Linkage of the murine steroid sulfatase locus, *Sts*, to sex reversed, *Sxr*: a genetic and molecular analysis

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ABSTRACT

We present genetic and molecular data demonstrating linkage of the gene for steroid sulfatase (*Sts*) to the mutation sex reversed (*Sxr*) definitively showing the existence of a functional allele for *Sts* mapping to the pseudoautosomal region of the mouse Y chromosome. Thus, in mouse, functional *Sts* genes are present in the pseudoautosomal region of both the X and Y chromosomes. This is in contrast to man where *Sts* has been mapped to the short arm of the X just centromeric to the pseudoautosomal region. Only a single recombinant separating *Sts* and *Sxr* was found out of 103 male meioses analyzed; double recombinants were not found between sex (*Tdy*), *Sts* and *Sxr*. If the rate of recombination in the pseudoautosomal region in male mice is equivalent to that in man and thus 7-10X higher than normal, then our data suggest that the distance between *Sts* and *Sxr* (or the telomere of the Y) is approximately 100 - 200 kb in length. Our data is in contrast to a recent report of a recombination frequency separating *Sts* and *Sxr* of as high as 6.2-9.8%.

INTRODUCTION

Steroid sulfatase (STS, EC 3.1.6.2) is an enzyme active against various natural (estrogen sulfate, dehydroepiandrosterone sulfate, cholesterol sulfate) and artificial (p-nitrophenyl sulfate and 4-methyl umbelliferone sulfate) steroid sulfates¹⁻⁴. STS is located in the microsomal fraction of various tissues (e.g., brain, liver, spleen, kidney, testes, pituitary, adrenals, ovaries, placenta) but not in red blood cells^{3,5-7}. Although the relationship between STS and arylsulfatase C, an enzyme of similar properties and tissue distribution, is unclear, genetic and biochemical evidence suggest that the activities attributed to both STS and arylsulfatase C is due to a single enzyme^{3,8}.

In man, STS deficiency is correlated with difficulties in parturition and X-linked ichthyosis vulgaris⁵. The human gene for STS (*Sts*) has been mapped to the short arm of the X chromosome (Xp22.3-Xpter) just centromeric to the pseudoautosomal region and has been shown to partially escape X inactivation^{5,9-11}. Recent cloning of the human *Sts* cDNA has confirmed its X location and revealed the presence of a

pseudogene on the long arm of the Y^{12,13}. In most cases of STS deficiency a deletion of the *Sts* gene or neighboring DNA sequences have been detected¹²⁻¹⁴.

In contrast to man, mouse inbred strains possessing low (A/J) or zero (C3H/An) steroid sulfatase activity are apparently normal^{2,3,6,15}. The murine *Sts* gene has been mapped to the X chromosome distal to the most terminal known X mutation, Cream (*Cr*) and indirectly to the pseudoautosomal region¹⁶⁻¹⁹. Although direct evidence is lacking, presence of a functional *Sts* allele on the Y chromosome, which undergoes regular exchange with its X linked allele during male meiosis, has been put forward¹⁷.

In the sex reversed mutation (*Sxr*) a minute pericentric region of the Y is believed to have been duplicated and transposed to a position distal to the pseudoautosomal region^{20,21}. The *Sxr* segment is believed to contain, at minimum, the testis-determining gene (*Tdy*), the gene coding for the H-Y histocompatibility antigen and the banded krait minor satellite (Bkm) related sequences²¹. Due to its telomeric location, *Sxr* undergoes recombination with the X at a frequency of 0.5. Since *Sxr* can be used as a marker for the telomere of the Y, linkage between *Sts* and *Sxr* would conclusively map the *Sts* locus to the pseudoautosomal region in the mouse. Using a combination of the STS deficient inbred strain C3H/An, the *Sxr* mutation, and a variety of recombinant DNA probes specific for the mouse X and Y chromosomes, we present direct evidence for a fully functional *Sts* allele in the pseudoautosomal region of the Y. Only one recombinant segregating *Sts* and *Sxr* was found among 103 mice tested and double recombinants were not found between *Tdy*, *Sts*, and *Sxr*.

MATERIALS AND METHODS

Recombinant DNA probes

The recombinant DNA probes used were:

- 1) pYCR8/B: a unique sequence mouse Y chromosome derived probe specific for the *Sxr* region of the mouse Y chromosome (Roberts et al., in preparation).
- 2) pCS316: a highly repeated Bkm-related drosophila probe which under certain conditions can be used to define the *Sxr* & region^{22,23}.
- 3) pY353/B and pY80/B: repeated mouse Y derived probes giving an X-Y differential pattern of hybridization. The Y located fragments are spread along the length of the Y but are absent from the *Sxr* region. Thus XX*Sxr* males give a female

hybridization pattern²⁴⁻²⁶. Although pY353/B was previously described as Y-specific, when labeled to high specific activity, it has been found to give a single X-Y common band.

