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The Search for the Male-Determining Gene

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Molecular Genetics of Sex Determination

I. Introduction

Several traits, including hairy ears, have been attributed to genes on the human Y chromosome, but sex determination was not included among them until recently. Less than 36 years ago, a prominent geneticist said: "That the (human) Y chromosome has a function of its own, is attested by its very existence. What it is still must be discovered" (Stern, 1957). In fact, the major function of the Y remained uncertain until 1959, when four papers appeared showing that the mammalian Y chromosome determines male sex: the report by Welshons and Russell (1959) showing that XO mice become females; the report by Jacobs and Strong (1959) describing the 47,XXY karyotype in a man with Klinefelter syndrome; the report by Ford *et al.* (1959b) describing the 45,X karyotype in a woman with Turner syndrome; and another by Ford *et al.* (1959a) describing two supernumerary chromosomes—an X and a 21—in a 48,XXY+21 man with Klinefelter and Down syndromes. Those papers provided strong evidence for male-determining genes on the Y chromosome. They indicated, moveover, that a single Y chromosome is sufficient to induce differentiation of the embryonic testis—even in the presence of two or more X chromosomes (so sex determination in the human is different from that in *Drosophila*). Indeed, 48,XXXY and 49,XXXXY males also have been described, severely retarded, but unmistakably male.

Today we know that the sex-determining action of the Y chromosome is limited in particular to the development of the testis (reviewed in Wolf *et al.*, 1992). Further male differentiation is due to the action of testicular hormones and their metabolites (Fig. 1). Thus in the presence of the Y, the gonad becomes a testis: the testis secretes testosterone, which governs timely differentiation of the epididymides, vasa deferentia, and seminal vesicles, and anti-Müllerian hormone, which blocks development of the uterus and fallopian tubes. Dihydrotestosterone, the 5α-reductase metabolite of testosterone, and a more potent androgen, induces orderly differentiation of the external genitalia. In the mutational absence of androgen or of the androgen receptor, the embryo develops the female phenotype, despite presence of the Y chromosome and of the testes (see Chapters 15 and 16, this volume).

It is a dictum of biology that genes of fundamental significance tend to be conserved. On that basis a number of genes and sequences have been proposed as candidates for the elusive Y-situated testis-determining gene, now called *TDF* for testis-determining factor. Among them are *HYS*, the determinant of serological histocompatibility-Y (H-Y) antigen; Bkm (banded krait minor) DNA, a satellite cloned origi-

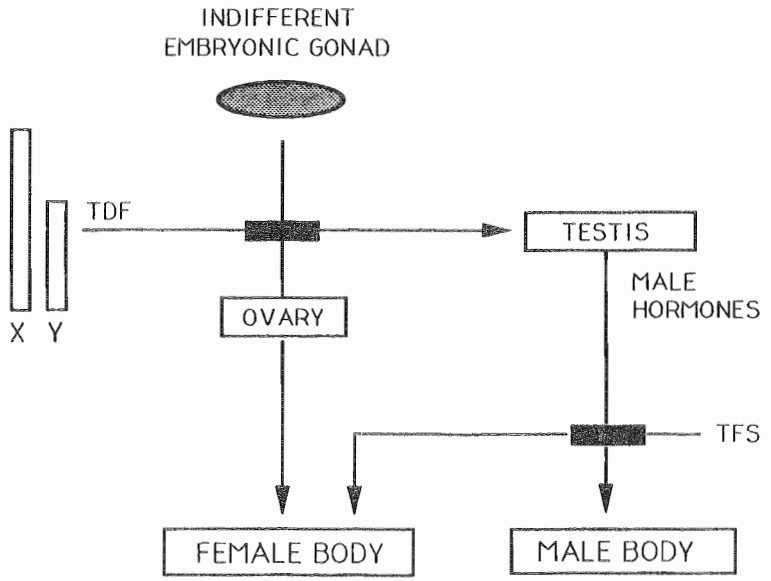

Figure 1 Sex determination in the human. In normal XX embryos, the gonad becomes an ovary and the body develops along female lines. In the presence of the Y-situated testis-determining gene, *TDF*, the gonad becomes a testis. The testis secretes testosterone, which masculinizes the Wolffian ducts, and anti-Müllerian hormone, which blocks formation of the female (Müllerian) ducts; the end result is the male phenotype. In cases of mutation of the X-linked gene encoding the androgen receptor, the response to testosterone (and its metabolite, dihydrotestosterone) is inadequate, and the embryo develops as a female despite presence of XY chromosomes and testes (TFS, testicular feminization syndrome).

nally from the sex-determining W chromosome of a snake; *ZFY* (zinc finger Y); and *SRY* (sex-determining region Y). In this chapter we shall discuss each of these candidates, with emphasis on *ZFY* and *SRY*. We shall consider the role of *TDF* in normal sex determination and the effect of perturbations of *TDF* in XX male syndrome, XX true hermaphroditism, and XY gonadal dysgenesis.

II. H-Y Antigen

Male-specific antigens have been identified by the methods of transplantation, cell-mediated cytotoxicity, and serology. The H-Y antigen that causes rejection of male skin grafts in female mice is probably the same as the male antigen identified in cell-mediated cytotoxicity tests, but there is evidence that it is different from the male antigen recognized by antibody from male-grafted females; the latter is sometimes

referred to as serological H-Y antigen and sometimes as SDM, for serologically detected male antigen (Simpson *et al.*, 1987).

