Analysis of the testes of H-Y negative XOSxr\textsuperscript{b} mice suggests that the spermatogenesis gene (Spy) acts during the differentiation of the A spermatogonia

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Summary

H-Y antigen negative XOSxr\textsuperscript{b} mice, like their H-Y positive XOSxr\textsuperscript{a} counterparts, have testes; but, in contrast to XOSxr\textsuperscript{a} males, XOSxr\textsuperscript{b} testes almost totally lack meiotic and postmeiotic stages of spermatogenesis. The quantitative analysis of the testes of XOSxr\textsuperscript{a} males and their XY±Sxr\textsuperscript{b} sibs, described in the present study, identified two distinct steps in this spermatogenic failure. First, there was a reduction in mitotic activity among T\textsubscript{1} prospermatogonia, so that approximately half the normal number of T\textsubscript{2} prospermatogonia were produced. Second, there was a dramatic decrease in the number of A\textsubscript{3} and A\textsubscript{4} spermatogonia and no Intermediate or B spermatogonia. These reductions were also largely due to decreased mitotic activity, there being a shortage of A\textsubscript{1} and A\textsubscript{2} spermatogonial divisions and no divisions among A\textsubscript{3} or A\textsubscript{4} spermatogonia. Mitotic activity among the T\textsubscript{2} prospermatogonia and the undifferentiated A spermatogonia was normal. This means that the spermatogonial stem cells, which are a subset of the undifferentiated A spermatogonia, are unaffected in XOSxr\textsuperscript{h} mice. Sxr\textsuperscript{b} is now known to have arisen by deletion of DNA from Sxr\textsuperscript{a}. It is clear from the present findings that a gene (or genes) present in the deleted DNA plays a major role in the survival and proliferation of the differentiating A spermatogonia.

Key words: mice, spermatogenesis, sex reversal, H-Y antigen negative, Spy.

Introduction

Sex-reversed (Sxr) is a factor that causes an inherited form of sex-reversal, such that XX and XO mice carrying Sxr develop as phenotypic males (Cattanach et al. 1971). In 1982 evidence was obtained that Sxr was in fact an extra copy of the testis-determining region of the mouse Y chromosome which had become located distal to the pairing and exchange region of the Y, so that it regularly crossed over onto the X chromosome during male meiosis (Singh and Jones, 1982; Evans et al. 1982; Burgoyne, 1982; Eicher, 1982; Hansmann, 1982).

In addition to testis-determining information, the original Sxr (now termed Sxr\textsuperscript{a} – McLaren et al. 1988) included information required for H-Y antigen expression (Bennett et al. 1977). In 1984 McLaren et al. discovered a variant of Sxr\textsuperscript{a} (originally designated Sxr\textsuperscript{2}, but now Sxr\textsuperscript{b}) that retained the testis-determining information, but which had lost the Y-chromosomal gene required for transplantation H-Y antigen expression (Simpson et al. 1981, 1986). This finding, recently confirmed by the separation of TDF from H-Y loci in humans (Simpson et al. 1987), negated the hypothesis of Wachtel et al. (1975) that H-Y antigen was the primary testis determinant (at least in so far as the transplantation H-Y antigen is concerned).

XXSxr males differ genetically from normal males not only in that they lack most of the Y chromosome, but also in having two X chromosomes. The presence of two X chromosomes is incompatible with male germ cell survival beyond the perinatal period (reviewed by McLaren, 1983) so that in order to investigate the effects of the Y-chromosomal deficiencies associated with Sxr\textsuperscript{a} and Sxr\textsuperscript{b}, it is necessary to produce Sxr males with single X chromosomes.

XOSxr\textsuperscript{b} mice were first described by Cattanach et al. (1971) and although all stages of spermatogenesis are represented in their testes, the later stages are severely depleted so that the testes are small and the mice are sterile. The majority of the spermatids are in fact diploid and the few sperm produced, whether haploid or diploid, are abnormal (Levy and Burgoyne, 1986a).

