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Inheritance of T-associated sex reversal in mice

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Summary

We previously identified a primary sex-determining locus, *Tas*, on mouse Chr 17 that causes ovarian tissue development in C57BL/6J $T^{hp}/+$ and $T^{Orl}/+$ individuals if the AKR/J Y chromosome is present. We hypothesized that *Tas* is located within the region of Chr 17 deleted by T^{hp} and T^{Orl} and that C57BL/6J carries a diagnostic *Tas* allele, based on the observation that ovarian tissue develops in XY mice when T^{hp} is on a C57BL/6J inbred strain background, whereas normal testicular development occurs when T^{hp} is on a C3H/HeSnJ inbred strain background. To test this hypothesis, we mated (C57BL/6J × C3H/HeSnJ)F1 females to C57BL/6J $T^{hp}/+$ hermaphrodites. As expected, half of the XY $T^{hp}/+$ offspring developed ovarian and testicular tissue while half developed exclusively testicular tissue. Unexpectedly, the inheritance of selected Chr 17 molecular loci was independent of gonadal development, as half of the male and hermaphroditic offspring inherited C3H/HeSnJ-derived Chr 17 loci and half inherited C57BL/6J-derived Chr 17 loci. We conclude that for ovarian tissue to develop in an XY $T^{hp}/+$ or XY $T^{Orl}/+$ individual (1) *Tas* must be present in a hemizygous state, which is accomplished by heterozygosity for the T^{hp} or T^{Orl} deletions; (2) the AKR/J-derived Y chromosome must be present; and (3) an additional locus involved in primary sex determination must be present in a homozygous C57BL/6J state. This newly identified gene may be one of the previously defined loci, *tda-1* or *tda-2*.

1. Introduction

The number and location of the genes responsible for determining whether the mammalian embryonic gonad will develop as an ovary or a testis remain elusive because mutations affecting either gonadal pathway are rare and, predictably, result in aberrant gonad development and often sterility. Despite these difficulties, evidence is overwhelming that at least one primary sex-determining locus, designated *Tdy* in the mouse (*TDF* in the human), resides on the mammalian Y chromosome (reviewed by Eicher & Washburn, 1986; Goodfellow & Darling, 1988; Eicher, 1988) and that normal testis determination in XY individuals depends on the proper interaction of this locus with autosomal primary sex-determining loci (reviewed by Eicher & Washburn, 1986; Eicher, 1988).

One autosomal locus that programs ovarian development in XY mice is *Tas* (T-associated sex reversal), so named because this sex reversal condition is expressed concordantly with hairpin tail (T^{hp}), a deletion that includes the brachyury gene (T) located on Chromosome (Chr) 17 (Washburn & Eicher, 1983; Herrmann, *et al.* 1990). Early investigations indicated that XY $T^{hp}/+$ mice develop either as females with two ovaries or as hermaphrodites with ovarian and testicular tissue, while all XY $+/+$ mice develop as normal males. Furthermore, we found that complete or partial sex reversal of developing gonads required the presence of the AKR/J Y chromosome (Y^{AKR}) and depended on the origin of the autosomal complement: ovarian tissue developed in XY $T^{hp}/+$ mice when T^{hp} was inherited on a C57BL/6J (B6) inbred background whereas testicular tissue developed in these mice when T^{hp} was present on a C3H/HeSnJ (C3H) inbred strain background. This inbred strain specificity suggested that the development of ovarian tissue in XY $T^{hp}/+$ mice was caused by the hemizygous expression of a B6-derived *Tas* allele.