When antibody from male-grafted female mice was found to react with cells from the heterogametic (XY) sex in mammals, birds, and amphibians, it was proposed that the H-Y serological antigen was the product of the mammalian testis-determining gene (Wachtel *et al.*, 1975). In fact, since the initial report of phylogenetic conservation of serological H-Y antigen, the molecule has been found in more than 80 species representing each of the major vertebrate classes (Table I).

We have already noted that the sex-determining function of the mammalian Y chromosome is limited to gonadal differentiation; hence embryonic castration leads to development of the female phenotype, even in the presence of a Y chromosome (Jost, 1947). It follows that the function of the *TDF* gene is also limited to gonadal differentiation. But that allows the prediction that the product of *TDF* should always be present in cases of testicular development, or, in terms of the serological H-Y antigen, that testicular differentiation in mammals should always occur in association with the H-Y^+ cellular phenotype, regardless of body sex or chromosomal sex.

To evaluate the association of serological H-Y antigen with testicular development, the molecule was therefore surveyed in cases of aberrant sex differentiation—especially cases of sex reversal such as XX male syndrome, XX true hermaphroditism, and XY gonadal dysgenesis. This is a standard practice in evaluation of candidates for *TDF*. In addition to providing information about the gene or sequence in question, this ap-

Table I
Phylogenetic Conservation of Serological H-Y Antigen

Class	Species	Sex chromosomes	H-Ys in
Mammalia	Human	XX/XY	Male
	Mouse	XX/XY	Male
Aves	Chicken	ZW/ZZ	Female
	Quail	ZW/ZZ	Female
Reptilia	Copperhead	ZW/ZZ	Female
	Alligator	TSD[a]	Male, female
Amphibia	Leopard frog	XX/XY	Male
	Clawed frog	ZW/ZZ	Female
Osteichthyes	Guppy	XX/XY	Male
Chondrichthyes	Horned shark	?	Male

[a] TSD, temperature-dependent sex determination.

proach can often provide insight concerning the etiology of the sex-reversed condition itself.

The initial experimental evidence seemed to support the idea that sex-specific antigens were responsible for male differentiation (Ohno *et al.*, 1979; Wachtel, 1983), but 9 years after the original proposal, McLaren *et al.* (1984) described XX male mice lacking the H-Y antigen in cell-mediated cytotoxicity tests, and more recently, Goldberg *et al.* (1991) reported that these mice also fail to express the male antigen identified in serological tests. Hence neither male antigen could be responsible for testicular development, and the search for *TDF* has been directed elsewhere.

Yet there may be more to the H-Y story. Serological H-Y induces seminiferous tubules in bovine fetal ovaries in culture (Ohno *et al.*, 1979). Thus when Behringer *et al.* 1990) found seminiferous tubules in the ovaries of transgenic female mice chronically expressing the human anti-Müllerian hormone (AMH), the question arose whether H-Y (SDM) might be related to AMH. H-Y antibodies *did* react with purified AMH in serological tests, and the reactions were inhibited by absorption of the antibodies with male cells (Müller *et al.*, 1993). H-Y (SDM) and AMH both are secreted by Sertoli cells and both may be encoded by autosomal genes under control of the Y chromosome. Since both can sex reverse ovarian cells *in vitro*, and both are implicated in sex reversal of ovarian cells *in vivo*, it was inferred that H-Y (SDM) and AMH are in fact similar molecules with a common epitope.

III. Bkm Satellite DNA

The human Y chromosome contains long repetitive DNA sequences known as satellites. These occur typically in the heterochromatic regions of the chromosome, which are in general genetically inert. Within these regions, the G : C and A : T nucleotide pairs are distributed unequally; for that reason, satellite DNA has a density that is different from the density of DNA in which the base pairs are equally represented. Satellite DNA thus can be identified and isolated from the main body of DNA by analytical centrifugation along a density gradient (Fig. 2). One of the more common patterns occurring in satellite DNA is the repetitive tetranucleotide GATA, which is highly conserved in evolution.

In snakes and birds, the female is the heterogametic sex, and carries the ZW chromosome pair. This is in contrast to the situation in mammals, in which the male is the heterogametic sex (XY). Like the Y chromosome, the W is largely heterochromatic, and contains many se-