XOSxr\textsuperscript{b} mice have a more severe spermatogenic impairment with only a few germ cells reaching early meiotic prophase (Burgoyne et al. 1986). The XO germ cells in an XO/XY/XXY mosaic male described by Levy and Burgoyne (1986b) suffered a similar fate despite a normal XY Sertoli cell environment. These
findings led Burgoyne et al. (1986) to suggest that Sxr<sup>a</sup> carries a spermatogenesis gene (Spy) that is lacking in Sxr<sup>b</sup>, and that Spy is expressed cell-autonomously in the germ line. Recent studies have shown that the Sxr<sup>b</sup> variant arose by deletion of DNA from Sxr<sup>a</sup> (Bishop et al. 1988; Mardon et al. 1989).

The purpose of the present study was to define the spermatogenic block in XO Sxr<sup>b</sup> mice by a quantitative analysis of germ cells in the two weeks following birth (when the block first becomes apparent) and from this deduce the function of Spy. During the course of the experiment, the finding of a significant body weight difference between XO Sxr<sup>b</sup> and XO Sxr<sup>a</sup> mice supported a hypothesis, under separate study, that a growth and development gene (dubbed Gdy) may also be deleted.

**Materials and methods**

**Mice**

XYSxr<sup>a</sup> males were mated with females heterozygous for the inversion In(X)/X. In(X)/X females produce some nullo-X eggs following crossing-over within the inversion (Evans and Phillips, 1975), and approximately 1 in 19 of the progeny from this cross have the XOSxr<sup>b</sup> genotype. The In(X)/X females were checked for vaginal plugs each morning, and coitus was presumed to have taken place at the midpoint of the previous dark cycle. Ages were calculated from conception, rather than birth, because it is known that the duration of pregnancy is affected by litter size. The majority of litters were born about 19 days post coitum (dpc), so in what follows this is equated with the day of birth. 157 litters were bred of which 59 included XOSxr<sup>b</sup> males. Litters were processed from 194 dpc (day of birth) through 301 dpc (11 days post partum), 324 dpc (13dpp) and 594 dpc (40dpp).

A similar breeding cross was set up to produce XO Sxr<sup>b</sup> males as controls for a possible XO effect. Data from 35 litters are included in this study. The litters were processed at 194, 214 through 244, 274, 294, 314 and 334 dpc.

Body weights were recorded at autopsy. Following exclusion of 'runts' (Burgoyne et al. 1983b), litters were evaluated provided at least one XOSxr and one XY±Sxr male was present. Since a qualitative analysis suggests that XY and XYSxr testes are not significantly different during the premeiotic stages (results not shown) XY and XYSxr males were not separately identified. 52 Sxr<sup>a</sup> and 35 Sxr<sup>b</sup> litters finally provided data.

**Karyotyping**

Mitotic spreads were prepared either by dissociating liver fragments (194 and 204 dpc) or by flushing out bone marrow cells (214 dpc onwards) in 0.04 % colcemid in Hapes-buffered Eagle's minimal essential medium, and incubating at 32°C for 60 min (liver) or 15 min (bone marrow). Cells were then treated with 0.56 % KCl for 20 min followed by five changes of 3:1 methanol:glacial acetic acid. The cells were then air-dried on slides and stained for 15 min in 2 % Giemsa in pH 6.8 buffer. XOSxr males were identified by scoring at least 5 consecutive spreads with 39 chromosomes and no evidence of a Y chromosome. XY±Sxr males were identified by 40 chromosomes, with a Y recognised by size and the presence of splayed short arms (Ford, 1966).

**Histology**

Both testes from each male were weighed using a Cahn electrobalance, and were then retained in Bouin's fixative awaiting the results of karyotyping. Testes from XOSxr and XY±Sxr littermates were dehydrated and cleared according to standard procedures, embedded in paraffin wax, serially sectioned at 3 μm and stained with haematoxylin and eosin.

**Quantitative analysis**

This analysis was carried out 'blind' with respect to genotype of the mice from which the sections were taken. The sampling was one tubule cross-section from every 20th section, or every 10th section for smaller testes, such that between 25 and 35 tubule cross-sections were analysed per testis. The procedure for selecting tubules for analysis was as follows: (1) A 0.25 mm square grid (R-4 grid, Graticules Ltd, Tonbridge, Kent) was 'stuck' to the bottom of the microscope slide with a film of water and a Chalkley grid (G52, Graticules Ltd) was inserted in the eyepiece. (2) When a section was selected, the square grid was focused under low power with a x10 objective and a square chosen at random. The central cross of the Chalkley grid was centered over the square and the section was brought back into focus. (3) The tubule cross-section adjacent to the central cross was analysed under oil immersion, provided it could be encompassed within the field of view.

