

SEX IN THE 90s: *SRY* and the Switch to the Male Pathway

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ABSTRACT

In mammals the male sex determination switch is controlled by a single gene on the Y chromosome, *SRY*. *SRY* encodes a protein with an HMG-like DNA-binding domain, which probably acts as a local organizer of chromatin structure. It is believed to regulate downstream genes in the sex determination cascade, although no direct targets of *SRY* are clearly known. More genes in the pathway have been isolated through mutation approaches in mouse and human. At least three genes, *SRY* itself, *SOX9*, and *DAXI*, are dosage sensitive, providing molecular evidence that the sex determination step operates at a critical threshold. *SRY* initiates development of a testis from the bipotential cells of the early gonad. The dimorphic male and female pathways present a rare opportunity to link a pivotal gene in development with morphogenetic mechanisms that operate to pattern an organ and the differentiation of its cells. Mechanisms of testis organogenesis triggered downstream of *SRY* include pathways of cell signaling controlling cell reorganization, cell proliferation, cell migration, and vascularization.

INTRODUCTION

Sex determination in different species can occur by a number of different mechanisms (reviewed by 12). Some species have an identical genetic makeup between the sexes and depend on external signals to control their sexual phenotypes, i.e. environmental sex determination (ESD). For example, in some fish sexual phenotype can be labile in adult life and is controlled by group dynamics that include hormonal and/or visual signals (40). In many reptiles (alligators and turtles), the sexual phenotype depends on the incubation temperature of the

embryo (116). In other species, chromosomal differences between the sexes provide an internal means of regulating sex determination. In this latter group, sex determination can be controlled by dosage-dependent mechanisms (DSD) or by the presence of a dominant gene (GSD), often on a heterogametic chromosome. Both *Drosophila* and *Caenorhabditis elegans* depend on a DSD system. In both cases the sex determination switch is controlled by the ratio of X chromosomes to autosomes. Although *Drosophila* has a Y chromosome, it is irrelevant for sex determination because XO individuals are male and XXY individuals are female.

Eutherian mammals belong to the GSD group: Females carry two X chromosomes, and males carry a single X chromosome and one Y chromosome that encodes a dominant gene that triggers male sex determination. Sexual development in mammals has been described as a three-step process: At the first step the genetic sex of the embryo is decided when the oocyte is fertilized by an X- or a Y-bearing sperm. In the second step, sex determination occurs when the fate of the bipotential gonad is determined by the expression of the Y-linked genic switch in the XY embryos. In the third step, male or female differentiation results from the hormonal secretions of the developing testis or ovary (108).

For most developmental processes, molecular information gained from work in non-vertebrate systems has been extremely valuable in rapidly advancing the field. Sex determination pathways in *Drosophila* and *Caenorhabditis elegans* have been worked out in molecular detail (23, 67, 113). In both organisms, the sex determination and dosage compensation pathways are linked, and many of the genes and proteins are known. However, no homology exists between these two systems or with any elements defined in vertebrate pathways, suggesting that the mechanisms for sex determination have evolved independently many times. This finding stands in sharp contrast to most other developmental pathways; for example, the homeobox gene control of body axis formation, where the proteins involved bear a high degree of homology from flies to mammals (48, 98). For this reason, work on *Drosophila* and *C. elegans* thus far has not shed much light on the mammalian sex determination system.

The existence of common primordia for alternative pathways of sexual development is an ancient and widespread phenomena in the animal kingdom (94). Furthermore, basic testis and ovary structure and function are similar in many vertebrates, suggesting that the basic pathways involved in building a testis or an ovary are conserved. It may be the proximal switch mechanisms that have evolved independently, giving rise to systems controlled primarily by temperature, hormones, dosage, or dominant genes, depending on differing developmental circumstances of the animals. Fish and other animals that live in water can rely on diffusion of chemical cues and presumably benefit from the ability to switch sex in adult life, depending on the optimal survival requirements

of the group. Among lizards and turtles, instances of temperature-dependent and genotypic sex determination have been documented (26). Some evolutionary biologists suggest that the genic switch on the mammalian Y chromosome has been superimposed on older systems of sex determination that were primarily temperature or hormone based (26, 147). Mammals, after all, cannot rely on environmental cues for dimorphic development, either hormone or temperature based, because they develop within a temperature-controlled uterine environment, bathed in maternal hormones.

This maternal hormone environment may explain why the fundamental developmental pathway in mammals is female. Jost showed that surgical removal of the gonads during embryonic development of the rabbit resulted in development of female sexual characteristics no matter what the chromosomal sex of the embryo (79). Mainly because of this experiment, the female developmental pathway has often been referred to as the default pathway, although this terminology may be misleading because it suggests that the female pathway is not an active, genetically controlled process. Jost's experiments demonstrated that male secondary sexual differentiation depends on the presence of a testis in eutherian mammals. Hormones produced and exported by the developing testis are responsible for differentiation of the male urogenital tract and all male secondary sex characteristics. In the absence of the dominant gene on the Y chromosome, the basic cascade of gene expression in the embryo leads to the development of an ovary from the bipotential gonad and consequent development of female secondary sex characteristics. So far, no ovary-determining genes have been clearly identified in this pathway, although they must exist. Indeed, some investigators argue that the male sex-determining gene may function to repress the female pathway (53, 78, 95).

In 1959, the Y chromosome in mammals was shown to carry a dominant genetic determinant, such that 48:XXX^Y individuals are male and XO individuals are female (37, 74, 143). Through the work of cytogeneticists, who linked the presence or absence of a testis to Y chromosome translocations, rearrangements, or deletions, the minimal portion of the Y chromosome required to specify maleness was successively narrowed down (reviewed in 46, 57). In 1990, the gene from the sex-determining region of the Y chromosome was isolated in human (*SRY*)¹ and mouse (*Sry*) and shown to be conserved on the Y chromosome of all mammals so far tested (52, 126). *Sry* is both necessary and sufficient to trigger male development. Mutations of this gene in humans, or deletion of this single gene from the Y chromosome in mice (*Y^{Tdym1}*) (54, 89), lead to female development. Conversely, the presence of this gene in

¹In accordance with convention, throughout this review *SRY* will be used to refer to the gene from humans and other animals whereas *Sry* will be used to refer to the gene from mouse. *SRY* will refer to the protein in all species.

Sry transgenic mice on an XX background leads to the initiation of testis development and complete sex reversal (33, 83). It is believed that the expression of *Sry* in XY embryos interrupts development along the ovarian pathway and initiates a pattern of testis development in the bipotential gonad (87, 88, 100).