Southern blot analysis

Fifteen µg of high molecular weight spleen genomic DNA was digested with Taq I or Eco RI, electrophoresed on 0.8% agarose gels, and transferred to Hybond-N nylon membranes (Amersham, U.K.) by capillary blotting²⁷. Transferred DNAs were fixed to the filters by placing the filters at 80°C for 2 hrs followed by UV irradiation. Blots were prehybridized (50% formamide, 5X SSC, 5X Denhardt's, 50 mM phosphate buffer, pH 7.0, 0.5% SDS and 0.2 mg/ml denatured, sonicated herring sperm) at 42°C for 14-16 hours then hybridized in the same solution but minus the denatured herring sperm and plus 5% dextran sulfate under the same conditions. Recombinant DNA probes, either as plasmids or inserts, were ³²P labelled by nick-translation or random priming²⁷⁻²⁹ to a specific activity of >10⁸ cpm/µg and used at a concentration of 1-2 x 10⁶ cpm/ml of hybridization solution. Hybridized filters were washed three times under low stringency (2X SSC, 0.1% SDS, 65°C, 30 min), once at high stringency (0.1X SSC, 1% SDS, 65°C, 15 min), then exposed for 6-48 hrs. to Kodak XAR5 film at -70°C using an intensifying screen.

Dot blots

Five-15 µg of DNA was diluted in 200 µl 10X SSC, denatured for 10 min at 100°C, then dotted on Hybond-N using a Hybri-Dot Manifold (BRL, U.K.). The filters were rinsed in 2X SSC, redenatured in 0.5 M NaOH, 1.5 M NaCl then neutralized in 1 M Tris, 3 M NaCl, pH 7.4. The filters were then processed as above.

Steroid sulfatase assay

The steroid sulfatase assay was a modification of a previously published protocol³⁰. In brief, portions of spleen or liver were minced with scissors in 0.5-2.0 ml ice-cold 0.1 M Tris buffer, pH 7.2, sonicated, then microfuged at 4° C for 10 min. Fifty µl of the supernatant, 500 µl 0.1 M Tris buffer, pH 7.2, and 25 µl of thrice-extracted ³H-dehydroepiandrosterone sulfate, ammonium salt (New England Nuclear, Mass.; 300,000-600,000 cpm) were combined and incubated for 4 hrs at 37° C. The reaction mixture was extracted with 700 µl of petroleum ether, the aqueous phase frozen on dry ice and the organic phase counted in scintillation fluid (Aqualyte, J.T. Baker Chemical, Holland). All experiments were run with known STS positive and negative samples as

controls. Protein determinations were performed using the Bio-Rad Protein Assay Kit (Bio-Rad Laboratories) using bovine serum albumin as the standard.

Mice

Male mice carrying the *Sxr* mutation (XYSxr) were obtained from Dr. B. Cattanaach (M.R.C, Harwell, U.K.) and the YSxr chromosome placed on the inbred129/Sv background by backcrossing. XYSxr of the third backcross generation (*Sts* +/*Sts* +) were mated to C3H/An (*Sts* -/*Sts* -) females and F1 XYSxr as well as their N2 XYSxr sons (both *Sts* -/*Sts* +) backcrossed to C3H/An females. Male and female progeny were sexed by presence of bilateral testes or ovaries, respectively.

RESULTS

Nine (C3H/An x XYSxr) F1 XYSxr male mice (*Sts* -/*Sts* +, *Sxr*) were backcrossed to C3H/An (*Sts* -/*Sts* -) females. Thirty female and 73 male progeny (total: 103) derived from this cross were studied. The progeny can be divided into four genotypes: normal XX females, sterile XXSxr males, fertile XYSxr males carrying the *Sxr* mutation (carriers), and fertile XY normal males (non-carriers)³¹. If a functional *Sts* allele exists in the pseudoautosomal region of the Y chromosome, linkage to *Sxr* is predicted, i.e., all XX females and XY males should be STS negative whereas XXSxr and XYSxr males should be STS positive (Fig. 1).