Investigation of the brachyury mutation T-Orleans (T^{Orl}) revealed that sex reversal also occurred in XY $T^{Orl}/+$ mice on the B6 inbred strain background. Significantly, T^{Orl} involves a deletion of Chr 17 that partially overlaps the deletion associated with T^{hp} (Silver *et al.* 1983; Herrmann *et al.* 1990) (Fig. 1). The

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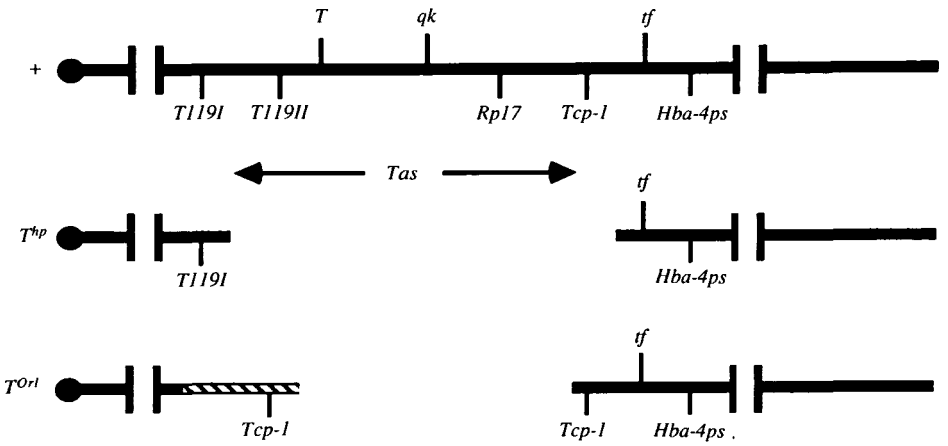

Fig. 1. Genetic maps of mouse Chr 17. The linkage relationship of the following loci are shown relative to gene order but not genetic distance for a wild-type (+) chromosome and mutant T^{hp} and T^{Orl} chromosomes: *D17Leh119I* and *D17Leh119II* (abbreviated as *T119I* and *T119II*, respectively) (Fox *et al.* 1985; Herrmann *et al.* 1986; Herrmann *et al.* 1987; Sarvetnick *et al.* 1986); *D17Her190* (DNA segment, Chr 17, Herrmann-190), the DNA sequence hypothesized to be the *T* locus and abbreviated as *T* (Herrmann *et al.* 1990); *qk*, quaking (Bennett, 1975; Erickson *et al.* 1978; King *et al.* 1989); *D17Rp17* (abbreviated *Rp17*; Mann *et al.* 1986); *Tcp-1*, t-complex protein-1 (Silver *et al.* 1979; Willison *et al.* 1986); *tf*, tufted (Bennett, 1975); and *Hba-4ps* (Leder *et al.* 1981). *D17Leh119II*, *T*, *qk*, and *D17Rp17* are deleted in T^{hp} and T^{Orl}; and *Tcp-1* is deleted in T^{hp} and duplicated in T^{Orl} (Willison *et al.* 1986). The hatched line on the T^{Orl} chromosome represents *t*-chromatin flanking this deletion (Alton *et al.* 1980; Silver *et al.* 1983). The proposed location of *Tas* is indicated by the double-headed arrow.

differentiation of ovarian tissue in XY $T^{Orl}/+$ mice supported the hypothesis that *Tas* is located on Chr 17 in the region deleted by T^{hp} and specifically defined this region as that commonly deleted by T^{Orl} and T^{hp}. In addition, we identified ovarian tissue in B6 $T^{Orl}/+$ mice when Y^{AKR} was present but normal testis development occurred when the B6 Y chromosome (Y^{B6}) was present. This result suggested that, as is the case for T^{hp}, the expression of sex reversal is coupled with an AKR-derived *Tdy* locus (Washburn & Eicher, 1989).

DNA markers spanning the T^{hp} deletion (Table 1 and Fig. 1) provide genetic tools to test the hypothesis that abnormal gonad development in XY $T^{hp}/+$ individuals is a consequence of a hemizygously expressed B6-derived *Tas* allele. If this assertion were true, XY $T^{hp}/+$ offspring produced from matings of B6-$T^{hp}/+$ sires to (B6 × C3H)F1 females would have normal testicular development when C3H-derived alleles on Chr 17 are inherited but ovarian or ovotesticular development when B6-derived alleles on Chr 17 are inherited. Exceptional mice would be expected as a consequence of the inheritance of a recombinant Chr 17 and would define the position of *Tas* relative to other Chr 17 markers. The data and conclusions from such an analysis are reported here.

