and their mitotic progeny should be androgen-insensitive if the X-coded RNA message and its eventual protein products do not function during spermatogenesis. To test the role of androgens in spermatogenesis, Lyon *et al.* produced chimeric mice that were $X^{Tfm}Y <---> XY$ (*27*). The androgen-insensitive $X^{Tfm}Y$ germ cells should not form sperm if testosterone is directly implicated at any stage in spermatogenesis. Most of the chimeric males did not form sperm derived from the $X^{Tfm}Y$ germ line and had mosaic gonads with undeveloped areas, probably consisting of androgen-insensitive tissue. But two male mice were produced that did pass on the Tfm gene through the male chromosome. The capability of Tfm cells to undergo spermatogenesis implied that the critical factor is the environment provided by the testosterone-sensitive Sertoli cells, and not the direct action of testosterone on the developing spermatozoa. Estrogens, produced by the ovary, do not have a major role in early characteristics of sex differentiation but do appear to play a role in oocyte development and the completion of meiosis.

The interaction between the germ cells and somatic elements of a gonad is critical for the successful fertility of the animal. The somatic elements must provide an environment where the germ cells can mature. In general, if germ cells populate a gonad of the sex opposite to their chromosomal sex, they will not mature to form functioning gametes. Since early developing genital ridge tissue may undergo functional sex reversal and function normally in its reassigned role, it is important to define factors that directly influence somatic cell differentiation of the gonad.

Testicular and Ovarian Organizing Substances

The existence of ovarian and testicular organizing substances has been proposed for many years. Investigators are searching for the organizing substances, as well as possible receptor or recognition sites on the surface of cells for these organizing substances. Any substance that directs somatic cell differentiation must have the following properties:

1) It must permit early gonadal differentiation without the presence of germ cells.

2) It should be diffusible and transferable between cells, and should be capable of being blocked by germ cells or gonadal organizing substance of the opposite sex.

3) It should have a receptor on the somatic cells that respond to testicular or ovarian organizing signals.

Investigators have mainly studied the existence of a masculinizing substance, since testicular differentiation appears to be under a more direct early embryologic control than ovarian development. The search for a testicular organizing substance intensified after the discovery in inbred mouse strains of a histocompatibility antigen that is responsible for the rejection of male skin grafts by female recipients (*28*). This antigen was related to the presence of the Y chromosome (*29*). A major breakthrough occurred when it was noted that serum from female mice that had rejected male skin had cytotoxic activity for male cells. This activity could be absorbed out of the serum by many types of male cells. A standard assay was then developed that measured residual cytotoxic activity of the absorbed serum against standard target cells (*30*).

The term "H-Y antigen" was originally applied to the transplantation antigen (that is, the antigen responsible for the rejection of male skin grafts), but as now often used the term includes antigens found only on male cells that induce the formation of serum antibodies. However, there may be a difference between these antigens in that examination of a mutant mouse has revealed a possible difference between the H-Y antigen detected by skin graft rejection and the one measured by an antiserum with cytotoxic capabilities. A male mouse lacking a Y chromosome (39X), but possessing

testes, was discovered among the offspring of an irradiated male. This mouse appeared to lack the H-Y transplantation antigen, but the serologically detectable H-Y antigen was present (*31*). The ability of the H-Y transplantation antigen to be recognized is determined by the antigen haplotype of MHC (major histocompatibility) antigens of the mouse. The possibility must be considered that a change in the MHC occurred so that the H-Y antigen could not be recognized. The investigators considered this possibility and performed skin grafting experiments, but no changes in the MHC antigens were detected. However, these experiments must be confirmed.

