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Developmental and Genetic Analysis of Primary Sex Determination in the Mouse

by

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ABSTRACT

Autosomal factors interact with the Y chromosome to cause incomplete testis development in the mouse manifested by a combination of ovaries, ovotestes and testes in the XY embryos.

I have demonstrated that the phenotypic distribution of types of gonad in the XY embryos from the B6.Y-POS consomic males is stable over days 14, 15 and 16 of gestation. The ovotestis is not a transient phenotype during this period of development. The Peru W-9 Y now falls into functional class 2 of *domesticus*-type Y chromosome along with AKR/J and CLA Y chromosomes based on the types of gonad observed in the XY embryos from the B6.Y-Peru W-9 consomic males. The T^{Orl} deletion has an interaction effect with the *poschiavinus* Y in a B6 × D2 hemizygous background. This effect is chromosome specific since three *Splotch* deletions/mutations on chromosome 1 did not increase the frequency of gonadal hermaphroditism. The incomplete testis development effect of the T^{Orl} deletion is different from the *Tda* trait. The WC/ReJ - DBA/2J and BALB/cByJ - DBA/2J differences in the *Tda* trait are due to alternate alleles at more than one locus.

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LIST OF ABBREVIATIONS

cM	Centimorgan
df	Degrees of freedom
EDTA	Ethylenediaminetetraacetic acid
g	Gram
het	Heterogeneity
kb	Kilobase
μl	Microlitre
ml	Millilitre
NaCl	Sodium chloride
NaOH	Sodium hydroxide
O	Ovary
OT	Ovotestis
rcf	Relative cumulative frequency
rcN	Relative cumulative number
SDS	Sodium dodecyl sulfate
Sry	Sex determining region of the Y
SSC	Saline sodium citrate
T	Testis
Tda	Testis determining autosomal
TE	Tris-EDTA
Tris	Tris(hydroxymethyl)aminomethane

Chapter 1. Introduction

Primary sex determination in mammals is the differentiation of a testis from the indifferent and apparently bipotential embryonic gonad. The genetic basis for testis determination was demonstrated to be the Y chromosome. Absence of the Y chromosome in either a single X or a two X chromosome mouse produced a normal fertile female (Welshons and Russell, 1959). The search for the minimum region of the Y chromosome that is necessary for primary sex determination has identified the *SRY* gene in humans and *Sry* in the mouse (Gubbay et al., 1990).

Deletion of the *Sry* region of the Y in the mouse, by spontaneous mutation ($Y^{Tdy.m1}$), results in XY female development and supports the hypothesis that *Sry* may be the testis determining gene (Lovell-Badge and Robertson, 1990). Deletion of Y chromosomal material outside the minimal testis determining region results in XY female mice (Capel et al., 1993). This suggests that there may be position effects on normal *Sry* function (Capel et al., 1993).

In the laboratory mouse, there are two major types of Y chromosome, defined by differences in repetitive DNA sequences outside of the *Sry* region (Nishioka, 1987; Nishioka and Lamothe, 1986). They are derived from the *musculus* and *domesticus* subspecies whose admixture formed the historical foundation of the common strains of the laboratory mouse (Silver, 1995).

If the Y chromosome is necessary, it may not be sufficient for normal testes development (Eicher and Washburn, 1986). When the Y chromosome of

poschiavinus, a local variety of *Mus musculus domesticus* from Switzerland and Northern Italy, was put into the background of the highly inbred C57BL/6J laboratory mouse strain, gonadal hermaphrodites were found (Eicher et al., 1982). During the construction of a C57BL/6J consomic strain carrying the *poschiavinus* Y chromosome, the gonads of embryos from the N6 and N7 backcross generations were assessed. From a total of 38 XY fetuses, 16 had bilateral ovaries, 9 had an ovary and an ovotestis, and 13 had bilateral ovotestes. None of the observed fetuses had bilateral, normal testes (Eicher et al., 1982).

A putative autosomal recessive allele at the *Tda-1* (testes-determining autosomal gene-1) locus was interpreted to be the cause of these observations (Eicher et al., 1982; Eicher and Washburn, 1983). It was suggested that the *poschiavinus* strain is homozygous for the dominant allele $Tda\text{-}1^a$ that permits normal functioning of the *poschiavinus* Y in its own genetic background. C57BL/6J has a recessive $Tda\text{-}1^b$ allele that, with its own *musculus*-type Y chromosome, gives normal XY males. However, the *poschiavinus* Y interacts with the recessive $Tda\text{-}1^b$ of C57BL/6J to give gonadal hermaphrodites in the first backcross (BC_1) and subsequent backcross generations. Genetic studies suggested linkage of *Tda-1* to chromosomes 2 or 4 (Eicher and Washburn, 1986), but subsequent attempts to map this gene failed. Therefore, the *Tda* or testis determining autosomal trait may be determined by more than one gene locus.

Y-specific DNA probes for repetitive sequences outside the *Sry* region of the mouse have been isolated and, when applied to different mouse strains, various

restriction-fragment length polymorphisms are detected (Nishioka, 1987; Nishioka and Lamothe, 1986). In particular, a 2.3-kb *Taq*I fragment with the AC11 probe has been identified in some *domesticus*-type Y chromosomes (Biddle et al., 1991; Nishioka and Lamothe, 1986). After the molecular analysis of the *domesticus*-type Y chromosome, the next logical step was to functionally assay the Y chromosomes through their interaction with the C57BL/6J female genetic background. Embryos from the first backcross generation (BC_1) were examined. Based on the phenotypic distribution of types of gonad, three functional classes of *domesticus*-type Y chromosome were identified (Biddle and Nishioka, 1988; Biddle et al., 1991). These are normal testis development (class 1), low frequency of gonadal hermaphrodites (class 2), and high frequency of gonadal hermaphrodites (class 3).

A polymorphic stretch of trinucleotide (CAG) repeats has been identified in the *Sry* gene of several *domesticus*-type Y chromosome (Coward et al., 1994). The number of glutamine (CAG) residues may be crucial for the proper functioning of a *domesticus*-type *Sry* allele in the C57BL/6J genomic background. This polymorphic tract may be associated with different degrees of gonadal hermaphroditism. The *Sry* allele of *poschiavinus* contains 11 CAG repeats and the strain belongs to class 3 (high frequency of gonadal hermaphrodites). The *Sry* allele of AKR/J, however, contains 13 CAG repeats and the strain belongs to class 2 (low frequency of gonadal hermaphrodites). Strains with *domesticus*-type Y chromosome that interact with the C57BL/6J background to produce XY embryos with only normal testes (class 1), contain 12 CAG repeats in their *Sry* allele.

Further assessment of the size of the CAG repeats in the *Sry* gene of other *domesticus*-type Y chromosomes suggests there may be no association between the number of repeats and the degree of gonadal hermaphroditism (Carlisle et al., 1996), but there were no empirical data on gonadal development to support the suggestion. As a result, the question of association between number of CAG repeats and frequency of gonadal hermaphrodites remains unanswered.

A genetic study was conducted to define the phenotype of the *Tda* trait, when homozygous, in C57BL/6J and DBA/2J. The DBA/2J strain, like C57BL/6J, has a *musculus*-type Y chromosome. Two strains, C57BL/6J.Y-POS and DBA/2J.Y-POS, were constructed to be consomic for the *poschiavinus* Y chromosome by repeated backcrosses to C57BL/6J and DBA/2J, respectively. Only normal XY males with testes were recovered from crossing DBA/2J females with the consomic DBA/2J.Y-POS males. Gonadal hermaphroditism was observed when C57BL/6J females were crossed to the consomic C57BL/6J.Y-POS males. The phenotypic distribution of types of gonad in day 14 XY embryos in the C57BL/6J.Y-POS consomic strain can be represented by a histogram (Figure 1) with five classes: bilateral ovaries; an ovary and an ovotestis; bilateral ovotestes; an ovotestis and a testis; bilateral testes. The liability to express incomplete testes differentiation in the C57BL/6J.Y-POS genotype is normally distributed (Figure 2) (Biddle et al., 1994). The frequencies of embryos in the five gonadal classes are the areas under the normal curve defined by the interclass intervals. The genetic difference between C57BL/6J and DBA/2J in their interaction with the *poschiavinus* Y

chromosome was assessed in standard genetic crosses (Biddle el al., 1994). The resulting backcross studies allowed the rejection of the single gene model, previously suggested (Eicher and Washburn, 1986), for the difference between C57BL/6J and DBA/2J. This genetic difference appears to be in four or five gene loci (Eisner et al., 1996).

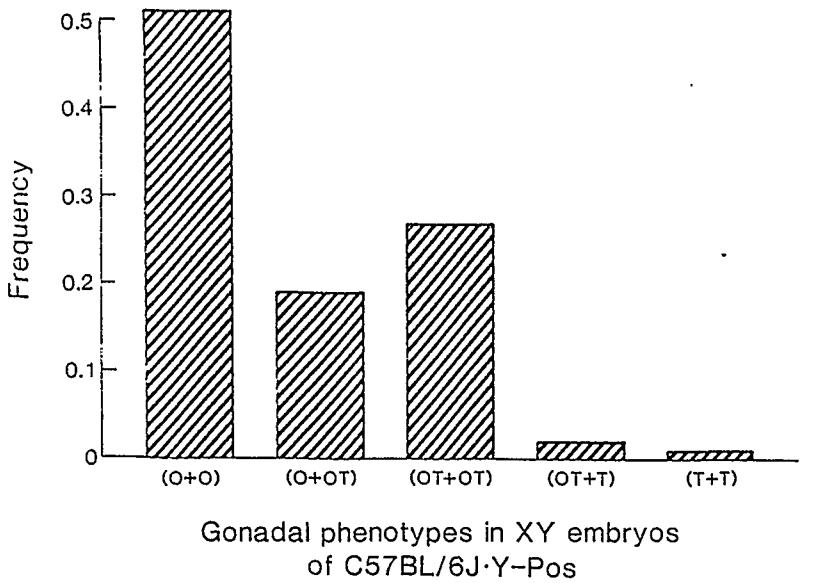

Figure 1. Histogram of the phenotypic distribution of day 14 XY embryonic gonads from C57BL/6J.Y-POS consomic strain (from Biddle et al., 1994).

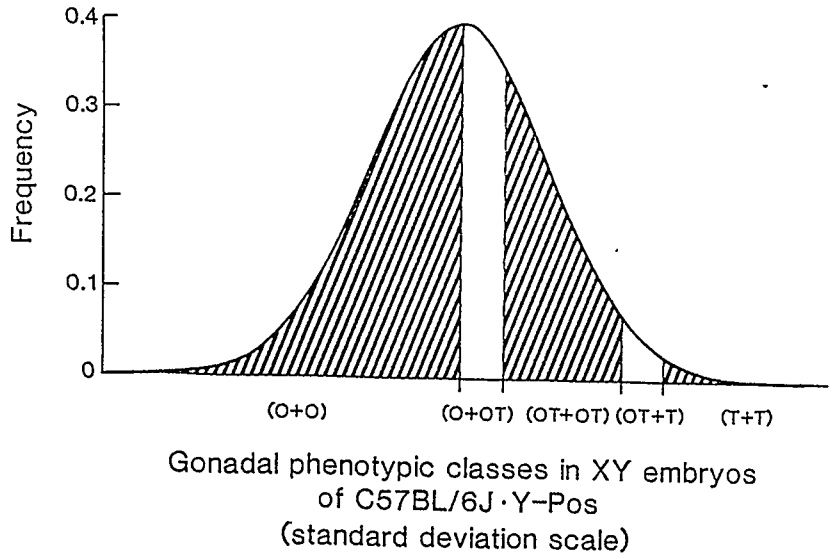

Figure 2. Phenotypic distribution of the C57BL/6J.Y-POS genotype as a threshold model (from Biddle et al., 1994).

