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The *Mus musculus domesticus Tdy* allele acts later than the *Mus musculus musculus Tdy* allele: a basis for XY sex-reversal in C57BL/6-Y^{POS} mice

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Summary

Consomic C57BL/6 males, carrying either the *Mus musculus musculus*-derived C57BL/6 Y chromosome or the *Mus musculus domesticus*-derived Poschiavinus Y chromosome, were outcrossed to females of the inbred strains C3H/Bi and CXBH/By and to females of the random bred strain MF1/Ola. In a study at 12.5 days *post coitum*, gonads of XY^{C57} and XY^{POS} fetuses were assessed for the presence of testicular cords. It was found that XY^{POS} fetuses had a later onset of testicular development than XY^{C57} fetuses. Limb development, which was monitored as a measure of overall development, was unaffected by the strain of Y present. These data were supported by a longitudinal study in which the increased growth rate of the testes relative to undifferentiated gonads, was also shown to be delayed in XY^{POS} fetuses. The extent of the delay was estimated to be approximately 14 h. It is concluded that this delay in the onset of testicular differentiation must be caused by differences between the two Y-chromosome types, most probably allelic differences in the testis determinant *Tdy*.

Key words: Sex-reversal, testis determination, Poschiavinus, *Tda-1*, mouse embryo.

Introduction

In 1982, Eicher *et al.* described a case of XY sex reversal in mice, resulting from backcrossing the *Mus musculus domesticus*-derived Poschiavinus Y chromosome onto a C57BL6/J background. Adult C57BL/6-Y^{POS} mice show a range of sexual phenotypes, including fully sex-reversed XY females, true hermaphrodites (i.e. they have both ovarian and testicular tissue) and males; although the males have small testes and are of poor fertility. Observations on fetal litters from this cross have revealed that the XY individuals at 16.5 days *post coitum* (dpc) have either ovaries, or ovotestes with a varying ratio of ovarian to testicular tissue. It seems clear that most ovotestes go on to form testes in adult life, the regression of the ovarian component accounting for the reduced testicular size.

Further studies have shown that a number of other (but not all) *domesticus*-derived Y chromosomes generate some XY females when placed on the C57BL/6 background (Eicher *et al.* 1982; Nagamine *et al.* 1987*a*; Biddle and Nishioka, 1988). Eicher and Washburn (1986) have suggested that the '*domesticus*' Y carries a *Tdy* allele which is later-acting than that on the '*musculus*'-derived Y chromosome of the C57BL/6 inbred strain and that this delay sometimes enables the process of ovary determination to pre-empt Y action. To explain the requirement for a C57BL/6 background, Eicher and Washburn (1983, 1986) proposed that C57BL/6 carries a recessive autosomal allele ($Tda\text{-}1^b$) which is in some way incompatible with the '*domesticus*' Y chromosome.

There are two possible models for how *Tda-1* is involved in this scheme (Eicher and Washburn, 1983). (1) This autosomal gene may be one element in the *Tdy*-initiated cascade of genes involved in testicular differentiation. (2) A second interpretation supported by Burgoyne (1988), proposes that it is an ovary-determining gene, with the C57BL/6 allele acting earlier than those of other inbred strains. When this is brought together with the late-acting '*domesticus*' *Tdy* allele, a 'timing-mismatch' may occur such that ovary determination may pre-empt testis determination. It is not hard to imagine how minor differences in the timing of expression of these two key genes could lead to the range of gonadal phenotypes observed in these mice. The 'timing mismatch' concept is discussed more fully in Burgoyne and Palmer (1991).

The aim of the present study was to test directly the hypothesis that the Poschiavinus Y chromosome is later acting than the C57BL/6 Y. Since C57BL/6 XY^{POS} fetuses develop ovaries or ovotestes, rather than testes, the effect of the Poschiavinus Y on the timing of testicular development was assessed in F_1 hybrids in which the paternal parent was C57BL/6 or C57BL/6-Y^{POS}. In these F_1 hybrids all the XY fetuses develop testes.

