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The use of specific DNA probes to analyse the *Sxr* mutation in the mouse

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Summary

The mouse Y chromosome plays a fundamental role in the control of primary sex determination and fertility. Both genetic and molecular biological evidence has shown that much of the necessary information is contained in a minute piece of the Y (the *Sxr* region) which has arisen by a duplication of the pericentric region of the normal Y and the transposition of one copy to the distal pseudoautosomal region. The present article describes the isolation of random Y-chromosome probes and their use to investigate this *Sxr* region at the molecular level. Total mouse Y-chromosome libraries were constructed from flow-sorted material and a *Sxr* regional library after specific microdissection and cloning. Transcription has been detected in the testis using both *Sxr*-specific and non *Sxr*-located genomic probes taken from these libraries. In addition, we have been able to confirm the presence of an active steroid sulphatase gene on the mouse Y. This gene is located in the distal portion of the pseudoautosomal region and is tightly linked to *Sxr*. Finally, using an *Sxr*-specific probe we can define multiple Y-chromosome haplotypes in the mouse showing that the region is evolving very rapidly.

Key words: DNA probe, sex reversal, mouse, *Sxr*, sex determination.

Introduction

The Y chromosome is the most specialized of mammalian chromosomes being involved almost exclusively in primary sex determination and fertility. Under the dominant influence of the Y, the bipotential fetal gonad develops along the testicular pathway even when multiple copies of the X are present (Jacobs & Strong, 1959; Russell & Chu, 1961). In the mouse, this process of testicular development, although undoubtedly initiated by the Y, involves a complex interaction with at least three autosomally located genes, *Tda-1*, *Tda-2* and *Tas* (Eicher & Washburn, 1986). At present, the way in which the Y controls this pathway is unknown. We favour the simplest explanation that there is primary sex-determining gene(s) located on the Y (*Tdy*) which is regulatory in action. An alternative hypothesis has been put forward, however, by Chandra (1985) based on a passive Y chromosome containing only noncoding DNA.

The most powerful and direct method to analyse the role of the Y in this process would be to identify, map and molecularly clone *Tdy*. This analysis has already started in man with the analysis of numerous XX males and XY females using Y-chromosome probes. In molecular terms, the human equivalent of *Tdy* (*TDF*) has been mapped to the short arm of the Y between the locus *MIC2* and locus *DXYS5* defined by random probe 47a (Vergnaud *et al.* 1986). Hence it is proximal to the telomeric X–Y recombination (or pseudoautosomal) region but distal to the centromere. In the mouse, an analysis of the *Sxr* mutation (see below), with a snake satellite DNA probe (Bkm) has led to the identification of the pericentric region of the Y as the putative sex-determining region (Singh & Jones, 1982). In this respect, it differs from the human in that it does not map near the telomeric

pseudoautosomal region. Using a variety of molecular probes, the *Sxr* mutation and a newly defined Y-chromosome rearrangement (Y*) Eicher and co-workers (Eicher, Phillips & Washburn, 1983; Eicher & Washburn, 1986) have been able to split the mouse Y into four functional regions. Region 1 contains the centromere, region 2 the Bkm-related sequences, *Tdy* and *H-Y*, region 3 a central region containing repeated viral sequences related to M720 and MuRVY and region 4 a telomeric region involved in homologous pairing and recombination with the X (Fig. 1). Keitges, Rivest, Siniscalco & Gartler (1985) have presented evidence for the presence of X- and Y-linked genes for steroid sulphatase mapping to this region.

The present paper summarizes recent work done in our laboratory in isolating DNA probes from the mouse Y chromosome (and in particular from the pericentric region) and their use to identify potential Y-located genes that may play a role in primary sex determination and/or fertility.

The origin segregation of the *Sxr* mutation

The *Sxr* (sex reversed) mutation was first discovered by Cattenach, Pollard & Hawkes (1971). Males carrying this mutation were able to sire male mice with an apparent XX karyotype. Although the mutation segregated as an autosomal dominant, extensive linkage analysis failed to map the locus. In 1982, Singh and Jones isolated minor satellite DNA sequences from a female banded krait (Bkm) which hybridized strongly to the mouse Y. Using this probe they were able to show that XX*Sxr* male mice did in fact contain a small portion of the Y in their genomes which presumably carried *Tdy*. Subsequent *in situ* hybridization data showed that Bkm hybridized to the pericentric region of the normal Y but to both the pericentric and telomeric region of the Y in XY*Sxr* carrier males. Further, unlike in normal features, a concentration of Bkm could be found in the telomeric region of one X chromosome in XX*Sxr* males. This has led to the proposed origin and segregation of *Sxr* as shown in Fig. 1 (Eicher, 1982; Hansmann, 1982; Burgoyne, 1982). It is proposed that the pericentric sex-determining region of the Y transposed from one chromatid to the telomere of the other, distal to the pseudoautosomal region. Hence during male meiosis this *Sxr* chromatin can be transferred to the distal region of the paternal X by recombination, making it function as a Y and inducing testis formation in XX*Sxr* mice. As indicated, four types of progeny can be produced: non-recombinant XX females and the XY*Sxr* carriers, and the recombinant XX*Sxr* sterile males and XY fertile males who have lost *Sxr* by recombination.