A strain survey was conducted by test-mating females of different inbred strains with C57BL/6J.Y-POS consomic males (Eales et al., 1996). Five groups were identified based on the degree of gonadal hermaphroditism observed. Group I contains C57BL/6J (high frequency of gonadal hermaphroditism with *poschiavinus* Y) and group V contains DBA/2J (XY males with normal testes with *poschiavinus* Y). Groups II (WC/ReJ), III (BALB/cByJ and NOD/Lt) and IV (C3H/HeSn.*Paf*, CDS/Lay, and CBA/FaCam) expressed gonadal hermaphroditism with *poschiavinus* Y but at a lower frequency than group I (Eales et al., 1996). The frequency distributions of gonadal hermaphrodites were significantly different from each other and from C57BL/6J (group I) and DBA/2J (group V) (Figure 3) (Eales et al., 1996). When the F_1 males from the WC/ReJ and BALB/cByJ females were tested

with their respective parental female strain, they produced the same distribution of XY gonadal hermaphroditism in BC_1 embryos as in the F_1 embryos (Eales et al., 1996). The groups II, III, and IV of mouse strains may represent some of the recombinant genotypes between the extremes of C57BL/6J and DBA/2J.

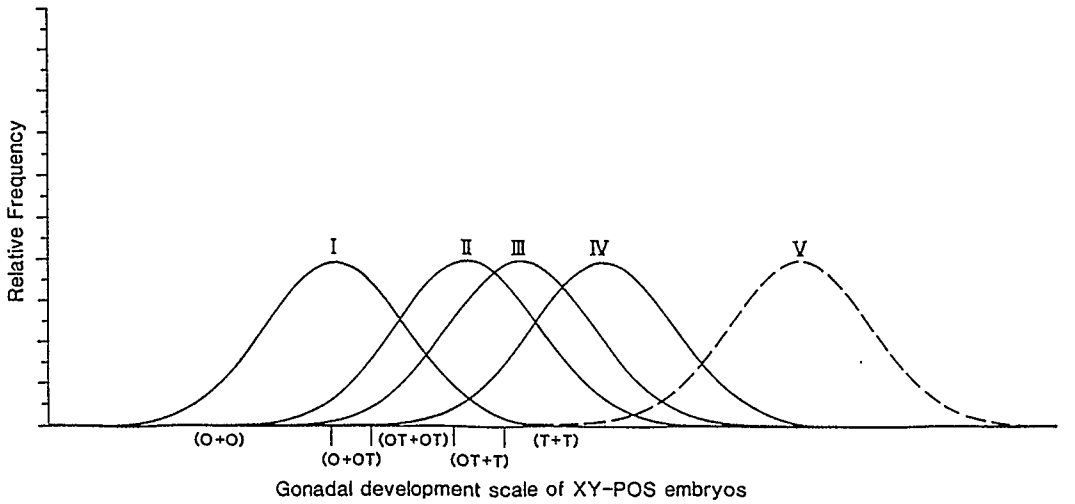

Figure 3. Normal distributions of the threshold model for the interaction of the *poschiavinus* Y chromosome in XY F_1 embryos from test-matings of females from strains of groups I to V with C57BL/6J.Y-POS males (from Eales et al., 1996).

Autosomal deletions of chromosomes 5 (W^{19H}, W^e) and 17 (T^{hp}, T^{Orl}) have been found to interact with the *poschiavinus* Y and AKR/J Y chromosomes to produce high frequencies of gonadal hermaphroditism (Eicher and Washburn, 1983; Cattanach, 1987; Washburn and Eicher, 1989; Burgoyne and Palmer, 1991). As previously mentioned, the *poschiavinus* Y chromosome belongs to class 3, which

produces a high frequency of gonadal hermaphrodites in the C57BL/6J genetic background, whereas the AKR/J Y chromosome belongs to class 2, which produces a low frequency of gonadal hermaphrodites in C57BL/6J. The autosomal deletions are partially monosomic for normal wild-type genes which may be exhibiting haploinsufficiency or dosage sensitivity for sex determination where only one copy of the normal allele is not enough for normal testes development. Burgoyne and Palmer (1991) proposed a model based on thresholds of development for the interaction of the autosomal deletions in the C57BL/6J background with the Y chromosome of *poschiavinus* and AKR/J. The deletions were suggested to move the distribution of incomplete testis development toward more ovary development. However, no analyses of empirical data were presented to define the interaction of the wild-type alleles and the deletions with the respective Y chromosomes.

Several research questions arise from this background information and form the foundation for the questions and hypotheses that will be tested in this thesis.

1. Developmental stability of the C57BL/6J.Y-POS embryonic gonadal phenotype over different gestational days.

It has been suggested that the clear zones observed in the ovotestes of some XY embryos are undifferentiated testicular tissue that will fill up with testicular tubules as development proceeds (Eicher, 1994). That is, the gonad may be a transient ovotestis. This suggestion can be tested by looking at the gonadal phenotypic distribution in the C57BL/6J.Y-POS consomic strain over three different

gestational days (14, 15, and 16). The research question is: Is the phenotypic distribution of types of gonad in the C57BL/6J.Y-POS consomic strain, which was defined by Biddle et al. (1994) using day 14 embryos, stable over different gestational days?

2. Definition of XY gonadal phenotype in C57BL/6J.Y-AKR/J, C57BL/6J.Y-CLA, C57BL/6J.Y-Peru W-9 consomic strains.

The gonadal phenotypes in day 15 embryos from C57BL/6J.Y-AKR/J, C57BL/6J.Y-CLA, and C57BL/6J.Y-Peru W-9 consomic strains will be assessed. These three strains are now consomic for two of the three different functional classes of *domesticus*-type Y chromosomes previously reported (Biddle and Nishioka, 1988). The second research question is: Now that these three consomic strains have been inbred for more than 30 backcross generations to C57BL/6J, do they still exhibit the same phenotypic differences that were previously reported (Biddle and Nishioka, 1988)? When analyzing the data, it will be taken into consideration that earlier studies could not unambiguously assign the Peru W-9 Y chromosome to either class 2 or 3 (Biddle and Nishioka, 1988). In addition, those earlier studies were conducted on the first backcross generation and the embryos were not chromosomally sexed by the presence of a Y chromosome.

3. Effects of deletions on testes development.

Certain deletions on chromosomes 5 and 17 have been reported to exacerbate gonadal hermaphroditism when they interact with *poschiavinus* or AKR/J Y chromosomes (Eicher and Washburn, 1983; Cattanach, 1987; Washburn and Eicher, 1989; Burgoyne and Palmer, 1991).

The T-Orleans (T^{Orl}) deletion on chromosome 17 is known to increase the frequency of gonadal hermaphrodites (Eicher and Washburn, 1983; Washburn and Eicher, 1989). I will use this deletion to test the threshold model of Burgoyne and Palmer (1991). I will assess the phenotypic distribution of XY embryos that are homozygous wild-type and compare it to that obtained for the $T^{Orl}/+$ genotype. I will quantitate the deletion effect using C57BL/6J.Y-POS and C57BL/6J.Y-AKR consomic males and ask the following: Does T^{Orl} in C57BL/6J have the same (additive) effect on *poschiavinus* Y and AKR/J Y chromosomes or does this deletion change the underlying developmental scale which then interferes with normal testes development? This might be another quantifiable assessment of the Y chromosome function.

If deletions on chromosomes 5 and 17 change the penetrance of XY gonadal hermaphroditism, do other deletions on other chromosomes have any effect? Is this phenomenon chromosome or gene specific? In order to test this hypothesis I will use deletions on chromosome 1 of the mouse. These are: *Splotch, Splotch delayed* and *Splotch retarded.*

4. Genetic difference between BALB/cByJ - DBA/2J and WC/ReJ - DBA/2J strain pairs.

The C57BL/6J and DBA/2J mouse strains differ by more than a single gene in their interaction with the *poschiavinus* Y chromosome (Biddle et al., 1994). I will assess the interaction between BALB/cByJ and DBA/2J as well as between WC/ReJ and DBA/2J. F_1 males will be produced from BALB/cByJ or WC/ReJ females and DBA/2J.Y-POS males. These resulting F_1 males (BALB × D2.Y-POS F_1 and WC × D2.Y-POS F_1) will then be test-mated to the respective female strain to obtain the backcross (BC_1) generation. The data obtained will then be analyzed and compared to the parental data previously reported (Eales et al., 1996) in order to test the single gene hypothesis for the difference between BALB/cByJ and DBA/2J as well as between WC/ReJ and DBA/2J.

Chapter 2. Materials and Methods

In this chapter, the general materials and methods used in all four projects are documented. The specific matings, hybrid mouse constructions and statistical tests are outlined in the chapter describing the specific project.

1. Mice and Test Matings

Tables 1, 2 and 3 provide a general summary of all mouse strains and stocks used in this study. The full name and an abbreviation (when needed) are given for each strain used. The year of import and the number of generations the strains have since been bred are also listed.

Table 1. Summary of inbred mouse strains.

Inbred strain	Abbreviation	Generation	Source and year imported
C57BL/6JBid	B6	F65	C57BL/6J, Jackson Laboratory, 1978
BALB/cByJBid	BALB	F45	BALB/cByJ, Jackson Laboratory, 1982
WC/ReJBid	WC	F20	WC/ReJ, Jackson Laboratory, 1990

Table 2. Summary of the mouse strains used for the deletion study.

Strain	Deletion	Chromosome	Generation	Source and Year imported
Splotch Retarded	Sp^r	1	Gen20	McGill University, 1990
Splotch	Sp	1	F18	McGill University, 1990
*Splotch delayed**	Sp^d	1	N18	McGill University, 1990
C57BL/6JEi.T^{Orl}	T-Orleans	17	N35	Jackson Laboratory, 1995

* The *Splotch delayed* is a point (missense) mutation. It is included in Table 2 since it belongs to the *Splotch* family.

Table 3. Summary of consomic mouse strains.

Consomic strain	Abbreviation	Generation	Stock
C57BL/6JBid. Y-POS	B6.Y-POS	N35	Males with the Y-POS backcrossed to C57BL/6JBid females
C57BL/6JBid. Y-AKR/J	B6.Y-AKR	N35	Males with the Y-AKR backcrossed to C57BL/6JBid females
C57BL/6JBid. Y-CLA	B6.Y-CLA	N38	Males with the Y-CLA backcrossed to C57BL/6JBid females
C57BL/6JBid. Y-Peru W-9	B6.Y-Peru W-9	N35	Males with the Y-Peru W-9 backcrossed to C57BL/6JBid females
DBA/2JBid. Y-POS	D2.Y-POS	N30	Males with the Y-POS backcrossed to DBA/2JBid females

The mouse colony was maintained on a standard 14 hour light and 10 hour dark cycle with lights on at 6:00 a.m. The mice used in this study were fed Purina "Mouse Diet 9F" and water *ad libitum* and the standard "shoe box" polycarbonate mouse cages contained kiln-dried "Aspen Chip" bedding (Biddle et al., 1994).

To produce test mating progeny for this study, females were placed with singly caged males in the late afternoon. The next morning, evidence of mating was detected by the presence of a copulation plug. The day of finding the copulation plug was considered as day zero of pregnancy.

2. Phenotypic Sexing of Embryonic Gonads and Tissue Collection

On the desired gestational day (day 14, 15 or 16), pregnant females were euthanized by cervical dislocation. The gravid uterus was removed and placed in physiological saline (0.9%). Individual embryos were examined under a dissecting microscope. A tissue sample (usually the upper limb) was taken and placed in a 1.5 ml centrifuge tube containing 0.1 ml TE (Tris.Cl-EDTA, pH 8.0) (Appendix A) for further DNA analysis.

Day 15 embryos were used for most studies because they were easier to handle under the microscope and their gonads were easily scored and assessed compared to the day 14 and 16 embryos. The gonads were removed from each embryo and put in a drop of saline on a glass slide and examined under a dissecting microscope with transillumination (Biddle and Nishioka, 1988). Gonads were

classified as ovaries, testes, or ovotestes or any combination based on the presence or absence of testicular tubules (Whitten et al., 1979).

