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Studies on the phylogenetic conservation of the *SRY* gene

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Summary. A probe from a conserved motif of the *SRY* gene *(sex-determining region Y)*, a prime candidate for the human testis-determinant, was hybridized to DNA from 23 species representing 5 vertebrate classes. Hybridization occurred in species with male or female heterogamety, in species with and without sex chromosomes and in those with temperature sex determination. Sex-specific signals were observed only in mammals. Conservation of sequences homologous with *SRY* through 400 million years of vertebrate evolution would indicate persistence of function. However, if *SRY* is the primary sex determinant in mammals, it is not clear that it has a similar function, or even one that is sex-related, in nonmammals.

Introduction

In the human, sex determination is governed by a gene on the Y-chromosome. In the presence of this gene, the embryonic gonad becomes a testis and further development is male. In the absence of this gene, the gonad becomes an ovary and further development is female. The gene, referred to as *TDF (testis-determining factor)*, is located in the short arm of the Y chromosome, proximal to the pseudoautosomal boundary.

Page et al. (1987) identified a gene called *ZFY (zinc-finger Y)* (Page 1988) that is located within a deletion interval consisting of 140 kb, or about 0.2% of the Y chromosome. Corresponding sequences were identified in the Y chromosome of every eutherian mammal studied, and it was inferred that *ZFY* was the same as *TDF*. Subsequent studies have brought that inference into question (Palmer et al. 1989; Koopman et al. 1989); more recently, Sinclair et al. (1990) have identified a new candidate for *TDF*, situated within 35 kb of the pseudoautosomal boundary in the human, and part of a family of at least 5 genes related by a conserved amino acid motif in the mouse (Gubbay et al. 1990). The new gene, called *SRY (sex-determining region Y)*, is the prime candidate for *TDF*: it is present in 46,XX males that lack *ZFY* (but see Ferguson-Smith et al. 1990), and it encodes a testis-specific transcript. The corresponding gene in the mouse, *Sry*, is present in the smallest region of the Y chromosome known to induce testicular differentiation *(Sxr^1)* and is absent in a mutant Y that has lost testis-determining function. It is expressed in somatic cells of the gonadal ridge just before differentiation of the testis (Koopmann et al. 1990).

Sequences hybridizing with a region within *SRY* were identified in males in 8 mammalian species (including mouse and human), and similar amino acid motifs were identified in the mating-type protein of the yeast, *Schizosaccharomyces pombe* (Sinclair et al. 1990; Gubbay et al. 1990). We therefore tested samples of DNA from a broad spectrum of vertebrate species, by Southern blot analysis, to determine whether genes homologous to SRY were present, and if so whether they were present in a particular sex.

Materials and methods

The probe used, designated *SRYcm (SRY conserved motif)*, was obtained as follows. DNA from a normal XY male human was amplified in the polymerase chain reaction (PCR) by use of oligonucleotide primers flanking the conserved region of *SRY*. The amplified DNA was then sequenced and found to correspond exactly to a 360-bp fragment of *SRY* including the 240-bp conserved motif (Sinclair et al. 1990). Sequences of the primers used for PCR were, from 5′ to 3′, CAGTGTGAAACGGGAGAAAACA and GTACAACCTGTTGTCCAGTTGC.

Samples of DNA were digested with *Eco*RI, *Hin*dIII or *Rsa*I, and Southern blotting and hybridization were performed according to standard procedures. Genomic DNA (10 μg) was digested, electrophoresed through a 0.8% agarose gel and transferred to Hybond N filter (Amersham) in 10 × SSC buffer (1 × SSC = 150 m*M* NaCl/15 m*M* sodium citrate, pH 7.0). The probe was labeled with ^{32}P, added to the filter in Church's buffer and hybridized for 16h at 55°C. The filters were washed at the following stringencies: 2 × SSC, 0.2% SDS, 55°C; 1 × SSC, 0.2% SDS, 62°C; 0.1 × SSC, 0.2% SDS, 65°C. Autoradiography was then performed.

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Results

In Southern blot analysis of DNA from normal human males, the probe identified a 2.1-kb *Hin*dIII fragment corresponding to pY53.3, which contains *SRY* (Sinclair et al. 1990). Other bands (e.g. the 1.6-kb *Hin*dIII fragment) were found in human males and females (Fig. 1), implying the presence of autosomal or X-linked homologs. At lower stringencies, fragments homologous with *SRYcm* were identified in each of the 23 species tested (Table 1). The number of fragments varied from species to species; polymorphism in individual bands was observed in organisms such as *Anthias squamipinnis* (data not shown) and channel catfish (Fig. 1). The sizes of the fragments were variable among species. Certain bands disappeared when the filters were washed at medium stringency. At the highest stringency (0.1 × SSC, 0.2% SDS, 65°C), most bands disappeared or were considerably reduced in intensity except for the male-specific fragment of the human. Similar results were obtained for DNA samples digested with *Eco*RI or *Rsa*I.