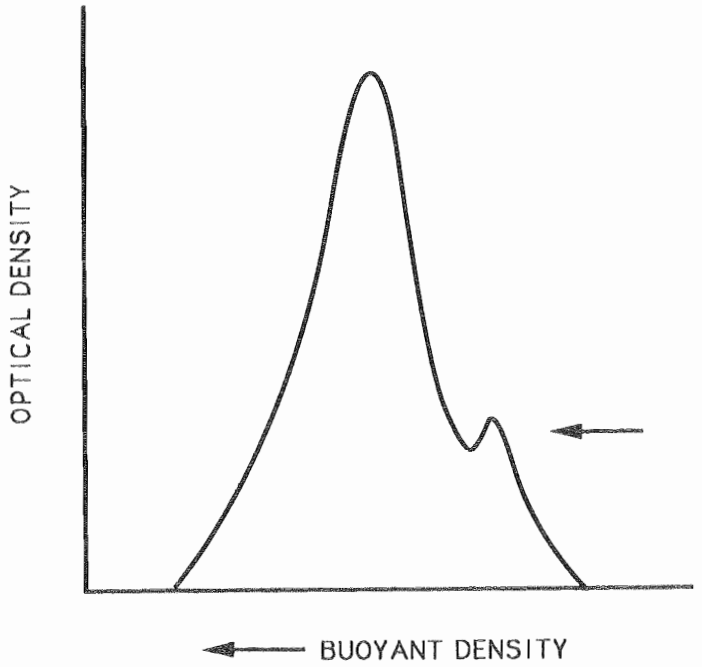

Figure 2 Satellite DNA. The buoyant density of double-stranded DNA—determined by centrifugation through a cesium chloride density gradient—is a function of G-C content. The larger peak above includes about 90% of the genome, centered about a value of 1.70 g cm^{-3} corresponding to an average G-C content of about 42% (which is typical of mammals). The smaller peak at the right (arrow), representing about 10% of the genome, is a *satellite* DNA with a G-C content of about 30% and a buoyant density of 1.69 g cm^{-3}. Satellites may have densities larger or smaller than the density of the main band.

quences of repetitive DNA. In fact several satellites have been isolated from the W chromosome of the banded krait, a snake. One of the satellites, consisting mostly of GATA repeats, was called banded krait minor satellite DNA (Singh *et al.*, 1981).

The Bkm satellite was found to be poorly represented among the autosomes of the banded krait and related ophidians: it was concentrated on the W chromosome instead. A comparative study of phylogenetically primitive, intermediate, and advanced snakes revealed that the association of Bkm with the W chromosome was correlated with the amount of heterochromatin on the W; this was related in turn to the evolution of sex chromosome heteromorphism within the snakes (Singh *et al.*, 1976).

The discovery of Bkm-related sequences in taxa as diverse as reptiles, birds, mammals, and even insects signaled widespread phylogenetic distribution of Bkm, and thus its potential involvement in sex determination (Table II). In the mouse Bkm was found on the autosomes in males and females. But rich clusters of Bkm were identified in the sex-

Table II
Conservation of Bkm

Class	Species	Mechanism of sex determination	Sex-specific fragments in
Mammalia	Human	XX/XY	—[a]
	Mouse	XX/XY	Male
Aves	Chicken	ZW/ZZ	Female
	Japanese quail	ZW/ZZ	Female
Reptilia	Green turtle[b]	TSD[c]	Male, female
	Kemp's ridley[b]	TSD	Male
	Banded krait	ZW/ZZ	Female
	Cobra[b]	ZW/ZZ	Female
Osteichthyes	Rainbow trout	XX/XY	—
	Channel catfish[b]	XX/XY	—
Insecta	Mealworm moth	ZW/ZZ	—
	Drosophila[d]	XX/XY[e]	—

[a] Although Bkm sequences are found throughout the genome, sex-specific bands are not evident in Southern blots.

[b] Heteromorphic sex chromosomes are not apparent in these species, although the W of the cobra can be identified by differential staining for heterochromatin.

[c] TSD, temperature-dependent sex determination.

[d] *Drosophila melanogaster*, the fruit fly.

[e] The sex of *Drosophila* is determined by the ratio of autosomes to X chromosomes.

determining region of the Y chromosome (Jones and Singh, 1981). This prompted the hypothesis that Bkm was involved in mammalian sex determination. The hypothesis was tested by study of the *Sxr* mutation (sex reversed), which causes XX mice to become phenotypic males with small, sterile, testes (Cattanach *et al.*, 1971; and see Chapter 4, this volume).

The condition is transmitted by carrier XY*Sxr* males, which produce four kinds of sperm, and thus four kinds of offspring: normal females, fertile XY noncarrier males, fertile XY*Sxr* carrier males, and sterile XX*Sxr* sex-reversed males. Of these, only the XX females lack the clusters of Bkm found in males (Table III). By using Bkm to probe preparations of fixed chromosomes (*in situ* hybridization), Singh and Jones (1982) showed that one of the X chromosomes in sex-reversed XX*Sxr* males contained a cluster of Bkm such as that normally found in the sex-determining region of the Y. It was inferred that *Sxr* sex reversal was due to a nonreciprocal recurrent Y–X translocation in the germline of the father. Because the translocation was small enough to have escaped detection by standard cytogenetic methods (Y–X crossover has

Table III
Four Kinds of Offspring Produced by XY*Sxr* Carrier Males

Sperm	Progeny	Sex phenotype	Bkm foci[a]
X	XX	Fertile female	None
Y	XY	Noncarrier male	Y
X*Sxr*	XX*Sxr*	Sex-reversed male[b]	X
Y*Sxr*	XY*Sxr*	Carrier male	Y

[a] Rich clusters of Bkm normally asociated with the sex-determining region of the mouse Y chromosome (Singh and Jones, 1982).
[b] XX males are sterile.

since been confirmed cytogentically), this indicated that the Bkm-rich area of the Y and the testis-determining gene were very closely linked or identical.

The work of Singh and Jones (1982) resolved the mechanism of XX sex reversal in the mouse and opened the door for similar studies in XX males of the human. As in the mouse, there was strong evidence for transfer of Y material to one of the X chromosomes in most human XX males (see Chapters 10 and 12). Yet later studies indicated that Bkm sequences were not likely to play a significant role in the sex determination of man, because they were found scattered throughout the genome and were not concentrated in the sex-determining region of the Y chromosome (Kiel-Metzger *et al.*, 1985). With the work on Bkm, however, the study of mammalian sex determination had now passed beyond the bounds of immunology and into the realm of molecular biology.