3. Chromosomal Sexing using Y specific DNA probe

Genomic DNA was prepared from the tissue sample from each embryo (Laird et al., 1991). The tissue was digested overnight in a water bath at 55°C after adding 0.5 ml Lysis buffer (Appendix A) and 10 µl Proteinase K (10 mg/ml). The next morning, DNA was precipitated by adding 0.5 ml isopropanol and vigorously shaking the centrifuge tubes. The samples were then spun for 1 min in a benchtop micro-centrifuge (approximately 13,000 ×g) to pellet the precipitated DNA. The supernatant was decanted and the DNA was left to air-dry for about one hour. Distilled deionized water (200 µl) was then added to redissolve the DNA and it was dot blotted onto a positively charged nylon membrane (Cat. No. 1209-299) according to the manufacturer's specification (Boehringer Mannheim, 1990).

The nylon membrane was wetted with 2× SSC before enclosing it in the manifold (Cat. No. 1050 MM, BRL) which was attached to a water vacuum. Each well was pre-wetted with 100 µl 0.4 N NaOH, 2× SSC (Appendix A). A 2.5 µl sample of DNA was put into each well of the manifold (total of 50 samples per blot). Each well was wetted with 100 µl 0.4 N NaOH, 2× SSC before increasing the water suction for 10 additional minutes. The membranes were then soaked in the 2× SSC solution before they were air-dried. They were put into a sealing bag (Cat. No. 6010A, Dazey) and UV cross-linked for 3 minutes. Pre-hybridization was

performed at 42°C for one hour using 10 ml of pre-hybridizing solution (Appendix A). Hybridization was then carried out overnight using the 145SC5 Y-specific DNA probe (Nishioka, 1988). The probe was uniformly labelled using a PCR technique (Boehringer Mannheim, 1994) (further discussion in the section to follow).

The DIG-labelled probe was denatured at 90°C for 5-10 min and 2-4 µl were added to 10 ml of hybridization solution. The membranes were hybridized overnight at 42°C. The next day, post-hybridization washes were performed. The membranes were washed twice for 5 min each in 2× SSC, 0.1% SDS at room temperature with shaking. They were then washed twice in a water bath at 68°C for 15 min each in 0.1× SSC, 0.1% SDS with shaking. The membranes were equilibrated for 1 min in a small dish in Buffer A (Appendix A) and soaked in a blocking buffer, Buffer B (Appendix A), for three hours at room temperature with shaking. The membranes were then incubated in a 1:5000 Anti-DIG solution (10 ml Buffer B, 2 µl Anti-DIG antibody) for 30 minutes at room temperature with shaking. Two washes, 15 min each, were carried out afterwards in Buffer A. The membranes were then soaked in Buffer C (Appendix A) for two minutes and wetted with 3 ml of Lumi-Phos (Cat. No.1275-470, Boehringer Mannheim), a chemiluminescent agent. The membranes were then resealed in the sealing bags and incubated in a heating oven at 37°C for 30 minutes. The membranes were then exposed for 5-10 minutes to a diagnostic film (Kodak X-OMAT AR) to detect the

chemiluminescent reaction of the Lumi-Phos with the probe. The presence of a Y chromosome was defined as a black circular spot on the film.

4. Probe Labelling

The 145SC5 probe is a 1.5 kb EcoR I fragment that hybridizes preferentially to male mouse DNA (Nishioka, 1988). It was originally isolated from a BALB/c male mouse (Nishioka, 1988), and it contains at least 200 repetitive sequences in the Y chromosome with very few copies present in the female mouse genome (Prado et al., 1992). Hence, the 145SC5 probe is a most appropriate molecular tool for sexing the mouse embryos used in this study.

The DNA was uniformly labelled by incorporating digoxigenin-labelled deoxyuridine triphosphate using the Polymerase Chain Reaction (PCR) method (Boehringer Mannheim, 1994). The dUTP is linked via a spacer arm to the steroid hapten digoxigenin (DIG-dUTP). After hybridization to the target DNA, the hybrids are then detected by enzyme-linked immunoassay using an antibody-conjugate (anti-digoxigenin alkaline phosphatase conjugate) and subsequent reaction with Lumi-Phos 530 substrate and exposure on X-ray film to record the chemiluminescent signal (Boehringer Mannheim, 1990).

The primer pair, used to synthesize the DIG-labelled probe, flank the 145SC5 genomic sequence (Prado et al., 1992). The primer pair sequences were supplied by Dr. Y. Nishioka (Department of Biology, McGill University, personal communication).

They are: 5'-AGGTCTACAGCCAGGTAGAA-3'
and
5'-CAAAGACCCATAGGTACCCA-3'

The PCR reaction was as follows:

Reagents	Volume (µl)
sterile water	25.5
PCR buffer without $MgCl_2$ 10× (vial 3)	5
$MgCl_2$ stock solution 25 mM (vial 4)	3
PCR DIG-mix 10× (vial 2)	5
Forward 145SC5 20 µM	5
Reverse 145SC5 20 µM	5
Template DNA (1/1000 Dilution)	1
Taq DNA Polymerase	0.5
Total volume	50 µl

Overlay with 75 µl of mineral oil and let run for 35 cycles under the following conditions:

denature at 94°C for 4 minutes - Start

denature at 94°C for 1 minute
anneal at 55°C for 1 minute
elongate at 72°C for 2 minutes
} Cycle

time delay at 72°C for 7 minutes - End

soak at 4°C

5. Data Analysis

Appropriate tests for statistical analysis were made using standard biometrical procedures (Siegel, 1956; Sokal and Rohlf, 1981). The individual procedures are described later, when they are used, in the appropriate chapter. In all comparisons, $\alpha \leq 0.01$ was used for significance.

Chapter 3. Stability of the Gonadal Phenotype in C57BL/6J.Y-POS XY embryos

1. Introduction

The *Tda* trait that is expressed in day 14 XY embryos of the C57BL/6J.Y-POS consomic strain can be defined as a normally distributed liability to express incomplete testis development (Biddle et al., 1994). The frequencies of embryos in the five gonadal classes (bilateral ovaries, an ovary and an ovotestis, bilateral ovotestes, an ovotestis and a testis, and bilateral testes) are the areas under the normal curve defined by the interclass intervals (see Figure 2, Chapter 1). Therefore, the distribution of gonadal types, rather than the presence/absence of any degree of incomplete testis development, is the expected phenotype of XY embryos of the C57BL/6J.Y-POS strain.

A fetal ovotestis is defined as a gonad with testicular tubules in the middle and clear areas at the cranial and/or caudal ends that are devoid of tubules (Whitten et al., 1979). It has been suggested that these clear areas are in fact undifferentiated testicular tissue that will eventually fill up with testicular tubules as gestation proceeds (Eicher, 1994). This hypothesis can be tested by looking at the gonadal phenotypic distribution of day 14, 15 and 16 XY embryos from the C57BL/6J.Y-POS consomic strain. If the clear areas in the ovotestis that are devoid of tubules do differentiate tubules as the embryo grows older, the types of XY gonad will be shifted towards more normal testis development. The research

question is: Is the phenotypic distribution of types of gonad in the B6.Y-POS day 14 XY embryos, which was defined by Biddle et al. (1994), stable over different gestational days? Furthermore, is there a consistency among various observers in classifying the gonads into one of the five phenotypic classes?

2. Materials and Methods

The C57BL/6J.Y-POS consomic strain was maintained as previously mentioned in Chapter 2 (Table 3) (Biddle et al., 1994). C57BL/6J females were timed mated with B6.Y-POS males and, on the desired gestational day (day 14, 15 or 16), the females were euthanized and embryonic gonads were assessed. A minimum sample of 100 XY embryos for each gestational day was collected. The phenotypic and chromosomal sexing of the embryos were fully addressed in Chapter 2.

3. Results

Table 1 of Appendix B summarizes the data for day 14, 15 and 16 embryos in the B6.Y-POS consomic strain. The phenotypic distributions of types of gonad in the XY embryos are summarized in Table 4 as frequency distributions and are compared to the previously identified distribution of day 14 XY embryos (Biddle et al., 1994).

The distributions of XY embryos in the five phenotypic classes obtained from day 14, 15, and 16 embryos are not significantly different from each other

Table 4. Frequency distribution (%) of types of gonad in day 14, 15, and 16 XY embryos from B6.Y-POS consomic males test-mated to C57BL/6J females and compared to a previously identified distribution of day 14 XY embryos in the B6.Y-POS consomic strain.

Male Strain/Day	O+O	O+OT	OT+OT	OT+T	T+T
B6.Y-POS 14	58	15	24	3	0
B6.Y-POS 15	47	22	26	4	1
B6.Y-POS 16	62	21	10	4	3
B6.Y-POS 14[1]	51	19	27	2	1

[1]Data from Biddle et al., 1994

when a Kolmogorv-Smirnov multiple pairwise analysis is conducted (Table 1, Appendix C).

When my day 14 XY distribution is compared to that previously obtained by Biddle et al. (1994), there is no significant difference (G het= 1.389, 3 df, $P > 0.5$).

A goodness-of-fit test to a 1:1 ratio for the XX to XY chromosomal sex ratio was performed on embryos from the three gestational days (Table 2, Appendix C). No significant deviations from the expected were observed.

4. Discussion and Conclusion

The phenotypic distribution of types of gonad in the B6.Y-POS genotype is stable over the three gestational days of 14, 15 and 16. If the ovotestis is filling up with tubules as the embryo is growing, it is not observed in this window of testis development. The distribution that we observe for day 14 B6.Y-POS XY embryos is the same as that observed for day 15 and day 16 XY embryos. There is no increase in the number of XY embryos in the bilateral testes or even in the ovotestis and testis phenotypic classes. Furthermore, the comparison of the previously identified phenotypic distribution for day 14 B6.Y-POS XY embryos (Biddle et al., 1994) with the phenotypic distribution that I had obtained from my day 14 B6.Y-POS XY embryos, showed no significant statistical difference. This indicates a consistent result between two different observers when classifying the gonads.

It would be a useful future study to look at the stability of the gonadal phenotypic distribution of XY embryos from the other functional class 2 Y chromosome (CLA and AKR/J Y chromosomes) that also produces XY gonadal hermaphrodites in C57BL/6J but at a lower frequency (Biddle and Nishioka, 1988). Does the distribution remain the same over the three different gestational days or does it change towards more normal testes development?

Chapter 4. Definition of XY Gonadal Phenotype in B6.Y-AKR/J, B6.Y-CLA, and B6.Y-Peru W-9 Consomic Strains

1. Introduction

Three functional classes of *domesticus*-type Y chromosome were identified by their interaction with the genetic background of C57BL/6J females (Biddle and Nishioka, 1988; Biddle et al., 1991). C57BL/6J females were crossed to males with different *domesticus*-type Y chromosome. The F_1 males were then backcrossed to C57BL/6J females. Based on the types of gonad observed, the functional classes in the BC_1 embryos are class 1 normal testis development, class 2 low frequency of XY gonadal hermaphrodites and class 3 high frequency of XY gonadal hermaphrodites.

The early studies described the XY gonadal phenotype in the first backcross generation with C57BL/6J females (Biddle and Nishioka, 1988). The XY embryos were not chromosomally sexed by the dot-blot method for the presence of a Y chromosome and XY embryos that might have expressed bilateral ovaries were not included in the analysis. The Peru W-9 Y chromosome could not be assigned unambiguously to either class 2 or 3.

Now that the Y chromosomes have been backcrossed into the genetic background of the C57BL/6J strain for over 30 backcross generations, the consomic strains are homozygous for the autosomal genes of the C57BL/6J. What is observed when a C57BL/6J female is mated to a consomic male is the interaction

between the specific Y chromosome and the female autosomes. It is now important to look at the interaction of the Peru W-9 Y chromosome with the C57BL/6J background. Can we assign this *domesticus*-type Y chromosome unambiguously to either class 2 or class 3? In order to test this hypothesis, the phenotypic distribution of day 15 XY embryos from the B6.Y-Peru W-9 consomic strain was compared to that obtained from the B6.Y-AKR/J and B6.Y-CLA consomic strains.

2. Materials and Methods

The consomic strains were maintained as previously mentioned (Table 3 in Chapter 2). C57BL/6J females were timed mated with B6.Y-AKR/J, B6.Y-CLA and B6.Y-Peru W-9 males. On day 15 of gestation, pregnant females were euthanized and the embryonic gonads were assessed. A minimum sample of 100 XY embryos for each cross was collected.