As in flies and worms, chromosomal differences between the sexes in mammals require gene dosage regulation. Dosage inequalities that result from the heterogametic system in mammals are resolved by the process of X-inactivation, which silences expression of most genes from one of the X chromosomes in XX individuals (4). It has been widely, but not universally, believed that X-inactivation and sex determination are independent processes in mammals (20). However, there is evidence in marsupials that the dosage of the X chromosome controls the differentiation of some dimorphic secondary sex characteristics (123), and there is also good evidence that dosage of a gene on the X chromosome is linked to sex determination in eutherian mammals (for review, see 111).

This review focuses on mammalian sex determination in mouse and human. Although *Sry* is often referred to as the mammalian sex determination gene, this is true only in the sense that *Sry* is the single Y-linked gene both necessary and sufficient to initiate male development. *Sry* operates as a molecular switch; however, it is clear that there are many critical genes required in both the male and female sex-determining pathways. Much of the recent progress in the field has related to *Sry* partly because it provides a unique and valuable access point for dissection of the sex determination pathway. Below, I summarize what is currently understood about the *Sry* gene, its transcript, and its protein. Other genes in the gonadogenesis/sex determination pathway discovered through expression and mutation approaches are described. Cell biology of the testis and the mechanisms operating downstream of *Sry* that trigger the testis developmental pathway in the bipotential gonad are also discussed.

SRY EXPRESSION

Linear Versus Circular Transcripts

Sry is expressed in the mouse gonad, in a narrow window of time between 10.5 and 12.0 days post-coital (dpc), immediately before the divergence of ovarian and testicular development is apparent, as the cells of the XY gonad organize into testis cords. Expression of *Sry* is limited to the gonadal portion of the urogenital ridge (ugr) from 10.5 to 12.0 dpc, as shown by in situ hybridization (84; A Swain & R Lovell-Badge, unpublished observations), polymerase chain reaction (76), and RNase protection techniques (55). Because of the low level of *Sry* expression, the gonadal transcript is undetectable on Northern blots. *Sry* expression has been analyzed only in the mouse during the critical period of gonadogenesis. RNase protection assays reveal that the mouse ugr *Sry* transcript is distinct. It initiates at a promoter specific to the ugr, and it includes

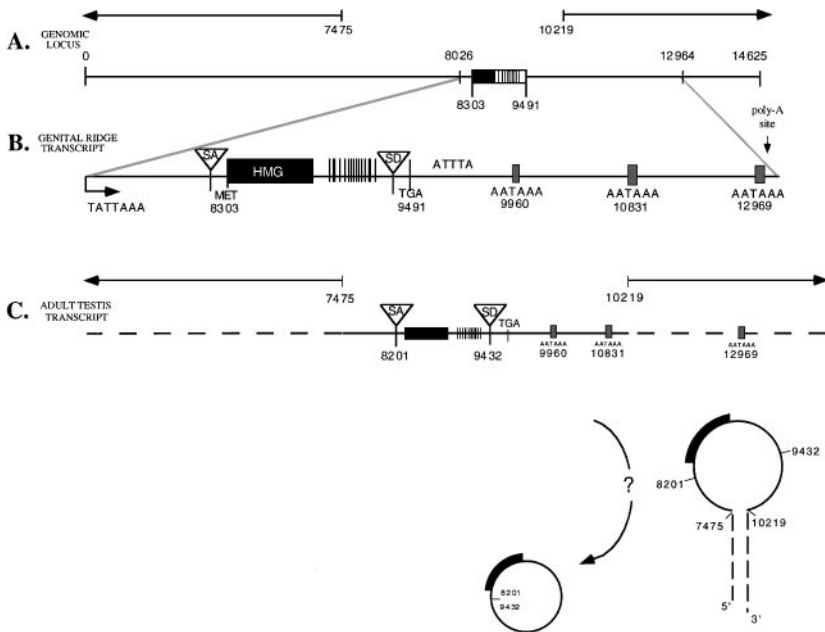


Figure 1 Structure of the mouse *Sry* transcript. **A.** Diagram of the *Sry* locus. The *Sry* gene sequence lies within a large inverted repeat that extends more than 15 kb on either side. **B.** The specific ugr transcript. The ugr transcript initiates at a gonad-specific promoter located inside the 5' arm of the inverted repeat. RNAase protection mapping shows that it extends as a single 5 kb exon, passing two potential poly-A sites, and terminating at the third. This transcript includes ≈ 1.2 kb of coding region plus 3.5 kb of 3' UTR. **C.** Primary and circular testis transcripts. The prominent adult testis transcript is a 1.2 kb circular RNA. RNAase protection analysis reveals a large minor transcript extending into the inverted repeat sequence at the 5' and 3' ends of the mouse *Sry* locus (*dashed lines*). The mechanism by which the circular transcript forms is not well understood, although it might arise as the result of an attack of a 3' splice donor (SD) against a 5' splice acceptor (SA). This event could be mediated by pairing between the homologous tails of the transcript.

3.5 kilobases of 3' untranslated sequence not present in transcripts reported in other tissues (Figure 1) (55).

In the adult mouse testis (and in some other heterologous sites), the *Sry* transcript is circular and probably results from cleavage of a larger primary transcript present at very low levels. The primary transcript is initiated at an unidentified promoter >1 kb 5' of the specific ugr promoter and continues beyond the ugr polyadenylation site (55) (Figure 1). The mouse *Sry* gene lies within a large inverted repeat extending more than 15 kb on either side of the unique coding region (54). The long primary transcript found in adult testis encodes homologous arms of this inverted repeat that could give rise to a secondary structure

capable of splicing to generate a circular transcript (18). In mouse, the circular transcript may be a consequence of the chromosomal situation of the gene. Experiments have shown that as few as 400 base pairs of the inverted repeats are sufficient for circularization of the *Sry* transcript in transfected COS cells (29). The circular RNA is thought not to be translated because it is not associated with polysomes (18). No inverted repeat surrounds the human *SRY* gene, and the circular transcript has not been found in humans or other mammals so far. If circularization is one means of disabling the *Sry* transcript when necessary, other species must disable the *Sry* transcript by other means.