Materials and methods

Experiment 1

C57BL/6Mcl-Y^{POS} stud males were produced by backcrossing C57BL/6J-Y^{POS} males to C57BL/6Mcl females for more than 10 generations. Inbred C3H/Bi females were mated to stud C57BL/6Mcl-Y^{C57} and to C57BL/6Mcl-Y^{POS} males in a room in which the dark period was 7pm to 5am. Pregnant mothers were killed at 12 days 15 h *post coitum* (*pc*) (mating was assumed to have taken place at 12:00 midnight) and the fetuses placed into Hepes-buffered Eagle's minimum essential medium (EMEM, ICN Flow Ltd). Amniotic membranes were removed for sex chromatin analysis. Each membrane was placed near the top of a conical-bottomed centrifuge tube and flushed down into the tube with 3:1 methanol:glacial acetic acid fixative. Fetuses were killed by decapitation, the hind limbs staged (see below) and the gonads removed. The gonads were photographed under EMEM using a Wild M400 Photomakroskop. Photographic prints of male and female gonads from both crosses were randomized and scored blind for the presence of testicular cords.

The hind limbs were staged according to the developmental series of McLaren and Buehr (1990), which covers the period spanning sexual differentiation in the mouse. This scheme was subdivided to provide maximum sensitivity and extended to cover the later stages found at this time point and the stages of the subsequent two days studied in experiment 2 (see Fig. 1).

The amniotic membranes were processed using an adaptation of the method of Evans *et al.* (1972) for yolk sac preparations (Burgoyne *et al.* 1983). Tubes containing the amniotic membranes were centrifuged briefly at 1000 revs min^{-1} and excess fixative decanted off (being careful to retain the amnion). The tubes were inverted on a paper towel and tapped on the bench to bring the amnion 1 or 2 cm away from the base of the tube. A single drop of 60% glacial acetic acid in distilled water was applied directly onto the amnion in order to dissociate the cells. After 60 s, the action of the 60% acetic acid was blocked by the addition of approximately 1 ml of fixative. The cells were then centrifuged at 1000 revs min^{-1} for 5 min. Excess fix was decanted off and the tube inverted for a few seconds to allow the tube to drain, without letting the cells dry completely. When the tube was righted again, the cells were resuspended in the small amount of fixative that ran down to the bottom from the sides of the tube. If this process was judged correctly, the cells could be concentrated into a single drop of fluid. This single drop was spotted onto a clean glass slide. When the cells had air-dried, one drop of 1% aqueous toluidine blue was spotted onto the cells and a coverslip pressed on. These preparations were scored immediately for the presence or absence of the sex chromatin (see Fig. 2).

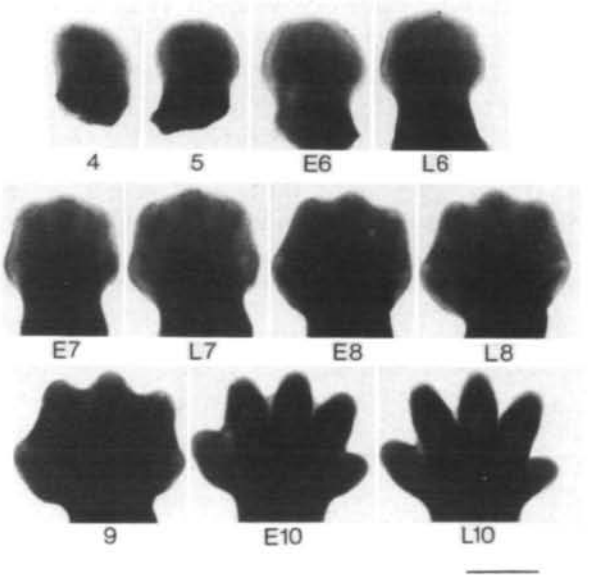

Fig. 1. Hind limb stages of fetuses between 11.5 and 14.5 days *post coitum*. E, early; L, late. In general, stages 4 and 5 were found during the 12th day of pregnancy, 5 to L7 during the 13th, L7 to 9 during the 14th and 9 to L10 during the 15th. Bar, 1 mm.