3. Results

Table 5 summarizes the phenotypic distribution (as percent frequency) of types of gonad in day 15 B6.Y-AKR/J, B6.Y-CLA, and B6.Y-Peru W-9 XY embryos. The complete summary of the data from this study is found in Appendix B, Table 2. Based on the Kolmogorov-Smirnov multiple pairwise analysis (Table 3, Appendix C), there are no differences among the three consomic strains.

Table 5. Frequency distribution (%) of types of gonad in day 15 XY embryos from B6.Y-AKR/J, B6.Y-CLA and B6.Y-Peru W-9 consomic males test-mated to C57BL/6J females.

Male Strain	O+O	O+OT	OT+OT	OT+T	T+T
B6.Y-AKR/J	0	1	18	23	58
B6.Y-CLA	2	1	30	27	40
B6.Y-Peru W-9	1	2	17	31	49

Based on the goodness-of-fit test (Table 4, Appendix C), there is no deviation from an expected 1:1 (XX:XY) chromosomal sex ratio within and among the three strains.

4. Discussion and Conclusion

The original studies that were conducted on the first backcross generation could not unambiguously place the Peru W-9 Y with either the *poschiavinus* or the CLA Y chromosomes (Biddle and Nishioka, 1988). Now these strains have undergone over 30 generations of backcross mating to C57BL/6J females and all the autosomes from the male parent strain have been eliminated by segregation. Therefore, what we observe now is the interaction between the C57BL/6J genome and the specific Y chromosome. The Peru W-9 Y chromosome falls clearly into functional class 2 with low frequency of gonadal hermaphroditism in the C57BL/6J genetic background.

A polymorphic stretch of trinucleotide (CAG) repeats has been identified in the *Sry* gene of several *domesticus*-type Y chromosome (Coward et al., 1994). This polymorphism may be associated with different degrees of gonadal hermaphroditism. The *Sry* gene of the *poschiavinus* Y contains 11 CAG repeats, and it belongs to class 3, high frequency of gonadal hermaphroditism. The *Sry* gene of AKR/J Y chromosome contains 13 CAG repeats, and the strain belongs to class 2, low frequency of gonadal hermaphroditism. Strains that give XY embryos with normal testes in the C57BL/6J genetic background (class 1), contain 12 CAG repeats in their *Sry* gene.

It would be useful to assess the number of CAG repeats in the Peru W-9 and CLA Y chromosomes. If the hypothesis holds true (Coward et al., 1994), these two strains should contain 13 CAG repeats, the same number of repeats as the AKR/J strain. Other polymorphisms have been found within *Sry* (Carlisle et al., 1996), but whether these have any effect on testis development will require quantitative assessment of gonadal development in XY embryos carrying these *Sry* polymorphisms.

Chapter 5. Effect of Autosomal Deletions on the Frequency of Gonadal Hermaphroditism.

1. Introduction

Several deletions of chromosome 5 (W^{19H}, W^{e}) and chromosome 17 (T^{hp}, T^{Orl}) have been reported to affect testis development (reviewed by Burgoyne and Palmer, 1991). Their effect has come to attention through their interaction with the *poschiavinus* Y or AKR/J Y chromosome. These deletions were observed to cause an increase in the frequency of gonadal hermaphroditism. Table 6 summarizes the deletion studies previously conducted, the Y chromosome with which each deletion interacts, and the type of XY individuals observed.

From these data, Burgoyne and Palmer (1991) proposed a model for the interaction of the specific deletions with the respective Y chromosomes. What they proposed is that there is an ovary determining factor which acts earlier on the gonadal developmental scale when the deletion is present. When that happens, all the XY embryos at that particular time of gestation develop as XY females with bilateral ovaries. Nevertheless, the study neglected to assess the types of gonad of the XY embryos that are obtained from the homozygote wild-type females. The embryos from the wild-type females would then be used as controls to which the embryos from the females, heterozygous for the deletion, would be compared. In addition, no in depth assessment of types of gonad into the five phenotypic classes was performed in order to obtain a quantitative measure of testis development.

Table 6. Autosomal deletions of chromosomes 5 and 17, their interaction with the respective Y chromosomes and the type of XY progeny observed (Burgoyne and Palmer, 1991).

Female	Male	Deletion	Chr.	del/+ Progeny
B6.T^{Orl}/+	B6.Y-AKR	T^{Orl}	17	6 XY ovaries
B6	B6-T^{hp}/+ Y-AKR	T^{hp}	17	15 XY ovaries 12 XY ovotestes
B6.W^{e}/+	B6.Y-POS	W^{e}	5	139 XX+XY females 4 XY hermaphrodites 10 XY males
B6.W^{19H}/+	B6.Y-AKR	W^{19H}	5	gonadal hermaphrodites observed, no data presented

The purpose of this study was to test Burgoyne's model by looking at the effect of the T^{Orl} deletion on the frequency of gonadal hermaphroditism. How does the deletion act with various *domesticus*-type Y chromosomes? Do we observe an additive effect or an interaction effect?

Furthermore, is there an effect of other autosomal deletions on the frequency of gonadal hermaphroditism? If so, is it an additive or an interactive effect? Three *Splotch* mutations of chromosome 1 of the mouse were available to me in order to test the above-mentioned hypothesis. These were *Splotch*, which is a small size deletion (Goulding et al., 1993; Strachan and Read, 1994); *Splotch delayed*, which is a point mutation (Goulding et al., 1993; Vogan et al., 1993;

Strachan and Read, 1994); and the *Splotch retarded*, which is a large size deletion that spans 14 cM of chromosome 1 (Epstein et al., 1991; Goulding et al., 1993; Strachan and Read, 1994). Wild-type and heterozygote females were timed mated with B6.Y-POS males and the frequency of gonadal hermaphroditism was assessed in day 15 XY embryos. If the hypothesis that any deletion on any chromosome exacerbates gonadal hermaphroditism is true, then we would expect a considerable shift in the distribution of gonadal phenotypes towards more XY ovaries when heterozygote females are used. Preliminary results have been presented (Nahas et al., 1996).

2. Materials and Methods

The T-Orleans mutation was transferred onto the genetic background of the C57BL/6J inbred strain and a C57BL/6J.T^{Orl} congenic strain was established (Washburn and Eicher, 1989). Considering that the B6.T^{Orl} strain was difficult to breed and that the heterozygote females were hard to obtain, a smaller sample size was collected for this particular study. The T^{Orl}/+ embryos were easily assessed by their short tails compared to the normal sized tails of the +/+ embryos.

The *Splotch delayed* mutation was transferred onto the C57BL/6J genetic background and a C57BL/6J.Sp^{d}/+ (B6.Sp^{d}/+) congenic strain was established (Dr. F. G. Biddle, University of Calgary, personal communication). For the purpose of this study, this strain will be referred to as *Splotch delayed* (+/+ and Sp^{d}/+, respectively). For the *Splotch delayed* and *Splotch retarded* studies, a minimum

sample size of 50 XY embryos from wild-type (+/+) and heterozygote (deletion/+) females was collected. For the *Splotch* study, a minimum sample size of 100 XY embryos was collected. The gonadal phenotypic distributions obtained were assessed for any increase in the frequency of gonadal hermaphroditism.

3. Results

Table 7 summarizes the T^{Orl} crosses; a complete summary of the data from this study can be found in Appendix B, Table 3. Two types of controls were used to compare the gonadal phenotypic distribution of XY T^{Orl}/+ embryos: the +/+ embryos from the B6.T^{Orl} +/+ females and the +/+ littermates of T^{Orl}/+ embryos from the B6.T^{Orl}/+ females.

A three-way test of independence was performed in order to test the independence of the three parameters: the type of cross, the genotype and the chromosomal sex of the embryos. For a level of significance of $\alpha = 0.01$, and 7 df, a G value of 7.854 was obtained and P was greater than 0.1. This implies that the three tested criteria (cross, chromosomal sex and genotype) are jointly independent.

For the B6.Y-POS study, when the phenotypic distribution of +/+ XY embryos from the +/+ females was compared to that obtained for the +/+ XY embryos from the T^{Orl}/+ females (Table 5, Appendix C), no significant difference was observed (Critical D = 0.3652 for $\alpha = 0.01$, Dmax = 0.1163). The phenotypic distribution of day 15 +/+ embryos from the +/+ females was not significantly

different from the distribution that I had obtained for day 15 B6.Y-POS XY embryos (Critical D = 0.2232 for α = 0.01, Dmax = 0.0198) (Table 6, Appendix C). As opposed to the normal distribution obtained for the +/+ XY embryos, all the T^{Orl}/+ XY embryos had bilateral ovaries. The results obtained from T^{Orl}/+ XY embryos are at the end of the developmental scale. It was impossible to determine whether the effect of the deletion is additive or interaction since we could not graph a relative cumulative frequency plot based on a single data point.

Table 7. Summary of the matings of B6.T^{Orl}/+ females and their +/+ female sibs to B6.Y-POS, B6.Y-AKR, and D2.Y-POS sires.

Cross	Genotype	Number of XY embryos					
		O+O	O+OT	OT+OT	OT+T	T+T	Σ
T^{Orl}/+ × B6.Y-POS	T^{Orl}/+	17	0	0	0	0	17
	+/+	14	6	4	1	0	25
+/+ × B6.Y-POS	+/+	48	19	27	4	0	98
T^{Orl}/+ × B6.Y-AKR	T^{Orl}/+	7	0	0	0	0	7
	+/+	0	0	3	5	0	8
+/+ × B6.Y-AKR	+/+	0	1	22	30	37	90
T^{Orl}/+ × D2.Y-POS	T^{Orl}/+	0	0	8	0	0	8
	+/+	0	0	0	0	4	4
+/+ × D2.Y-POS	+/+	0	0	0	0	11	11

A goodness-of-fit test to a 1:1 ratio for the XX to XY chromosomal sex ratio was performed on embryos from B6.Y-POS sires and no significant difference was observed (Table 7, Appendix C). There was no effect of resorption on data analysis using a G-test of heterogeneity (G het = 0.455, 1 df, P = 0.5) (Table 8, Appendix C).

The phenotypic distribution of +/+ XY embryos from the +/+ females that were mated to B6.Y-AKR males was compared to that obtained for the +/+ XY embryos from the T^{Orl}/+ females and no significant difference was observed (Critical D = 0.6014 for α = 0.01, Dmax = 0.4111) (Table 9, Appendix C). The phenotypic distribution of day 15 +/+ embryos from the +/+ females was not significantly different from the distribution that I had obtained for day 15 B6.Y-AKR XY embryos (Critical D = 0.2331 for α = 0.01, Dmax = 0.1683) (Table 10, Appendix C).

All the T^{Orl}/+ XY embryos with the AKR/J Y chromosome had bilateral ovaries whereas the +/+ XY embryos fell into the bilateral testes and the ovotestis and testis classes (Table 7). The results from the T^{Orl}/+ XY embryos with the AKR/J Y are at the end of the developmental scale, similar to the results with the *poschiavinus* Y chromosome. It was impossible to determine whether the effect of the deletion is additive or interaction.

A goodness-of-fit test to a 1:1 (XX:XY) chromosomal sex ratio was performed on embryos from the B6.Y-AKR males. No significant difference was observed (Table 11, Appendix C). A G-test of heterogeneity was performed to test

whether or not there is a difference in resorption rate from the $T^{Orl}/+$ and +/+ females mated to B6.Y-AKR males. The value obtained was statistically significant (G het = 14.069, 1 df, $P < 0.001$) (Table 12, Appendix C). This significant difference may be an effect of the deletion interacting with the specific AKR/J Y chromosome or it could be due to chance. A duplication of the sample would be necessary to determine whether or not the resorption rate is due to chance. However, the resorption rate had no effect on the XX and XY chromosomal segregation ratio of $T^{Orl}/+$ and +/+ embryos from the $T^{Orl}/+$ females that were mated to B6.Y-AKR males.