Since much of our present knowledge about a serologically detected H-Y antigen is based on immunologic assays, a brief description of the current assays and their limitations is of interest. In cytotoxic assays, antigen can be measured directly or indirectly by absorption of the antiserum. Antiserum that kills male cells has been produced in both mice and rats by grafting male skin onto females, or by injecting male spleen cells into females. Also serum from multiparous females appears to have H-Y antibody. Target cells include sperm, tail epidermal cells, and fetal testis cells (*30, 32*). In most of the cytotoxic assays cells from the same species are used so that the killing assays may be very specific. In one assay, a line of human male Burkitt lymphoma cells, called Raji cells, is used as the cytotoxic target for the H-Y antiserum raised in rats (*33*). Other assays depend on localizing antiserum bound to cells. This usually involves exposing reacting cells to hemagglutination reactions, fluorescent reagents, protein A, or peroxidase reagents (*30, 34, 35*). In all of these assays, there is the potential of detecting cross-reacting antibodies (*30-33*).

An exciting observation has been the discovery that the serologically detectable H-Y antigen is ubiquitous in that it has been found on all normal male tissue of mature animals so far tested. We do not yet know whether there is more than one cross-reacting antigen or how similar in structure and function different cross-reacting antigens might be. The conservation and widespread distribution of the serologically detected antigen has occurred because it has a function. Because the antigen is associated with the presence of a heterogametic gonad, it has been implicated as a director of gonadal differentiation. In mammals, its appearance is generally correlated with the presence of a Y chromosome or the presence of testes in the case of XX sex-reversed males. The presence of the H-Y antigen in sex-reversed situations—for example, the freemartin cattle, polled goats, and sex-reversed mice—make it an excellent candidate for a testicular inducer (*36, 37*).

The H-Y antigen is not species-specific. H-Y antigen assays performed on tissues from animals that have autosomal recessive sex-reversed conditions (goats and dogs) can be interpreted as indicating intermediate levels of serologic H-Y antigen in the heterozygous females (*37, 38*). Here, a quantitative determination is being made with an indirect assay. Further studies that detect the number of molecules of H-Y antigen on a cell surface may give additional information. Comparing different species is somewhat risky, even if sex differentiation occurs under similar controls. The events are differently timed, and exact correlations cannot be drawn between the amount of serologically detectable H-Y antigen and the degree of sex reversal resulting in testicular tissue.

After serologic assays for H-Y antigen were developed, it became possible to examine the timing of its appearance, its secretion, and its interaction with other cells, as well as its biochemical properties. Serologic detection of H-Y antigen has been reported on mouse eight-cell embryos (*1*). After a group of eight-cell morulae was exposed to H-Y antiserum and complement, 50 percent of the embryos lost viability or died. Those embryos that were killed in this way were possibly male embryos, and the female embryos survived (*1*). Control antiserum that had been extensively absorbed with male cells did not kill embryos but antiserum absorbed with female cells did kill embryos. However, it was not shown that only females survived, because the remaining embryos were not implanted into foster mothers to determine the sex of the survivors. Even in immunizations with syngeneic animals, massive absorptions are required to remove nonspecific antibodies that react against embryos (*39*). Willison and Stern pointed out the need for using monoclonal antibodies when studying the antigenic determinants on an early morula, and they have found a stage-specific antibody on eight-cell embryos (*40*).

Other work on the timing of the appearance of H-Y antigen has involved the transplantation H-Y antigen. Immunologic tolerance to H-Y antigen can be induced by injections of male tissues into neonatal females. Immunologic tolerance has not been induced by injections of tissue from 8-day embryos, but it can be produced by injecting tissue from 11-day embryos into neonatal females (*41, 42*). The H-Y antigen recognized by histocompatibility may not have appeared in mice until after germ cell migration has started, but has appeared by the time of genital ridge differentiation. It is not clear whether the cells lack these antigens or whether there are insufficient antigens to induce tolerance. The precise timing of serologically detectable H-Y antigen in mice may be better defined when specific monoclonal antibodies become available.