IV. The Zinc Finger Y Gene (*ZFY*)

Aberrant inheritance of the Xg(a) phenotype in human XX males (see de la Chapelle *et al.*, 1964, and Chapter 11 this volume) could be due to X–Y crossover involving the male-determining gene of the Y and the *Xg* gene of the X. On that basis, Ferguson-Smith (1966) proposed that XX male syndrome and XX true hermaphroditism both are the result of Y to X translocation (Fig. 3) (and we have seen that this is in fact the case in XX*Sxr* males of the mouse). The difference between XX male syndrome and XX true hermaphroditism in the human could accordingly be due to variable X-chromosome inactivation. If the X;Y translocation chromosome (the one carrying *TDF*) were activated in most cells in one gonad, that gonad would become a testis, and if the normal X

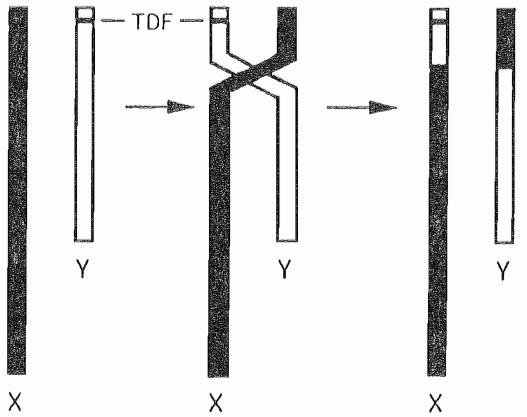

Figure 3 Translocation of the male-determining gene. Crossover of a portion of the Y chromosome to the X during meiosis. If the translocated portion contains the testis-determining gene, *TDF*, and if an egg is fertilized by the sperm carrying the X;Y translocation chromosome, the resulting embryo becomes an XX male. X–Y crossovers have been confirmed in most, but not all, 46,XX males.

were activated in most cells in the other gonad, that one would become an ovary.

Y–X translocation has now been confirmed—at least in the majority of XX males that have been studied (Petit *et al.*, 1987)—and this has enabled geneticists to construct deletion maps of the Y chromosome, for if Y–X crossover involved different amounts of DNA in different XX males, sequences closer to *TDF* should be found in a greater proportion of XX males than should those that are farther away.

By flow cytometry, Müller *et al.* (1986) obtained populations of chromosomes enriched for the Y. The Y-chromosome DNA was digested with restriction enzymes to obtain a series of Y fragments that could be used as probes to detect corresponding Y-specific sequences in XX males. As expected, the different fragments were found in different frequencies among the several XX males surveyed. For example, sequences Y-156 and Y-190 were found in about half of the XX males, whereas Y-280 and Y-286 were found in about three-fourths of the XX males (Table IV). Müller (1987) thus inferred that Y-280 and Y-286 are closer to the *TDF* gene than are Y-156 and Y-190; several other Y sequences were mapped in this way (see Fig. 1 in Chapter 10, this volume). A similar map was constructed by Vergnaud *et al.* (1986) using an alternative series of Y-specific probes in samples of DNA from 27 patients, including XX males and XX true hermaphrodites.

By this method, Page *et al.* (1987) constructed a map of the short arm of the Y chromosome, consisting of 13 deletion intervals. One of the

Table IV
Examples of Hybridization of Y-Specific Sequences in DNA from 12 XX Males

Probe	460	462	548	756	775	693	GM2626	GM2670	102	510	GM1189	481
Y-156	+	+	+	+	+	+	−	−	−	−	−	−
Y-190	+	+	+	+	+	+	−	−	−	−	−	−
Y-223a	+	+	+	+	+	+	+	+	−	−	−	−
Y-228	+	+	+	+	+	+	+	+	−	−	−	−
Y-280	+	+	+	+	+	+	+	+	+	+	−	−
Y-286	+	+	+	+	+	+	+	+	+	+	−	−
Y-198	−	−	−	−	−	−	−	−	+	−	−	−
Y-253	−	−	−	−	−	−	−	−	+	−	−	−
Y-202	−	−	−	−	−	−	−	−	−	−	−	−
Y-221	−	−	−	−	−	−	−	−	−	−	−	−
Y-294	−	−	−	−	−	−	−	−	−	−	−	−

Note: Modified after the table in Müller (1987). Reprinted with permission of the author and the Company of Biologists, Ltd. The data in this table can be used to generate maps such as the one in Fig. 1 in Chapter 10, this volume.

intervals—called "1A2" and comprising 140 kb (140,000 DNA base pairs, or about 0.2% of the Y chromosome)—was found to be present in an XX male who carried only 300 kb or about 0.5% of the Y, and absent in an X,t(Y;22) female (a woman with a Y;22 translocation) who lacked only 160 kb or about 0.3% of the Y. The authors concluded that interval 1A2 contained "an essential portion if not all" of the testis determining gene *TDF*. They reckoned that the gene might be pinpointed by a search for highly conserved DNA sequences within 1A2.

They accordingly prepared a series of "Noah's ark blots." Probes representing collectively the entire 1A2 deletion interval were developed and tested in Southern blots with samples of DNA from males and females of a variety of species. Many of the probes identified corresponding sequences in DNA from humans and great apes, but under stringent conditions, did not identify sequences from more distant mammals. But one of the probes—pDP1007, consisting of a 1.3-kb *Hin*dIII fragment—identified a single- or low-copy DNA sequence in males from every mammal tested, including gorilla, rhesus monkey, owl monkey, rabbit, dog, goat, horse, and bovine. This signaled occurrence of a sequence corresponding to pDP1007 on the Y chromosome in each of these species.

