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Evidence that the testis determination pathway interacts with a non-dosage compensated, X-linked gene

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ABSTRACT In a number of mammals, including mouse and man, it has been shown that at equivalent gestational ages, males are developmentally more advanced than females, even before the gonads form. In mice, although some strains of Y chromosome exert a minor accelerating effect in pre-implantation development, it is a post-implantation effect of the difference in X chromosome constitution that is the major cause of the male/female developmental difference. Thus XX females are retarded in their development by about 1.5 h relative to X^MO females or XY males; however, they are more advanced than X^PO females by about 4 h. It has been suggested that this early developmental difference between XX and XY embryos may "weight the dice" in favour of ovarian and testicular development, respectively, although expression of *Sry* will normally overcome any such bias. Here we test this proposal by comparing the relative frequencies of female, hermaphrodite and male development in X^PO, XX and X^MO mice that carry an incompletely penetrant *Sry* transgene. The results show that testicular tissue develops more frequently in XX,*Sry* transgenics than in either of the two types of XO transgenics. Thus the incidence of testicular development is affected by X dosage rather than by the developmental hierarchy. This implies there is a non-dosage compensated gene (or genes) on the X chromosome, which interacts with the testis-determining pathway. Since the pseudoautosomal region (PAR) is known to escape X-inactivation, penetrance of the *Sry* transgene was also assessed in X^MY^{*X} mice that have two doses of the PAR but have a single dose of all genes proximal to the distal X marker *Amel*. These mice showed similar levels of testicular development to X^MO mice with the transgene; thus the non-dosage compensated X gene maps outside the PAR.

KEY WORDS: *Sry penetrance, testis determination, X-Y homologous genes, X gene dosage, XO mice.*

I spent an enjoyable 13 years working with Anne McLaren at the Mammalian Development Unit in London and will always be grateful for her tolerance (even encouragement) of my never-ending sideline projects. Many of these eventually died a natural death, but others led into interesting new avenues of research. Since moving to The National Institute for Medical Research, sideline projects have continued and the study reported here falls squarely into this category.

(Preface by P.S. Burgoyne)

Introduction

The demonstration that XY mice deleted for *Sry* are female (Gubbay *et al.*,1990), while XX mice carrying an *Sry* transgene are male (Koopman *et al.*, 1991), established that testis determination in mammals is triggered by the action of a single Y chromosomal gene, *Sry*, rather than being due to an accelerating effect of 'Y heterochromatin' on gonadal growth as had been proposed by Mittwoch (Mittwoch, 1969). Nevertheless, it does seem that the earliest measurable consequence of *Sry* action is an increase in proliferation of cells in the coelomic epithelium overlying the gonadal primordium (Schmahl *et al.*, 2000).

It has now been established for a number of mammalian species that males are more advanced than females at equivalent gestational ages and that this difference precedes gonadal sex differentiation, thus precluding gonadal hormone effects (Scott and Holson, 1977; Pedersen, 1980; Seller and Perkins-Cole,

Abbreviations used in this paper: AMH/MIS, the Müllerian regression factor Anti-Müllerian Hormone or Müllerian Inhibiting Substance; *Paf*, the X-linked *Patchy-fur* mutation; PAR, pseudoautosomal region; *Sry*, the testis determining gene *Sex-determining region Y*; tg, transgene; X^M, maternally-derived X; X^P paternally-derived X.

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1987; Burgoyne *et al.,* 1995). As an adjunct to her proposition that increased gonadal growth is central to the testis determination process, Mittwoch has suggested that the early developmental advantage of XY males over XX females may predispose XY gonads to testicular development, perhaps because XY gonads will be larger than XX gonads at the time gonadal sex determination occurs (Mittwoch, 1989, 1993). However, a detailed analysis of these XX v. XY developmental differences in mice has shown that while XY fetuses are indeed larger than XX fetuses prior to gonadal sex determination, this size difference is adequately explained by the fact that the XY fetuses are more developmentally advanced (Burgoyne *et al.,* 1995). Thus, there is no reason to suppose that prior to *Sry* action, XY gonads are bigger than XX gonads, if adjustments are made for developmental stage (Palmer and Burgoyne, 1991).