The time of appearance of serologically detected H-Y antigen has not been studied in fetal rats. The data obtained by examining germ cells from the testes of the newborn suggest that serologically detectable H-Y antigen is not present on spermatogonia until the spermatogonia mature (*43*). If germ cells can direct gonadal differentiation, they do so by a system different from the H-Y antigen.

Early somatic cells of the testis, the Sertoli cells, secrete a substance that has properties of serological H-Y antigen and is bound by prepubescent Leydig cells. Further, the rat Sertoli cells seem to be able to release serologically detectable H-Y antigen at all ages, but the Leydig cells can only bind it after puberty, that is, at 30 to 40 days of age (*43*). The secreted serologically detected antigen, which permits the conversion of fetal tissue, has been studied in cattle, mice, and rats; each of these animal systems has its own characteristic developmental properties that allow specific function to be studied. Rat gonadal differentiation was studied by Turner (*44*) who showed that fetal ovaries would develop testicular elements if grafted beneath tunica albuginea of the testes but not if grafted under a male kidney capsule, implying that masculinizing substance was present in rat testes. The bovine system is historically very important and still provides much information.

Freemartins in cattle provide a classic example of gonadal sex reversal in mammals. Freemartins are genetic female cattle, which in twin or multiple pregnancies develop in parabiosis with at least one male twin. Because of the ability of multiple placentas to fuse and establish vascular anastomosis, early exchange of fetal blood occurs. Modification of the females results in masculinized ovaries that frequently contain sterile seminiferous tubules. The active masculinization of internal and external ducts very probably depends entirely on the amount of active testicular tis-

sue formed during the sex reversal process (*45*). Serologically detected H-Y antigen has been found in the gonads of freemartin cattle (*46*). Work in the laboratory of one of us (S.O.) has been directed toward further defining the process of freemartin gonadal formation. The serologically detectable H-Y antigen that is used in these experiments does not come from epididymal fluid or Sertoli cell secretion but from the culture media in which Daudi cells have been growing. Daudi cells are a line of human male Burkitt's lymphoma cells which have reduced levels of histocompatibility (HLA) antigens on their surface, and which are associated with an undetectable level of β_2 microglobulin. This microglobulin interacts with HLA-A and HLA-B antigens to form dimers that potentially serve as anchorage sites for organogenetic directing antigens (*47*). Freemartin gonads can be induced with partially purified fractions of supernatant from growing Daudi cell cultures. These freemartin gonads develop seminiferous tubules and a tunica albuginea, as was determined by histological studies (*48*). When bovine ovarian cells are disassociated and permitted to reaggregate, exposure of the disassociated cells to the concentrated Daudi extracted proteins causes the free suspension of fetal ovarian cells to form seminiferous tubule-like structures (*48*). Other workers have done similar experiments with rat gonadal tissue and in addition were able to block normal testicular aggregation with H-Y antiserum (*50*). Further, when epididymal fluid has induced tubular aggregation in Moscona reaggregation experiments (*51*), the cells acquire LH/HCG receptors, characteristic of fetal Leydig cells. Wachtel and Hall found that not only did H-Y antiserum prevent serological reaggregation directed by H-Y antigen but also that a diffusible factor from newly differentiated fetal ovary of the dog is able to prevent the binding of H-Y antigen to ovarian cells (*52*). The ability of H-Y antiserum to block H-Y antigen activity in vitro implies an interaction between the H-Y antigen and the target cells to produce masculinization. Blocking of testicular differentiation may, but does not necessarily, imply ovarian differentiation. Antiserum to H-Y and fetal ovarian extract block testicular differentiation in an XY genital ridge and permit varying degrees of ovarian differentiation. An ovarian organizing determinant could be an active substance or could be an unreacted receptor for the testicular-inducing substance. One of the problems with many of these experiments is that, although the antiserum used to block testicular reaggregation is known to react with serologically detectable H-Y antigen, the antiserum has not been rendered inactive by absorption with male cells and has not been shown to lose its ability to block testicular formation at the same time.