Besides hybridizing with the male-specific fragment, pDP1007 hybridized with a second fragment present in mammalian males and females. The second fragment was presumed to be X-chromosomal because more (twice as much?) was present in females than in males. (The pDP1007 probe also hybridized with DNA from male and female of the chicken.) The amino acid sequence identified by pDP1007 was compared with previously determined amino acid sequences and was found to resemble closely the sequences of proteins with multiple tandemly repeated "finger" domains. Finger proteins are regulatory; some finger proteins bind DNA and regulate transcription (conversion of the DNA sequence into messenger RNA).

On the basis of these observations, Page *et al.* (1987) speculated that the finger protein encoded by pDP1007 was the product of the mammalian Y-linked testis-determining gene. They suggested that the pDP1007 protein might bind to DNA in a sequence-specific manner, thereby regulating transcription of another gene in the pathway leading ultimately to differentiation of the male gonad. The gene was named *ZFY*, for zinc finger Y, because zinc cations form complexes with the finger domains reminiscent of the manner in which iron porphyrin forms complexes with the subunits of hemoglobin. The corresponding X-linked homolog was called *ZFX*, and it was duly noted that any *ZFY*-based model for sex determination in mammals would have to accommodate the presence of an X-linked homolog in males *and* females.

The discovery of *ZFY* was followed by a flurry of experiments aimed at ascertaining the function of the putative testis determinant. In one study, Bull *et al.* (1988) used the pDP1007 probe to look for related sequences in reptiles with genetic sex determination and in reptiles in which the sex of the embryo is determined by the temperature of the incubating egg. Positive hybridization, indicating presence of *ZFY*-like sequences, was obtained in Southern blots, but the results were the same for male and female. Was the *ZFY*-like sequence *expressed* in both sexes? To answer that question, messenger RNA was extracted from embryos of the snapping turtle maintained at female-producing and at male-producing temperatures (the sex of the snapping turtle is determined by temperature). As in the other tests, the pDP1007 probe hybridized with mRNA from both sexes, indicating transcription of the *ZFY*-like sequence in male and female (Table V).

Sinclair *et al.* (1988) studied *ZFY* in several marsupials including kangaroo, wallaby, and others. In these animals, as in the eutherian mammals, sex is determined by a gene on the Y chromosome, but when marsupial DNA was tested with the pDP1007 probe, sequences corresponding to *ZFY* were found in males and females. *In situ* hybridization

Table V
Conservation of *ZFY* and *SRY*

Class	Species	Mechanism of sex determination	*ZFY* hybridization in	*SRY* hybridization in
Mammalia	Human	XX/XY	Male (female)[a]	Male
	Mouse	XX/XY	Male (female)[a]	Male
	Kangaroo	XX/XY	Male, female	Male
	Wallaby	XX/XY	Male, female	Male
Aves	Chicken	ZZ/ZW	Male, female	Male, female
	Cockatiel	ZZ/ZW	Male, female	NT[b]
Reptilia	Leopard gecko	TSD[c]	Male, female	NT
	Alligator	TSD	Male (female?)[d]	Male, female
	Cottonmouth	ZW/ZZ	Male, female	NT
	Crotalus atrox[e]	ZW/ZZ	NT	Male, female
Osteichthyes	Catfish	XX/XY	Male, female	Male, female
	Wreckfish[f]	Sex changing	Male, female	Male, female
Agnatha	Lamprey	?	NT	Male, female

[a] The related sequence, *ZFX* (*Zfx*), is found on the X chromosome in these species.
[b] NT, not tested.
[c] TSD, temperature-dependent sex determination.
[d] The female was not tested.
[e] Western diamondback rattlesnake.
[f] *Anthias squamipinnis*, a coral reef species in which all fish begin life as females.

confirmed that *ZFY*-homologous sequences are autosomal in marsupials. Given the phylogenetic relationship of marsupials and eutherian mammals, this was an unexpected finding. It was inferred that *ZFY* is not the primary sex-determining gene in marsupials, and it was concluded that the genetics of sex determination are different in marsupials and eutherians, or, if they were the same, that *ZFY* could not be *TDF* in man (Table V).

In the mouse, two *ZFY* homologs were found on the Y chromosome (called *Zfy-1* and *Zfy-2*) and one on the X (*Zfx*) (Page, 1988; also see Nagamine *et al.*, 1989, and Chapter 5 this volume). The *Zfy-1* and *Zfy-2* genes are both present in XX*Sxr* males. However, only *Zfy-1* is present in XX*Sxr'* (or XX*Sxr*^{b}) males, which carry a deleted, albeit male-determining, *Sxr* region. It could be inferred that *Zfy-2* is not necessary for testicular development in the mouse (Page, 1988). More recently, Koopman *et al.* (1989) found that *Zfy-1* but not *Zfy-2* is transcribed during organogenesis of the mouse testis, and that *neither* gene was expressed in mutant embryonic testes lacking germ cells. These findings seemed to imply that neither gene functioned in development of the murine testis per se, but they did not exclude a role for *Zfy-1* (and *ZFY*) in development of the male germ cell.