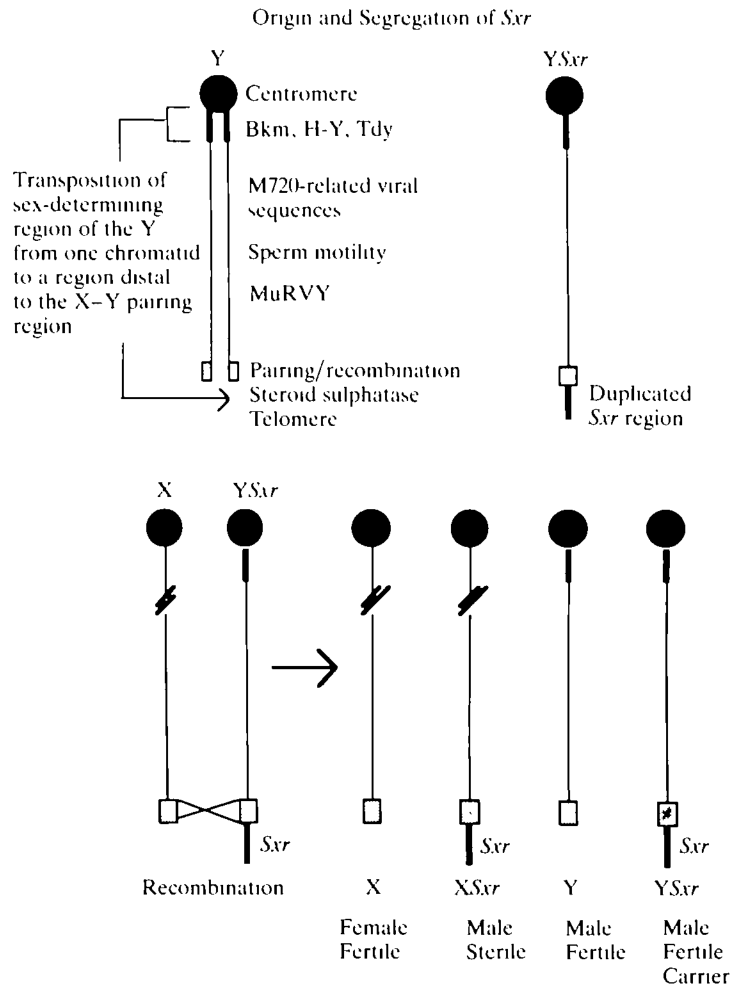

Fig. 1. Diagram showing the proposed origin and segregation of *Sxr*.

This inheritable mutation offers great potential for molecular analysis as XX*Sxr* mice carry a minute but critical portion of the Y bearing not only *Tdy* but also *H-Y* as defined by the cytotoxic T cell assay (Simpson *et al.* 1981). Recent evidence suggests that *H-Y* may be involved in spermatogenesis (Burgoyne, Levy & McLaren, 1986; Levy & Burgoyne, 1986). In addition, an aberrent *Sxr* type has been identified (designated *Sxr'*), which, although it still retains *Tdy*, no longer expresses *H-Y* (McLaren *et al.* 1984), representing another potential source of genetic material for analysis.

Isolation of DNA sequences derived from the mouse Y chromosome

(A) *Flow sorting*

The potential of random DNA probes for analysing a similar, although generally sporadic, condition in the human–XX maleness has been well proven. Hence we decided to use this approach to analyse the mouse