For the study with D2.Y-POS sires, all the +/+ XY embryos from the +/+ females and the +/+ XY embryos from the $T^{Orl}/+$ females had normal bilateral testes (Table 7). This is what is expected of a B6 × D2 F_1 hybrid. Since DBA/2J is dominant to C57BL/6J in the *Tda* trait (Biddle et al., 1994), the XY embryos from the F_1 generation are expected to have only normal testes like the DBA/2J parent. However, all the $T^{Orl}/+$ XY F_1 embryos expressed only bilateral ovotestes. The number of +/+ and $T^{Orl}/+$ XY F_1 embryos from the $T^{Orl}/+$ females that were found in the two different gonadal classes were assessed by a Fisher exact probability test (Siegel, 1956). The difference was significant at the 0.01 level (one-tailed) and the exact probability is $p = 0.002$. These results show us that there is an effect of the deletion on testis development and suggest it is due to an interaction rather than an additive effect.

A goodness-of-fit test to a 1:1 (XX:XY) chromosomal sex ratio was performed on embryos from the D2.Y-POS sires. No significant difference was observed (Table 13, Appendix C). A G-test of heterogeneity was performed to test whether or not there is a resorption effect on data analysis. The value obtained was statistically not significant (G het = 0.72, 1 df, $P > 0.1$) (Table 14, Appendix C).

A goodness-of-fit test to a 1:1 (XX:XY) chromosomal segregation ratio was performed on $T^{Orl}/+$ and +/+ embryos from the $T^{Orl}/+$ females that were mated to B6.Y-POS, B6.Y-AKR, and D2.Y-POS males (Table 15, Appendix C). No significant difference was calculated indicating an equal transmission rate of XX to XY in $T^{Orl}/+$ and +/+ embryos in all three crosses.

In the assessment of the effect of the *Splotch* mutations and deletions on testis development, there was no simple genetic marker to distinguish *Splotch*/+ from +/+ day 15 embryos. The mating of +/+ *Splotch* females with B6.Y-POS males produced all +/+ embryos whereas the mating of *Splotch*/+ females with B6.Y-POS produced both *Splotch*/+ and +/+ embryos. If there is an effect of *Splotch* deletion or mutation on XY gonadal hermaphroditism, the *Splotch*/+ females should produce a higher frequency of XY gonadal hermaphrodites which are shifted more towards bilateral ovaries. Table 8 summarizes the results obtained from the *Splotch* mutation study as percent frequency distributions. A more detailed summary can be found in Appendix B, Table 4.

Table 8. Frequency distribution (%) of types of gonad in day 15 XY embryos from the three *Splotch* heterozygote females and their +/+ female sibs that were mated to B6.Y-POS males.

Mutation	O+O	O+OT	OT+OT	OT+T	T+T
Splotch retarded +/+	29	20	33	15	3
Splotch retarded Sp^r/+	25	13	42	13	7
Splotch delayed +/+	43	16	30	6	5
Splotch delayed Sp^d/+	32	23	30	9	6
Splotch +/+	23	25	26	20	6
Splotch Sp/+	9	19	32	30	10

The Kolmogorov-Smirnov goodness-of-fit test was performed on each strain to compare the distributions of gonadal phenotypes of XY embryos from the +/+ and deletion/+ females. Table 16 in Appendix C shows no significant difference between the distributions of XY embryos obtained from *Splotch retarded* +/+ and Sp^r/+ females (Critical D for α = 0.01 is 0.3031, Dmax = 0.110). Table 17 in Appendix C shows no significant difference between the distributions of XY embryos obtained from *Splotch delayed* +/+ and Sp^d/+ females (Critical D for α = 0.01 is 0.299, Dmax = 0.107). Table 18 in Appendix C shows no significant difference between the distributions of XY embryos obtained from *Splotch* +/+and Sp/+ females (Critical D for α = 0.01 is 0.229, Dmax = 0.200). This implies that the three *Splotch* mutations, whether point mutation or major deletions, do not

exacerbate the frequency of gonadal hermaphroditism compared to their homozygous wild-type control. Therefore, the effect of deletions, when heterozygous at the W locus (chromosome 5) or T locus (chromosome 17), may be chromosome or gene specific.

A goodness-of-fit test to a 1:1 (XX:XY) chromosomal sex ratio was conducted on embryos from all three *Splotch* studies (Table 19, Appendix C), no significant difference was observed, indicating an equal rate of transmission of the XX and XY chromosomes in the wild-type and heterozygote females of the three studies.

4. Discussion and Conclusion

The effect of the T^{Orl} deletion on testis development was first tested using B6.Y-POS males. In the C57BL/6J background, all of the XY $T^{Orl}/+$ embryos with the *poschiavinus* Y chromosome had bilateral ovaries. There was no evidence for any degree of testis development in the sample size that was assessed. Since the response of the XY embryos from the B6.Y-POS males was at the end of the developmental scale, the only statement we can make from these results is that there is an effect of the deletion, but we can not determine whether this effect is additive or is due to an interaction.

Since the *poschiavinus* Y belongs to class 3, producing a high frequency of gonadal hermaphrodites in C57BL/6J, and since we observed the severe effect of the T^{Orl} deletion with that specific Y chromosome, we used the AKR/J Y

chromosome, which belongs to class 2 and produces a low frequency of gonadal hermaphroditism in C57BL/6J. Perhaps the T^{Orl} deletion would have a less severe effect with the AKR/J Y chromosome so that the gonadal distribution will be measurable on the developmental scale.

The XY T^{Orl}/+ embryos from the B6.Y-AKR males all had bilateral ovaries. The results are still at the end of the gonadal developmental scale and the only conclusion we can make from this study is that there is an effect of the deletion with the AKR/J Y chromosome but we still can not determine whether this effect is additive or an interaction.

Since we could not define the type of effect that the deletion has when it acts with the *poschiavinus* Y and the AKR/J Y chromosomes in the C57BL/6J background, we decided to test the effect that the deletion might have when put in a B6 × D2 F_1 hybrid. All the +/+ XY F_1 embryos from both the +/+ females and T^{Orl}/+ females had normal bilateral testes which is expected since DBA/2J is dominant to C57BL/6J in the *Tda* trait.

The most interesting result was observed with the T^{Orl}/+ XY F_1 embryos. They expressed only bilateral ovotestes and no other phenotypic class was observed. These results suggest that there is an interaction effect, not an additive effect of the deletion with the respective Y chromosome.

To simplify the argument for the effect of the deletion being an interaction, let us suppose that the type of gonads obtained in +/+ F_1 XY embryos from +/+ females mated to D2.Y-POS males can be represented by a normal distribution

(distribution V, Figure 3, Chapter 1). When $T^{Orl}/+$ females are mated to D2.Y-POS males, +/+ and $T^{Orl}/+$ F_1 XY embryos are obtained. If the effect of the T^{Orl} deletion was additive and the single gonadal class that we observed is at the end of the normal distribution from the left-hand side (distribution I, Figure 3, Chapter 1), we would expect to see some gonads in the bilateral ovaries and ovary-ovotestis classes. If it was an additive effect and the single gonadal class that we observed is at the end of the normal distribution from the right-hand side (distribution IV, Figure 3, Chapter 1), we would expect to see some gonads in the ovotestis-testis and bilateral testes classes. If it was an additive effect and the single gonadal class that we observed is at the middle of the normal distribution (distribution II, Figure 3, Chapter 1), we would expect to see some gonads in the two classes that are adjacent to the bilateral ovotestes class. Since none of these expectations was observed, the effect of the T^{Orl} deletion on testis development appears to be an interaction rather than an additive effect. This suggests that the T-Orleans deletion has a qualitatively different effect on testis development compared with what has been observed with the *Tda* trait. The deletion changes the underlying scale of testis development.

An important genetic fact emerges from the interaction effect of the T^{Orl} deletion with the *poschiavinus* Y chromosome. The $T^{Orl}/+$ F_1 embryos from the B6.$T^{Orl}/+$ × D2.Y-POS cross are hemizygous for the DBA/2J wild-type (+) gene on chromosome 17. This hemizygosity was not enough to prevent incomplete testis development in the $T^{Orl}/+$ XY F_1 embryos. Whether this interaction effect is due

to haploinsufficiency of wild-type alleles or a dominant mutation linked to the breakpoint of the deletion on chromosome 17 can not be readily determined. Genetic markers (phenotypes), independent segregation of the mutation (if present) from the T^{Orl} deletion and other deletions on chromosome 17 are the tools by which these questions can be answered.

The three *Splotch* studies demonstrated that the deletion effect on the frequency of gonadal hermaphrodites is chromosome specific. Two deletions and a point mutation of chromosome 1 did not change the frequency of gonadal hermaphrodites with respect to the wild-type distribution. The female mice were mated to B6.Y-POS males, with the Y chromosome belonging to class 3 which produces a high frequency of gonadal hermaphrodites in the C57BL/6J female background. It might be a useful test to assess the effect of the *Splotch* mutation on the gonadal hermaphrodites when it interacts with a class 2 Y chromosome such as AKR/J, CLA, or Peru W-9. If the *Splotch* mutations/deletions cause a change in the gonadal phenotype of class 2 Y chromosome, this would demonstrate a functional difference between class 2 and class 3 *domesticus*-type Y chromosome.

A further important study would be to look at other autosomal deletions on other chromosomes (other than chromosomes 5, and 17) and how they interact with class 2 and class 3 *domesticus*-type Y chromosome. The quantitative analytical method that is based on a threshold model appears to provide a clear framework to assess the developmental effects of the deletions.

Chapter 6. Analysis of the genetic difference between BALB/cByJ-DBA/2J and WC/ReJ-DBA/2J strain pairs.

1. Introduction

The interaction between the *poschiavinus* Y chromosome and the C57BL/6J female genetic background was suggested originally to follow a recessive single gene model (Eicher et al., 1982). Further work assessed the net genetic difference between the two mouse strains C57BL/6J and DBA/2J by their interaction with the *poschiavinus* Y chromosome (Biddle et al., 1994). Normal testis development in DBA/2J with *poschiavinus* Y is dominant to incomplete testis development in C57BL/6J. Backcrosses to the recessive C57BL/6J strain (Biddle et al., 1994) and an F_2 generation (Eisner et al., 1996) rejected the single locus model. A segregation analysis of the second backcross generation suggested that the difference between C57BL/6J and DBA/2J in the *Tda* trait is due to at least four or five gene loci (Eisner et al., 1996).

A strain survey was conducted to define the interaction of the *Tda* trait of several female genetic backgrounds with the *poschiavinus* Y chromosome (Eales et al., 1996). Three additional types of XY gonadal hermaphroditism were observed to fit in between the C57BL/6J and DBA/2J strains (see Figure 3, Chapter 1). The shapes of the distributions were similar to that of C57BL/6J (same variance), but the means of the distributions were shifted towards normal testes. A directional

dominance model in the direction of more complete testis development was suggested for the *Tda* trait (Eales et al., 1996).

The purpose of this study was to determine the genetic difference between the strain pairs BALB/cByJ - DBA/2J and WC/ReJ - DBA/2J in their interaction with the *poschiavinus* Y chromosome.

2. Materials and Methods

Two types of F_1 males were used in this study, BALB × D2.Y-POS and WC × D2.Y-POS. Five BALB × D2.Y-POS males were backcrossed to BALB/cByJ females and a minimum of ten litters from each male was collected. Five WC × D2.Y-POS males were backcrossed to WC/ReJ females and a minimum of ten litters from each male was collected. The day 15 embryos were phenotypically and chromosomally assessed as described in Chapter 2.

3. Results

Tables 5 and 6 in Appendix B summarize the data for the backcross studies. I compared the backcross data to the previously collected estimates of the BALB/cByJ and WC/ReJ strains with *poschiavinus* Y and the respective F_1 matings of these strains with DBA/2J.Y-POS males (Eales et al., 1996). The comparisons were performed in Tables 20 and 21 in Appendix C in order to test the single gene hypothesis for the difference between BALB/cByJ - DBA/2J and WC/ReJ - DBA/2J strain pairs. The observed gonadal distribution in XY embryos from each

backcross was compared to an expected phenotypic distribution for a single gene model with dominance that was calculated from the original estimated parental and F_1 data (Tables 20 and 21, Appendix C).