The substance that Daudi cells secrete behaves very much like serologically detectable H-Y antigen, although its properties have not yet been clearly defined (*48*). It exists naturally as a series of polymers, and dissociation into 18,000-dalton (about) subunits occurs when a Sephadex G200 fraction of the media in which Daudi cells have grown is exposed to sodium dodecyl sulfate (SDS) and 5 percent mercaptoethanol. Ohno has previously proposed that the β_2-microglobulin associated with the MHC antigens (H-2D and H-2K of the mouse and HLA-A and HLA-B of man) functions as one of the plasma membrane anchorage sites for H-Y antigen (*47*). Antiserum to H-Y can only be produced in the presence of specific MHC haplotypes. One interpretation of this MHC restriction is that the H-Y antigen is coupled with β_2-microglobulin major histocompatibility antigen dimers to form hybrid antigens on the male plasma membrane (*53*). Evidence supporting this theory comes from the inability of β_2-m(−), HLA(−) human male Burkitt cells stably to maintain H-Y antigen on their plasma membrane (*46, 48, 49*). However, experiments with individually produced antiserum directed against H-2K, H-2D, and H-Y antigens, demonstrated that the H-Y antigen and H-2 antigens are somewhat independent since they did not cocap on the male lymphocyte plasma membrane (*54*).

If H-Y antigen and the H-2 antigen form complexes, they do so in such a way that the H-2 sites when coupled with H-Y antigen are rendered unrecognizable by available antibodies.

Thus we venture to suggest that the cell-bound complex that contains H-Y antigen could be the testicular organizer. Of note is the observation that male Sertoli cells can be killed in a cytotoxic reaction when exposed to antiserum and complement. Although ovarian cells can bind H-Y antigen from epididymal fluid, this bound antigen does not give the cytotoxic properties to ovarian cells (*51*). The specific binding of H-Y antigen implies the existence of a cell surface receptor for this product. If the proposed H-Y antigen-receptor complex is the active form of the testicular-inducing substance, the receptor must be able to bind either free or cell-bound H-Y antigen.

Genetics of Testicular Differentiation and Male-Specific Antigens

For many years the Y chromosome was thought to be largely inert. XO individuals without either a Y chromosome or a second X were viable, and this seemed to support the idea of an inactive Y chromosome. With time, maleness was shown to be determined by the Y chromosome, and therefore the phrase "sex-linked," which originally meant X-linked, is now applied to both X-linked and Y-linked genes.

An H-Y antigen is normally found by both transplantation immunology and serology when the Y chromosome is present. Mapping of a regulator locus for H-Y antigen expression to the Y has been attempted, and there is now evidence that the regulator control for serologically expressed H-Y antigen maps to the short arm of the Y chromosome (*55, 56*). Regulation of sex differentiation may be controlled by the Y chromosome, but the structural locus or loci for specific inducers of gonadal masculinization have not been precisely mapped.

The potential role of the X chromosome in male sex differentiation has been emphasized for some time (*57*). Another gene which causes the testicular feminization syndrome of androgen insensitivity is also located on the X chromosome (*26, 27*). Indeed, the fertile XY female wood lemming is H-Y antigen-negative in the presence of an X-linked mutant and the normal Y. In humans, X-linked structural abnormalities may influence H-Y antigen expression. Two recent reports implicate the X chromosomes. Cells from a patient with an aberrant X chromosome, in the presence of a Y chromosome, have been shown not to have serologically detectable H-Y antigen.