Discussion

In the present study, sequences cross-hybridizing with *SRYcm* were found in both sexes of animals representing a variety of sex-determining mechanisms. Hybridization occurred in animals with male heterogamety and in those with female heterogamety, in animals with and without sex chromosomes, and in others with environmental sex determination. The same is true of *ZFY*, the earlier candidate for the testis-determining gene. Sequences hybridizing with probe pDP1007, which identifies *ZFY*, were found in males and females in the chicken (Page et al. 1987) and in males and females in three major reptilian groups, squamata, turtles and crocodilians, including species with environmental sex determination (Bull et al. 1988). Sequences hybridizing with pDP1007 were also found in males and females in two fishes, sturgeon and trout (Ferreiro et al. 1989).

The amino acid sequence of the conserved motif of *SRYcm* is similar to amino acid sequences in DNA-binding proteins (Jantzen et al. 1990). It follows that the protein product of *SRY* regulates a gene, possibly on another chromosome, as postulated for the protein encoded by *ZFY* (Page et al. 1987). Thus, *SRY* may be the primary regulator of genes responsible for the first stages of testicular devleopment.

If *SRY* is the primary determinant of the testis in mammals (Berta et al. 1990; Jäger et al. 1990), it is not clear that *SRY* homologs have a similar function or even one that is sex-related in the nonmammals, because these genes occur in males and females. However, the genes might be involved in a pathway that leads ultimately to the differentiation of male and female. A pathway shared among vertebrates and including *SRY*-homologous genes might be activated at particular temperatures in species with temperature-dependent sex determination, or by a particular gene in species with genetic sex determination.

Table 1. *SRY* homology in vertebrates

Class	Species	Sex-determining mechanism	*SRYcm* hybridization pattern
Agnatha	*Petromyzon marinus*	?	F, M[c]
	Eptatretus stouti	?	F, M
Osteichthyes	*Polyodon spathula*	?	F, M
	Lepisosteus oculatus	?	F, M
	Amia calva	?	F, M
	Dorosoma cepedianum	?	F, M
	Ictalurus punctatus	XX/XY	F, M
	Oncorhynchus mykiss	XX/XY	F, M
	Micropterus salmoides	?	F, M
	Tilapia aurea	ZZ/ZW	F, M
	Anthias squamipinnis	Sex-changing[a]	F, M
Reptilia	*Chelonia mydas*	TSD[b]	F, M
	Alligator mississipiensis	TSD	F, M
	Sceloporus undulatus	Genetic	F, M
	Eublepharis macularius	TSD	F, M
	Pituophis melanoleucus	ZZ/ZW	F, M
	Crotalus atrox	ZZ/ZW	F, M
Aves	*Balearica pavonina*	ZZ/ZW	F, M
	Gallus domesticus	ZZ/ZW	F, M
	Coturnix coturnix japonica	ZZ/ZW	F, M
	Meleagris gallopavo	ZZ/ZW	F, M
	Ara ararauna	ZZ/ZW	F, M
	Cacatua moluccensis	ZZ/ZW	F, M
Mammalia	Tiger[d]	XX/XY	MSB[g]
	Mouse[e]	XX/XY	MSB
	Rabbit[d]	XX/XY	MSB
	Pig[d]	XX/XY	MSB
	Bovine[d]	XX/XY	MSB
	Horse[d]	XX/XY	MSB
	Chimpanzee[d]	XX/XY	MSB
	Homo sapiens[d,f]	XX/XY	MSB

[a] *A. squamipinnnis* is protogynous hermaphrodite
[b] Temperature-controlled sex determination
[c] M, male; F, female
[d] Sinclair et al. (1990)
[e] Gubbay et al. (1990)
[f] Present study
[g] MSB, male-specific bands; other bands were noted in male and female DNA (Sinclair et al. 1990; Gubbay et al. 1990; and present study). We tested DNA samples from 75 animals