In the landmark paper describing the sex reversal of XX fetuses carrying *Sry* transgenes (Koopman *et al.,* 1991), one transgenic line (32.10) was described as being incompletely penetrant and this is now known to be due to low levels of transcription of the transgene (Hacker *et al.,* 1995; Lovell-Badge and Hacker, 1995). In this paper we test Mittwoch's proposal that there is a linkage between pregonadal developmental stage differences and the incidence of testicular development, by introducing this incompletely penetrant *Sry* transgene into X^PO, XX and X^MO mice. X chromosome constitution is the major factor contributing to the early developmental differences between XY males and XX females and this is reflected in the fact that X^MO fetuses (that like XY fetuses have a single maternally derived X chromosome) are more advanced than XX fetuses by about 1.5h (Thornhill and Burgoyne, 1993; Burgoyne *et al.,* 1995); X^PO fetuses, on the other hand, are developmentally retarded by about 4h hours relative to XX fetuses (Burgoyne *et al.,* 1983). According to Mittwoch's proposal, the incompletely penetrant transgene should trigger testicular development more frequently in X^MO than XX mice and more frequently in XX than in X^PO mice.

Results

For details of the crosses used, genotyping and the basis for assessing sexual phenotype, see Materials and Methods. Because genetic background was already known to affect the penetrance of the *Sry* 32.10 transgene, it was considered important to generate the three genotypes X^PO, XX and X^MO in the same litters. All mice in the litters were typed by PCR for the presence of the Y and for the presence of the transgene. The type of gonad present on each side in fetal life was inferred from an examination of the whole reproductive tract postweaning.

Table 1 gives the incidence of females, hermaphrodites and males, in X^PO, XX and X^MO mice carrying the incompletely penetrant Sry transgene, derived from three different crosses. From the initial cross (using In(X)1H heterozygous mothers), it soon became apparent that the X^PO,*Sry* transgenics were much more frequently developing as females than their XX,*Sry* transgenic sibs and a chi-square test showed this to be highly significant (χ^2 = 13.4, P<0.0005). The results for the X^MO mice were inconclusive due to low numbers. A second cross was carried out using XO mothers in order to rule out any possible effect of the In(X)1H inversion that will have been present in half the XX and X^MO progeny but not in the X^PO progeny. Once again a clear X^PO,*Sry* v. XX,*Sry* difference was observed, with all the X^PO,*Sry* progeny from this cross developing as females. The frequency of X^MO,*Sry* was again low, but pooling the data from the two crosses indicated that X^MO,*Sry* may also be more likely to develop as females than their XX,*Sry* sisters.

For the third cross, the *Paf* mutation was backcrossed to C3H since our records showed that this was associated with a higher frequency of X^MO offspring. This did indeed increase the X^MO frequency and analysis of the pooled data from the first three crosses now shows a significantly lower level of testicular development for both types of XOs carrying the transgene (X^PO,*Sry* v. XX,*Sry*; χ^2 =23.9, P <<0.0005; X^MO,*Sry* v. XX,*Sry*,χ^2=10.1, P= 0.005-0.001).

The results in Table 1 suggest the surprising conclusion that the penetrance of the transgene is increased by the presence of two X chromosomes. An X dosage effect on the penetrance of the *Sry* transgene implies that there is a gene (or genes) on the X that impinges on the testis determination pathway and that this gene escapes X inactivation.

Genes mapping to the PAR are expected on theoretical grounds to escape X-inactivation (Burgoyne, 1982) and this is indeed true for the mouse PAR gene *Sts* (Salido *et al.,* 1996). The Y^{*X} chromosome - a recombinant product of XY* males (Hale *et al.,* 1991) - comprises a complete PAR and the adjacent X-specific region up to just proximal to *Amel* (Burgoyne *et al.,* 1998, and unpublished observations). It thus includes *Sts* and any other as yet unidentified gene in the mouse PAR. A fourth cross was therefore set up in which C3H XO females were mated to C3H $X^{Paf}Y^*$ males in order to allow a comparison of XX, X^MO and X^MY^{*X} transgenics. A deficiency of the comparisons in the first three crosses is the fact that the paternal and maternal Xs are not from the same strain; this deficiency is corrected in this cross by using C3H XO mothers (produced by mating the C3H $X^{Paf}Y$ males to C3H XX females). Unfortunately, these inbred XOs proved to be very poor breeders. A fifth cross was therefore set up in which the C3H XO females were replaced with C3H XX females and the efficiency of the cross was increased by using C3H $X^{Paf}Y^*$ males that were homozygous for the *Sry* transgene. Table 2 shows the incidence of testicular development in XX, X^MO and X^MY^{*X} females carrying the transgene for crosses 4 and 5. The X^MO,*Sry* v. XX,*Sry* difference, is now even more marked with 92% of X^MO,*Sry* mice developing as females (n=13) whereas 82% of the XXs were hermaphrodite or male (n=17). The X^MY^{*X} mice carrying the transgene

TABLE 1

THE NUMBERS OF FEMALE, HERMAPHRODITE AND MALE MICE IN X^PO, XX AND X^MO MICE CARRYING AN INCOMPLETELY PENETRANT *SRY* TRANSGENE