No function has been assigned to the circular transcript through genetic analysis. It is expressed primarily in germ cells in the adult testis; however, no cell-autonomous effect on sperm development has been observed in mutants. The original XY-XY^{Tdym1} mosaic male transmitted its Y^{Tdym1} chromosome deleted for the *Sry* locus normally (54, 89). Similarly in humans, a mosaic father transmitted a mutant *SRY* allele containing a premature termination codon to two XY daughters (1, 62). In both cases, the mutant sperm developed normally in the absence of a functional *SRY* gene. However, it is possible that neighboring sperm with a normal *SRY* gene, developing in the same mosaic testis, compensated for the loss of a functional SRY protein in some cells.

The specific ugr transcription initiation site eliminates the 5' end of the inverted repeat present in transcripts in adult testes and other heterologous cell types (55). This may account for the absence of the circular transcript in the fetal gonad. The 3' region of the ugr transcript may also contain regulatory information. Detailed RNAase protection studies have defined a 3.5 kb untranslated region (UTR), 3' of the open reading frame (ORF) in the mouse ugr transcript (55). It seems likely that the UTR is involved in mediating post-transcriptional control: stability of the transcript, splicing, and/or translation. Regulation of *Sry* at the post-transcriptional, translational, and post-translational levels probably requires other interactive proteins. Cell lines derived from the genital ridge (where *Sry* is normally expressed and functional) have been established for in vitro studies of the regulation of *Sry* and of its interactions with other proteins normally expressed at the same time and in the same cell type (16).

Because human fetal tissue is not easily available, detailed analysis of the *SRY* transcript in humans is based largely on the testis transcript and transcripts generated in heterologous cell types in culture. Whether the human ugr transcript is different outside the ORF is unknown. The ORF of both mouse and human transcripts is encoded within a single exon common to the testis and ugr transcripts, and there is evidence that splicing does not occur within this ORF (22). *SRY* mRNA expression in humans (22) and in some other mammals occurs over a longer time period than the ≈ 40 h window of expression during mouse gonadogenesis (55). This gene is expressed in tissues other than those

of the gonad and testis in marsupial, human, and mouse (22, 59, 151), but no role has been ascribed to expression of *Sry*/*SRY* in other tissues. For example, in humans with mutations in *SRY*, no defects other than sex reversal have been described (121). In the absence of detailed antibody studies, it is not known whether expression of the protein parallels expression of the transcript in any species. Controls of translation and nuclear localization may exist.

Regulation of the Level of the Sry Transcript

The transcriptional regulation of *Sry* is still uncharacterized, although efforts to define the limits of the 5' regulatory region are under way in transgenic animals. Several problems have impeded progress of these experiments. For instance, efforts to devise a transcriptional reporter construct [e.g. with varying amounts of *Sry* 5' sequence driving β -galactosidase (β -gal)] have not been successful (V Navarez & R Lovell-Badge, personal communication); thus the efficacy of the transgene has relied on assays of sex reversal in transgenic animals. An *in vitro* assay for *Sry* expression would be valuable for defining the regulatory elements of the gene. *Sry* has been expressed from exogenous promoters in cell lines but has not yet been expressed from its own promoter sequences.

From transgenic studies where *Sry* is activated normally in a genetic female, it is assumed that all upstream genes required for activation are present on an XX genetic background. Several genes have been identified that appear to act upstream of gonad formation in both sexes, but none has been directly linked to activation of *Sry*. Based on the loss of gonads in null mutant mice, steroidogenic factor 1 (*Sf1*), the Wilms' tumor gene (*Wt1*), and two homeobox genes, *Lim1* (also known as *Lmx1*) and *Emx2*, all appear to be required for genital ridge development. In *Sf1*^{-/-} mutant mice, initial steps in gonad formation occur, but between 11.5 and 12.5 dpc the gonadal primordium regresses by apoptosis (90). *Sry* is still expressed in *Sf1*^{-/-} mutants (B Capel, unpublished results), showing that SF1 is not required for the transcriptional activation of *Sry*. In *Wt1*^{-/-} mutants, the genital ridge also forms initially, but thickening of the coelomic epithelium does not occur; by 14.5 dpc, no remnant of the gonad remains (85). At present, *Wt1* is not a likely candidate for an activation factor for *Sry* (P Koopman, personal communication). Most *Lim1*^{-/-} embryos die before gonad development begins (124). In the few *Lim1*^{-/-} mice that survive to birth, both gonads and kidneys are missing, but it is not known whether they never formed or regressed as in *Sf1*^{-/-} and *Wt1*^{-/-} mutants (R Behringer, personal communication). In *Emx2*^{-/-} mutant mice, development of the gonad becomes aberrant after 11.5 dpc. Regression of the gonad may be a result of defects in proliferation, differentiation, and/or survival of mesonephric or gonadal cells (104).

Many experiments suggest that the level of *Sry* expression is at a critical threshold for sex determination and thus is highly sensitive to dosage effects.

For example, in mutants in which regions of the Y chromosome between *Sry* and the centromere are deleted (*Sry*^{del}), the expression of *Sry* is depressed, resulting in mice that are sex reversed (17). In *Sry* transgenics, the level of *Sry* expression correlates with the frequency of sex reversal in different strains (A Hacker, N Vivian & R Lovell-Badge, unpublished results). Recent results in other sex-reversed mice also support this view (see below; C Nagamine, unpublished results). In humans, 10 to 20% of all 46 XY females harbor a mutation in *SRY* (15). De novo mutations in *SRY* almost always result in a complete failure of testicular differentiation and unambiguous female development, but in some families the unaffected father harbors the same mutation as the XY daughters. Several explanations for this finding include gonadal mosaicism in the father and variable penetrance of the mutation. Because the timing and expression level of *Sry* is critical in mouse, it is possible that these conditional sex reversal cases result from variable genetic backgrounds affecting the level of *SRY* expression in humans (15). A case of partial sex reversal has been found associated with deletion of sequences 3' of the *SRY* ORF. This deletion has been postulated also to affect the level of *SRY* expression (96).

THE *SRY* PROTEIN

Sry Encodes a Protein with a Conserved High Mobility Group (HMG) Box DNA-Binding Domain

Sry encodes a protein with an HMG box DNA-binding domain that is believed to regulate transcription of downstream genes (58, 88, 126). The HMG box region of *Sry* is highly conserved across all mammalian species sequenced. Mutations found within the DNA-binding domain of the human *SRY*, in cases of XY sex reversal, provide strong evidence that *SRY* is the sex-determining gene (58, 62–65). Many mutations have now been described in sex-reversed males within the HMG box of human *SRY* (Figure 2) (15, 63) and one located 5' of the ORF (97). No polymorphisms have been found in the HMG domain of normal males (15).