There were other reasons for believing that *ZFY* is not the same as *TDF*. In their original paper, Page *et al.* (1987) noted that "all XX true hermaphrodites and a few XX males" that they had studied lacked the 1A2 interval, including the sequences identified by pDP1007. They suggested that testicular differentiation in such cases was secondary to mutation in autosomal or X-linked testis-determining genes. Later, Verga and Erickson (1989) described another XX male lacking *ZFY*. In view of the homology of *ZFY* and *ZFX*, those authors suggested that maleness in this case was the result of expression of both copies of *ZFX* (because of partial failure of X-inactivation). They mentioned the alternative possibility that *ZFY* was not the critical sex-determining gene, which took on added significance with the discovery that *ZFX* escapes X-inactivation (Schneider-Gädicke *et al.*, 1989).

There still remained the possibility of mutation of downstream autosomal or X-linked genes. But XX males with downstream mutations would be expected to lack evidence of Y–X crossover, so the discovery of four *ZFY*-negative XX males who carried Y-specific sequences next to the pseudoautosomal boundary now seemed to eliminate *ZFY* as a candidate for *TDF*, and redefined the region of the Y chromosome in which *TDF* must lie (Palmer *et al.*, 1989). A search of that region, comprising some 35 kb adjacent to the pseudoautosomal boundary, yielded a new gene, found to be Y-specific and, like *ZFY*, conservative across a broad

spectrum of mammalian species. The new gene was called *SRY* (sex-determining region Y) in the human and *Sry* in the mouse.

V. The Sex-Determining Region Y Gene (*SRY*)

The *SRY* gene was discovered in the laboratory of Peter Goodfellow in London (Sinclair *et al.*, 1990). Initially DNA from the 35-kb region near the pseudoautosomal boundary was digested and subcloned into fragments of about 4 kb. Then each of the 4-kb fragments was cut into smaller pieces in the range of about 500–1000 bp (0.5–1 kb). Of the fragments generated in this manner, 50 were used as probes in samples of DNA from males and females of the human, mouse, and bovine, and from human–hamster somatic cell hybrids containing the human X or Y chromosome. Seven of the probes identified single-copy male-specific sequences in *Eco*R1-digested human DNA, but only one could also identify male-specific sequences in the mouse and bovine. This probe was designated pY53.3.

A 0.9-kb subclone of pY53.3—which hybridized most conspicuously with Y-specific DNA—was used in the ensuing search for *TDF*. First, Sinclair *et al.* (1990) probed "Noah's Ark" blots containing male and female DNA from human, chimpanzee, rabbit, pig, horse, bovine, and tiger. Unique male-specific sequences were detected in each species (although at reduced stringency, other fragments were detected in males and females, signaling occurrence of pY53.3-related autosomal or X-linked fragments). Next the sequence of the 2.1-kb fragment was determined, whereby two open reading frames were identified.

Analysis of the protein encoded by the nucleotide sequence of one of the open reading frames (223 amino acids) revealed a "striking" homology to part of the Mc mating-type protein of the fission yeast *Schizosaccharomyces pombe,* and a conservative DNA binding motif found in the nuclear high-mobility-group proteins HMG1 and HMG2. The conservative motif—spanning 80 amino acids—was compared to the protein that would be predicted by the nucleotides of the corresponding rabbit sequence. Of the 80 conservative amino acids in the human, 64 (80%) were also present in the rabbit, but only 54% homology was found outside of the conservative motif. Finally, the 0.9-kb *Hin*cII fragment of probe pY53.3 was used to evaluate presence of RNA transcripts in samples of human ovary, testis, and male lung and kidney. In a Northern blot, pY53.3 identified a 1.1-kb transcript in testis but not in the other tissues (see also Su and Lau, 1993).

Taken together, these observations confirmed occurrence of the new

gene (*SRY*) in the testis-determining region of the human Y chromosome. Given its location, the conserved DNA binding motif, and its testis-specific expression, the authors proposed that *SRY* was in fact *TDF* (Sinclair *et al.*, 1990). They cautioned that there was room for other genes in the 35-kb region containing *SRY*, and that final proof of identity of *SRY* and *TDF* would rest on the analysis of *SRY* in women with XY gonadal dysgenesis, or on the production of an *SRY*-transgenic XX male (see Chapter 2, this volume).

In an accompanying paper, Gubbay *et al.* (1990) described the murine homolog of *SRY*. When the human sequence, pY53.3, was used to probe Southern blots containing DNA from male and female mice, a strongly hybridizing 3.5-kb male-specific fragment was identified. This fragment was also present in XX*Sxr* and XXSxr^b male mice. Sxr^b, a deletion variant of *Sxr*, is the smallest part of the mouse Y chromosome known to contain the male-determining gene. Since in addition to *Zfy-1* and repetitive sequences such as Bkm only *Sry* was found within the Sxr^b region, pY53.3 had now defined a new gene in the minimal region of the Y chromosome known to determine sex in mouse and human. Moreover, the pY53.3 probe failed to hybridize with DNA from a line of XY female mice carrying a mutation within *Tdy* (the murine homolog of *TDF*): the 3.5-kb band was absent in Southern blots, indicating deletion of the sequence with which pY53.3 had hybridized in males.

When the mouse homolog of pY53.3 was cloned, it was found to contain a 237-bp sequence characterized by 80% sequence homology with pY53.3. As with the corresponding human sequence, the mouse clone exhibited a remarkable similarity to the highly conserved 80 amino acid motif in the Mc protein of the yeast *S. pombe*. RNA transcripts of *Sry* were found in adult testis and in urogenital ridge from 11.5-day-old male embryos, but not in liver or in urogenital ridge from female embryos. Gubbay *et al.* (1990) thus inferred that *Sry* is "a good candidate" for *Tdy* (see Chapter 3, this volume).