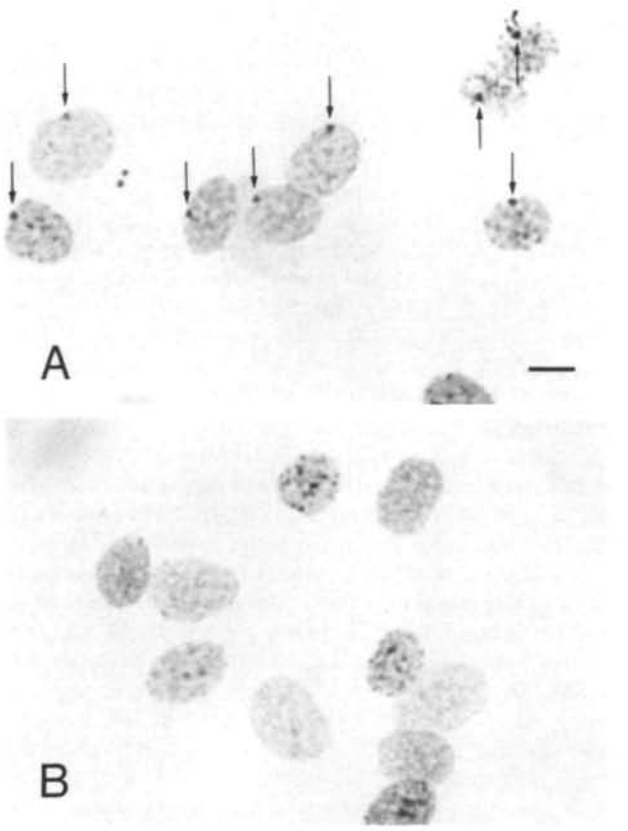

Fig. 2. Disaggregated amnion cells (A) positive and (B) negative for sex chromatin (arrows). Bar, 10 μm.

Experiment 2

Consomic C57BL/6Mcl-Y^{C57} and C57BL/6Mcl-Y^{POS} stud males were mated to females of the inbred strain CXBH/By and to females of the random-bred albino stock MF1/Ola. Once again the dark period was 7pm to 5am and mating was assumed to have taken place at 12:00 midnight. In order to study the gonadal growth rates in the fetuses produced by these crosses, pregnant females were killed at time points between 11.5 and 14.5 d*pc*. Amniotic membranes were fixed for sexing and the hind limbs were scored as before.

Gonads were dissected out and scored under the dissecting microscope for the presence of testicular cords. The length and breadth of each gonad was measured to provide continuous variables to study the relative growth rates. However, we found that the length measurements were highly

variable at the earlier stages due to difficulties in delineating the ends of the gonads. The breadth was better defined and showed a relatively smooth exponential growth curve. This was therefore chosen as the representative parameter for gonadal growth. Breadth was measured by gently holding the mesonephros with forceps so that the gonad was uppermost. The width across the widest part was measured using a graticule fitted in the eyepiece of the dissecting microscope.

Results

Experiment 1. Transverse study: fetal litters examined at 12 days 15 h pc

9 litters of fetuses produced from the cross C3H× C57BL/6-Y^{C57} were found to contain 19 individuals typed as sex-chromatin negative. In the C3H×C57BL/ 6-Y^{POS} cross, a total of 8 litters yielded 15 sex-chromatin-negative individuals. These sex-chromatin-negative fetuses are assumed to be XY although in rare instances they could be XO. All but 2 of the XY^{C57} males were scored as having visible testis cords whereas none of the XY^{POS} fetuses showed any sign of testicular differentiation at this time point. The probability of this occurring by chance (χ^2=26.8, 1 degree of freedom) is P=0.00014.

When these data are plotted against hind limb stage (see Fig. 3), individuals that are at the same stage of limb development show a clear difference in testicular development dependent upon the source of the Y chromosome. A comparison of the distributions of the hind limb stages demonstrates that, as far as the sensitivity of this measure will allow, there is no significant difference between the two male populations with respect to overall fetal development (χ^2=3.01 4df. P=0.56).