This selection procedure ensures that all regions of the gonad have an equal chance of being sampled. Once a tubule was selected, all cells within the tubule cross-section were classified as to cell type except dead or dying cells which could not be classified. Sertoli cells were scored as being in interphase or division. Gonia were scored as being in interphase of division, and were also classified as to stage (i.e. T<sub>1</sub> spermatogonia, T<sub>2</sub> spermatogonia, undifferentiated A spermatogonia, differentiating A<sub>1</sub> or A<sub>2</sub> spermatogonia, A<sub>3</sub> or A<sub>4</sub> spermatogonia, Intermediate or B spermatogonia) using the criteria described by Clermont and Perey (1957), Oakberg (1971), Hilscher et al. (1974), Hilscher and Hilscher (1976), Bellve et al. (1977), Huckins and Oakberg (1978) and Klun and de Rooij (1981). It was often difficult to assign divisions to specific spermatogonial stages and in these cases they were classified according to the adjacent interphase stages in the same tubule. A category existed for cells that could not be classified. This group formed less than 0.5% of germ cells scored and have been omitted from the analysis. It should be pointed out that these cell counts are crude counts, uncorrected for cell sizes and thickness of the sections.

**Results**

The body weight data for the Sxr<sup>a</sup> and Sxr<sup>b</sup> litters are given in Table 1. The best estimates for the body weights of the four genotypes (XOSxr<sup>a</sup>, XY±Sxr<sup>a</sup>, XOSxr<sup>b</sup>, XY±Sxr<sup>b</sup>) at the various ages studied are provided by the means of litter means. In order to compare the two genotypes in each cross, mean weighted differences between these genotypes and the significance of these differences have been calculated from 'within litters' as described by Burgoyne et al. (1983b). From these mean weighted differences it is clear that XOSxr<sup>a</sup> mice are underweight when compared with XY±Sxr<sup>b</sup> mice. Despite the limited number of mice at each age, the difference is significant for 5/13 age groups, and pooling across age groups (the mean weighted differences are similar throughout the age
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Table 1. Mean body weights for (A) XOSxrb and XY±Sxrb, and (B) XOSxra+Sxra mice and the estimated difference between them for the period 19¹—33¹ dpc

(A) Days post coition

<table>
<thead>
<tr>
<th>No. of Mice</th>
<th>Mean±SEM body weights (g)*</th>
<th>Mean±SEM weighted difference (g)</th>
<th>Significance of difference (P)</th>
</tr>
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<tbody>
<tr>
<td>XOSxrb</td>
<td></td>
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<tr>
<td>XY±Sxrb</td>
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<tr>
<td>19¹</td>
<td>3              5           1.570±0.03     1.693±0.08                  -0.120±0.046   NS</td>
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<tr>
<td>21¹</td>
<td>4              12          2.197±0.10     2.200±0.10                  -0.024±0.0070  NS</td>
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<tr>
<td>23¹</td>
<td>7              16          2.346±0.12     2.862±0.11                  -0.516±0.070   &lt;0.005</td>
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<tr>
<td>25¹</td>
<td>11             14          3.297±0.24     3.645±0.30                  -0.347±0.197   0.05—0.025</td>
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<tr>
<td>27¹</td>
<td>6              19          3.857±0.16     4.240±0.13                  -0.384±0.108   &lt;0.005</td>
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<tr>
<td>29¹</td>
<td>5              19          4.094±0.19     4.932±0.26                  -0.842±0.108   &lt;0.005</td>
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<tr>
<td>31¹</td>
<td>3              8           5.130±0.66     5.500±0.79                  -0.330±0.266   NS</td>
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<tr>
<td>33¹</td>
<td>4              8           5.290±0.25     5.525±0.16                  -0.234±0.144   &lt;0.005</td>
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<tr>
<td>35¹</td>
<td>4              7           6.385±0.11     6.500±0.10                  -0.115±0.194   NS</td>
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<tr>
<td>37¹</td>
<td>4              6           6.618±0.66     6.675±0.47                  -0.029±0.284   NS</td>
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<tr>
<td>39¹</td>
<td>4              6           7.505±0.71     8.025±0.67                  -0.504±0.332   NS</td>
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<tr>
<td>41¹</td>
<td>4              6           8.060±0.50     8.563±0.53                  -0.508±0.407   NS</td>
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</table>