The sex-determining role of *SRY* (*Sry*) was borne out in subsequent studies. For example, several laboratories set out to find mutants within *SRY* in women with XY gonadal dysgenesis (in whom the testes fail to develop and the gonads are represented by inert streaks of fibrous tissue). After studying 11 cases of XY gonadal dysgenesis, Berta *et al.* (1990) described such two mutations: both were single base changes within the conservative domain. One was a *de novo* mutation and one was shared by the father of the patient. It was concluded that the latter was "fortuitously associated with, or predisposing towards sex reversal." A similar finding was reported by Jäger *et al.* (1990): four nucleotides were deleted from within the conservative motif in 1 of 12 women with

XY gonadal dysgenesis (see Pivnick *et al.*, 1992, and Chapter 13, this volume).

As for timely expression of the *Sry* gene, Koopman *et al.* (1990) found that transcription of *Sry* was limited to the genital ridge of the mouse fetus, first occurring with the onset of testicular organogenesis (10.5 days), and becoming less pronounced at about 12.5 days. *Sry* transcripts were not detected thereafter. Transcription did not require the presence of the fetal germ cells. Moreover, expression of *Sry* in mutant W^e/W^e (germ cell negative) mouse embryos was no different from that in wild-type embryos, unlike the situation described for *Zfy-1*.

In our own laboratory, Tiersch *et al.* (1991) reacted a probe from the conserved motif of the *SRY* gene with DNA from 23 different species from five different classes representing 400 million years of vertebrate evolution. Cross-hybridization occurred in each case—in species with genetic modes of sex determination and in species with temperature-regulated sex determination (Table V; Fig. 4). The data were compatible with persistence of a critical function, but there was no evidence of sex-specificity outside of the mammals (see Coriat *et al.*, 1993), so it would be informative to determine whether *SRY* homologs are *expressed* in both sexes in these animals.

VI. *SRY* and the Search for *TDF*

It would seem that the search for the testis-determining gene has come to an end, for *SRY* evidently satisfies the requirements of that gene. It is widely conserved in evolution; a homolog is present in the marsupial Y (Foster *et al.*, 1992); it is located in the smallest region of the Y chromosome that is sex-determining; it is present in most XX males; mutations within *SRY* have been identified in sex-reversed XY women, and the *Sry* homolog is deleted in a line of XY females of the mouse; it exhibits the characteristics of a regulatory gene; it is expressed in the gonadal ridge in the mouse embryo and precisely at the time of testicular organogenesis; its transcription is curtailed after formation of the seminiferous tubules; its expression is correlated with a somatic cell lineage in the

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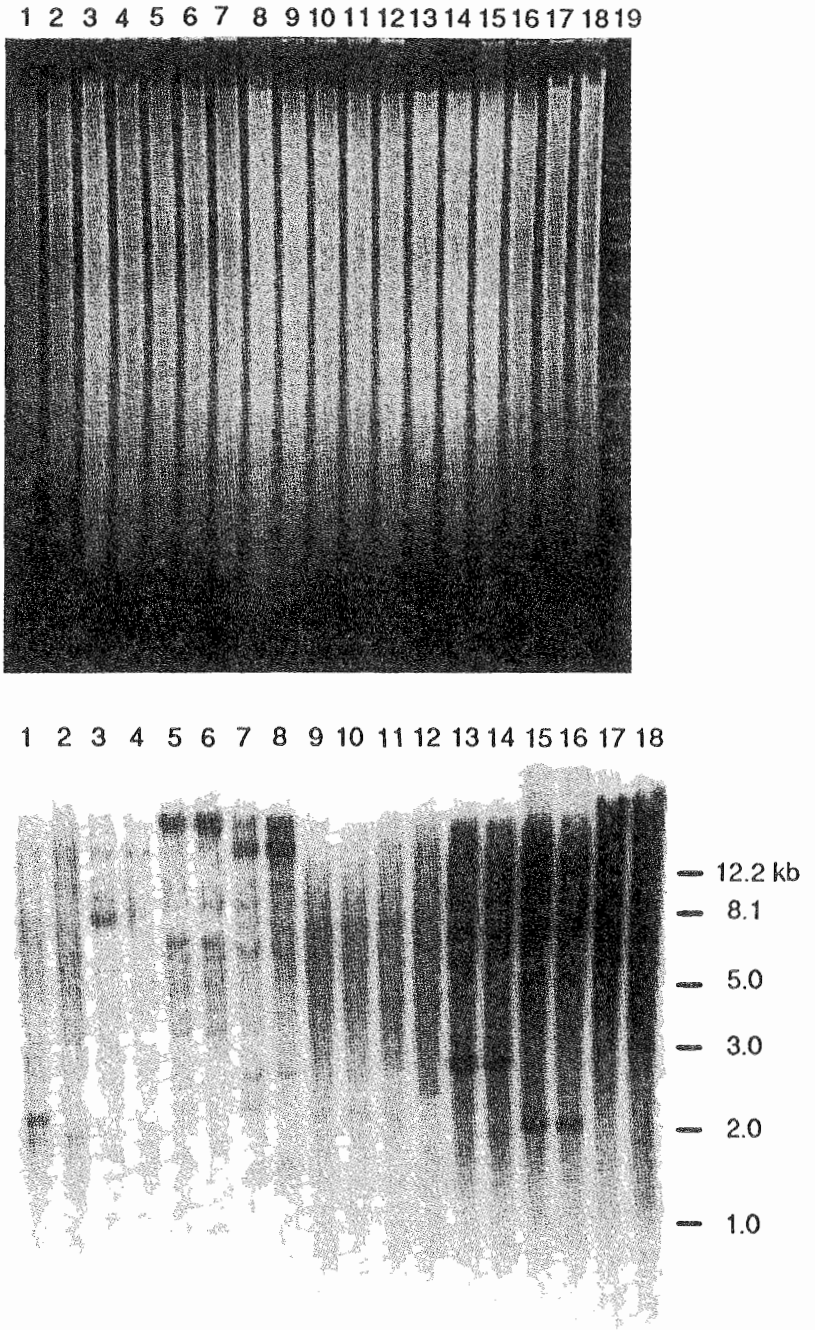
Figure 4 Conservation of sequences corresponding to *SRY*. Bottom: Southern blot of *Hin*dIII-digested DNA from males and females of nine different vertebrate species, showing hybridization with a 320-bp fragment amplified from *SRY*. In each pair of animals, the male DNA was on the left. Lanes 1,2: human; lanes 3,4: moluccan cockatoo; lanes 5,6: chicken; lanes 7,8: Japanese quail; lanes 9,10: American alligator; lanes 11,12: green sea turtle (*Chelonia mydas*); lanes 13,14: channel catfish; lanes 15,16: gizzard shad; lanes 17,18:



sea lamprey. A male-specific fragment of 2.1 kb was identified in the human, but sex-specific fragments were not observed in any of the nonmammals. Differences in hybridization patterns in male and female catfish were caused by polymorphisms and were not associated with gender. Differences in intensity of bands in males and females of the birds (lanes 3,4,7,8) were not found consistently. Top: agarose gel used to prepare Southern blot, after staining with ethidium bromide. Lane 19 contains size markers. See Table V. From Tiersch *et al.* (1991), by permission.

developing gonad, not with the germ cells; and finally, although sex reversal did not occur in every case, microinsertion of a 14-kb segment containing *Sry* was sufficient to induce development of testes in at least some transgenic XX embryos (Koopman *et al.*, 1991).

VII. *TDF* in Normal and Abnormal Sex Determination

The precise role of the Y-linked testis-determining gene remains to be elucidated. It would seem likely that the protein product of *TDF* is a regulatory factor, probably a DNA binding element that regulates expression of a secondary or "downstream" testis determinant. The downstream locus could be X-linked or autosomal. Assuming identity of *SRY* and *TDF*, this would account for the apparent absence of mutations within *SRY* in most cases of XY gonadal dysgenesis and the apparent absence of *SRY* in certain XX males and XX true hermaphrodites (Ferguson-Smith *et al.*, 1990). Existence of downstream sex-determining genes would also account for autosomal and X-linked forms of XY gonadal dysgenesis (Simpson, 1989; Tommerup *et al.*, 1993; and see Chapter 13, this volume).

Perhaps the earliest recognizable event in the embryology of the male gonad is the appearance of the Sertoli cells. The Sertoli cells make physical contact with one another. They aggregate and surround the germ cells, thereby forming the seminiferous cords that characterize the newly differentiated testis. Further differentiation involves the appearance of Leydig cells from the interstitial cells of the gonad, and the development of the tunica albuginea, the fibrous outer covering of the testis. We have said that the Sertoli cells secrete the anti-Müllerian hormone and the Leydig cells secrete testosterone. It follows that the product of the *TDF* gene initiates a cascade of events, the first of which is differentiation of the Sertoli cells. But how that first differentiative event is governed is still a mystery.

Yet we have gained certain insights into the etiology of abnormal sex differentiation. We can say, for example, that many cases of XY gonadal dysgenesis are not due to mutations within the conservative motif of *SRY*, but are due to mutations outside of the conservative motif, or alternatively to mutations in other X-linked or autosomal testis-determining genes (Pivnick *et al.*, 1992; but see Hawkins *et al.*, 1992). Constitutive activation of these "secondary" genes would explain sex reversal in the few XX males in whom *SRY* sequences have not been identified (five such males have been described by Ferguson-Smith *et al.*, 1990).

As a concluding point, the etiology of XX true hermaphroditism is

enigmatic, for *SRY* sequences have not been detected in the great majority of XX true hermaphrodites that have been tested, and the presumptive X-linked and autosomal genes of the testis-determining pathway have yet to be identified. Although absence of *SRY* in blood cells need not rule out differential expression of *SRY* in *gonadal* tissue, the same would be true of a downstream product induced by *SRY*. A mutant X-linked gene, subject to inactivation, would readily explain male development in at least some Y(−) true hermaphrodites and Y(−) XX males. A mutant autosomal gene with variable penetrance could explain male development in some others.

VIII. Summary

The *SRY* gene seems to satisfy the requirements for *TDF*. The various abnormalities of sex differentiation manifest in conditions such as XX male syndrome, and XY gonadal dysgenesis can accordingly be explained as transcriptional or translational errors within *SRY*. For example, testicular differentiation in XX males is due to Y–X crossover and transfer of *SRY* to the X or, more rarely, to constitutive mutation (activation in the absence of a regulatory signal) of a secondary, downstream, testis determinant. And failure of male differentiation in women with XY gonadal dysgenesis is due to mutation or deletion in *SRY* itself or in downstream autosomal or X-linked genes required for testicular development. But the etiology of true hermaphroditism remains to be clarified.

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