Steroid sulfatase activity of progeny

In no assay did values for STS positive animals overlap values for STS negative animals. Values obtained in a single experiment (CPM - Bkg hr⁻¹) were as follows: STS positive mice (n=11) 129±76, STS negative mice (n=12) 4540±945, STS positive recombinant mouse (see below) 3504. In keeping with the presence of an *Sts* locus on the X and with the hypothesis that in the *Sxr* mutation XX females are derived from the X chromatid that had not undergone meiotic recombination (Fig. 1), all 30 females were STS negative^{17,21,32}. Forty-nine males were STS positive, the remaining 23 males being STS negative (Table 2).

To differentiate XXSxr from XY and XYSxr males the DNA of male mice was analysed for the presence of the entire Y chromosome using pY353/B and pY80/B. Since neither probe recognizes the *Sxr* region of the Y, Southern blots of XXSxr males will possess the female pattern of hybridization (Fig. 2a,b; lanes 2-4)^{24,26}. Twenty-one of the males were negative with both probes and were thus XXSxr males.

Figure 1. Segregation of *Tdy*, *Sts*, and *Sxr* in (C3H/An x XY Sxr) F1 XY Sxr males (*Sts* - / *Sts* +, *Sxr*). Homologous pairing and recombination in the pseudoautosomal region (illustrated by the cross) during male meiosis results in the transfer of *Sxr* from one Y chromatid to the X. Thus four types of sperm are produced in these males: two from the non-recombined chromatids (X, Y Sxr) and two from the recombined chromatids (X Sxr, Y). When these males are backcrossed to C3H/An (*Sts* - / *Sts* -) females, four classes of progeny are predicted if a single recombination occurs proximal to *Sts* (second row). If recombination occurs distal to *Sts*, *Sts* and *Sxr* are separated and two additional recombinant classes of progeny are predicted (third row).

As predicted, all the XX Sxr males were STS positive. Patterns of hybridization did not vary either between XY and XY Sxr males (Fig. 2a,b; lanes 5-10) or XX females and XX Sxr males (Fig. 2a,b; lanes 1-4).

Normally XY and XY Sxr males can be differentiated only by progeny testing.

Figure 2. A-C: Southern blot analysis of 11 C3H/An x (C3H/An x YYSr) F1 YYSr backcross mice. Lane 1 contains DNA from a normal female; lanes 2-11 contain DNA from mice of male phenotype (YYSr, YYSr, XY). In A, probing with pY353/B shows that in lanes 2-4, the mice lack the characteristic male hybridization pattern shown by mice in tracks 5-11 and instead possess the female pattern of hybridization (lane 1). Hence DNA in lanes 2-4 were derived from YYSr males and those in lanes 5-11 from YYSr or XY males. In B, this result was confirmed by reprobing with pY80/B after blot dehybridization.

In Fig. 2C, a dosage analysis of the same blot using pYCR8/B. The quantity of DNA in each track was verified using the steroid 21-OH genomic probe. Hence the underloading of DNA seen in lane 7 can be normalized. It can be seen that there is no reaction of the Sxr specific probe pYCR8/B with the female (lane 1) and that the 3 YYSr males (lanes 2-4) are shown to have one copy of pYCR8/B. Lanes 5-7 possess two copies of the probe and are thus YYSr carrier males whereas the remaining males (lanes 8-11) carry only one copy and are thus XY males. Confirmation of this dosage analysis was obtained by progeny testing. DNA in lane 11 was obtained from the XY, STS positive recombinant male (see text). In Fig. 2D, a dot blot analysis for YYSr males in the progeny sired by 4 males judged to be YYSr carriers by pYCR8/B dosage. Five µg of DNA from 4 male (M) and 1 female (F) progeny were dot blotted along with C57BL/6 (BL6) male and female controls. As can be seen, each test male sired at least one YYSr male as judged by a negative reaction with pY353/B. The DNA of the controls were serially diluted (2.5-0.6 µg) to illustrate the sensitivity of the technique.

However, as there are two copies of the *Sxr* region in YYSr males versus only one in XY males, a dosage analysis of their DNA with the *Sxr* specific probe pYCR8/B allowed us to distinguish these two categories. Southern blots of non-YYSr males were thus probed with pYCR8/B and the signal intensity normalized to that obtained with single copy probes (e.g. , 21-hydroxylase, chromosome17; p45, X chromosome)^{33,34} (Fig. 2c). This approach was validated by mating 7 males

Table 1. Results of linkage analysis between *Tdy*, *Sts* and *Sxr*.