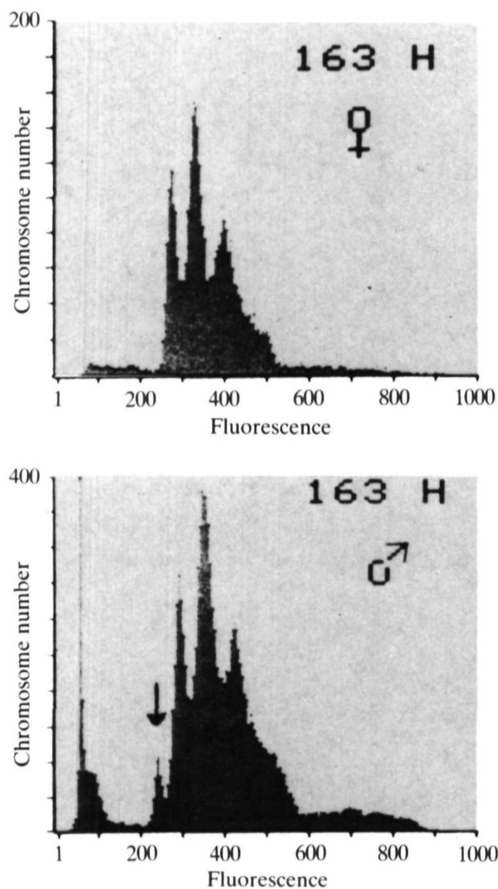

Fig. 2. Flow-sorting karyotype of the male mouse fibroblast line (163SV40/Pas) used for isolating the mouse Y chromosome. The Y peak is arrowed. Chromosome 19 which usually contaminates this peak has been removed by the use of the 9:19 Robertsonian translocation.

Y at the molecular level. We first constructed chromosome libraries highly enriched for the Y by flow sorting (Baron *et al.* 1986). In this technique, isolated metaphase chromosomes are stained with ethidium bromide (or other DNA fluorochromes) and passed through a laser. The emitted fluorescence, which is proportional to DNA content, allows individual chromosomes to be separated by size. Preliminary flow karyotype analysis showed that, although the Y could be separated from other chromosomes, it could not be resolved from chromosome 19. To circumvent this problem, a male cell line homozygous for the Rb(9:19) Robertsonian translocation was used. Due to its now increased size the 19 was removed from the Y peak (Fig. 2). After collection of this peak, the DNA was extracted (approximately 70 ng representing 650 000 chromosomes), half was digested with *Eco*RI and half with *Hin*dIII. In this way, two different libraries representing many genome equivalents of the Y were constructed in phage lambda 1149. From these libraries, a large

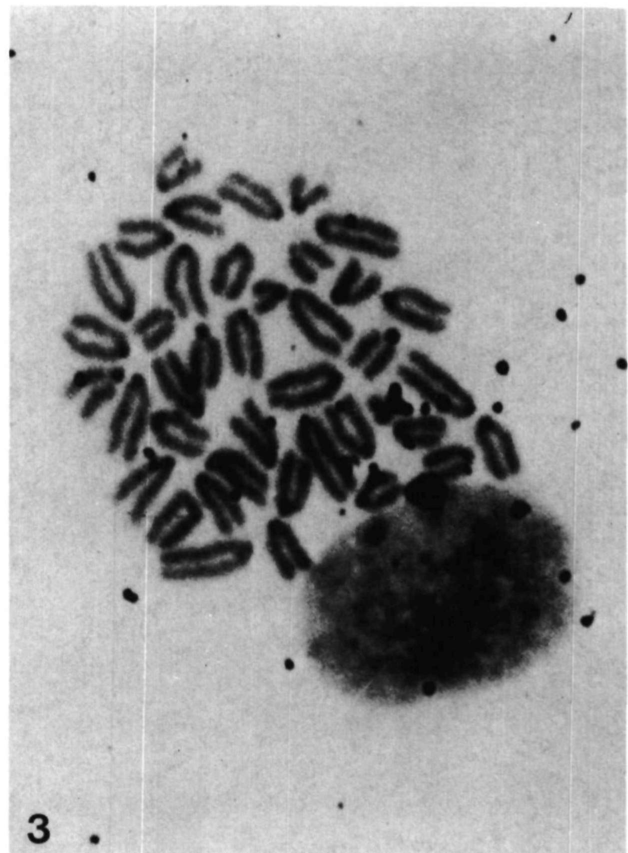

Fig. 3. *In situ* hybridization of the Bkm probe to metaphase chromosomes from a male mouse carrying the pericentric Y chromosome. The concentration of grains on the Y are localized to the short arm.

number of Y-derived clones from all regions of the Y were obtained.

(B) *Microdissection and microcloning*

As the *Sxr* DNA represents only a small portion of the Y, to obtain sequences specific for this region a large number of clones must be screened. An alternative strategy is to microdissect the region of interest from metaphase chromosomes and microclone the picogram quantities of DNA obtained. This technique has previously been applied successfully to mouse chromosomes 17, X and 1 (Rohme *et al.* 1984, Fisher, Cavanna & Brown, 1985; Weith *et al.* 1987). For this technique, it is necessary to have a marker chromosome which can be easily and unambiguously identified without any staining. This is not possible with the normal mouse Y. However, Winking has recently reported a metacentric Y formed by a pericentric inversion. The short arm of this chromosome represents between 25–30% of the Y. Male mice carrying this easily recognizable Y are fully fertile. We confirmed by *in situ* hybridization of Bkm that the putative sex-determining region was located on the short arm. As shown in Fig. 3, an intense