Breg *et al.* (*58*) described a series of patients with aberrant karyotypes, gonadal dysgenesis, and serologically active H-Y antigen. One of us (F.P.H.) has reevaluated the patients described by Breg *et al.* (*58*) and expanded the study to include more patients with an isochromosome for the long arm of X [i(Xq),] as well as patients with a deletion of the long arm of X and a patient with an X-10 balanced translocation. The cells of nine of eleven patients studied with an i(Xq) absorbed H-Y antiserum activity. Three patients with a long arm deletion of the X chromosome and one patient with an X-10 balanced translocation involving the long arm of X, have serologically detectable H-Y antigen. All patients were phenotypic females, with varying de-

grees of gonadal dysgenesis. Our observations are also being confirmed in other patients with X chromosomal abnormalities (*58, 59*). The cells from these patients have a cell surface antigen that cross-reacts in the H-Y antigen assay system but may not be active as a testicular organizing substance. Although no experiment has been performed on human gonads to show the presence of serologic H-Y antigen receptors, serologically detectable H-Y antigen in man may mature to lose its gonadal receptor binding ability, as measured on bovine fetal ovarian cells, but keep its antiserum-binding ability (*60*). Some phenotypic females, who have come to medical attention because they have gonadal dysgenesis, were found to have a 46,XY karyotype and be positive for H-Y antigen. Some of these patients may have a mutation in the receptor on genital ridge cells that binds the testicular-inducing substance, thereby preventing the formation of testes.

Thus, it would appear that the location of masculinizing substances, the H-Y antigen structural gene or genes, is not only on the Y chromosome. Regulatory genes for the expression of H-Y antigen are probably on the X as well as on the Y chromosome. A structural gene for H-Y antigen may be on either the X chromosome or an autosome. Both autosomal dominant and recessive forms of sex reversal occur and direct part of the testis organizing function normally assigned to the Y. According to Hamerton (*56*), these autosomal genes are mutational mimics of the Y-linked regulatory gene for testicular organogenesis (*56, 57*). Recent work in the laboratory of one of us (F.P.H.) has identified three individuals with 46,XY karyotype, who show testicular tissue and who are H-Y antigen negative. These patients may exemplify the situation in which the masculinizing substance that has been called H-Y antigen has lost its antigenic properties while retaining its testicular organizing properties. In these individuals the germ cells presumably were 46,XY and may have been the primary testicular organizers. The regulation of H-Y antigen in these individuals is not clear (*61*).

Reports of H-Y antigen activity in mosaic cell lines are puzzling. Most of the studies are performed on patients that are mosaic with a line of 45,X cells. The other mosaic line may have a Y chromosome or a structurally rearranged X chromosome. At the moment, there is no obvious explanation for the wide spectrum of serologically detectable H-Y antigen levels that have been found. A few H-Y antigen-positive cells could convert a whole culture, but that does not explain lines of 45,X mosaic cells which have been identified that are H-Y antigen-positive at levels comparable to normal male cells, with less than 3 percent of their cells mosaic for another karyotype. Since it is possible that the normal regulation for H-Y antigen is first expressed quite early, that is by the eight-cell embryo, H-Y antigen is constitutively expressed in a cell, and if nondisjunction occurs after the initiation of H-Y antigen expression, then all the cells will produce H-Y antigen regardless of their karyotype. The implication is that once a chromosome (X or an autosome) has started to express H-Y antigen, all of the descendants of that cell will continue to express H-Y antigen. The degree of H-Y antigen expression in a population may be an indication of the timing of the nondisjunctional event that produced mosaicism and its relationship to the timing of H-Y antigen expression (*53*).

There is a tantalizing hint on the nature of the above-noted Y-linked regulatory gene that is needed for the expression of H-Y antigen. The specific repeated DNA sequence conserved in evolution has been found on the W chromosome of snakes and birds as well as on the Y chromosome of mammals (*62*). While such a repeated DNA sequence may not specify a protein such as H-Y antigen, it may have a regulatory function and activate the H-Y structural gene.