If the single gene model is correct, then we would expect to see, in the BC_1 distribution, ½ the parental and ½ the F_1 distributions ($BC_1 = ½P + ½F_1$) (Tables 20 and 21, Appendix C). The Kolmogorov-Smirnov test (Siegel, 1956) rejected the single gene model for the difference between BALB/cByJ and DBA/2J and between WC/ReJ and DBA/2J in the *Tda* trait. Therefore, BALB/cByJ and WC/ReJ each differs from DBA/2J in the *Tda* trait by more than one gene locus.

When the segregation ratio of XX:XY embryos from the five males of each study was analyzed (Tables 22 and 23, Appendix C), no deviations were observed indicating a 1:1 transmission ratio.

The resorption number among the five WC × D2.Y-POS F_1 males varied from 15 to 34 resorptions per male (15-30%). A G-test of heterogeneity was performed (Table 24, Appendix C), but there is no significant difference among the test matings of the five F_1 males (G het = 8.487, 4 df, $P > 0.05$). The wide range in the resorption rate appears to be due to chance and is independent of the *Tda* trait. The resorption number among the five BALB × D2.Y-POS F_1 males ranged from 13 to 19 resorptions per male (10-17%). Since the range was not wide enough to necessitate further analysis, no G-test of heterogeneity was performed.

4. Discussion and Conclusion

The BALB/cByJ and the WC/ReJ mouse strains differ from the DBA/2J by more than one gene in their interaction with the *poschiavinus* Y chromosome (Figures 4 and 5, respectively). The first backcross generation from the BALB × D2.Y-POS F_1 males had a lower observed number of gonads in the bilateral ovotestis and ovotestis-testis classes than would be expected for a single gene model. This deficiency was compensated for by an increase in the number of gonads observed in the bilateral testes class. The BC_1 embryos from the WC × D2.Y-POS F_1 males had less than the expected number of gonads in the bilateral ovaries, ovary-ovotestis, and bilateral ovotestes classes. However, the ovotestis-testis and bilateral testes classes contained more gonads than would be expected for a single gene model. Both BC_1 gonadal phenotypic distributions are shifted towards more normal testes development. The BALB × D2.Y-POS and the WC × D2.Y-POS studies both rejected the single gene model for the genetic difference between BALB/cByJ and DBA/2J and between WC/ReJ and DBA/2J in their interaction with the *poschiavinus* Y. The results obtained point towards a multi-locus model for the *Tda* trait. This is an exciting result, since BALB/cByJ and WC/ReJ are intermediate between C57BL/6J and DBA/2J on the gonadal developmental scale with lower frequency of gonadal hermaphrodites than C57BL/6J with the *poschiavinus* Y chromosome (see Figure 3, Chapter 1).

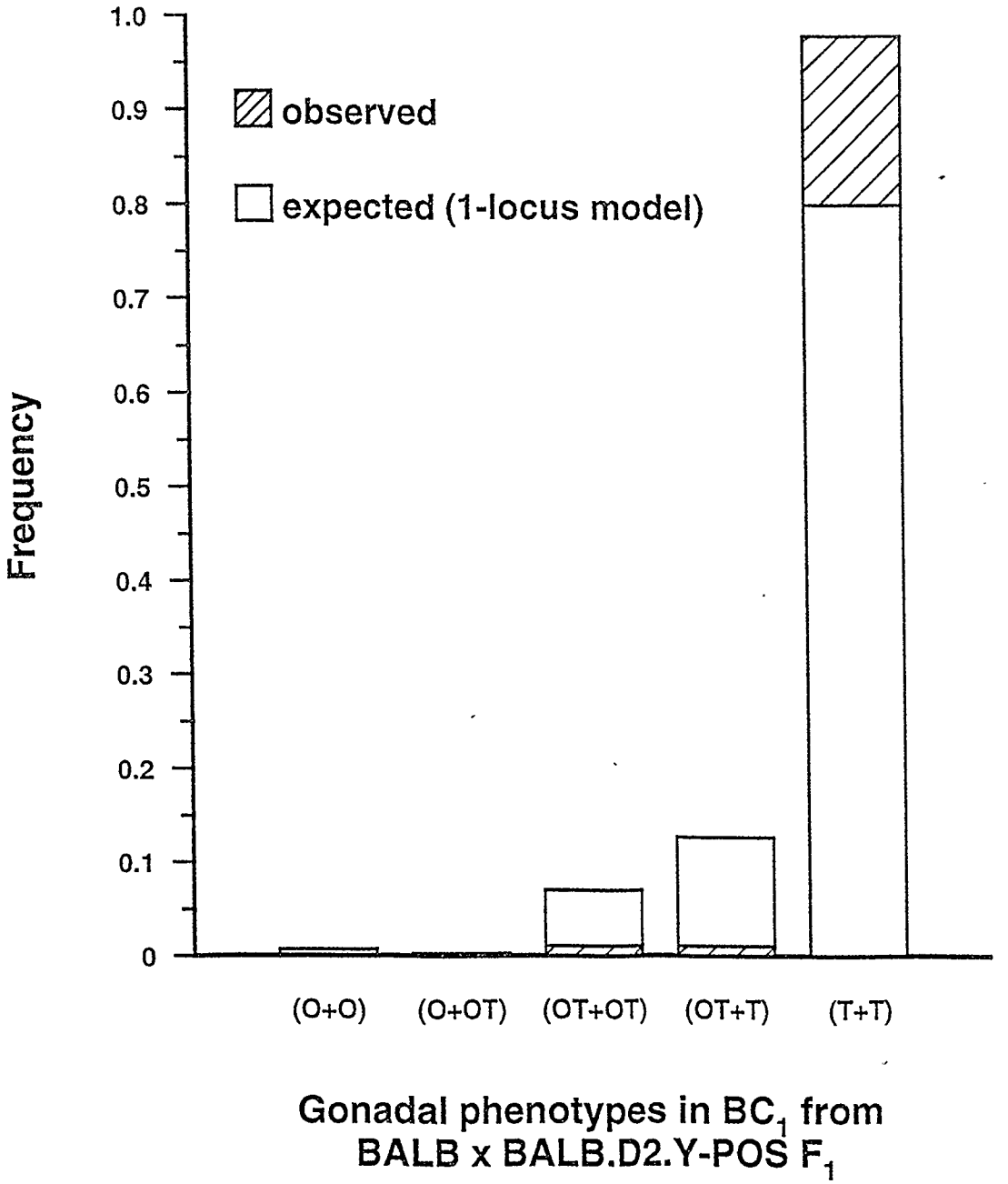

Figure 4. Comparison of the observed and expected gonadal distribution for the BC_1 from the BALB × BALB.D2.Y-POS F_1 cross.

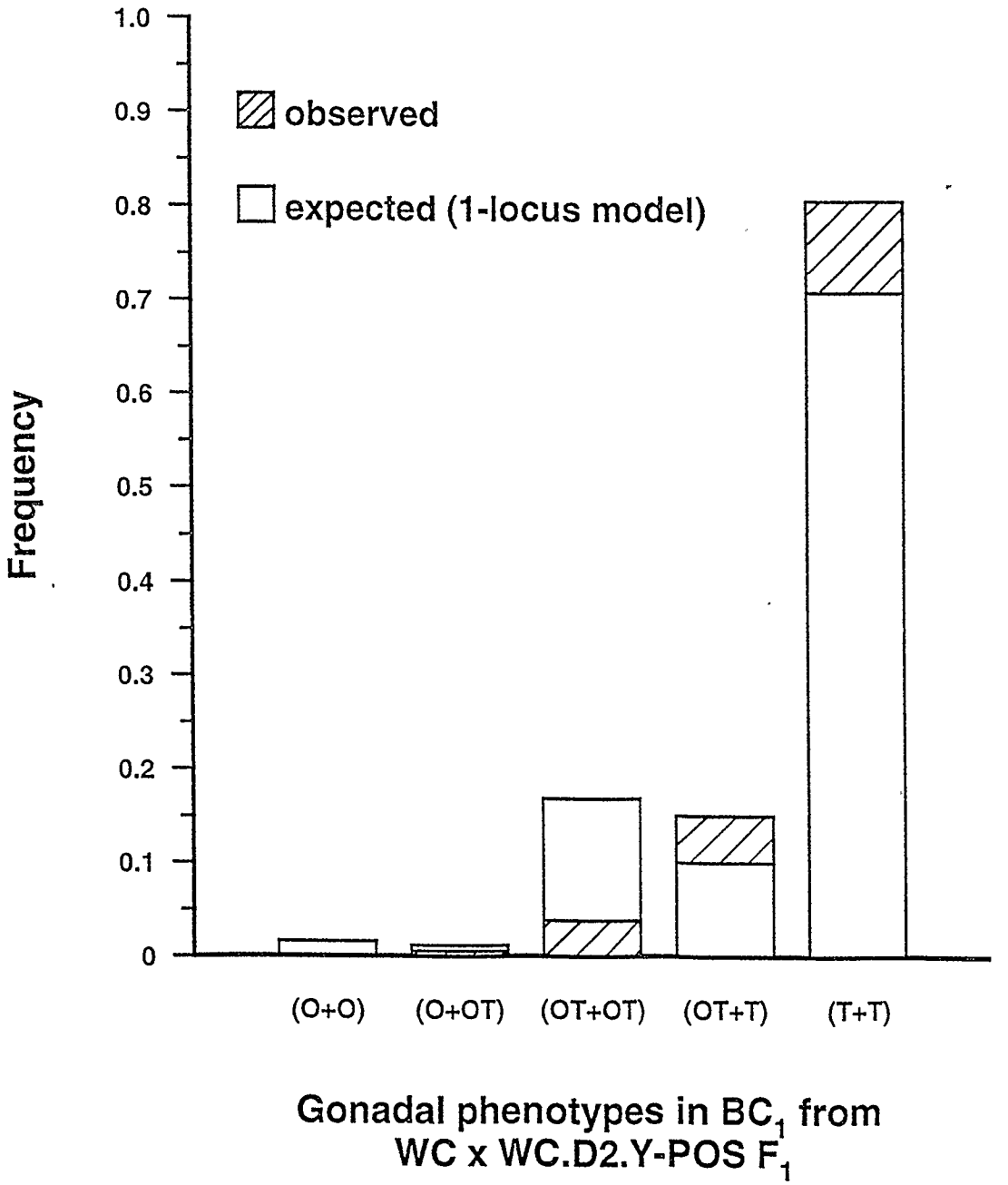

Figure 5. Comparison of the observed and expected gonadal distribution for the BC_1 from the WC × WC.D2.Y-POS F_1 cross.

These strains may be showing us various allelic recombinations of the *Tda* trait and its interaction with the *poschiavinus* Y chromosome to cause the different classes of incomplete testes development.

Important future studies would be to look at the genetic difference between the remaining class (group IV which includes C3H/HeSn.*Paf*, CDS/Lay and CBA/FaCam) (Eales at al., 1996) and DBA/2J in their interaction with the *poschiavinus* Y chromosome. A long term goal would be to look at other pairwise comparisons between each of the middle classes and the C57BL/6J strain as well as a pairwise comparisons among the middle classes themselves. Strain-pairs that differ by one autosomal factor in the *Tda* trait will be the key to mapping the chromosomal locations of the multi-locus *Tda* trait.

Chapter 7. Summary

The understanding of primary sex determination in mammals has improved over the past decade. In the mouse, the notion that the Y chromosome was solely responsible for male development has changed. It is now believed that autosomal factors, represented by the *Tda* trait, interact with the *Sry* region on the Y chromosome. Some *domesticus*-type Y chromosomes interact with the *Tda* trait of the C57BL/6J background and produce incomplete testis development. The *poschiavinus* Y, a local variety of *Mus musculus domesticus*, interacts with the *Tda* trait of certain female genetic backgrounds (see Figure 3, Chapter 1) causing incomplete testis development.

This thesis has answered several important research questions pertaining to primary sex determination in the mouse:

1. The phenotypic distribution of types of gonad in the B6.Y-POS genotype is stable over gestational days 14, 15, and 16. There was no increase in the bilateral testes class as gestation proceeded. The hypothesis that the ovotestis is a transient gonadal phenotype and will fill up with testicular tubules as the embryo grows (Eicher, 1994) does not hold true for the B6.Y-POS genotype. Histological staining of the adult male gonads will provide definite proof whether the ovotestis is a transient or stable phenotype.