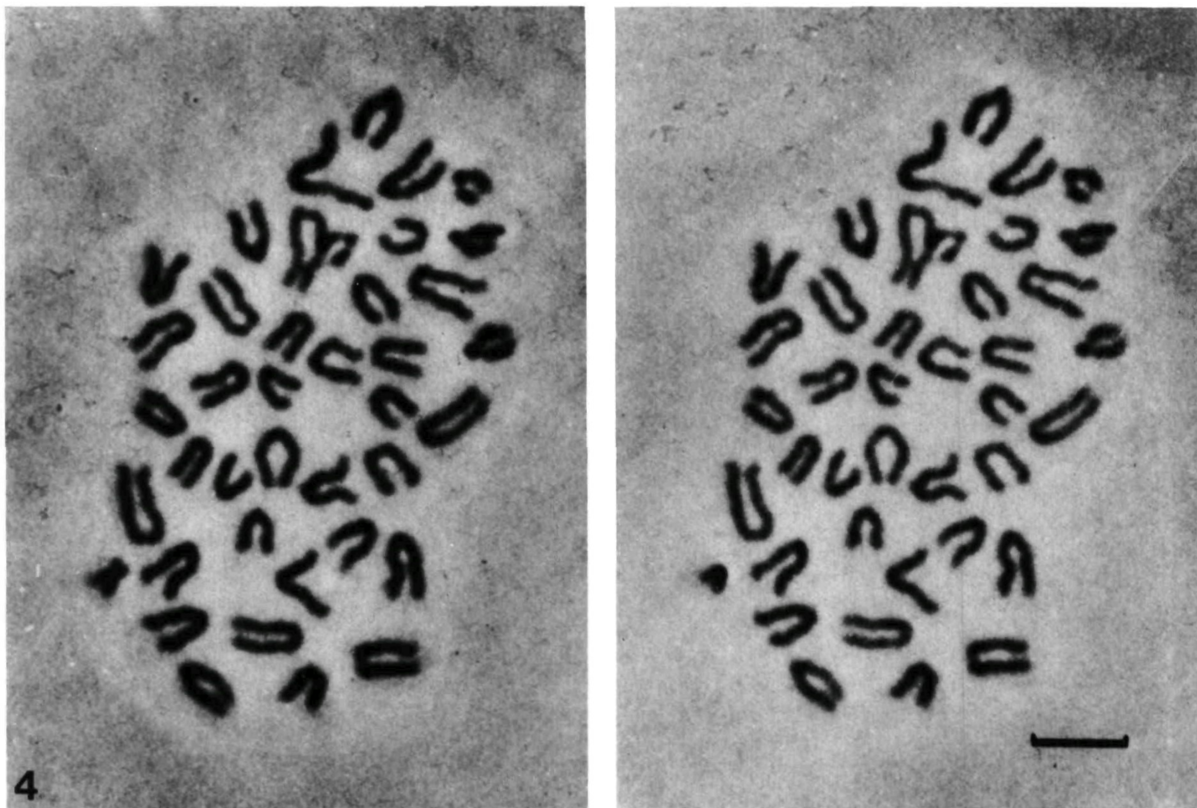

Fig. 4. Microdissection of the short arm of the pericentric. Y. Left panel, before microdissection the Y can be seen at 8 o'clock (unstained preparations from embryonic yolk sac viewed under phase contrast microscopy). Right panel, same preparation after microdissection of the short arm.

hybridization could be detected exclusively on the short arm of the Y. We then performed a microcloning experiment by dissecting this short arm from approximately 100 metaphase spreads as shown in Fig. 4. The DNA (approx. 3 pg) was cloned into the immunity insertion vector NM1149 and 502 independent recombinants were obtained. The analysis of this bank is now under way and to date from 91 inserts tested we have obtained 4 unique or low copy number probes mapping to the *Sxr* region.

Transcription from the mouse Y chromosome

(A) *Non*-Sxr *transcription*

Using pools of Y-derived probes taken from the flow-sorted Y libraries to probe Northern blots of testis mRNA we identified a Y-specific sequence (pY353/B) which detected a specific transcript in the testis (Fig. 5) (Bishop & Hatat, 1987). The genomic sequence itself is repeated about 250 times and *in situ* hybridization studies suggest that it maps along the entire length of the Y (unpublished observations). It does not react with XX*Sxr* DNA, however, showing that it is absent from this region. The 1·3 kb transcript appears to be testis specific as it could not be detected