Experiment 2. Longitudinal study

The delay in the onset of testicular differentiation is also found in the XY fetuses produced from crosses of the consomic C57BL/6-Y^{C57} and C57BL/6-Y^{POS} males with CXBH/By or MF1/Ola females (see Fig. 4). These data also show that all the XY^{POS} fetuses in both crosses develop testes from late 12.5 d*pc* onwards and no signs of XY sex reversal were found in a total of 47 sex-chromatin-negative individuals between 13.5 and 14.5 d*pc*.

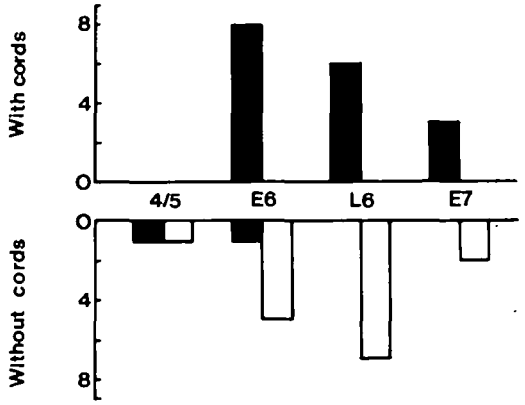

Fig. 3. Frequency of sex-chromatin-negative individuals with and without visible testis cords at 12 days 15 h *post coitum*, plotted against hind limb stage. Solid bars, C3H× C57BL/6-Y^{C57} F_1; open bars, C3H×C57Bl/6-Y^{POS} F_1.

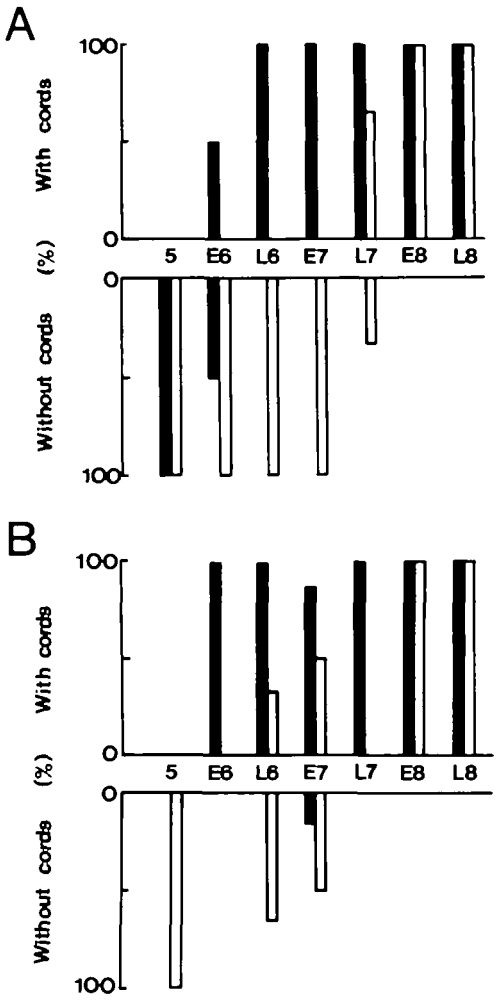

Fig. 4. Percentage of sex-chromatin-negative individuals with and without visible testicular differentiation at a range of hind limb stages. Solid bars, XY^{C57} fetuses; open bars, XY^{POS} fetuses. (A) F_1 males produced from CXBH outcross; 33 XY^{POS} and 28 XY^{C57} fetuses. (B) F_1 males from MF1 outcross; 28 XY^{POS} and 56 XY^{C57} fetuses. Missing bars indicate that no sex-chromatin-negative individuals were found with that hind limb stage. The dip in the XY^{C57} data at E7 in the MF1 cross is caused by a single individual. It is possible that this fetus was mistyped or may have been a rare XO.