Mating	X^PO,*Sry*tg[1]			XX,*Sry*tg[2]			X^MO,*Sry*tg[3]		
(strain background)	♀	♀*	♂	♀	♀*	♂	♀	♀*	♂
In(X)X x $X^{Paf}Y$ tg (mixed) (mixed)	35	4	0	54	14	21	7	0	1
XO x $X^{Paf}Y$ tg (CBA/MF1) (MF1/CBA)	22	0	0	29	2	13	6	0	1
XO x $X^{Paf}Y$ tg (CBA/MF1) (C3H)	6	0	0	11	1	3	6	0	0
Totals	63	4	0	84	17	37	19	0	2

[1] The X^PO mice from all crosses carry the X-linked marker *Paf.*
[2] The XX mice from all crosses are heterozygous for *Paf.* In the first cross, half the XX females will also be heterozygous for the X inversion In(X)1H.
[3] The X^MO mice from these crosses are wildtype at the *Paf* locus.

TABLE 2

THE NUMBER OF FEMALE, HERMAPHRODITE AND MALE MICE IN XX, X^MO AND X^MY^{*X} MICE CARRYING AN INCOMPLETELY PENETRANT *SRY* TRANSGENE

Mating (strain background)	XX,*Sry*tg[1]			X^MO,*Sry*tg[2]			X^MY^{*X},*Sry*tg[2]		
	♀	♀	♂	♀	♀	♂	♀	♀	♂
XO x $X^{Paf}Y^*$ tg (C3H) (C3H)	3	2	3	7	0	0	2	0	0
XX x $X^{Paf}Y^*$ tgtg (C3H) (C3H)	0	3	6	5	1	0	3	0	0
Totals	3	5	9	12	1	0	5	0	0

[1] The XX mice from both crosses are heterozygous for *Paf.*
[2] The X^MO and X^MY^{*X}mice from both crosses are wildtype at the *Paf* locus.

were all female (n=5) and chi-square analysis with Yates' correction (to compensate for the small n) showed that the X^MY^{*X} transgenics have significantly less testicular development than the XX transgenics (χ^2=8.04, P=0.005-0.001), but are indistinguishable from X^MO transgenics. This serves to map the non-dosage compensated X gene (or genes) to outside the PAR.

Discussion

The present results do not support the suggestion by Mittwoch (1989, 1993) that the developmental advantage of males over females before the gonads form, predisposes towards testis development. However, the results do show that the presence of two X chromosomes, rather than one, increases the penetrance of the *Sry* transgene.

An X dosage effect on *Sry* transgene penetrance implies that a non-dosage compensated X gene promotes the testis determination process. Since normal males have a single X chromosome this X-dosage effect seems at first sight to have no relevance to the normal process. However, of the four genes known to escape X inactivation in the mouse, one maps to the PAR (*Sts*: Salido *et al.,* 1996) while the other three have closely related Y homologues (*Smcx*: Agulnik *et al.,* 1994; *Utx*: Greenfield *et al.,* 1998; *Eif2γx*: Ehrmann *et al.,* 1998); thus in these four cases males do in fact have a 'double' dose, and in the case of the X-Y homologous genes, the Y-linked copy could be a stronger 'allele'. There is a fifth X-linked gene, *Sox-3*, that may also partially escape X-inactivation, at least in the somatic component of the fetal gonad (Collignon *et al.,* 1996). *Sox-3* is thought to have been the progenitor of *Sry* (Stevanovic *et al.,* 1993; Graves, 1995), so it would be intriguing if *Sox-3* proved to be the non-dosage compensated X gene that promotes *Sry* function.

Materials and Methods

Mice

The strains referred to in the crosses below are random bred MF1 and the inbreds CBA/Ca and C3H/He (all NIMR maintained stocks).

Cross 1: In(X)X x $X^{Paf}Y$,*Sry*tg (mixed genetic background in both parents). In this cross females heterozygous for the large X inversion In(X)1H that produce a reasonable frequency of 'O' eggs (Evans and Phillips, 1975), were mated to males carrying the incompletely penetrant *Sry* transgene 32.10 (Koopman *et al.,* 1991) and the X linked mutation *Paf* that leads to the production of a reasonable frequency of 'O' sperm (Lane and Davisson, 1990). The *Paf* mutation also serves as an X-linked marker that allows the three female genotypes to be distinguished; X^PO: hemizygous *Paf* with very little hair; XX: heterozygous *Paf* showing patchy hair; X^MO and XY - wildtype (distinguishable by Y-specific PCR - see below). Half the 'XX' mice in this cross carry the inversion.