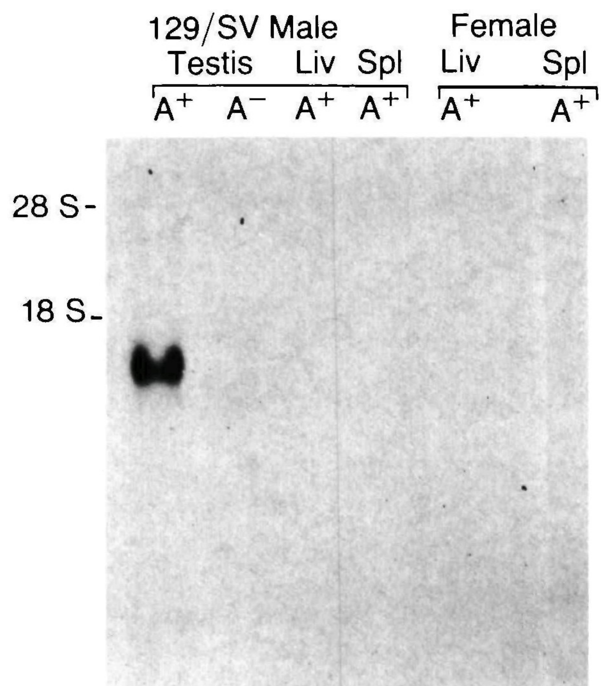

Fig. 5. Northern blot analysis of the probe pY353/B. An approximately 1·3 kb transcript can be detected in the testis but not in male or female liver or spleen.

in RNA from liver, spleen, lung, heart, kidney or brain tissues. Sequence analysis of the cloned cDNA suggested that it was not retroviral in origin and contains a potential open reading frame of 760 bases. Although we have not as yet been able to assign a function to the transcript, it is possible that it might play a role in sperm motility.

(B) *Transcription from the* Sxr *region*

In an effort to identify transcription from this region, we probed our flow-sorted Y libraries at low density with a mixture of total mouse DNA, Bkm, M720, pY353/B and a mixture of anonymous Y-repeated sequences. The negative plaques were then replica-plated and screened with cDNA probes synthesized from male testis, male liver and female liver. Five clones which were positive with testis cDNA but negative with that from the liver were identified. Probing genomic blots with these clones identified pCRY8/B, which is a 2·2 kb *Eco*RI fragment. As can be seen in Fig. 6 (left panel), it hybridizes to male but not female DNA from the inbred strain C57BL/6 showing the bands to be Y located. In addition, all the bands can be seen to react with XX*Sxr* DNA showing them to be contained within this critical region. An examination of DNA from a family segregating *Sxr* (Fig. 6 right panel) shows that the probe reacts with XY*Sxr*, XY, XX*Sxr* but not the normal female XX. An increased intensity of hybridization can be seen on the XY*Sxr* DNA as compared to XY or XX*Sxr* showing the sequence has probably been duplicated in XY*Sxr*. More careful dosage studies have confirmed this result and are consistent with the proposed origin of *Sxr* by duplication.

As this sequence was initially identified with cDNA synthesized from testis mRNA it should be transcribed. Preliminary data probing Northern blots of mRNA from testis and male and female liver show that pCRY8/B detects a single-band transcript of approximately 3·5 kb in the testis but not in the liver (Fig. 7). At present we are trying to clone the corresponding cDNA and to analyse this potentially important result further.

Identification of a functional Y-located gene for steroid sulphatase and linkage to *Sxr*

Evidence has recently been put foreward by Keitges *et al.* (1985) for the X and Y linkage of the gene for steroid sulphatase in the mouse. This was based on the apparently paradoxical situation which showed that STS was definitely X-linked but segregated as if it were an autosomal gene. They postulated that, if there were functional X- and Y-linked genes in the pseudoautosomal region, the alleles could recombine during male meiosis and mimic an autosomal segregation pattern. As the observed crossover frequency with respect to sex was 50 %, the genes should map very close to the telomere. In the Y*Sxr* chromosome one copy of the *Sxr* region is situated distal to the X–Y recombination (or pseudoautosomal) region. Hence on this chromosome these two very interesting