2. Materials and methods

(i) *The* T^{hp} *mutation*

T^{hp} (Fig. 1) arose spontaneously in the AKR/J inbred strain (Dickie, 1965) and shares several characteristics with other brachyury mutations, including shortening of the tail in the presence of the wild-type allele ($T^{hp}/+$), taillessness in the presence of a number of *t* haplotypes (T^{hp}/t), and early embryonic lethality in the homozygous state (T^{hp}/T^{hp}) (Bennett, 1975; Silver, 1975). Because heterozygous $T^{hp}/+$ offspring are viable if T^{hp} is paternally inherited but inviable if T^{hp} is maternally inherited (Johnson, 1974, 1975; Winking & Silver, 1984; Babiarz *et al.* 1988), all stocks of mice containing T^{hp} carry the AKR Y chromosome. Approximately half of the XY $T^{hp}/+$ individuals from the B6-T^{hp} strain develop exclusively ovarian tissue, i.e., they are females, and half develop ovarian and testicular tissue, i.e., they are true hermaphrodites (Washburn & Eicher, 1983). Because some XY $T^{hp}/+$ hermaphrodites develop sufficient testicular tissue to breed as males, genetic crosses can be established to investigate the inheritance of the sex reversal condition.

(ii) *Genetic crosses*

F1 females, obtained by crossing B6 females with C3H males, were mated to B6 $T^{hp}/+$ hermaphrodites (see Fig. 2). The B6 $T^{hp}/+$ hermaphrodites used as breeders were at the 15th and 16th (N15 and N16, respectively) generation of transferring T^{hp} to the B6 inbred strain. Seventy-six fetuses were collected at $14\frac{1}{2}$–15 days of development and classified for tail phenotype (normal-tailed or short-tailed) and gonadal development (ovary, testis, ovotestis) as previously defined (Eicher *et al.* 1980; Washburn & Eicher, 1983). The sex chromosome complement (XX *versus* XY) of each $T^{hp}/+$ and +/+ fetus that showed exclusively ovarian development was ascertained from

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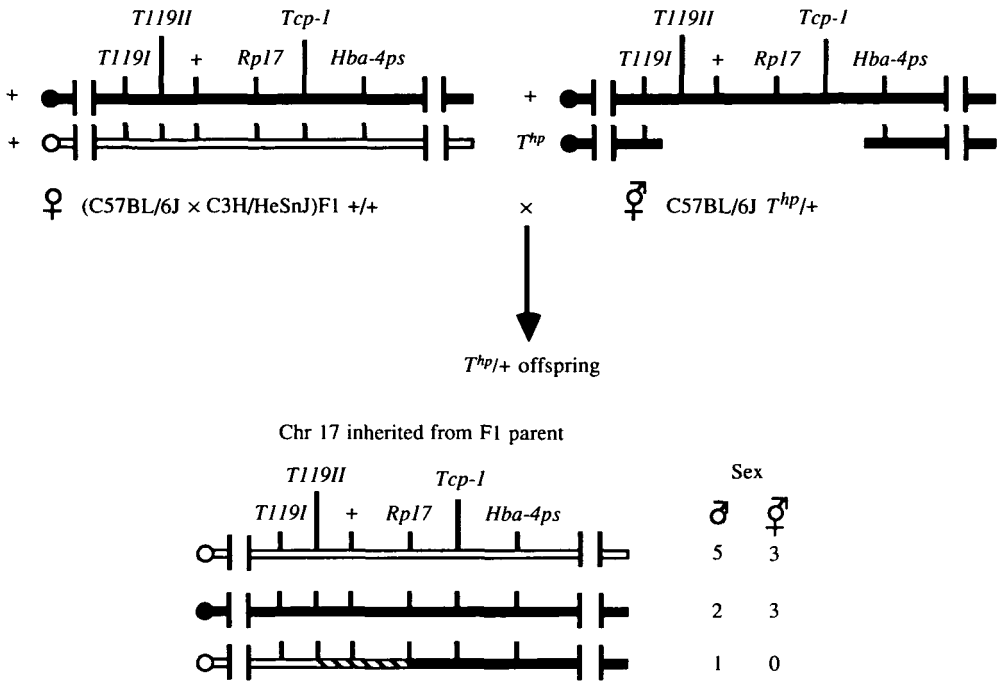