NUMBER OF MICE N= 103	PHENOTYPE	PRESENCE OF ENTIRE Y CHROMOSOME		COPIES OF <i>SXR</i> BY DOSAGE PYCR8	STEROID SULFATASE	GENOTYPE
		pY80	pY353			
30	F	-	-	0	-	XX
21	M	-	-	1	+	XXSxr
23	M	+	+	1	-	XY
28	M	+	+	2	+	XYSxr
1*	M	+	+	1	+	XY

possessing 2 copies of pYCR8/B (putative XY Sxr) and four males possessing 1 copy of pYCR8/B (putative XY) to 129/Sv or C3H/An females. The criterion for the designation of an XY Sxr carrier was the presence of at least one XX Sxr male as well as an abnormal sex ratio among its progeny; the criterion for an XY non-carrier male was the absence of XX Sxr males after testing 14-16 male progeny as well as the presence of a normal sex ratio among its progeny. DNA from male progeny was dot blotted and analyzed for the presence of XX Sxr males by probing with pY353/B (Fig. 2d). All 7 males designated to have 2 copies of pYCR8/B were indeed found to be XY Sxr carrier males, while all 4 males designated to possess 1 copy of pYCR8/B were indeed of XY genotype (Table 2).

Having verified the methodology, all non-XX Sxr males were dosed with pYCR8/B and the results correlated with STS activity. Twenty-eight of the 52 non-XX Sxr males were found to possess 2 copies of pYCR8/B and thus of XY Sxr genotype. Significantly, all 28 of these males were STS positive (Table 1). Twenty-four males were found to possess 1 copy of pYCR8/B and thus of XY genotype. Twenty-three of these 24 males were STS negative. A summary of all the linkage data

Table 2. Results of the test breeding of putative XYSxr and XY males and the XY, STS positive male to 129/Sv and C3H/An females.

MOUSE No.	STS ACTIVITY	No. LITTERS	MALE:FEMALE RATIO	No. XYSxr MALES No. MALES TESTED	GENOTYPE
3	+	5	25:3	2/6	XYSxr
7	+	4	11:3	2/11	"
17	+	4	19:1	1/3	"
31	+	2	13:6	4/8	"
37	+	3	18:5	4/8	"
38	+	1	7:2	2/7	"
40	+	3	10:2	2/10	"
5	-	3	15:15	0/15	XY
35	-	4	16:18	0/16	"
36	-	4	15:13	0/15	"
39	-	4	14:21	0/14	"
2	+	7	17:24	0/15	XY (recombinant)

is presented in Table 1. As can be seen, only 1/103 mice was not in agreement with the hypothesis that *Sts* and *Sxr* are linked. Thus a functional *Sts* allele exists in the pseudoautosomal region of the Y chromosome in the mouse. The single non-concordant male is believed to represent a single recombinant between *Sts* and *Sxr* (see below).

Recombination within the pseudoautosomal region

A single XY male, its genotype confirmed by pYCR8/B dosage (Fig. 2c, lane 11) and progeny testing (Table 2, male 2) was found to be STS positive on both liver and spleen samples (Table 2, male 2). The simplest explanation is that although recombination normally occurs proximal to *Sts*, in this case recombination had taken place distal to *Sts*, thus separating *Sts* and *Sxr* and giving an XY, *Sts* - / *Sts* + genotype (Fig. 1).

However, all data to date, including the data presented here, suggest that the site of recombination between the X and Y chromatids is centromeric to *Sts*. For segregation of *Sts* and *Sxr* to occur, recombination distal to *Sts* must have occurred. This raised the question of whether this new site of recombination was permanent. We

crossed the recombinant XY male (*Sts* +/*Sts* -) to a C3H/An female (*Sts* -/*Sts* -) and analyzed the resulting progeny for STS activity. If the new site of recombination is permanent and *Sts* no longer crosses over to the X chromatid, all male progeny should be STS positive while all female progeny should be STS negative. In fact, when 4 male and 20 female progeny were tested for STS activity, 1 male was found to be STS negative and 11 females were found to be STS positive. We conclude that a permanent new site of recombination distal to *Sts* is not a characteristic in this male.

An alternative explanation for the XY, *Sts* -/*Sts* + male is that a deletion/mutation involving both the testis determining gene and the sequences recognized by pYCR8/B within the *Sxr* region had occurred such that *Sxr*, although still present, is now incapable of inducing sex reversal. Precedence for a deletion/mutation event in *Sxr* is the recent discovery of the *Sxr'* mouse which lacks H-Y antigen³⁵. In an effort to see if any part of the *Sxr* region was still present on the telomere of the Y chromosome in the recombinant mouse, the DNA from 10 of its female progeny were probed with pCS316, the latter detecting the highly repeated Bkm-related sequences known to comprise a large part of the *Sxr* region²². Our reasoning was that if the *Sxr* region was still present but incapable of inducing sex reversal, it is likely that the Bkm-related sequences would still be present and transmitted to 50% of the XX progeny. No Bkm-related sequences could be detected (data not shown). We thus conclude that the *Sxr* region is indeed absent.