Conclusion

Mammalian sex differentiation now appears to be regulated by several processes. Primordial germ cells may influence the development of the early gonad and might be able to override other differentiation signals. One substance that has met some of the criteria for a gonadal organizer is serologically detectable H-Y antigen. This antigen can apparently direct testicular organization without germ cells; it is present when testicular development has occurred, is recognizable as an active diffusible substance, and its action can be blocked. A receptor that binds H-Y antigen is being defined. Without specific recognizable masculinizing signals the cells will undergo early ovarian differentiation. With multiple directors of sex differentiation, germ cells, H-Y antigen, and a receptor for masculinizing substances, a good deal of work lies ahead to define their functions and precise methods of interaction.

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The Hormonal Control of Sexual Development

Jean D. Wilson, Fredrick W. George, James E. Griffin

Summary. Male and female human embryos develop identically during the first phase of gestation. The indifferent gonads then differentiate into ovaries or testes and soon begin to secrete their characteristic hormones. If ovaries (or no gonads) are present the final phenotype is female; thus no gonadal hormones are required for female development during embryogenesis. Two hormones of the fetal testis—Müllerian regression hormone and testosterone—are responsible for the formation of the male phenotype. Analysis of fibroblasts from the skin of patients with abnormalities of sexual development due to single gene defects shows that testosterone is responsible for virilization of the male internal genital tract, that its derivative dihydrotestosterone causes development of the male external genitalia, and that both hormones act in the embryo by the same receptor mechanisms operative in postnatal life.

Human embryos of both sexes develop in an identical fashion for the first 2 months of gestation, and only thereafter do anatomical and physiological development diverge to result in the formation of the male and female phenotypes. The fundamental mechanism of sexual differentiation was elucidated between 1947 and 1952 by Alfred Jost (*1*). He established that the castrated mammalian embryo develops as a female. Male development is induced in the embryo only in the presence of specific hormonal signals arising from the fetal testis. According to the Jost formulation—now the central dogma of sexual development—sexual differentiation is a sequential, ordered, and relatively simple process. Chromosomal sex, established at the time of conception, directs the development of either ovaries or testes. If testes develop, their hormonal secretions elicit the development of the male secondary sex characteristics, collectively known as the male phenotype. If an ovary develops or if no gonad is present, anatomical development is female in character.

Stimulated by this paradigm, subsequent investigators have sought to identify the specific hormones that are secreted by the fetal testis and to elucidate the control mechanisms that regulate the rates of secretion of these hormones at the crucial moment in embryonic development. They have also attempted to characterize, at the molecular and genetic level, the mechanisms by which the testicular hormones act to induce the conversion of the sexually indifferent embryo into the male phenotype. As a consequence, the original formulation of Jost has been refined and expanded, and insight has been obtained into the pathogenesis of many derangements of sexual development in humans which result from single gene defects that impede either the formation or the cellular actions of the hormones of the fetal testis.

Other authors have described the chromosomal basis for sex determination (*2*) and the mechanism by which the X and Y chromosomes cause the differentiation of the gonad into a testis or ovary (*3*). In this article we describe current concepts of the processes by which the fetal gonads acquire the capacity to function as endocrine organs and of the mechanisms by which the endocrine secretions of the fetal testis modulate male development. We focus first on the anatomical events involved in the formation of the sexual phenotypes and then on the factors that mediate this development.

Formation of the Sexual Phenotypes

The temporal relation between the differentiation of the ovary and testis and the development of the sexual phenotypes in the human embryo is shown schematically in Fig. 1. The germ cells do not originate in the embryo itself but rather in the yolk sac (*4*). By about the stage at which the embryo reaches 10 to 20 millimeters in crown-to-rump length, the germ cells migrate to their ultimate destination in the genital ridges of the embryo. After this migration, the primitive gonads in male and female embryos appear identical, and each such gonad has three components: (i) the primordial germ cells, (ii) the mesenchyme of the genital ridge, and (iii) a covering layer of epithelium. Histological differentiation begins when the germ cells in the testis

The authors are in the Department of Internal Medicine at the University of Texas Southwestern Medical School, Dallas 75235.

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