Fig. 2. Inheritance of *Tas* and Chr 17 DNA markers. The matings involved crossing (C57BL/6J × C3H/HeSnJ)F1 females to C57BL/6J $T^{hp}/+$ hermaphrodites and analyzing $14\frac{1}{2}$–15 day XY $T^{hp}/+$ fetuses for inheritance of five Chr 17 markers. The results for the 8 male and 6 hermaphroditic XY $T^{hp}/+$ fetuses are shown. A solid bar represents the B6-derived Chr 17 and a hollow bar the C3H-derived Chr 17. The T^{hp} chromosome is deleted for the *D17Leh119II* (abbreviated *T119II*), *D17Rp17* (abbreviated *Rp17*), and *Tcp-1* loci. The *D17Leh119I* (abbreviated *T119I*) and *Hba-4ps* loci flank the deletion. The hatched region represents the interval on Chr 17 that underwent recombination in the F1 female parent and was recovered in one of the normal male offspring.

Table 1. *Probes used to determine inheritance of Chr 17 markers flanking and deleted from* T^{hp}

			Fragment size (kb)[a]		
Locus	Probe	Enzyme	C57BL/6J	C3H/HeSnJ	Reference for probe
D17Leh119I	p119AR	*Msp* 1	2·6	4·4	Herrmann *et al.* 1986
D17Leh119II	p119AR	*Bam* 1	5·8, 3·0	6·7, 4·3	Herrmann *et al.* 1986
D17Rp17	pMK174	*Bam* 1	6·4	6·1	Mann *et al.* 1986
Tcp-1	pB1·4	*Taq* 1	3·0	1·1	Willison *et al.* 1986
Hba-4ps	α–ϕ4	*Taq* 1	3·4	3·7	Leder *et al.* 1981

[a] Although multiple fragments were detected with each probe, we have only listed the fragments used to distinguish a B6-derived *versus* C3H-derived allele.

G-banded metaphase chromosomes prepared from the liver according to the method of Eicher & Washburn (1978).

(iii) *Chr 17 DNA markers*

B6 and C3H carry distinct alleles for DNA loci, including *D17Leh119I* (DNA segment, Chr 17, Lehrach-119I), *D17Leh119II* (DNA segment, Chr 17, Lehrach-119II), *D17Rp17* (DNA segment, Chr 17, Roswell Park-17), *Tcp-1* (t-complex protein-1), and *Hba-4ps* (hemoglobin alpha-4, pseudogene). As diagrammed in Fig. 1, *D17Leh119I* and *Hba-4ps* are located proximal and distal, respectively, to the T^{hp} deletion, whereas *D17Leh119II*, *D17Rp17*, and *Tcp-1* are deleted from the T^{hp} chromosome. The probes used to detect these loci and the restriction fragment sizes diagnostic of B6-derived and C3H-derived alleles are listed in Table 1.

(iv) *DNA isolation and hybridization conditions*

Genomic DNA was prepared from individual spleens or fetuses according to the method of Jenkins *et al.* (1982). Ten μg of each DNA were digested with the appropriate restriction enzyme (see Table 1) using conditions recommended by the supplier (BRL), electrophoresed in 1 % agarose (Seakem, FMC Corp.) at 30 V for 18–24 hrs, and transferred to Zeta-Probe nylon filters (Bio-Rad Laboratories) by blotting overnight in 0·4 M NaOH (Reed & Mann, 1985).