The gonadal growth rate data are shown in Fig. 5. The mean gonad breadth measurements are log-transformed to improve the linearity of the resulting regression lines, decreasing the residual variance. Regression lines and their associated errors were computed using litter means of gonadal breadth for each sex, weighted according to the number of females or males in the litter. For the males, only the data between 12.5 and 14.5 d*pc* are included to be as certain

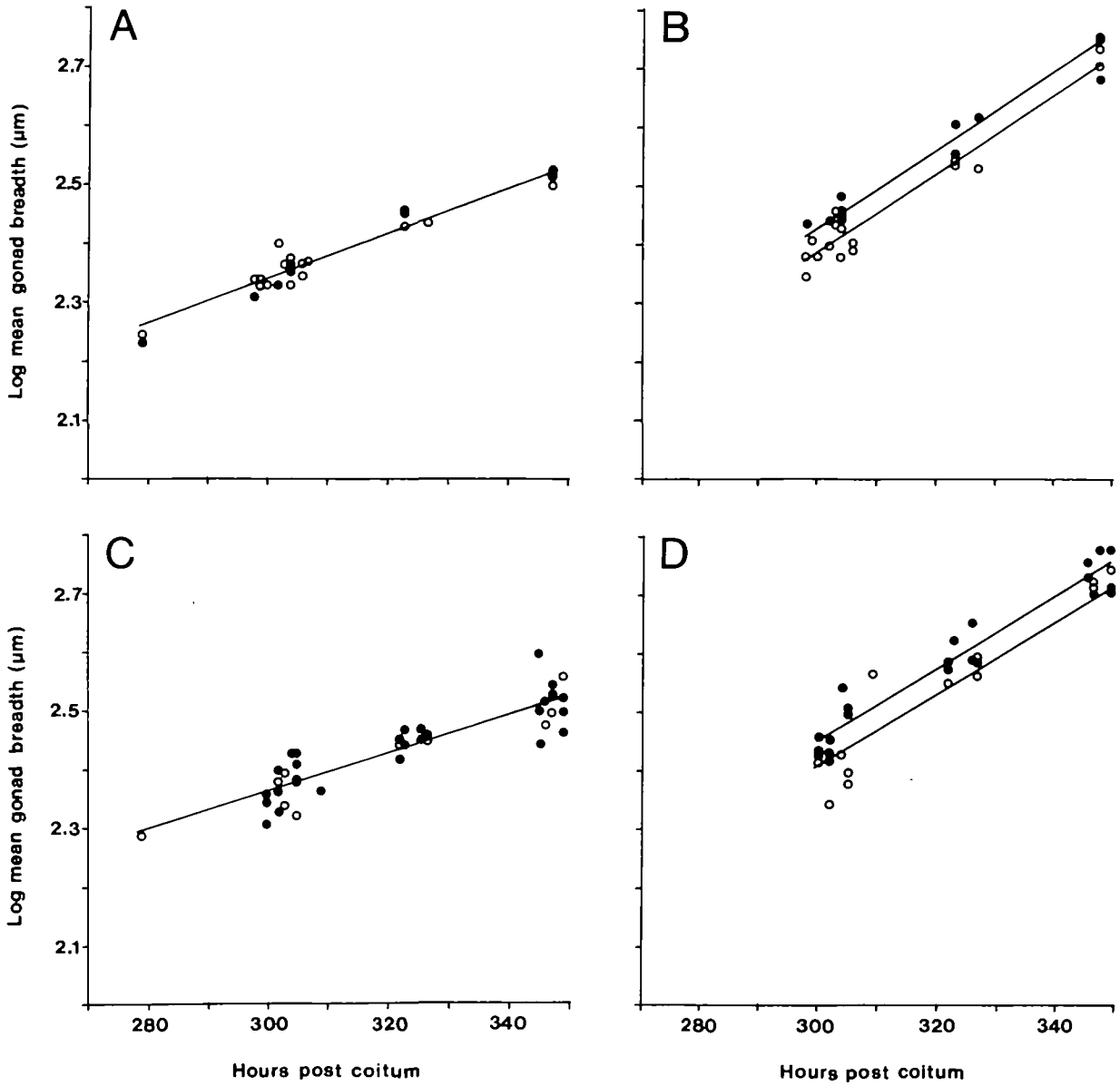

Fig. 5. Regression lines of gonadal growth in males and females between 11.5 and 14.5 days *post coitum*. Closed circles, litters fathered by C57BL/6-Y^{C57} males; open circles, litters fathered by C57BL/6-Y^{POS} males. Each point is the litter mean (for that sex) of the individual gonadal breadth means. Regression data are compared in each case with respect to the Y-chromosome-type of the father using an analysis of covariance. The residual variances are not significantly different in any of the four tests. (A) Females produced from CXBH outcross. Difference between slopes, $P=0.59$; between elevations, $P=0.86$. Data are pooled to produce one regression line. (B) Males produced from CXBH outcross. Difference between slopes, $P=0.67$; between elevations, $P=0.00009$. The combined slope is used in plotting the two regression lines. (C) Females produced from MF1 outcross. Difference between slopes, $P=0.321$; between elevations, $P=0.286$. Data are pooled to produce one regression line. (D) Males produced from MF1 outcross. Difference between slopes, $P=0.203$; between elevations, $P=0.00126$. The combined slope is used in plotting the two regression lines.

as possible that the points fall during the phase of increased growth that is characteristic of testes. Some outliers have been removed using a test for outliers in regression analysis. Regression lines of gonadal growth in XY^{C57} and XY^{POS} fetuses were compared for both the CXBH and the MF1 outcross using an analysis of covariance. This test compares separately the variance from the mean due to the elevation, the slope and the residual error.

The regression lines for the XX fetuses produced by the two consomic fathers were also compared for each outcross. These data (see Fig. 5 legend) demonstrate that, for both the MF1 cross and the CXBH cross, the females produced by the two consomic males show no significant difference in the residual variance, the variance due to the slope or the variance due to the elevations. This is to be expected since there is no genetic difference between the F_1 females produced by

each paired cross. It is therefore justified to pool these two populations within each outcross.