2. The phenotypic gonadal distribution obtained for the Peru W-9 Y chromosome was not different from that observed with the AKR/J and CLA Y chromosomes. The Peru W-9 Y now falls into functional class 2 which causes low frequency of gonadal hermaphrodites in C57BL/6J. Over thirty generations of backcross mating to C57BL/6J females have eliminated the male autosomal factors. The interaction that we now observe is between the specific Y chromosome and the female autosomal background.

3. The T^{Orl} deletion has an interaction effect with the *poschiavinus* Y chromosome in a B6 × D2 F_1 background. All the T^{Orl}/+ XY F_1 embryos in the B6.T^{Orl}/+ × D2.Y-POS cross had bilateral ovotestes. Important questions arise: What is the phenotype of these embryos as adults? Do they look like normal males? What do their gonads look like? Do they still show ovarian tissue at either end of the gonad?

It was mentioned earlier that the DBA/2J strain is dominant to the C57BL/6J in the *Tda* trait. The F_1 embryos are expected to have the same gonadal phenotype as the DBA/2J strain. That was true only for the +/+ XY F_1 embryos, not for the T^{Orl}/+ XY F_1 embryos. Hemizygosity for the D2 wild-type allele on chromosome 17 was not able to salvage incomplete testis development. The interaction of the deletion with the *poschiavinus* Y chromosome is different from the *Tda* trait. Whether this effect is due to haploinsufficiency of wild-type alleles or to a dominant mutation cosegregating with the T^{Orl} deletion is not known. Other

deletions of chromosome 17 and segregation analysis may shed light on these questions.

The T^{Orl}/+ XY embryos from B6.T^{Orl}/+ females mated to B6.Y-POS and B6.Y-AKR males all had bilateral ovaries. Do these embryos resemble normal XX females as adults? Do they cycle and produce ova? Are they fertile and able to reproduce?

The *Splotch* study demonstrated that the deletion effect is chromosome specific. Two deletions and a point mutation did not increase the frequency of gonadal hermaphroditism with the B6.Y-POS males. The phenotypic distributions of XY embryos from the +/+ females were not significantly different from those obtained from the deletion/+ females. An important future study is to assess the effect of these deletions/mutations on class 2 *domesticus*-type Y chromosome. If there is an effect, this would be another functional assay of this class. Would the Peru W-9 Y chromosome behave similarly to the AKR/J and CLA Y chromosomes?

4. The genetic difference between the BALB/cByJ - DBA/2J and WC/ReJ - DBA/2J strain pairs was demonstrated to be in more than a single gene locus. An expected BC_1 distribution was calculated based on the parental and F_1 distributions of each cross and the assumption of a 1:1 allelic segregation ratio in the *Tda* trait. The observed phenotypic distribution of the BC_1 embryos in both crosses differed

significantly from the distributions expected of a single gene model. The results obtained support a multi-locus model for the *Tda* trait.

Deletion of the *Sry* putative testis determining region in the mouse results in fertile XY female development (Lovell-Badge and Robertson, 1990) and deletion of Y chromosomal material outside the *Sry* region results also in fertile XY female mice (Capel et al., 1993). In other words, deletions in two regions of the Y chromosome, whether they be in the *Sry* region or not, silence testis development. Positional effects on normal *Sry* function were suggested (Capel et al., 1993). From these observations, one can not help but wonder which of these regions is the correct region for testis determination on the Y chromosome? What is the gonadal phenotype of XY embryos when both deletions occur on the Y chromosome? Does the *Tda* trait interact with one or the other or even with both of these Y factors to cause incomplete testis development? Once these questions have been answered it would be of importance to investigate the Y chromosomal factor(s) with which the T^{Orl} deletion is interacting to cause the uniquely observed gonadal phenotype.

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APPENDIX A

BUFFERS AND REAGENTS

TE

10 mM Tris.Cl (pH 8.0)	5 ml of 1 M
1 mM EDTA	1 ml of 0.5 M
distilled deionized water	494 ml
Total volume	500 ml

Lysis Buffer

100 mM Tris.Cl (pH 8.5)	50 ml of 1 M
5 mM EDTA	5 ml of 0.5 M
0.2% SDS	10 ml of 10%
200 mM NaCl	5.844 g
distilled deionized water	430 ml
Total volume	500 ml

0.4 N NaOH, 2 × SSC

0.4 N NaOH	60 ml of 4 N
2 × SSC	60 ml of 20 × SSC
distilled deionized water	480 ml
Total volume	600 ml

Hybridization solution

5 × SSC	25 ml of 20 × SSC
1% Blocking reagent	1 g (Cat.No. 1096176)
0.1% N-Laurylsarcosine	500 µl of 20%
0.02% SDS	200 µl of 10%
50% formamide	50 ml
distilled deionized water	24.3 ml
Total volume	100 ml

2 × SSC, 0.1% SDS

2 × SSC	200 ml of 20 × SSC
0.1% SDS	20 ml of 10%
distilled deionized water	1780 ml
Total volume	2000 ml

0.1 × SSC, 0.1% SDS

0.1 × SSC	100 ml of 20 × SSC
0.1% SDS	10 ml of 10%
distilled deionized water	890 ml
Total volume	1000 ml

Buffer A

100 mM Tris.Cl	31.52 g
150 mM NaCl	17.40 g
distilled deionized water	2000 ml

Adjust to pH 7.5 with 5 ml of 5 N NaOH

Buffer B

2% (w/v) Blocking reagent	2 g
Buffer A	100 ml

Store at 4°C

Buffer C

0.1 M Tris.Cl (pH 9.5)	15.76 g
0.1 M NaCl	5.84 g
50 mM $MgCl_2.6H_2O$	10.18 g
5 N NaOH	10 ml
distilled deionized water	990 ml
Total volume	1000 ml

20 × SSC

3 M NaCl	175.3 g
0.3 M Sodium Citrate	88.2 g
distilled deionized water	1000 ml
adjust to pH 7.0 with 5 N NaOH	

1 M Tris.Cl pH 8.0

Trizma.HCl	78.8 g
distilled deionized water	500 ml
adjust to pH 8.0 with 5 N NaOH	

0.5 M EDTA pH 8.0

EDTA.2H_2O	18.61 g
NaOH pellets	2 g
distilled deionized water	80 ml

APPENDIX B

DATA SUMMARY

Table 1. Summary of the matings of B6.Y-POS sires with C57BL/6J females over days 14, 15 and 16 of gestation.

B6.Y-POS	No. of litters	Resorbed (%)	Live Embryos						
			XX	XY					
				O+O	O+OT	OT+OT	OT+T	T+T	Total XY (%)
day 14	32	44/279 (16)	128	62	16	26	3	0	107 (46)
day 15	41	48/301 (16)	136	55	26	30	5	1	117 (46)
day 16	39	63/310 (20)	134	70	24	11	4	4	113 (46)
Total	112	155/890 (17)	398	187	66	67	12	5	337 (46)

Table 2. Summary of the test-matings of B6.Y-AKR/J, B6.Y-CLA and B6.Y-Peru W-9 sires with C57BL/6J females.

Strain	No. of litters	Resorbed (%)	Live Embryos						
			XX	XY					
				O+O	O+OT	OT+OT	OT+T	T+T	Total XY (%)
B6.Y-AKR/J	35	62/283 (22)	114	0	1	19	25	62	107 (48)
B6.Y-CLA	29	40/263 (15)	120	2	1	31	28	41	103 (46)
B6.Y-Peru	28	45/253 (18)	108	1	2	17	31	49	100 (48)
Total	92	147/799 (18)	342	3	4	67	84	152	310 (48)

Table 3. Summary of the matings of B6.T^{Orl}/+ females and their +/+ female sibs to B6.Y-POS, B6.Y-AKR, and D2.Y-POS sires.

Cross	No. of litters	Resorbed (%)	Genotype	XX	Live Embryos XY					
					O+O	O+OT	OT+OT	OT+T	T+T	Σ
T^{Orl}/+ × B6.Y-POS	13	15/104 (14)	T^{Orl}/+	18	17	0	0	0	0	17
			+/+	29	14	6	4	1	0	25
+/+ × B6.Y-POS	27	28/238 (12)	+/+	112	48	19	27	4	0	98
T^{Orl}/+ × B6.Y-AKR	8	23/67 (34)	T^{Orl}/+	17	7	0	0	0	0	7
			+/+	12	0	0	3	5	0	8
+/+ × B6.Y-AKR	25	28/215 (13)	+/+	97	0	1	22	30	37	90
T^{Orl}/+ × D2.Y-POS	3	1/31 (3)	T^{Orl}/+	9	0	0	8	0	0	8
			+/+	9	0	0	0	0	4	4
+/+ × D2.Y-POS	5	3/38 (8)	+/+	24	0	0	0	0	11	11

Table 4. Summary of the test-matings of the three different *Splotch* mutations with B6.Y-POS sires.

Splotch Strain	No. of litters	Resorbed (%)	Live Embryos XX	XY					
				O+O	O+OT	OT+OT	OT+T	T+T	Total XY (%)
Retarded +/+	15	26/152 (17)	65	18	12	20	9	2	61 (48)
Retarded Sp^r/+	14	25/142 (18)	62	14	7	23	7	4	55 (47)
Delayed +/+	16	19/151 (13)	69	27	10	19	4	3	63 (48)
Delayed Sp^d/+	19	25/152 (16)	71	18	13	17	5	3	56 (44)
Splotch +/+	29	18/225 (8)	105	23	26	27	20	6	102 (49)
Splotch Sp/+	24	19/223 (9)	104	9	19	32	30	10	100 (49)
Total	117	132/1045 (13)	476	109	87	138	75	28	437 (48)

Table 5. Summary of the test-matings of BALB × D2.Y-POS sires with BALB/cByJ females.

BALB × D2.Y-POS sire	No. of litters	Resorbed (%)	Live Embryos XX	XY					
				O+O	O+OT	OT+OT	OT+T	T+T	Total XY(%)
1	11	15/135 (11)	51	0	0	0	0	69	69 (58)
2	10	13/128 (10)	59	0	0	0	0	56	56 (49)
3	10	16/124 (13)	52	0	0	0	0	56	56 (52)
4	10	15/127 (12)	53	0	0	1	0	58	59 (53)
5	10	19/113 (17)	45	0	0	2	3	44	49 (52)
Total	51	78/627 (12)	260	0	0	3	3	283	289 (53)

Table 6. Summary of the test-matings of WC × D2.Y-POS sires with WC/ReJ females.

WC × D2.Y-POS sire	No. of litters	Resorbed (%)	Live Embryos						
			XX	XY					
				O+O	O+OT	OT+OT	OT+T	T+T	Total XY (%)
1	10	15/103 (15)	50	0	0	2	8	28	38 (43)
2	10	19/91 (21)	40	0	0	2	6	24	32 (44)
3	10	20/99 (20)	39	0	0	1	5	34	40 (51)
4	10	17/94 (18)	38	0	1	1	2	35	39 (51)
5	11	34/113 (30)	42	0	0	1	7	29	37 (47)
Total	51	105/500 (21)	209	0	1	7	28	150	186 (47)

APPENDIX C

DATA ANALYSIS

Table 1. Kolmogorov-Smirnov multiple pairwise comparisons of the observed distributions of gonadal phenotypes in the B6.Y-POS study.

Strain (day)		XY embryos					
		O+O	O+OT	OT+OT	OT+T	T+T	Σ
I) B6.Y-POS (14)		62	16	26	3	0	107
	rcN	62	78	104	107	107	
	rcf	0.579	0.729	0.972	1	1	
II) B6.Y-POS (15)		55	26	30	5	1	117
	rcN	55	81	111	116	117	
	rcf	0.470	0.692	0.949	0.991	1	
III) B6.Y-POS (16)		70	24	11	4	4	113
	rcN	70	94	105	109	113	
	rcf	0.619	0.832	0.929	0.965	1	
I & II | Δrcf |		0.109	0.037	0.023	0.009	0	
I & III | Δrcf |		0.040	0.103	0.043	0.035	0	
II & III | Δrcf |		0.149	0.140	0.020	0.026	0	

$\alpha = 0.01$ $\alpha' = 1 - (1-\alpha)^{1/k}$ with k= number of comparisons (3)
$\alpha' = 0.0033$

D_α for $\alpha' = 0.0033$ is
* 0.239 for I & II with $n_1 = 107$ and $n_2 = 117$
  Dmax = 0.109 Not Significant
* 0.241 for I & III with $n_1 = 107$ and $n_2 = 113$
  Dmax = 0.103 Not Significant
* 0.236 for II & III with $n_1 = 117$ and $n_2 = 113$
  Dmax = 0.149 Not Significant

Table 2. A goodness-of-fit test to the 1:1 chromosomal segregation ratio (XX:XY) in the B6.Y-POS study over three different gestational days.