Pooled mean weighted difference
-0.359±0.059 <0.005

(B) Days post coition

<table>
<thead>
<tr>
<th>No. of Mice</th>
<th>Mean±SEM body weights (g)*</th>
<th>Mean±SEM weighted difference (g)</th>
<th>Significance of difference (P)</th>
</tr>
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<tbody>
<tr>
<td>XOSxra</td>
<td></td>
<td></td>
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<tr>
<td>XY±Sxra</td>
<td></td>
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<tr>
<td>19¹</td>
<td>3              6           1.467±0.04     1.450±0.01                  +0.010±0.050   NS</td>
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<tr>
<td>21¹</td>
<td>4              6           2.727±0.27     2.927±0.01                  -0.214±0.103   0.05—0.025</td>
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<tr>
<td>23¹</td>
<td>5              14          3.142±0.43     3.148±0.45                  -0.006±0.102   NS</td>
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</tr>
<tr>
<td>25¹</td>
<td>4              12          3.448±0.14     3.465±0.25                  +0.004±0.082   NS</td>
<td></td>
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</tr>
<tr>
<td>27¹</td>
<td>4              12          4.123±0.31     4.213±0.17                  -0.090±0.219   NS</td>
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<tr>
<td>29¹</td>
<td>4              10          5.985±0.40     6.273±0.34                  -0.288±0.162   0.025—0.010</td>
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</tr>
<tr>
<td>31¹</td>
<td>4              17          6.658±0.45     6.305±0.32                  +0.352±0.230   0.025—0.010</td>
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</tr>
<tr>
<td>33¹</td>
<td>4              15          7.918±0.38     8.285±0.47                  -0.367±0.275   NS</td>
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<tr>
<td>35¹</td>
<td>4              12          9.145±0.62     9.295±0.33                  -0.092±0.157   NS</td>
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</tr>
</tbody>
</table>

Pooled mean weighted difference
-0.044±0.069 NS

* Mean of litter means.

range studied) gives an overall estimated weight deficit of -0.359±0.059g (P<0.005). XOSxra mice are not significantly underweight when compared with XY±Sxra mice (pooled mean weighted difference = -0.044±0.069 g).

The testis weight data for the Sxra and Sxrb litters are given in Table 2. XOSxra testes (Table 2B) are not underweight when compared with XY±Sxra litter mates, but XOSxrb testes (Table 2A) are significantly underweight for 9/13 of the ages studied. Since XOSxrb mice are underweight, this testis weight deficit could simply be a reflection of the overall reduction in body weight. The XOSxrb testes were therefore corrected by dividing by individual body weight and multiplying by the mean XY±Sxrb body weight for the relevant litters. The mean weighted XOSxrb—XY±Sxrb differences for these corrected testis weights are plotted in Fig. 1. XOSxrb testes are significantly underweight by 23¹ dpc and the weight deficit rapidly increases thereafter.

Fig. 1. Mean weighted differences in testis weights (corrected for body weights) for XOSxrb and XY±Sxrb mice for the period 19¹—324 dpc. Where error bars are shown the differences are significant (t-test, 1-tailed).

The reason for the reduced testis weight in XOSxrb mice is apparent in Fig. 2, which gives the mean number of germ cells and Sertoli cells per tubule cross-section in XOSxrb and XY±Sxrb mice, throughout the period...
studied. As expected, there is a marked increase in the number of germ cells per tubule cross-section in XY±Sxrb mice, but by contrast there is no increase in XOSxrb mice. There is no deficiency of Sertoli cells in XOSxrb mice. Indeed the mitotic index for Sertoli cells drops to less than 0.3% after 24dpc in both genotypes. Clearly, the testis weight deficiency in XOSxrb mice is due to germinal failure.