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Filters were prewashed in 0·1 X SSC and 0·5% sodium dodecyl sulphate (SDS) for 1 hr at 65 °C, then incubated in 100 ml prehybridization solution containing 4 X SSCP (480 mM NaCl, 60 mM Na_2Citrate, 60 mM Na_2HPO_4, 18 mM NaH_2PO_4), 10 X Denhardt's solution (0·2% Ficoll, 0·2% polyvinylpyrrolidone, 0·2% bovine serum albumin), and 1·0% SDS for 4 hrs at 65 °C. Probes were nick-translated according to Rigby *et al.* (1977) with (α^{32}P)-dCTP (3000 Ci/mM) to a specific activity of $> 2 \times 10^8$ cpm/μg. The prehybridized filters were incubated overnight at 65 °C with 1–5 X 10^6 cpm/ml of denatured, ^{32}P-labelled probe in 20 ml hybridizing solution (4 X SSCP, 2 X Denhardt's solution, 1% SDS, and 1 mg/ml denatured sonicated salmon sperm DNA). After hybridization, the filters were washed at 65 °C in 4 X SSC, 0·1% SDS for 15 min and 30 min, followed by two 30 min washes and one 15 min wash in 1 X SSC, 0·1% SDS. Autoradiography was performed using Kodak XAR-5 film for various times (see Fig. 2) at −70 °C with DuPont Cronex intensifying screens.

Used filters were stripped of labelled probe with two successive 20 min washes in a 0·1 X SSC; 0·1% SDS solution heated to 95 °C and reprobed as described above.

3. Results

Of the 76 fetuses recovered from mating (B6 × C3H)F1 females to B6 $T^{hp}/+$ hermaphrodites, 38 were $+/+$ and 38 were $T^{hp}/+$, as determined by tail morphology. Twenty-four $T^{hp}/+$ and 24 $+/+$ fetuses developed exclusively ovarian tissue, 14 $+/+$ fetuses developed normal testicular tissue, 8 $T^{hp}/+$ fetuses developed exclusively testicular tissue, and 6 $T^{hp}/+$ fetuses developed both ovarian and testicular tissue. Chromosomal analysis of the 24 $T^{hp}/+$ and 24 $+/+$ fetuses having exclusively ovarian tissue indicated that all contained a normal XX chromosome complement.

The 14 XY $T^{hp}/+$ fetuses were analyzed for inheritance of 5 Chr 17 loci: *D17Leh119I*, *D17Leh119II*, *D17Rp17*, *Tcp-1*, and *Hba-4ps* (data are presented in Fig. 2 and representative results are presented in Fig. 3). Of the 6 $T^{hp}/+$ hermaphrodites, 3 inherited B6-derived Chr 17 markers and 3 inherited C3H-derived Chr 17 markers. Among the 8 $T^{hp}/+$