SRY sequences outside the HMG-binding domain bear little homology between species, either at the nucleotide or amino acid levels. For example, the glutamine-rich repeat region located 3' of the HMG box in the mouse protein is absent in human *SRY*. Activation of a target reporter gene in assays performed in heterologous cell types was shown to depend on the presence of the glutamine repeat sequence. The human protein did not activate the target in this assay (30). One possibility is that additional proteins associate with the human protein and serve as activator or repressor domains at binding sites. *SRY* may act as a repressor, an activator, or both in vivo, and its activity could be determined by a combinatorial complex at the binding site (82, 88).

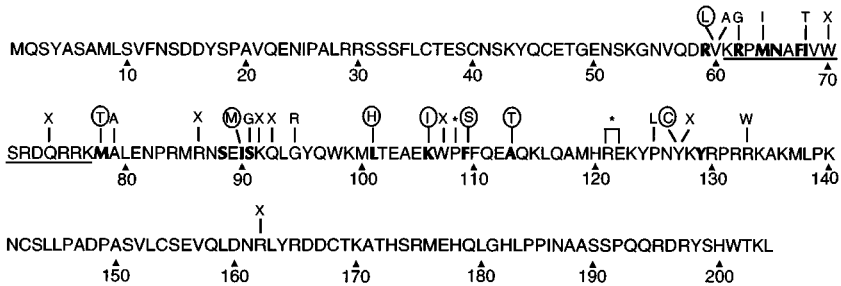


Figure 2 Amino acid sequence of the human SRY protein. Known mutations leading to amino acid changes causing 46:XY male to female sex reversal are indicated. Asterisks represent 1 or 4 bp deletions resulting in frameshifts. Mutations expected to destabilize the protein structure are circled; amino acids that contact the DNA appear in bold. The nuclear localization signal is underlined.

The regions of *Sry* outside the DNA-binding domain evolved rapidly (136, 137, 146), a process that might tune SRY for interactions with other proteins. This finding has led to the hypothesis that the rapid evolution of *Sry* may contribute to speciation (146). However, recent data reveal little variation between species in marsupial *Sry* sequences outside the HMG box DNA-binding region, indicating that speciation is not necessarily associated with divergence of *Sry* sequences (50). The rapid evolution of these sequences could also reflect few sequence-specific restraints on these regions, coupled with the location of *Sry* on the Y chromosome, which, lacking a pairing partner, is known to evolve rapidly.

A screen of an 8.5 dpc embryonic mouse cDNA library, using the *Sry* HMG domain as a probe, produced a number of *Sry*-related HMG box-containing SOX genes, members of a large family of genes all related through >60% homology in the HMG DNA-binding domain (25). So far, they all recognize and bind to the same DNA target sequence in site-selection assays in vitro, albeit with some differences in affinity (28). These proteins probably play highly specific roles in development. Their specificity likely depends on limited expression in specific cell types, nuclear localization, and interaction with different protein partners required for their activities as regulators. Efforts are under way to produce chimeric proteins between different SOX proteins and between SOX proteins and *Sry* to determine which regions of SOX genes regulate the specificity of transcriptional activation (V Harley, E Eicher & R Lovell-Badge, personal communications).

SRY Is Thought to Be Localized in the Nucleus, Where It Binds and Bends DNA

An antibody that detects nuclear localization of the human SRY protein has been reported. Nuclear localization is dependent on the presence of a nuclear

localization signal in the HMG domain (118). Efforts to obtain antibodies against mouse SRY have been unsuccessful; therefore, it has not been possible to study SRY protein localization during development. Entry into the nucleus may be a further level of regulation of SRY activity: Nuclear localization signals might be masked by regulatory proteins, limiting the access of HMG proteins to the nucleus under some conditions. Other SOX proteins also contain nuclear localization sequences (131). SOX9 is expressed in both male and female gonadal cells; however, soon after Sry expression begins in the male, SOX9 is transported to the nucleus of pre-Sertoli cells as their differentiation process initiates (27).

The HMG proteins, originally isolated as a heterogeneous group of non-histone components of the nucleosome complex (47), all contained a DNA-binding domain that became known as the HMG box. SRY and the SOX proteins contain the HMG-binding domain; however, they belong to a subset of the HMG proteins that display site-specific DNA-binding properties. Like other site-specific HMG proteins, they bind the minor groove of the DNA helix. They recognize bent or pre-structured DNA, and they bind linear DNA and induce a bend when they bind (36). Most data suggest that the SOX proteins and SRY function as local organizers of chromatin structure, rather than as classic activators or repressors (117). It is thought that HMG proteins play an architectural role in facilitating the assembly of nucleoprotein complexes at the target site (51). Studies of another HMG family member, LEF1, suggest that the bend induced in DNA may juxtapose other transcription factors bound at distant sites, resulting in the activation or repression of transcription of a target gene (43).

Comparisons of normal SRY and mutant SRY proteins from humans with 46 XY sex reversal in *in vitro* binding assays have distinguished mutations that affect DNA binding or bending properties (58). Molecular modeling of the interaction of SRY with chromatin has identified amino acids important to the structural stability of the protein and those involved in critical contact points with DNA (144) (Figures 2 and 3). Many of the mutations identified in XY sex-reversed individuals fall into one of these categories.

The HMG family member LEF1 interacts with β -catenin, a protein with a well-defined role linking the cytoskeleton with adhesion junctions. Interaction between β -catenin and LEF1 results in the nuclear localization of the complex and the activation of downstream targets of LEF1 (6, 68). Expression patterns and deficiencies in *Lef1*^{-/-} mice both suggest a functional role for LEF1 in tissues undergoing mesenchymal/epithelial inductions (140). It is often the case that cells embarking on a new transcriptional differentiation program also change cell shape and associations with other cells. The interaction of β -catenin, a cell structural protein, with an HMG protein known to regulate