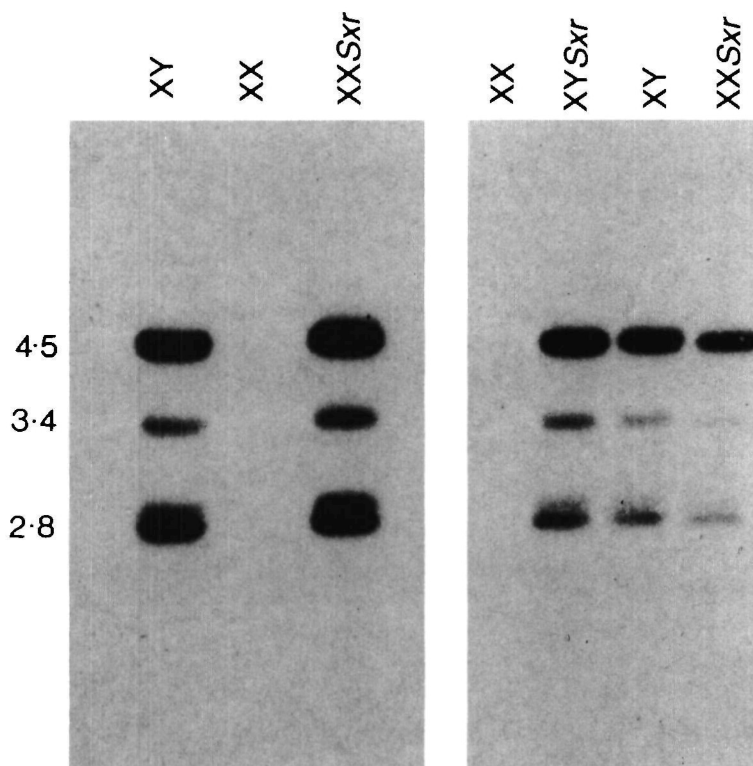

Fig. 6. Southern blot analysis (*Taq*I restricted) using *Sxr*-specific probe pYCR8/B. Left panel, four Y-specific bands can be detected in male (XY) but not female (XX) C57BL/6 DNA. These same bands can be seen in sex-reversed male (XX*Sxr*) DNA showing them to be contained within the *Sxr* region. Right panel, reaction of the probe with DNA from a family segregating *Sxr*(129Sv/Pas N4).

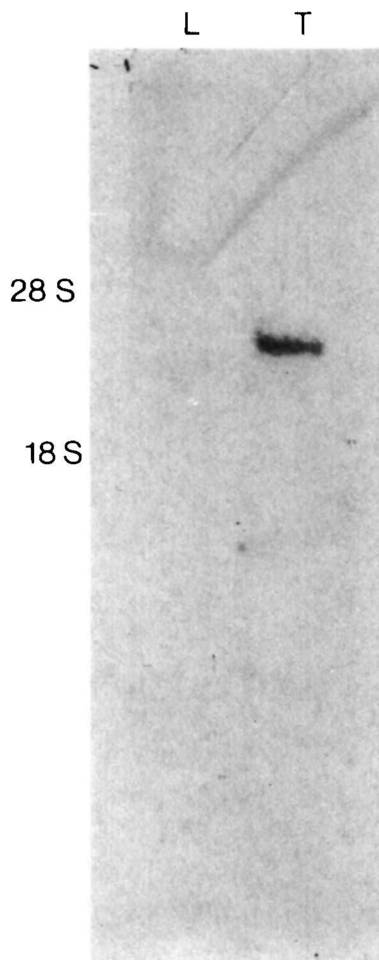

Fig. 7. Northern blot analysis of poly(A)$^+$RNA from adult male liver (L) and testis (T) (strain C57BL/6) using probe pYCR8/B. A transcript of approx. 3·5 kb can be seen in the testis but not liver.

regions must be physically very close and *Sxr* itself can be used as a marker for the telomere. We therefore set up crosses using the steroid-sulphatase-deficient mouse strain C3H/an and *Sxr* to test the hypothesis. At the same time we were able to analyse the progeny of the cross with our Y-derived DNA probes to look for new rearrangements involving this region of the Y.

The following cross was set up: *STS*−/*STS*− × (*STS*−/*YSxr*)F_1. If there is a telomeric Y-located *STS* gene linked to *Sxr* then four types of progeny should result: female XX (*STS*−/−), male XX*Sxr* (*STS*−/+), male XY (*STS*−/−) and male XY*Sxr* (*STS*−/+)(cf. Fig. 1). The results of analysing 47 backcross mice can be seen in Table 1. Of the 11 XX female mice found, all were STS negative and of the 11 XX*Sxr* males all were STS positive. In order to distinguish XY males from XY*Sxr* carrier males, we have selected 10 mice at random for progeny testing. Preliminary results show that as predicted the STS-positive males segregate *Sxr* whereas the STS-negative males do not. These results clearly show that the Y carries a fully functional *STS* gene and as it is tightly linked to *Sxr* it must be located in the distal X–Y pairing region. To date one exceptional male mouse which is STS positive but which does carry *Sxr* has been found. The simplest explanation is that *STS* and *Sxr* can occasionally be separated by recombination and that *Sxr* must be more distal than *STS* on the Y. Experiments are in progress to try and assess the recombination frequency. Careful DNA analysis suggests that it is possible to distinguish XY males from XY*Sxr* carrier males by dosage with *Sxr* probe pYCR8/B. Hence the tedious process of progeny testing can probably be avoided in future.

Finally, no obvious rearrangements were found at the DNA level using Y-derived repeated (non-*Sxr* located) probes pY353/B or pY80/B or *Sxr* located probes pCR8/B or pYC66/B.