The conserved domain of *SRY* defines a novel group of genes that code for DNA binding proteins, although some of them may not participate in sexual differentiation. Homologous protein domains comprising 80 amino acids were found to be conserved among *SRY* (human-Y), *Sry* (mouse-Y), and four autosomal genes (identified by the cDNA sequences designated as *mouse-a1*, *mouse-a2*, *mouse-a3* and *mouse-a4)* (Gubbay et al. 1990). An early representative of this group could have been se-

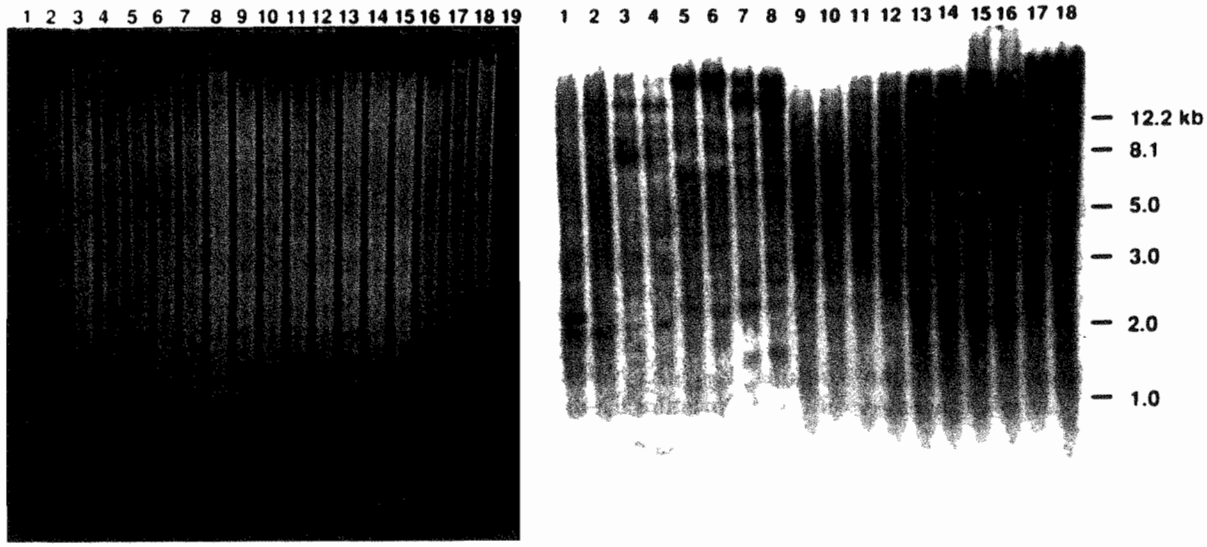

Fig. 1. Phylogenetic conservation of sequences corresponding to *SRY*. *Right* Southern blot of *Hin*dIII-digested genomic DNA from male-female pairs of 9 vertebrate species showing hybridization with *SRYcm*, a 360-bp fragment of *SRY*. Filters were washed at the following stringency: 2 × SSC, 0.2% SDS, 55°C. In each pair of animals, the male is on the *left: lanes 1, 2* human; *lanes 3, 4* moluccan cockatoo *(C. moluccensis); lanes 5, 6* domestic chicken *(G. domesticus); lanes 7, 8* Japanese quail *(C. c. japonica); lanes 9, 10* American alligator *(A. mississipiensis); lanes 11, 12* sea turtle *(Chelonia mydas); lanes 13, 14* channel catfish *(I. punctatus); lanes 15, 16* gizzard shad *(D. cepedianum); lanes 17, 18* sea lamprey *(P. marinus)*. The probe identified a male-specific fragment in humans (2.1 kb) but sex-specific fragments were not found in the nonmammalian species. Differences in intensity of bands in males and females of the birds *(lanes 3, 4, 7, 8)* were not observed in all cases. Differences in hybridization patterns in male and female catfish are the results of polymorphisms and are not associated with gender; these were not consistently observed in other catfish. *Left* agarose gel, used to prepare blot, stained with ethidium bromide. *Lane designations* are the same as those given above; *lane 19* contains size markers. Hybridization was obtained in all 23 species; see Table 1

questered on the chromosome destined to become the mammalian Y, and could thereby have assumed a fundamental role in determination of the testis.

Because the sex-determining mechanisms of lower vertebrates are labile (amphibians and fishes can be functionally sex-reversed by application of hormones, for example), it might be informative to determine whether *SRY* transcripts are produced in both sexes in those animals, and whether expression of the gene can be modulated during experimentally-induced sex change. The *SRY* gene might provide a useful starting point for the localization of sex-determining genes in vertebrate species that lack heteromorphic sex chromosomes.

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