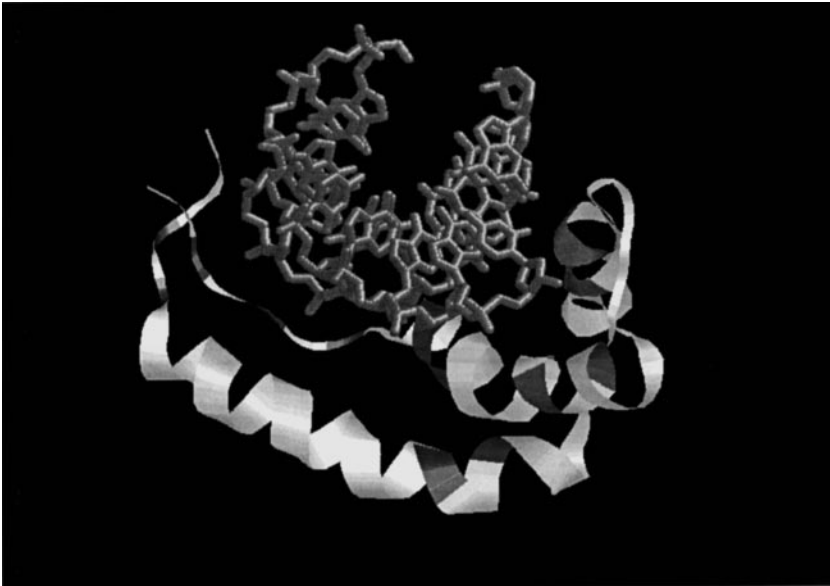


Figure 3 The HMG box of SRY bound to the DNA sequence 5' dGTTTGTGC. *Light areas* indicate three α helices of the SRY HMG box. Studies of human sex reversal (XY females) have implicated mutations affecting the residues highlighted in *gray*. Most of these mutations affect residues that contact the DNA (144). The image was rendered by C Mitchell & V Harley using RasMol2.6 (120). Molecular coordinates are from the Brookhaven Protein Data Base.

transcription provides a molecular link between cell shape changes and control of transcription. Given its role in cytoskeletal structure, β -catenin could be recruited to the nucleus to play some role in establishing and stabilizing chromatin domain structure induced by HMG proteins. It is not yet known whether this paradigm can be extended from LEF1 to other HMG proteins. Association between SRY (or any other SOX protein) and β -catenin has not been reported.

Specific Downstream Targets of SRY Have Been Difficult to Identify

One problem in identifying downstream targets of SRY is that no in vitro cell culture system has been available for conducting the assays. The battery of interacting proteins involved in regulating SRY and downstream targets may be present only in the cells in which *Sry* is natively expressed. Although this missing co-factor hypothesis seems likely, and has precedents in related pathways (125), it is also possible that SRY action is more global than gene specific. For example, if SRY establishes expression domains in chromatin, it

may not activate a classical promoter construct in transfection assays. It is also possible that the right pathway has not been tested.

An approach to finding genes downstream of *Sry* is to identify early differences in expression between male and female gonads using differential screens. This approach has revealed differences, but genes isolated from these screens are frequently abundantly expressed [e.g. α -globin and 3- β -hydrosteroid dehydrogenase (3- β -HSD)] (109; B Capel, unpublished data) and not particularly informative. Apart from differences in the expression of enzymatic steroid pathways, several early differences in expression between male and female gonads have been characterized downstream of *Sry* in the genes *Amh* (55, 105), *Sfl* (70), *Desert hedgehog* (*Dhh*) (10), and *Bmp8* (150).

Anti-Müllerian Hormone (AMH), otherwise known as Müllerian-inhibiting substance (MIS), is expressed by Sertoli cells and is an important factor in male differentiation; however, it is not required for testis formation. In the *Amh*^{-/-} mouse, Müllerian ducts persist, which leads to infertility due to ductal defects; however, testis development occurs normally (7, 8). SRY induces expression of *AMH*, but *AMH* is unlikely to be a direct binding target of SRY because mutation of the SRY-binding site 5' of *AMH* does not affect activation (56). However, this result could be explained if SRY promoted assembly of an activation complex at the *AMH* promoter, even if binding affinity were reduced by the mutation (145).

Differences in the expression of *Sfl* have been reported between male and female gonads after 12.5 dpc (70, 91). In addition to its role in the early formation of the gonad, SF1 acts at later stages of testis development to control androgen synthesis pathways in Leydig cells and to activate *AMH* in Sertoli cells (125). It may also have other roles in Sertoli cell differentiation that are critical to testis formation. A conditional *Sfl*^{-/-} mouse would be required to address this point since the early loss of the gonad in the null mutant precludes examination of later steps in testis development. This is also true for *Wtl*, which may also have a second role in testis development based on human mutations that lead to gonadal abnormalities (see below).

Desert hedgehog (*Dhh*) and *Patched* (*Ptc*) expression are involved in signaling pathways between Sertoli and germ cells (10), and *Bmp8* is also required within the testis for proper regulation of spermatogenesis (150). Although all three genes show expression differences between male and female gonads, neither *Dhh* nor *Bmp8* is required for testis morphogenesis because the testis develops normally in both deletion mutants. SRY also activates *Fra1* in vitro (24), but *Fra1* is not expressed at the appropriate developmental time in the mouse gonad, thus excluding a role for *Fra1* in sex determination (77) and illustrating the hazards of relying on data obtained in heterologous cell types.

MUTATIONS AFFECTING SEX DETERMINATION

Another approach to identify genes in the sex determination cascade is to study naturally occurring mutations known to affect sex determination in mouse and humans (see Table 1).

Mutations That Affect Sex Determination in Mouse

This analysis in mouse has centered on the Y^{POS} effect. The Y^{POS} chromosome functions normally within the *Mus musculus Poschiavinus* strain, but when crossed onto other genetic backgrounds (in particular, C57BL6), this Y chromosome leads to a high incidence of ovotestes or complete sex reversal and has been used as a classic genetic hypomorph to reveal other genes in the pathway

Table 1 Sex-reversal syndromes associated with gonadal dysgenesis

	Chromosome	Gene
Mouse		
Y^{POS}	Y	Sry
T^{Orl} (deletion)/ Y^{AKR}	17/Y	Brachyury (+/−)
T^{hp} (deletion)/ Y^{AKR}	17/Y	Brachyury (+/−)
W^{19H} , W^e	5	c-Kit
$S1^d$	10	Mgf^{SL-d}
Tda1	2 ctr	?
Tda2	4 dist	?
Tda3	5 ctr	?
XY^{Tdym1}	Y	Sry
XY^{del}	Y	?
Human		
XY gonadal dysgenesis	Y (+?)	~15% SRY
DSS, duplication	Xp21	DAX1
Campomelic dysplasia	17q	SOX9
Denys-Drash	11p	WT1
Frasier	11p	WT1
Deletions	9pter	?
Deletions	10qter	?
Short-rib polydactyly type IV	?	?
X-linked alpha thalassemia/ mental retardation (ATR-X)	Xq13	XH2
Smith-Lemli-Opitz	7q32.1 (?)	?