DISCUSSION

We initiated this study to demonstrate: 1) presence of a functional *Sts* gene in the pseudoautosomal region of the Y chromosome by demonstrating linkage to *Sxr* which can be used as a marker for the Y telomere, 2) to search for rearrangements within the X and Y chromosomes and *Sxr* region with recently isolated X and Y differential and *Sxr* specific DNA probes, and 3) to examine the frequency of recombination in the pseudoautosomal region utilizing *Tdy*, *Sts*, and *Sxr* as markers

Our data clearly demonstrate linkage of *Sts* to *Sxr*. The most logical explanation in keeping with all data to date is that XXSxr, STS positive males are the result of the transfer of *Sts* and *Sxr* from the Y (*Sts* +, *Sxr* +) chromatid to the X (*Sts* -) chromatid during meiotic pairing and crossing-over (Fig. 1). Since XXSxr and XYSxr males possess STS activity, whereas XY male siblings and XX females do not, the data

demonstrate that the *Sts* locus on the Y chromosome is functional.

No rearrangements of the X and Y chromosomes could be detected among XXSxr, XY and XYSxr males. Thus the additional *Sxr* region in XXSxr and XYSxr males did not result in new hybridization patterns with the probes and enzymes used in this study.

The presence of a single recombinant segregating *Sts* and *Sxr* out of 103 male meioses gives a frequency of recombination of 0.01 or approximately 1 centiMorgan. If one assumes that frequency of recombination in man and mouse are comparable, then 1 centiMorgan is equivalent to about 1000 -2000 kb of DNA³⁶. However, in both mouse and man, rates of recombination in the pseudoautosomal region in male meioses have been calculated to be 7 to 10 times higher than equivalent X-X rates of recombination, in their respective species probably due to the size limit of the X-Y pairing region^{37,38}. This would mean that the data from the present study maps *Sts* only about 100 -200 kb from the X and Y telomeres. Its very distal location in the pseudoautosomal region is in keeping with previous data showing *Sts* having a recombination frequency of 0.5 with regards to *Tdy*¹⁷. In contrast the Mov 15 proviral insert which is located more proximally in the pseudoautosomal region as compared to *Sts* has a recombination frequency of 0.1-0.2 with respect to *Tdy*³⁹ suggesting a gradient of recombination in the pseudoautosomal region of the mouse, as is observed in man³⁷.

Recently Keitges et al.⁴⁰ in a cross nearly identical to ours obtained a rate of a single recombination event between *Sts* and *Sxr* to be 10 times higher than that found in the present study (5/51, 9.8% vs. 1/103, 0.01%) and, in addition, the presence of a double recombinant among *Tdy*, *Sts* and *Sxr* (1/51). The most obvious explanation for the discrepancy is the presence of a gene or genes affecting recombination frequency in the pseudoautosomal region. In the present experiment, although the *Sxr* mutation was being placed on the 129/Sv genetic background, this had only progressed as far as the N3 backcross generation before C3H/An genes were introduced. Thus we do not believe that 129/Sv genes played a role. A closer examination of the X chromosomes used in the crosses, however, suggests a possible explanation. In the cross used by Keitges et al.⁴⁰, an XO *Ta* +, *Sts* - female was used to generate XYSxr (*Ta* +, *Sts* -/*Sts* +) sires. Since the *Sts* - genotype is present only in the C3H/An inbred strain, which itself lacks the *Ta* mutation, an XO *Ta* +, *Sts* - female

must possess an X chromosome of at least two different origins. In contrast, in the present experiment the entire X chromosome is of C3H/An origin. It is possible that this difference in the X chromosomes, even the *Ta* marker itself, may have resulted in the very different rates of recombination between the two studies. Indeed, an analysis of the STS positive : STS negative ratio reveals a slightly lower value for STS negative progeny in the study by Keitges et al. ⁴⁰ (35:24 vs. 50:53 in the present study) suggesting a possible difference in X and Y recombination.

The use of the recombinant DNA probes pY353/B and pY80/B in the identification of XXSxr progeny and pYCR8/B in the segregation of XY and XYSxr progeny proved invaluable in the present study. The genotypes of the experimental animals could be determined accurately on mice as young as newborns without resorting to karyotyping, the utilization of other, possibly deleterious, mutations, waiting for the animals to reach puberty to examine testes size, or progeny testing. Thus considerable time and animal maintenance costs were saved.

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