Polymorphism in the *Sxr* region

We have previously reported using the Y-specific repeated probe pY353/B that the Y chromosomes of the European semi-species *Mus musculus musculus* and *Mus musculus domesticus* can be distinguished on the basis of RFLP (restriction fragment length polymorphisms). Further analysis of the DNA from common laboratory strains showed that despite containing *domesticus* type mitochondrial DNA only SJL and AKR/J carried a *domesticus*-derived Y chromosome (Bishop *et al.* 1985 and unpublished results). The other strains tested including C57BL/6 carried a *musculus*-derived Y. We have subsequently confirmed this observation with numerous Y-derived probes both repeated and single copy (unpublished observations). When the *domesticus* Y chromosome is placed on the C57BL/6 background XY sex-reversal results generating both XY females and hermaphrodites (reviewed by Eicher & Washburn, 1986). They suggest that this may result from an improper interaction between different *musculus*- and *domesticus*-derived alleles of the Y-located *Tdy* and autosomally located *Tda*-1 and *Tas* genes. Recently Nagamine & Koo (1987) have presented evidence that the X–Y pairing region may be involved in *Tda*-1 inherited sex reversal. In addition, they report finding different degrees of fetal sex reversal when the Y from SJL and AKR are placed on the C57BL/6 background (Nagamine, Taketo & Koo, 1987). They suggest that although both SJL and AKR carry a *domesticus* type Y they may carry different alleles at *Tdy* or at other genes involved in the testis differentiation pathway.

We have recently used our *Sxr*-specific probe pCRY8/B to investigate the polymorphisms that might exist in this region of the Y. Such a study might

Table 1. *Linkage analysis of steroid sulphatase (STS) and* Sxr

Number of mice $n = 47$	Phenotype	Presence of entire Y chromosome p80	p353	Presence of *Sxr* region pY8	pC66	Steroid sulphatase	Genotype
11	F	–	–	–	–	–	XX
11	M	–	–	+	+	+	XX*Sxr*
15	M	+	+	+	+	–	XY or XY*Sxr*
10	M	+	+	+	+	+	XY*Sxr* or XY

STS analysis was performed using [^{3}H]dehydroepiandrosterone sulphate on spleen or liver samples. Phenotype was based on examination of external genitalia. *Taq*I restricted DNA from spleen was used for Southern blot analysis Presence of the Y chromosome was detected using repeated (non *Sxr* located) Y-chromosome probes (PY80/B and pY353/B thus distinguishing normal and XX*Sxr* males. The presence of the *Sxr* region was detected using *Sxr*-specific probes by pCRY8/B and pYC66/B.