In view of the normal numbers of Sertoli cells in XOSxrb mice, in the more detailed analysis of the germ cell deficiency that follows, germ cell numbers are expressed per 100 Sertoli cells, rather than per tubule cross-section.

Fig. 2. Mean number of Sertoli cells (SC) and germ cells (GC) per tubule cross-section in XOSxr and XY±Sxrb mice for the period 19–33dpc. The numbers in parentheses are the numbers of litters scored at each age. Asterisks indicate XOSxr points which are significantly different from controls (t-test, 2-tailed). The significantly higher numbers of Sertoli cells in XOSxr tubules at 29 and 32 dpc is a scoring artifact: at these ages some large tubule cross-sections from the controls had to be excluded because they would not fit in the field of view, resulting in an underestimate of the numbers of Sertoli cells and germ cells for controls at these ages.

In Fig. 3, germ cell numbers are plotted against age for the various classes of germ cells identified in the scoring procedure. The numbers of T1 prospermatogonia are indistinguishable in XOSxr and XY±Sxrb mice. However, XOSxr mice clearly have fewer T2 prospermatogonia than the controls and pooling over the period 20–24dpc reveals that XOSxr have only 39% of the control value. By contrast, XOSxra mice have 91% of the control value. Since T2 prospermatogonia are expressed per 100 Sertoli cells, rather than per tubule cross-section.

Table 2. Mean testis weights for (A) XOSxrb and XY±Sxrb, and (B) XOSxra±Sxra mice and the estimated difference between them for the period 19–33dpc
(A) Days post coitum No. of mice Mean±S.E.M. testis weights (mg)* Significance of Mean±S.E.M. weighted XOSxrb−XY±Sxrb difference (mg) Significance of XOSxrb−XY±Sxrb difference (P)

(B) Days post coitum No. of Mice Mean±S.E.M. testis weights (mg)* Significance of Mean±S.E.M. weighted XOSxra−XY±Sxra difference (mg) Significance of XOSxra−XY±Sxra difference (P)

* Mean of litter means.
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Fig. 3. Number of germ cells per 100 Sertoli cells for each germ cell stage during the period 19½–32½ dpc. The asterisk denotes occasional XOSxr<sup>b</sup> zygotene or pachytene cells.
Spermatogonia are assumed to be the progenitors of the undifferentiated A spermatogonia, a deficit of undifferentiated A spermatogonia is expected in XOSxr^b mice, and is indeed observed (XOSxr^b is 54% of XY±Sxr^b). Similarly, there is the expected deficit of differentiating A1/A2 spermatogonia (XOSxr^b is 42% of XY±Sxr^b). The number of A3/A4 spermatogonia, however, is reduced much more than expected (XOSxr^b is 7% of XY±Sxr^b) and there are no Intermediate or B spermatogonia.

This pattern of germ cell deficiency in XOSxr^b mice is largely accounted for by observations on mitotic index (Fig. 4). That is to say, there is a shortage of dividing T1 prospermatogonia, accounting for the drop in the number of T2 prospermatogonia; a reduced frequency of divisions among A1/A2 spermatogonia accounting for the much more severe shortage of A3/A4 spermatogonia; and no dividing A3/A4 spermatogonia accounting for the absence of In/B spermatogonia.

During the scoring procedure the gonia with the morphological characteristics of A1 and A2 spermatogonia were pooled, although it is assumed that they are distinct generations of spermatogonia, as in the adult. When the mitotic index of the A1/A2 spermatogonia is plotted against age (Fig. 5), there is no marked shortage of divisions in XOSxr^b mice until 25½ dpc, raising the possibility that it is the A2 rather than the A1 spermatogonia that are affected.

If A1/A2 spermatogonia rarely divide to give A3 or A4, but the undifferentiated A spermatogonia continue to divide, one might expect a 'piling up' of A1/A2 stages. This is not observed, implying that the cells that fail to divide are degenerating. This is supported by observations on the germ cell degeneration index (Fig. 6), which has been calculated on the assumption that all the dying cells observed were germ cells. The degeneration index is very low in XOSxr^b and XY±Sxr^b mice. Nevertheless, from 26½ days onwards XOSxr^b mice clearly have more degenerating cells than controls, which is consistent with the increased degeneration of A1/A2 spermatogonia. It is tempting to suggest that the increased degeneration index in XOSxr^b mice at 22½ days is similarly due to the death of T1 prospermatogonia that failed to divide.