Most entries are self-explanatory. T^{Orl} and T^{hp} are both deletions in the *Brachury* region of chromosome 17 (more than one gene may be deleted). Effects are revealed in association with a Y chromosome from the AKR strain. XY^{del} are a set of deletions of repeat sequences between *Sry* and the centromere that affect *Sry* expression. XY gonadal dysgenesis is a complex set of syndromes of which ~15% are associated with mutations in the HMG domain of *SRY*.

(34). These data implicate testis-determining autosomal (*Tda*) loci, termed *Tda1*, *Tda2*, *Tda3*, etc, that affect sex determination. Backcross mapping has shown a high correlation of sex reversal with chromosomes 2, 4, and perhaps 5 (35).

Levels of *Sry* expression have been examined in a variant of this allele: The Y^{TIR} allele: The level of *Sry* expression is depressed in sex-reversed mice carrying this allele and appears to recover near normal in crosses that relieve the effect. This finding suggests that at least part of the Y^{POS} effect is due to a genetic interaction that affects the level of *Sry* expression (C Nagamine, personal communication). One of the interactive loci appears to be a protein involved in the transcriptional regulation or stability of *Sry* RNA. A combined transgenic and comparative sequence approach indicates that this effect does not map to the 5' regulatory region, the coding region of the Y^{POS} *Sry* gene, or the proximal 3' region. By process of elimination, this maps the Y^{POS} effect to the distal 3' region of the gene (K Albrecht & E Eicher, personal communication).

Several other mouse mutations give a high incidence of male-to-female sex reversal revealed by a cross in which the Y chromosome from the AKR/J inbred strain is placed on a C57BL/6J background carrying either of two alleles at the T locus on mouse chromosome 17 (T^{Ori} and T^{hp}) (32, 142), or alleles at the dominant white spotting locus *W(cKit)*, or its ligand *Steel* (13, 19, 107). These cases usually result in ovotestes; however, the basis for sex reversal is not known.

Mutations That Affect Sex Determination in Humans

One gene involved in sex determination identified through a human mutation approach is *SOX9*, which encodes a *SRY*-related HMG box-containing protein. *SOX9* was identified by cloning the chromosomal breakpoints of translocations in patients with campomelic dysplasia, a disabling bone disease associated with a high incidence of male-to-female sex reversal (38, 141). In addition to its role in bone morphogenesis, *Sox9* is closely associated with testis development in mouse, and it may be essential for Sertoli cell differentiation. Expression of the gene is upregulated in the XY gonad soon after expression of *Sry* begins, and expression of *Sox9* is conserved in testis formation in other mammals and in chick (27, 81, 122). Genetic evidence suggests that *SOX9* and *SRY* operate in the same pathway to specify male development because female development is not affected in patients with campomelic dysplasia. Evidence from human mutational analysis indicates that *SOX9* is haplo insufficient. Sequenced mutations are present in a single allele and are predicted to result in loss of function rather than to act as dominant-negative proteins (38). These data suggest that the dosage level of *SOX9*, *SRY*, and other proteins in the sex determination pathway is critical for normal male development.

A region of the short arm of the X chromosome, Xp21, can cause male-to-female sex reversal when duplicated or translocated to an autosome. It is postulated that a dosage-sensitive gene, *DSS* (dosage-sensitive sex reversal), encoded in Xp21 can cause female development, even in the presence of a functional copy of *SRY* on the Y chromosome (5, 110).

This finding suggested that the dosage of the X chromosome is important for sex determination in mammals, even though the process normally depends on the dominant action of *SRY*. Because XXY individuals are male, it is assumed that levels of *DSS* are usually regulated by X chromosome inactivation mechanisms ensuring that XX cells produce a single allele dose. This dosage control is bypassed by duplication within the active X chromosome or translocation of the *DSS* to an autosome. The usual inactivation of all but one X chromosome in mammalian cells would normally mask any effects resulting from X chromosome dosage differences between males and females.

In eutherian mammals, all secondary sex characteristics are downstream of the development of a testis or ovary and probably depend on hormonal secretions of the developing gonad; however, in marsupials, some sex-specific secondary characteristics develop prior to the development of a testis. An *SRY* gene has been isolated in marsupials that is likely the pivotal testis determination signal (39); however, the initial development of a pouch or a scrotum is believed to be controlled by the ratio of X chromosomes to autosomes (123). This finding implies that several mechanisms of sex determination may be superimposed in mammals. Eutherian mammals and marsupials have evolved a mode of sex determination dependent on a dominant genic determinant present on the Y chromosome. However, marsupials appear to have retained a partial dependence on the X:autosome ratio. It could be that a dosage mechanism underlies genic sex determination in eutherian mammals as well but is normally masked by X inactivation that controls the dosage of the X-linked determinant. In males, the single X chromosome was originally thought not to be inactivated, but a recent report indicates that some somatic cells of the testis do inactivate their X chromosome during critical stages of testis determination (75). These data suggest a different model in which inactivation of the single X chromosome in Sertoli cell precursors permits the testis-determining gene *SRY* to initiate the male pathway.

An X-linked gene, *DAX1* (*DSS-AHC* critical region on the X gene 1), has been isolated from the interval Xp21. Although several other genes are present in this interval, *DAX1* is the leading candidate for the gene responsible for dosage-sensitive sex reversal syndrome (106, 148, 149). Mutations in *DAX1* lead to adrenal hypoplasia. Expression of *Dax1* in the mouse gonad is also consistent with a role in sex determination: Expression is coincident with *Sry* expression at 11.5 dpc, it continues in the ovary, but turns off in testis after

the initiation of cord development about 12.0 dpc (132). *Dax1* and *Sfl*, both of which encode orphan nuclear receptors, might act in a common pathway of endocrine development since their expression co-localizes in multiple cell lineages (71, 73). Similar to *Sfl*, *Dax1* may act at more than one stage of development. At the bifurcation of the developmental pathways of ovary and testis, *DSS* probably acts either as a dosage-sensitive competitor of the male pathway or as an initiator of the female pathway. One might have predicted that *DSS* would be conserved on the X chromosome of all mammals, although this is not a requisite for dosage control. In marsupials and monotremes, the entire syntenic cluster in Xp21, including *DAX1*, is located on an autosome and is probably not part of the ancient X chromosome (50). Efforts to overexpress *Dax1* in transgenic mice to determine whether an increased dose of the gene can trigger female development of an XY embryo are under way (A Swain & R Lovell-Badge, personal communication).