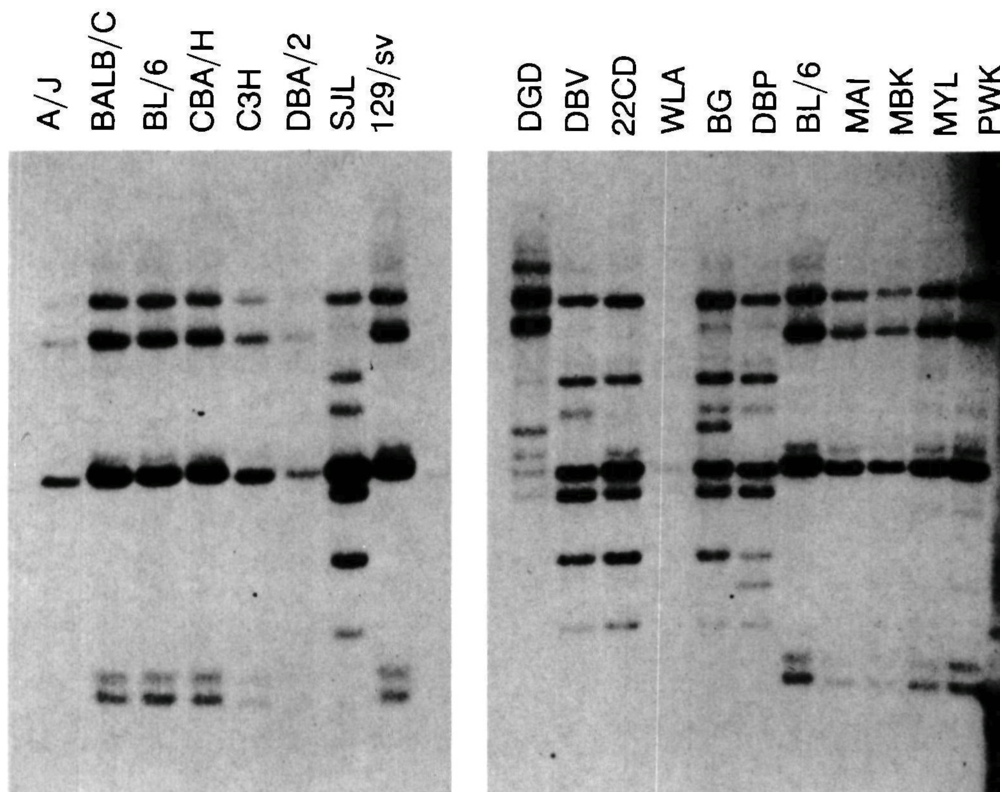

Fig. 8. Southern blot analysis (*Msp*I restricted) of laboratory and wild-derived mouse strains using probe pYCR8/B. Left panel, a clear polymorphism can be seen between laboratory strain SJL/Pas which carries the domesticus type Y and the other strains which all carry a *musculus* derived Y. Right panel, no polymorphisms could be seen within the *musculus* mouse group (MAI,MBK,MYL,PWK and C57BL/6) although extensive polymorphisms can be detected within the *domesticus* group (DGD,DBV,22CD,WLA,BG and DBP). The weak signal seen in WLA was due to underloading.

throw some light on these complex XY sex-reversed phenomena. Fig. 8 shows that when Y8 was used to probe *Msp*I digested DNA from laboratory inbred strains a *musculus/domesticus* RFLP could be seen with only the SJL strain carrying the *domesticus* type Y. When different *domesticus* and *musculus* mice originating from several locations in Europe were tested (for the origins of these mice see Bishop *et al.* 1985, Bonhomme & Guenet, 1987) we were unable to detect any RFLP within the *musculus* group but within the *domesticus* mice six Y haplotypes could be distinguished. A more detailed analysis using more enzymes, mice and probes is now under way. These preliminary results indicate however that the *Sxr* region of the Y is evolving at a surprisingly rapid rate and may well correlate with the type and degree of inherited XY sex reversal associated with the *domesticus* Y chromosome.

Prospectives

The isolation of unique or low copy number DNA sequences which detected specific DNA fragments within the *Sxr* sex-determining region of the mouse makes possible a direct analysis of this region at the molecular level. Further, as the *Sxr* region is linked to the pseudoautosomal region in both XY*Sxr* and XX*Sxr* mice and the isolation of DNA from this region has already been reported (Harbers, Soriano, Muller & Jaenisch, 1986) the simultaneous analysis of both regions becomes possible. The development of pulsed-field gel electrophoresis (Carle & Olson, 1984, Schwartz & Cantor 1984) has allowed long-range mapping of DNA over distances well in excess of a million base pairs (Brown & Bird, 1986, Van Omen *et al.* 1986). The application of this technique to an analysis of the *Sxr* region of the mouse will be an important first step and is underway in our laboratory. One of the questions we want to answer is how big is the region and how much of it is made up of the GATA/GACA tandem repeats? To date we have isolated five *Sxr* clones from our flow-sorted and microcloned libraries. These probes range from 0·6–2·2 kb. Some of them detect multiple fragments in the *Sxr* region which, on the basis of Southern blot analysis, appear not to be contiguous. Hence, it may be possible to clone a significant portion of the region simply by using them to isolate large genomic fragments from an XX*Sxr* cosmid library.

Of course, one of the main goals is to isolate functional genes from this region. Our preliminary data show that pYCR8/B detects a 3·5 kb transcript in the testis originating from this region but further analysis will have to await the isolation of the cDNA. Further transcripts can be sought by using isolated cosmids to probe Northern blots of testis RNA. This approach has its problems, however, for although *H-Y* has been shown to be ubiquitously expressed, perhaps *Tdy* may not be expressed in adult testis. Possibly expression for a relatively short time during embryogenesis is all that is required to initiate testis development. Perhaps a more effective approach is to look for sequence conservation between man and mouse as such sequences often represent expressed genes (Monaco *et al.* 1986). *Sxr*-located cosmids that are also crossreactive with the short arm of the human Y and present in the genome of XX(Y^+) males would surely be good candidates. McLaren has described a *Sxr'* mouse that does not express *H-Y*. Although it is unknown if this arose through a small deletion or point mutation which would be difficult to detect it would certainly be worthwhile examining this mouse with *Sxr*-specific probes.

Finally, the fact that the region appears to be highly polymorphic and must be evolving at a very rapid rate is intriguing. Further study may well reveal a correlation between the Y haplotypes detected in the wild-derived strains and their ability to produce sex-reversal when interbred to laboratory strains.

In conclusion, the isolation of numerous DNA probes from the *Sxr* region of the mouse Y chromosome represents a significant advance in the study of primary sex determination and fertility. The use of these tools in conjunction with recent advances such as pulsed-field gel electrophoresis and transgenic mice should now allow rapid progress in this area of research.

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