Although no Intermediate or B spermatogonia were scored during the quantification, very rare patches of these spermatogonia, and also of early meiotic stages, can be found in 32½ dpc and adult (59½ dpc) XOSxr^b testes. They occur without the normal hierarchy of stages, and in small patches, as if an occasional A3/A4 spermatogonium divides and the products proceed via the usual stages up to early pachytene.

**Discussion**

The present results show that XOSxr^b testes have normal numbers of germ cells at birth, but become
severely deficient in germ cells in the ensuing two weeks. During the same period the numbers of Sertoli cells remain normal. These findings are consistent with the view of Burgoyne et al. (1986) and Levy and Burgoyne (1986a) that the spermatogenic failure in XO^Sx^a mice is due to the loss of a gene (Spy) that acts cell autonomously in the germ line.

The quantitative analysis of the germ cell deficiency in XO^Sx^a mice revealed a reduction in mitotic activity among T1 prospermatogonia, which resulted in a shortage of T2 prospermatogonia, and consequently a reduced pool of undifferentiated A spermatagonia. However, mitotic activity among the undifferentiated A spermatagonia, which include the spermatogonial stem cells, was found to be normal. It was during the early differentiating spermatogonial stages that the spermatogenic block occurred, with mitotic failure leading to an almost complete absence of Intermediate and B spermatagonia and subsequent meiotic stages.

XO female mice are developmentally retarded in early pregnancy (Burgoyne et al. 1983b) and are significantly underweight postnataally (Burgoyne et al. 1983a). It was anticipated that XO^Sx^a mice would also be underweight from birth, and this proved to be the case. Unexpectedly, however, the XO^Sx^a mice originally included as controls for this 'XO effect' showed little, if any, postnatal weight deficit. Coincidentally, the genetic basis for the early developmental advantage of XY over XX embryos (Tsunoda et al. 1985; Seller and Perkins-Cole, 1987) was being investigated in this laboratory, concurrently with the present study of XO^Sx^a mice, and the findings may provide an explanation for this difference in postnatal weight between XO^Sx^a and XO^Sx^b mice. Briefly, it was shown that the Y chromosome carries a factor that accelerates the early growth and development of XY embryos, and it appears that this factor (Gdy) may be present in Srx^a (P. S. Burgoyne, S. Kalms, E. P. Evans, K. Holland and M. J. Sutcliffe, unpublished) but deleted from Srx^b (P. S. Burgoyne and C. E. Bishop, unpublished). Thus it may be that the 'XO effect' is ameliorated by the presence of Gdy in XO^Sx^a but not XO^Sx^b mice.

The deletion of Y-chromosomal material involved in the generation of Srx^b has thus removed genetic information required for H-Y antigen expression (McLaren et al. 1984), for spermatogenesis (Burgoyne et al. 1986) and for an early acceleration of growth and development (P. S. Burgoyne et al. unpublished). Burgoyne et al. (1986) pointed out that the spermatogenesis gene (Spy) and the gene controlling H-Y expression (Hya) might be one and the same, and this possibility still holds. Similarly, Gdy may not be a separate gene from Hya and/or Spy. At the molecular level, it has been shown that Zfy-2, one of the Y-chromosomal copies of a gene encoding a zinc finger protein, present along with Zfy-1 in Srx^a, has been deleted from Srx^b (Roberts et al. 1988; Mardon et al. 1989; Nagamine et al. 1989a). Because it is strongly expressed in testes, probably in germ cells (Mardon and Page, 1989; Nagamine et al. 1989b), it is an obvious candidate for Spy.

As to the function of the 'spermatogenesis gene' Spy, we have clearly shown that the spermatogenic failure seen in XO^Sx^b mice is due to a failure of proliferation during the differentiating A spermatogonial stages, and so by definition Spy is important for the survival/proliferation of these spermatogonial stages. Whether the deficiency of T1 or prospermatogonial divisions in XO^Sx^b mice is also a consequence of the deletion of Spy, or whether it is due to the deletion of a gene separate from Spy, remains to be determined.

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References


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