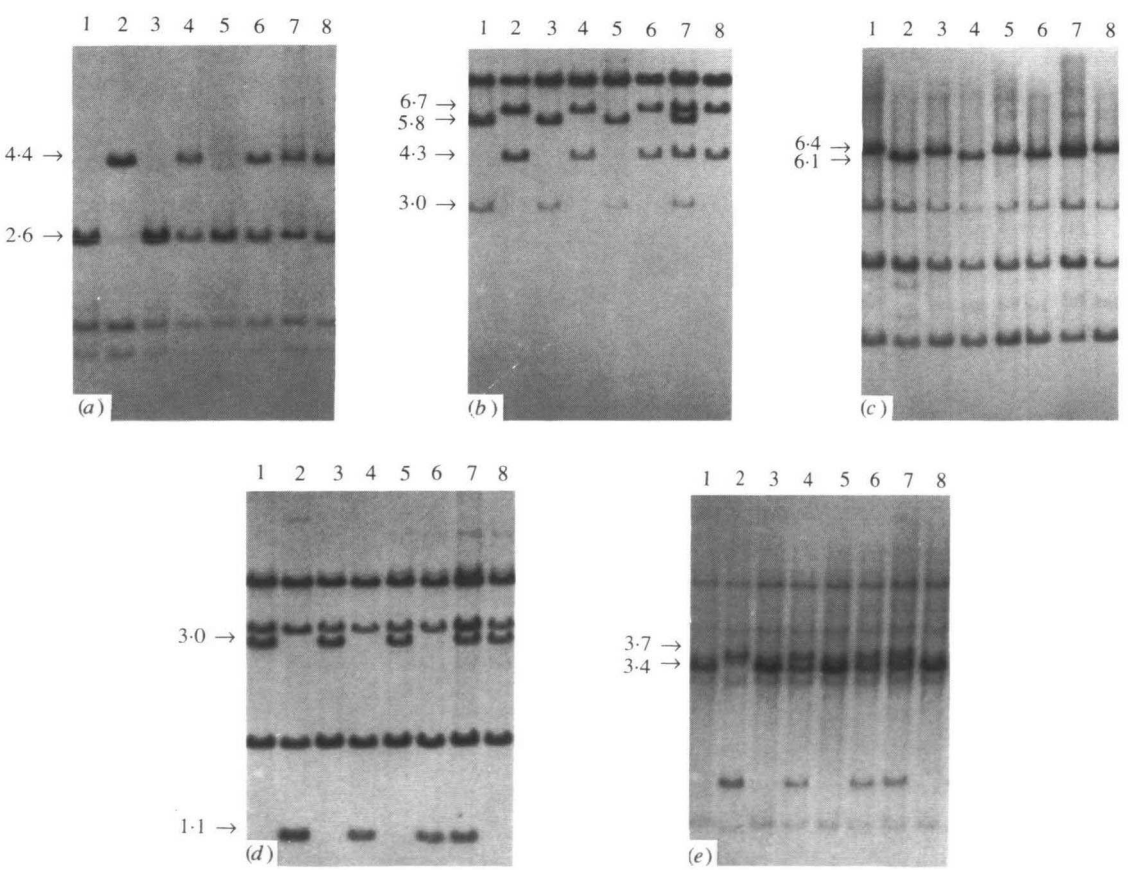

Fig. 3. Hybridization patterns for five Chr 17 molecular markers. The loci scored in the Southern blots are: (*a*) *D17Leh119I*, (*b*) *D17Leh119II*, (*c*) *D17Rp17*, (*d*) *Tcp-1*, and (*e*) *Hba-4ps*. Fragments used to distinguish B6-derived from C3H-derived alleles are noted. The DNAs in each blot are: lane 1, B6 male; lane 2, C3H male; lanes 3–4, backcross XY $T^{hp}/+$ hermaphrodites; lanes 5–6 and 8, backcross XY $T^{hp}/+$ male, and lane 7, (B6 × C3H)F1 female. Note that the DNA in lane 8 is from the backcross XY $T^{hp}/+$ male that inherited a recombinant Chr 17 (between *D17Leh119II* and *D17Rp17*). Refer to Table 1 for diagnostic fragments used to identify B6-derived and C3H-derived alleles.

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normal males, 2 inherited B6-derived Chr 17 markers, 5 inherited C3H-derived Chr 17 markers, and 1 inherited a recombinant Chr 17 with the crossover located between *D17Leh119II* and *D17Rp17*.

4. Discussion

We previously reported that partial or complete sex reversal occurs in B6 $T^{hp}/+$ or B6 $T^{Orl}/+$ mice carrying Y^{AKR}. This sex reversal phenomenon is dependent on genetic background. Ovarian tissue develops when T^{hp} or T^{Orl} is present on a B6 inbred strain background that includes Y^{AKR}, whereas normal testicular tissue develops when T^{hp} is on a C3H inbred strain background that includes Y^{AKR} or when T^{Orl} is on the B6 inbred background with Y^{B6}. One explanation to account for the background effect is that the gene responsible for this inherited sex reversal, *Tas*, is located within the region of Chr 17 deleted in the T^{hp} and T^{Orl} mutations and the development of ovarian tissue is a consequence of a hemizygous expression of the B6-derived *Tas* allele interacting abnormally with the *Tdy* allele on Y^{AKR} (Eicher & Washburn, 1986; Washburn & Eicher, 1989). If this hypothesis were correct, then ovarian tissue (with or without accompanying testicular tissue) would develop in an XY $T^{hp}/+$ individual when the wild-type Chr 17 is B6-derived; when the wild-type Chr 17 is C3H-derived, only testicular tissue would form. To test this hypothesis, we mated (B6 × C3H)F1 females to B6 $T^{hp}/+$ hermaphrodites and determined the inheritance of five Chr 17 molecular markers in the XY $T^{hp}/+$ fetal offspring (see Fig. 2 and Fig. 3).