Sox3, the SOX gene most closely related to *Sry* itself, maps to a region of the X chromosome believed to be part of the original X chromosome shared by all mammals. It has been suggested that the SOX3 gene was originally present on the X and Y chromosomes, but the Y-linked allele *SRY*, once isolated from recombination, evolved rapidly (25, 50). Because all the *Sry*-related SOX genes recognize the same DNA-binding target sequence in vitro, it has been speculated that *SRY* could compete with SOX3. If *SRY* gradually evolved away from SOX3, it could acquire a function as a male-determining gene by competing for a binding site and interfering with an ancient X-linked function of SOX3 to initiate the female pathway (49, 69). There is no evidence for this hypothesis.

Sox3 is expressed in early neural tissue and in somatic cells of the developing gonad of both sexes (25). Human mutations in this gene are associated with an X-linked mental retardation syndrome but show no defect in primary testis development. Larger deletions of this region of the X chromosome (Xq26-27) are associated with premature ovarian failure, but there is no reason to believe that *SOX3* is responsible, nor is there any association of *SOX3* with primary ovarian developmental defects (129). Because *Sox3* likely plays a critical role early in embryonic development (M Parsons & R Lovell-Badge, personal communication), a conditional null mutant mouse and/or a mouse overexpressing this gene may be required to resolve this issue.

Two human disorders, Denys-Drash syndrome (DDS) and Frasier syndrome, are associated with genitourinary defects. DDS is due to mutations in the *WT1* gene that include an amino acid substitution affecting a protein-DNA contact point, premature termination of protein translation, or the elimination of a splicing isoform. In each case, the DDS patient is heterozygous for the mutation, suggesting that there is also a critical dosage or dominant-negative

effect of this protein in the sex determination pathway (60). For any one of these mutations, there is a wide range of phenotypic variation among DDS patients (21, 60, 114). Frasier syndrome similarly includes a range of genitourinary abnormalities, including chronic renal failure, gonadal dysgenesis, and a high incidence of gonadoblastoma. In five patients diagnosed with Frasier syndrome, mutations were found in the WT1 splice donor site in intron 9; these mutations are predicted to remove the KTS motif between the last two zinc fingers (K McElreavey, personal communication).

Other disorders associated with sex reversal include X-linked alpha thalassemia/mental retardation (ATR-X) and Smith-Lemli-Opitz (SLO). ATR-X has been mapped to the *XH2* gene on Xq13 (42, 72, 128). *XH2* is a homologue of a *Drosophila* gene implicated in general transcriptional domain activation (134). SLO has been mapped to 7q32.1, although no candidate gene has been identified (2). This disorder impairs cholesterol biosynthesis (80), perhaps leading to deficiencies in steroid hormone production. The genes responsible for several other human syndromes associated with gonadal dysgenesis are still unknown. Table 1 presents a summary of these findings.

CELL BIOLOGY OF THE DEVELOPING TESTIS

The gonad arises as an identical primordium in male and female embryos. If *Sry* is not expressed, ovarian development is initiated. If a Y chromosome is present, *Sry* is expressed at a critical time in development and acts as a genetic switch to divert development of the undifferentiated gonad from the female to the male pathway. After *Sry* is expressed, the gonad grows in size and the cells reorganize into cord-like structures. In the absence of *Sry* expression, little change in size or cellular organization is apparent. Thus *Sry* apparently triggers mechanisms that lead to the divergence of organ architecture. Based on genetic analysis of XX-XY chimeras, the gene is required only in the supporting cell lineage, which gives rise to the Sertoli cells in the testis (14, 112).

Sry is thought to act as a cell fate determinant in pre-Sertoli cells, which probably recruit other cell types in the developing testis to the male pathway by cell-cell interactions (87, 92). In addition, *Sry* acts as a morphogenetic switch, initiating cell-cell reorganization processes such as extracellular matrix remodeling, cell associations, cell movements, and vascularization. These changes are apparent 12–24 h after *Sry* expression reaches its peak. It is not clear to what extent these two functions of *Sry* are separable, because the full differentiation of Sertoli cells may depend on the structural reorganization of the gonad (31, 66, 135). Differentiation of mammary epithelial cells, for example, requires contact with a laminin subunit normally present in the basal lamina (3, 130).

At least three somatic cell lineages participate in gonadal differentiation in addition to germ cells: the supporting cell lineage (Sertoli cells in the male; follicle (granulosa) cells in the female); the steroid-producing lineage (Leydig cells in the male; theca cells in the female); and connective tissue cells. The primordial germ cells are the only lineage whose origin is clearly established (44, 45); however, they are believed to play no role in determining the structure of the testis (i.e. in organizing the somatic cell lineages). In mutants lacking germ cells, the testis forms normally (99, 103). In females, however, germ cells are required to maintain the follicular structure of the ovary (101, 133).

The supporting and steroidogenic cells are thought to be present in the undifferentiated gonad by 11.5 dpc (102). It is not known whether they are distinct from the earliest stages of gonadogenesis. The expression of SF1 in a population of cells that segregate to the adrenal gland and the gonad during development of the adreno-genital primordium suggests that steroid lineage cells of these two organs originate together (61), but it does not address the issue of whether these cells are precursors of Leydig and Sertoli cells. Sertoli and Leydig cells may arise as a homogeneous population that takes on distinct differentiation characteristics through signaling processes similar to those that occur in other embryonic systems, for example by lateral inhibition signaling (86) or by mesenchymal/epithelial inductive interactions (9). They are distinguished clearly only after cord formation, when Sertoli cells aggregate inside cords and Leydig cells are partitioned to the interstitium. The third somatic cell group is a collection of cell types that comprise the connective tissue cells, about which little is known. These cells include endothelial cells and fibroblasts in both testis and ovary. The peritubular myoid cell, however, is specific to the male gonad, where it directly abuts Sertoli cells, surrounds the testis cords, and separates the Sertoli cells from the steroid-secreting Leydig cells in the interstitial tissue. In contrast, ovarian follicle cells are directly apposed to the steroid-secreting theca cells.