A comparison of the regression lines for the males (XY^{C57} *versus* XY^{POS}), on the other hand, although revealing no significant difference in residual variance or slope, shows a highly significant difference between elevations in both crosses (see Fig. 5 legend). This supports the conclusions of experiment 1 and shows that the late onset of testis cord formation in XY^{POS} fetuses is correlated with a delayed onset of increased growth.

Discussion

Both experiments demonstrate that the Poschiavinus Y chromosome causes a later onset of testicular development than the C57BL/6 Y chromosome. The simplest explanation for this disparity is a difference between the two *Tdy* alleles. This may be caused by differences in the regulatory elements or the structural sequence of *Tdy*. This is currently being tested by examining the structure and expression of the Poschiavinus *Sry* since it is now established that *Sry* is *Tdy* (Koopman *et al.* 1991).

These findings provide an explanation for the results of Nagamine *et al.* (1987*b*) who found that when C57BL/6 males with a *domesticus*-derived Y chromosome are outcrossed to various inbred females, a proportion of the F_1 male fetuses produced show 'abnormalities' of testicular development. In the light of results presented here, it seems likely that these 'abnormalities' are due to a delay in testicular cord formation caused by a late-acting *Tdy* allele.

From the growth rate data, it is possible to model the growth kinetics of male and female gonads and thereby estimate the timing difference between the action of the two Y chromosomes (see Fig. 6). If *Tdy* is the first gene to influence male and female gonads differentially, it is logical that prior to *Tdy* action the growth rates of the indifferent XX and XY gonads should be the same. After *Tdy* expression, however, the gonadal growth of XY fetuses increases dramatically but the gonads of XX fetuses continue to grow at a slower rate. The gonads of XY fetuses with delayed Y action would be expected to continue growing at the same rate as female gonads until expression of the delayed *Tdy* has occurred. Therefore, to estimate the timing difference it is necessary to find where the XY^{C57} and XY^{POS} testicular growth curves depart from the XX curve. Calculations based on this model show that the points of intersection for the CXBH outcross are: XY^{C57} fetuses, 269 h *post coitum* (h*pc*); XY^{POS} fetuses, 283 h*pc*; and for the MF1 outcross XY^{C57}, 270 h*pc*; XY^{POS}, 284 h*pc*. The estimate for the timing difference between the action of the two Y chromosomes is therefore 14 h. The estimated points of intersection should be regarded with caution since they result from extrapolations that may misrepresent the true shape of the curves. In spite of this caution, the timing for the C57BL/6 Y (early on the 12th day) fits well with the timing of expression of *Sry* which begins to