Cross 2. $CBA/MF1_{F1}$ XO x $MF1/CBA_{F1}$ $X^{Paf}Y$,*Sry*tg. In this cross the In(X)/X females in cross 1 are replaced by XO females in order to rule out an effect of the inversion, and both parents are on a similar more defined genetic background.

Cross 3. $CBA/MF1_{F1}$ XO x C3H $X^{Paf}Y$,*Sry*tg. In this cross the *Sry* transgene and *Paf* have been backcrossed to C3H because the frequency of X^MO offspring was thought to be higher from $X^{Paf}Y$ males on a C3H background (this proved to be the case).

Cross 4. C3H XO x C3H $X^{Paf}Y^*$,*Sry*tg. In this cross the Xs and the genetic background are now all C3H. The inclusion of the variant Y* chromosome means that X^MY^{*X} offspring are also produced. The Y^{*X} chromosome is produced following crossing over within the compound PAR of Y*, and comprises a PAR with a small adjacent X-specific region that includes the wild type allele of *Paf* and the distal X gene *Amel*, together with a non-Y (almost certainly an X) centromere (Burgoyne *et al.,* 1998, and unpublished observations). The presence of Y* in the father further increases the frequency of X^MO offspring (Burgoyne and Evans, 2000). However, litter size for the XOs on this inbred background proved to be very small and very few X^PO offspring were produced.

Cross 5. C3H XX x C3H $X^{Paf}Y^*$,*Sry*tgtg. This again is an all C3H cross and the mother is XX, which markedly improves litter size and the father is homozygous for the *Sry* transgene. This cross produces no X^PO offspring.

Genotyping

In all crosses *Paf* status was assessed at 7-14 days. At this age hemizygous *Paf* mice have sparse or absent hair, while heterozygous *Paf* mice have hair loss in patches; both can thus be clearly distinguished from wildtype.

All offspring were typed from DNA obtained from tail biopsies at weaning, by a triplex PCR using primers Ymtfp1 (5' CTG GAG CTC TAC AGT GAT GA 3') and Ymtrp1 (5' CAG TTA CCA ATC AAC ACA TCA 3') that amplify YMT2/B-related members of the multiple copy *Ssty* gene family from the mouse Y long arm (Bishop and Hatat, 1987), primers Tan3' (5' CTC AGT GTG GAA TTC ATC TGC 3') and Tan5' (5' GAG GGC ATG GTC AGT TGA AC 3') that are specific for the *Sry* transgene (Nigel Vivian, pers. comm) and primers Om1a and Om1b (Wright *et al.,* 1989) that amplify the autosomal gene encoding myogenin (amplification control). As a check for mis-typing with the Y PCR, the testis size of all males was assessed at autopsy - XO and XX transgenic males have very small testes due to germinal failure, whereas XY males with or without the transgene have normal-sized testes. No false positives or false negative males were identified by this criterion. Furthermore, all males or hermaphrodites typed as Y negative were positive for the transgene. Two females repeatedly typed as weakly Y positive, one of which was mated and found to produce XO and XX but not XY daughters. It was concluded that these were probably XO/XY mosaics.

In the early phase of the project (crosses 1 and 2) all phenotypically wild type but Y-negative mice (presumed X^MO) were karyotyped from bone marrow and all proved to be 39,XO as expected. All Y-negative wildtype mice from crosses 4 and 5 were also karyotyped from bone marrow in order to distinguish the X^MO and X^MY^{*X} mice since they are both Y negative and wild type at the *Paf* locus.

Sexual phenotype

Intersexual gonads (ovotestes) can only be reliably scored in fetal life because the ovarian component usually regresses due to a 'toxic' effect of AMH/MIS on fetal oocytes (Burgoyne and Palmer, 1992). However, the development of the reproductive ducts and descent of the gonad from just below the kidney towards the scrotal region are locally mediated effects of testicular hormones and thus provide a guide to the type of gonad present on each side in fetal life (Swain and Lovell-Badge, 2000). In the present study, a gonad located close to the kidney and associated with an oviduct and uterus, was presumed to have been an ovary in fetal life, while a pelvically located gonad associated with epididymis and vas deferens was presumed to have been a testis in fetal life (this latter criterion will

exclude ovotestes with a relatively minor ovarian component). A gonad associated with an anteriorally inhibited female duct system (lacking oviduct and the anterior portion of the uterine horn), or with a mixed duct system (part of the uterus present together with an epididymis and a segment of vas deferens), was classified as an ovotestis irrespective of position (they were frequently partially descended). A partially descended gonad associated with grossly normal oviduct and uterus was also classified as an ovotestis.

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