The data obtained in this study are consistent with previous observations that *Tas* segregates as a single Mendelian locus: Eight of the 14 backcross XY $T^{hp}/+$ fetuses developed normal testes and 6 developed both ovarian and testicular tissue. The data, however, do not support the suggestion that T-associated sex reversal is due to a specific interaction of the *Tas* allele carried by B6 and the *Tdy* locus located on Y^{AKR}. Of the 6 hermaphrodites recovered, 3 inherited exclusively B6-derived loci and 3 inherited exclusively C3H-derived loci, and of the 8 normal males recovered, 2 inherited B6-derived loci, 5 received C3H-derived loci, and 1 inherited a recombinant Chr 17 (Fig. 2). These data indicate that the type of gonadal development in an XY $T^{hp}/+$ fetus is independent of whether B6-derived or C3H-derived Chr 17 loci are inherited.

At this juncture, a review of some of the observations gathered on sex reversal in XY $T^{hp}/+$ mice is useful. The first occurrence of sex reversal associated with T^{hp} was noted at the N3 and N4 generations of backcrossing T^{hp} onto the C57BL/6J inbred strain when XY $T^{hp}/+$ hermaphrodites and females were identified at weaning. A more rigorous analysis then focused on a time during fetal development when minor amounts of one gonadal tissue can be easily identified in a majority of the opposite gonadal tissue (Eicher *et al.* 1980). This analysis revealed that all C57BL/6J XY $T^{hp}/+$ individuals develop at least some ovarian tissue, with half developing exclusively ovarian tissue (completely sex reversed) and half developing both ovarian and testicular tissue (partially sex reversed) (Washburn & Eicher, 1983). The fact that we did not observe this phenomenon until the N3-N4 backcross generation suggested either that we failed to previously note such animals or that sex reversal did not occur until the B3-N4 backcross generation, at which time one or more unlinked genes of B6 origin were fixed in the homozygous state.

The findings presented here, that half of the XY $T^{hp}/+$ fetuses recovered from matings of (B6 × C3H)F1 females to B6 $T^{hp}/+$ hermaphrodites developed ovarian as well as testicular tissue, suggests that the transfer of the T^{hp} mutation onto the B6 inbred strain background is not an absolute requirement for ovarian tissue to develop in XY $T^{hp}/+$ individuals as these offspring were effectively a second backcross generation (N2). Rather, these data suggest that development of ovarian tissue occurs in XY $T^{hp}/+$ fetuses if they are homozygous for a B6-derived locus unlinked to T^{hp}. In addition, the fact that none of the XY $T^{hp}/+$ fetuses in this study developed exclusively ovarian tissue suggests that the transfer of T^{hp} onto the B6 inbred strain background for at least three generations may be a requirement for obtaining XY $T^{hp}/+$ females, implicating a second B6-derived locus for complete sex reversal of XY $T^{hp}/+$ individuals. Either of these loci could be identical to two previously identified primary sex-determining loci, *tda-1* (testis determining, autosomal-1) and *tda-2* (testis determining, autosomal-2) (Eicher *et al.* 1982; Eicher and Washburn, 1983, 1986). Although we have identified additional primary sex-determining loci in this study, T^{hp} and T^{Orl} (or their associated deletions) together with the presence of the Y^{AKR} remain critical for development of ovarian tissue.