Organ culture experiments suggest that the myoid cells arise from a population of cells that migrate into the genital ridge from the mesonephros after 11.5 dpc. These experiments showed that the placement of a filter membrane between the mesonephros and gonad blocked cord formation (11, 102). The organ culture system has been refined by the use of a transgenic mouse that ubiquitously expresses β -gal (ROSA26) (41). When a mesonephros from a "blue" mouse is placed alongside a gonad from a "white" mouse (wild-type CD1) and cultured *in vitro*, blue cells move from the mesonephros to the gonad (93). Cell migration from the mesonephros to the gonad is a male-specific mechanism dependent on downstream responses to *Sry* activity within the genital ridge (Figure 4A). Cells can be induced to migrate into a female gonad by placing a small piece of a male gonad against the outside surface of the female gonad, demonstrating that the signal is an active rather than permissive chemoattractant

signal. When cells are induced to migrate into a female gonad, cord organization is also induced in the female tissue. The identity of the migrating cell types has been hampered by the absence of markers specific to the early cell lineages of the gonad; however, three migrating cell types have been distinguished.

Many cells that migrate from the mesonephros into the male gonad are associated with the vasculature. Some of these cells can be identified with a marker for endothelial cells, PECAM. Others are closely associated with the endothelial cells and are thought to be a myoepithelial cell type. The physiological

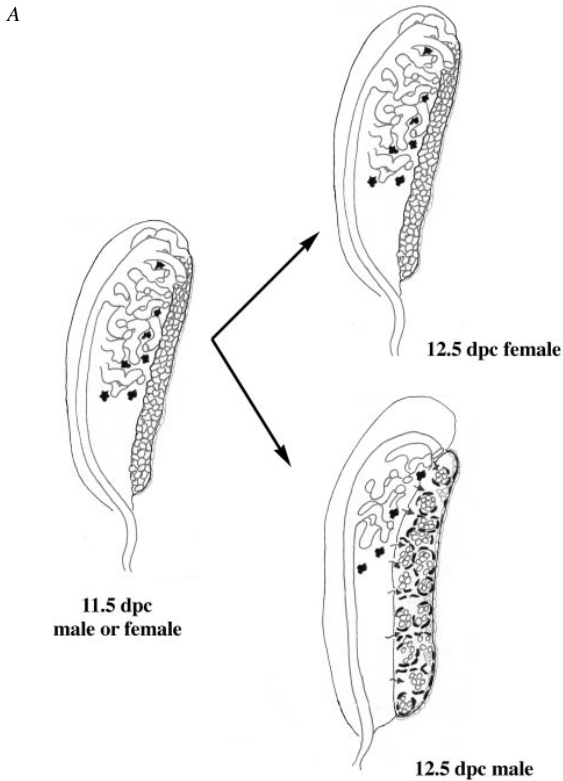


Figure 4 A. Migrating cells partition the cells in the gonadal primordium into testis cords. At 11.5 dpc, cells competent to respond to a migratory signal exist in the mesonephros of male and female gonads. Between 11.5 and 12.5 dpc, in response to a signal from the XY gonad, cells migrate into a male but not a female gonad. B. Models of cellular interactions critical for cord formation. Migrating cells may contribute structural components to the development of the basal lamina surrounding Sertoli cell cords. Alternatively, or additionally, migrating cells may provide lateral signals leading to the differentiation of Sertoli and peritubular myoid cells.

B

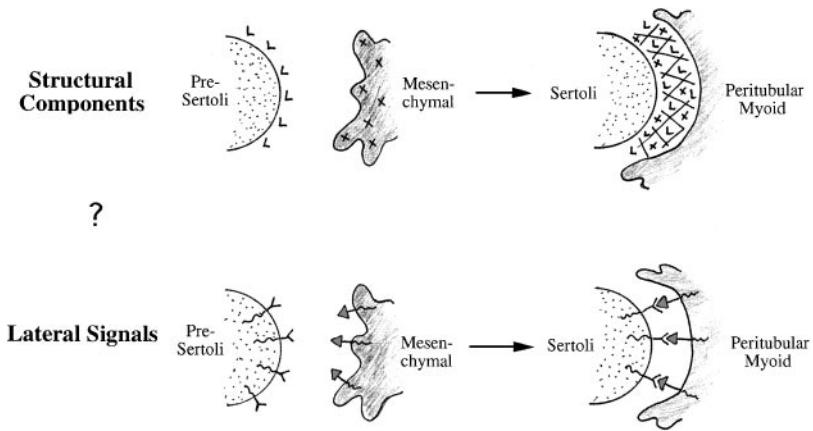


Figure 4 (Continued)

significance of this finding is not clear; however, it may be that the vasculature must be fully functional at an earlier stage in the testis than in the ovary. The male gonad is growing more rapidly at this stage, so that oxygen requirements are higher. Alternatively, the testicular vasculature may be required to export testosterone and AMH to masculinize the embryo, whereas the female embryo has no pressing requirement for the export of embryonic estrogens. Whether growth of the vasculature plays an architectural role in the floor plan of the organ is not known. There are no markers for peritubular myoid cells; however, some of the cells migrating into the gonad are located in direct apposition to Sertoli cells, bind the testis cords, and do not label with endothelial-specific markers. Based on work on cells of the adult testis (127, 138), peritubular myoid cells are believed to associate with Sertoli cells and participate in the formation of the testis cords. One possibility is that the arrival of this cell type triggers the polarization of the pre-Sertoli cell, possibly by bringing into the gonad a particular component required for the deposition of the basal lamina (115, 119, 139). Alternatively, or in addition, these cells may provide lateral signals that promote further differentiation of the Sertoli cell and other cell types already present in the gonad (see Figure 4B).

SUMMARY

The decision to enter the male developmental pathway in mammals is controlled by *Sry*, the dominant genetic switch on the Y chromosome. Much remains to be learned about the regulation of the *Sry* gene. SRY acts as a DNA-binding protein that probably plays a role in establishing higher-order chromatin structure.

While the direct targets of SRY have not been identified, SRY controls Sertoli cell differentiation and testis morphogenesis in the supporting cells of the gonad. Its activity may involve both activation and repression of downstream targets to divert development from the female pathway. Thus far, at least three genes in the pathway—*SRY*, *SOX9*, and *DAX1*—have been shown to be dosage sensitive, suggesting that this system is operating at a finely balanced threshold between male and female development. Studies of induced mouse mutations that impair sex determination and identification of human mutations associated with sex reversal have provided insight into the sex determination pathway. Because of the dimorphic nature of the male and female pathways, this system provides a rare opportunity to identify molecular and cellular mechanisms that link the expression of a pivotal gene in development with morphogenetic mechanisms that operate to pattern the development of an organ and the differentiation of its cells. In addition to the intrinsic interest in how mammalian sexual differentiation is controlled, the study will have broad relevance to our understanding of how the formation of organs is genetically controlled.

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