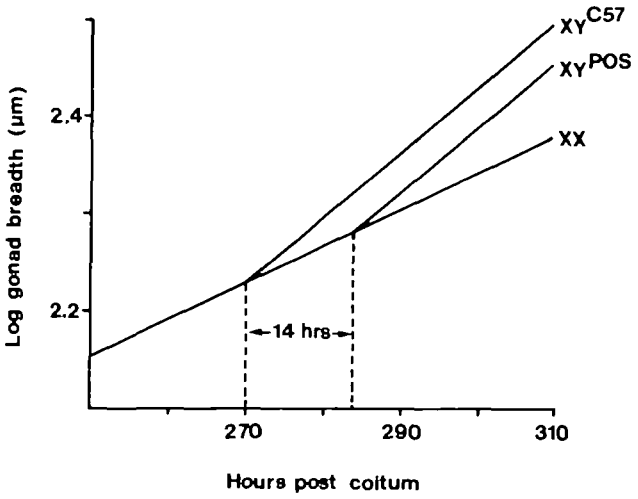

Fig. 6. Model of gonadal growth in females and in males carrying either the Y^{C57} or the Y^{POS} chromosome. The initial single line represents the growth rate of the indifferent XX or XY gonads. After expression of either of the two *Tdy* alleles the testicular growth rate increases and departs from that of the female gonad. The testicular growth rate is the same in Y^{C57}-bearing and Y^{POS}-bearing fetuses, only the time of departure differs. The points of intersection are calculated from the regression lines shown in Fig. 5. For two lines with slopes m_1 and m_2 and elevations c_1 and c_2, at the point of intersection $X = (c_2 - c_1) \div (m_1 - m_2)$.

be expressed during the 11th day, peaks on the 12th and declines during the 13th day (Gubbay *et al.* 1990; Koopman *et al.* 1990).

XY fetuses have been shown to have an overall developmental advantage over their XX siblings (Seller and Perkins-Cole, 1987), males being about 1½ hours ahead of females (Burgoyne *et al.* unpublished). This developmental advantage is now known to be a consequence of a retarding effect of the paternally imprinted X chromosome in XX fetuses, rather than an accelerating effect of the Y chromosome (Thornhill and Burgoyne, unpublished). Because it is not Y-linked, this developmental advantage of XY fetuses should not affect the estimate for the difference in timing of Y^{POS} and Y^{C57} action. However, it does complicate the interpretation of XX *versus* XY gonadal size differences.

Measurements of gonadal volume in rat fetuses have shown that XY gonads are larger than XX gonads prior to the formation of testis cords (Lindh, 1961; Mittwoch *et al.* 1969). This led Mittwoch (1969, 1989) to propose that an early growth advantage of XY gonads, rather than a single gene 'switch', forms the basis of the sex-determining mechanism in mammals. However, there is no previous evidence to show that these 'within litter' differences between XX and XY gonadal volume are not simply a manifestation of the fact that males are ahead of females. Our data, on the other hand, indicate that the increased growth, characteristic of testes, precedes testis cord formation. Nevertheless, this increased growth is after the onset of *Sry* expression so

it remains a moot point whether the increased gonadal size is simply a consequence of *Sry* expression, perhaps caused by the differentiation and growth of Sertoli cell precursors (Jost *et al.* 1973; Magre and Jost, 1980), or whether it is a requirement for determining the gonad as a testis.

The results presented here appear to be contradicted by a recent study on the onset of Mullerian inhibitor, AMH (or MIS), production in fetal testes (Taketo *et al.* 1991). The authors compared the onset of AMH production in SJL XY^{dom} fetuses with control SJL XY^{C57} fetuses and found no difference. It is possible that the assay system used and time intervals of 24 h, were not sensitive enough to detect a timing difference of only 14 h.

The delay in action of the Poschiavinus Y chromosome supports, in part, the hypothesis that sex-reversal in C57BL/6-Y^{POS} individuals is caused by a developmental mismatch involving a late-acting *Tdy* allele in conjunction with an early-acting ovarian program. It remains to be seen whether the other component of C57BL/6-Y^{POS} sex reversal, the recessive C57BL/6 allele of *Tda-1*, is a relatively early-acting gene in the ovary-determining pathway.

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