We hypothesized that for normal gonadal development to occur in an XY individual, the primary signal for differentiation of testicular tissue, encoded by a gene(s) on the Y chromosome, must occur before a genetic signal for ovarian development is initiated (Eicher & Washburn, 1986). If this temporal requirement is not maintained, that is, if the gene for testis differentiation is not activated early enough or functions abnormally, both ovarian and testicular tissue or exclusively ovarian tissue differentiates. One could imagine that two types of mutations could affect gonadal differentiation in XY individuals: (1) mutations that affect cellular structure within the developing gonad and (2) mutations that delay the process of gonadal differentiation by narrowing the temporal window sufficiently to allow development of both ovarian and testicular or exclusively ovarian tissue.

Cattanach has proposed that the partial or complete

sex reversal observed with T^{hp} and T^{Orl} is a consequence of the associated deletions rather than hemizygosity for actual primary sex-determining genes (Cattanach, 1987; see discussion in Eicher, 1988). He has hypothesized that T^{hp} and T^{Orl} cause a delay in fetal development during the time when gonadal differentiation is taking place and this delay results in ovarian tissue development in XY individuals. Although Cattanach's suggestion is similar to our hypothesis, in that a temporal element is involved in primary sex determination, Cattanach's hypothesis does not recognize the need for specific loci, whereas we assume that primary sex-determining genes are involved (Eicher & Washburn, 1986). Furthermore, if the deletions associated with T^{hp} and T^{Orl} were themselves causative, we would not expect that partial or complete sex reversal would be affected by inbred strain background or Y chromosome origin. In fact, T^{hp} shows no associative sex reversal, partial or complete, on the C3H background when the AKR Y chromosome is present nor does partial or complete sex reversal occur in XY $T^{Orl}/+$ mice when T^{Orl} is present on a B6 inbred background that includes the B6 Y chromosome.

It is important to genetically map the gene(s) that causes sex reversal in XY^{AKR} $T^{hp}/+$ and XY^{AKR} $T^{Orl}/+$ mice when present in the homozygous B6 state. Although we have assumed that this gene(s) is located on an autosome, the X chromosome remains a viable candidate for the location of a primary sex-determining gene given the hypothesized evolution of the mammalian X and Y chromosome from an autosomal pair (Ohno, 1967). Clearly, the Y chromosome contains a DNA sequence(s) that is indisputably sex-determining. Thus, it is plausible that the X chromosome also contains a gene or genes involved in one or both primary sex determination pathways (Page *et al.* 1987; German, 1988; Ferguson-Smith, 1988). Alternatively, it is conceivable that the newly identified sex reversal locus is identical to *tda-1* or *tda-2*. Genetic mapping of these loci will resolve the identities.

Our contribution to the issue of *Genetical Research* in honour of Mary Lyon has particular significance for us. The hairpin tail mutation was identified at The Jackson Laboratory in 1962 in an AKR/J male. Because mutations are difficult to maintain on a AKR/J inbred strain background due to inherited leukemia, a $T^{hp}/+$ male was mated to a C57BL/6J female. Resulting F1s were intercrossed to produce F2s, etc., and the T^{hp} mutation was maintained thereafter by crossing normal females to sib hairpin tail males. Unfortunately, a recessive mutation at the pudgy locus (*pu*) occurred spontaneously in this hairpin strain, and because *pu/pu* mice have a phenotype similar to that observed in severely affected $T^{hp}/+$ mice, a *pu/pu* male was inadvertently used to establish the next generation resulting in loss of T^{hp} from the stock.

In 1977 Mary kindly returned T^{hp} to The Jackson Laboratory. At this time we transferred T^{hp} onto the C57BL/6J inbred strain background, and there the sex reversal 'tale' begins. Mary has shared in our excitement in locating a sex-determining gene in one of her favourite pastimes, the *t* complex, and she has shared in our attempts to fathom the genetic network involving *T* deletions, *Tas*, and the Y chromosome. It is a special privilege and honour to dedicate this paper to her.

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