B6.Y-POS	Live Progeny			df	G	P	
	XX	XY	Total				
day 14	128	107	235	1	1.880	>0.1	NS
day 15	136	117	253	1	1.430	>0.1	NS
day 16	134	113	247	1	1.790	>0.1	NS
Total G				3	5.100	>0.1	NS
Pooled G	398	337	735	1	5.070	>0.01	NS
G het				2	0.030	>0.1	NS

NS = Not significant for $\alpha = 0.01$.

Table 3. Kolmogorov-Smirnov multiple pairwise comparisons of the observed distributions of gonadal phenotypes in the class 2 Y chromosome study.

Strain		XY embryos					
		O+O	O+OT	OT+OT	OT+T	T+T	Σ
I) B6.Y-AKR/J		0	1	19	25	62	107
	rcN	0	1	20	45	107	
	rcf	0	0.0093	0.1869	0.4206	1	
II) B6.Y-CLA		2	1	31	28	41	103
	rcN	2	3	34	62	103	
	rcf	0.0194	0.0291	0.3301	0.6019	1	
III) B6.Y-Peru		1	2	17	31	49	100
	rcN	1	3	20	51	100	
	rcf	0.010	0.030	0.200	0.510	1	
I & II | Δ rcf |		0.0194	0.0198	0.1432	0.1813	0	
I & III | Δ rcf |		0.0100	0.0207	0.0131	0.0894	0	
II & III | Δ rcf |		0.0094	0.0009	0.1301	0.0919	0	

$\alpha = 0.01$ $\alpha' = 1 - (1-\alpha)^{1/k}$ with k = number of comparisons (3)
$\alpha' = 0.0033$

D_α for $\alpha' = 0.0033$ is
* 0.2469 for I & II with $n_1 = 107$ and $n_2 = 103$
Dmax = 0.1813 Not Significant
* 0.2488 for I & III with $n_1 = 107$ and $n_2 = 100$
Dmax = 0.0894 Not Significant
* 0.2511 for II & III with $n_1 = 103$ and $n_2 = 100$
Dmax = 0.1301 Not Significant

Table 4. A goodness-of-fit test to the 1:1 chromosomal segregation ratio (XX:XY) in the class 2 Y chromosome study.

Strain	Live Progeny			df	G	P	
	XX	XY	Total				
B6.Y-AKR/J	114	107	221	1	0.222	>0.1	NS
B6.Y-CLA	120	103	223	1	1.297	>0.1	NS
B6.Y-Peru W-9	108	100	208	1	0.308	>0.1	NS
Total G				3	1.827	>0.1	NS
Pooled G	342	310	652	1	1.571	>0.1	NS
G het				2	0.256	>0.1	NS

NS = Not significant for $\alpha = 0.01$.

Table 5. Kolmogorov-Smirnov goodness-of-fit test of the observed distributions of gonadal phenotypes from the +/+ embryos from the +/+ and T^{Orl}/+ females mated to B6.Y-POS males.

Origin of +/+ embryos		XY embryos					
		O+O	O+OT	OT+OT	OT+T	T+T	Σ
+/+ female		48	19	27	4	0	98
	rcN	48	67	94	98	98	
	rcf	0.4898	0.6837	0.9592	1	1	
T^{Orl}/+ female		14	6	4	1	0	25
	rcN	14	20	24	25	25	
	rcf	0.56	0.8	0.96	1	1	
	| Δ rcf |	0.0702	0.1163	0.0008	0	0	

Critical D for $\alpha = 0.01$ is 0.3652 for $n_1 = 98$ and $n_2 = 25$

Dmax = 0.1163 Not Significant.

Table 6. Kolmogorov-Smirnov goodness-of-fit test of the observed distributions of gonadal phenotypes from the +/+ embryos from the +/+ × B6.Y-POS cross and the B6 × B6.Y-POS cross.

Cross		XY embryos					
		O+O	O+OT	OT+OT	OT+T	T+T	Σ
+/+ × B6.Y-POS		48	19	27	4	0	98
	rcN	48	67	94	98	98	
	rcf	0.4898	0.6837	0.9592	1	1	
B6 × B6.Y-POS		55	26	30	5	1	117
	rcN	55	81	111	116	117	
	rcf	0.470	0.692	0.949	0.991	1	
	| Δ rcf |	0.0198	0.0083	0.0102	0.009	0	

Critical D for $\alpha = 0.01$ is 0.2232 for $n_1 = 98$ and $n_2 = 117$

Dmax = 0.0198 Not Significant.

Table 7. A goodness-of-fit test to the 1:1 chromosomal segregation ratio (XX:XY) in the +/+ × B6.Y-POS embryos and T^{Orl}/+ × B6.Y-POS embryos.

Cross	Genotype	Live Progeny			df	G	P	
		XX	XY	Total				
T^{Orl}/+ × B6.Y-POS	T^{Orl}/+	18	17	35	1	0.029	>0.1	NS
	+/+	29	25	54	1	0.297	>0.1	NS
+/+ × B6.Y-POS	+/+	112	98	210	1	0.934	>0.1	NS
Total G					3	1.260	>0.1	NS
Pooled G		159	140	299	1	1.208	>0.1	NS
G het					2	0.052	>0.1	NS

NS = Not significant for $\alpha = 0.01$.

Table 8. G-test of heterogeneity between live and resorbed embryos in the $T^{Orl}/+$ and +/+ crosses to B6.Y-POS males.

Cross	Resorption		
	Live	Resorbed	Total
$T^{Orl}/+$ × B6.Y-POS	89	15	104
+/+ × B6.Y-POS	210	28	238
Total	299	43	342

G het = 0.455 1 df P = 0.5 Not Significant for $\alpha = 0.01$

Table 9. Kolmogorov-Smirnov goodness-of-fit test of the observed distributions of gonadal phenotypes from the +/+ embryos from the +/+ and T^{Orl}/+ females mated to B6.Y-AKR males.

Origin of +/+ embryos		XY embryos					
		O+O	O+OT	OT+OT	OT+T	T+T	Σ
+/+ female		0	1	22	30	37	90
	rcN	0	1	23	53	90	
	rcf	0	0.0111	0.2556	0.5889	1	
T^{Orl}/+ female		0	0	3	5	0	8
	rcN	0	0	3	8	8	
	rcf	0	0	0.375	1	1	
	$\lvert \Delta \text{rcf} \rvert$	0	0.0111	0.1194	0.4111	0	

Critical D for $\alpha = 0.01$ is 0.6014 for $n_1 = 90$ and $n_2 = 8$

Dmax = 0.4111 Not Significant.

Table 10. Kolmogorov-Smirnov goodness-of-fit test of the observed distributions of gonadal phenotypes from the +/+ embryos from the +/+ × B6.Y-AKR cross and the B6 × B6.Y-AKR cross.

Cross		XY embryos					
		O+O	O+OT	OT+OT	OT+T	T+T	Σ
+/+ × B6.Y-AKR		0	1	22	30	37	90
	rcN	0	1	23	53	90	
	rcf	0	0.0111	0.2556	0.5889	1	
B6 × B6.Y-AKR		0	1	19	25	62	107
	rcN	0	1	20	45	107	
	rcf	0	0.0093	0.1869	0.4206	1	
	$\lvert \Delta \text{rcf} \rvert$	0	0.0018	0.0687	0.1683	0	

Critical D for $\alpha = 0.01$ is 0.2331 for $n_1 = 90$ and $n_2 = 107$

Dmax = 0.1683 Not Significant.

Table 11. A goodness-of-fit test to the 1:1 chromosomal segregation ratio (XX:XY) in the +/+ × B6.Y-AKR embryos and T^{Orl}/+ × B6.Y-AKR embryos.

Cross	Genotype	Live Progeny			df	G	P	
		XX	XY	Total				
T^{Orl}/+ ×	T^{Orl}/+	17	7	24	1	4.296	>0.025	NS
B6.Y-AKR	+/+	12	8	20	1	0.805	>0.1	NS
+/+ ×	+/+	97	90	187	1	0.262	>0.1	NS
B6.Y-AKR								
Total G					3	5.363	>0.1	NS
Pooled G		126	105	231	1	1.912	>0.1	NS
G het					2	3.451	>0.1	NS

NS = Not significant for $\alpha = 0.01$.

Table 12. G-test of heterogeneity between live and resorbed embryos in the $T^{Orl}/+$ and +/+ crosses to B6.Y-AKR males.

Cross	Resorption		
	Live	Resorbed	Total
$T^{Orl}/+$ × B6.Y-AKR	44	23	67
+/+ × B6.Y-AKR	187	28	215
Total	231	51	282

G het = 14.069 1 df $P < 0.001$ Significant for $\alpha = 0.01$

Table 13. A goodness-of-fit test to the 1:1 chromosomal segregation ratio (XX:XY) in the +/+ × D2.Y-POS embryos and T^{Orl}/+ × D2.Y-POS embryos.

Cross	Genotype	Live Progeny			df	G	P	
		XX	XY	Total				
T^{Orl}/+ ×	T^{Orl}/+	9	8	17	1	0.059	>0.1	NS
D2.Y-POS	+/+	9	4	13	1	1.974	>0.1	NS
+/+ ×	+/+	24	11	35	1	4.946	>0.025	NS
D2.Y-POS								
Total G					3	6.979	>0.05	NS
Pooled G		42	23	65	1	5.636	>0.01	NS
G het					2	1.343	>0.1	NS

NS = Not significant for $\alpha = 0.01$.

Table 14. G-test of heterogeneity between live and resorbed embryos in the $T^{Orl}/+$ and +/+ crosses to D2.Y-POS males.

Cross	Resorption		
	Live	Resorbed	Total
$T^{Orl}/+$ × D2.Y-POS	30	1	31
+/+ × D2.Y-POS	35	3	38
Total	65	4	69

G het = 0.72 1 df $P > 0.1$

Not Significant for $\alpha = 0.01$

Table 15. A goodness-of-fit test to the 1:1 chromosomal segregation ratio (XX:XY) in the $T^{Orl}/+$ and +/+ embryos from the $T^{Orl}/+$ females mated to B6.Y-POS, B6.Y-AKR and D2.Y-POS males.

Cross	Genotype	Live Progeny			df	G	P	
		XX	XY	Total				
$T^{Orl}/+$ ×	$T^{Orl}/+$	18	17	35	1	0.029	>0.1	NS
B6.Y-POS	+/+	29	25	54	1	0.297	>0.1	NS
$T^{Orl}/+$ ×	$T^{Orl}/+$	17	7	24	1	4.296	>0.025	NS
B6.Y-AKR	+/+	12	8	20	1	0.805	>0.1	NS
$T^{Orl}/+$ ×	$T^{Orl}/+$	9	8	17	1	0.059	>0.1	NS
D2.Y-POS	+/+	9	4	13	1	1.974	>0.1	NS
Total G					6	7.460	>0.1	NS
Pooled G		94	69	163	1	3.849	>0.025	NS
G het					5	3.611	>0.1	NS

NS = Not significant for $\alpha = 0.01$.

Table 16. Kolmogorov-Smirnov goodness-of-fit test of the observed distributions of gonadal phenotypes in the *Splotch retarded* study.

Strain		XY embryos					
		O+O	O+OT	OT+OT	OT+T	T+T	Σ
Sp^r (+/+)		18	12	20	9	2	61
	rcN	18	30	50	59	61	
	rcf	0.295	0.492	0.819	0.967	1	
Sp^r/+		14	7	23	7	4	55
	rcN	14	21	44	51	55	
	rcf	0.254	0.382	0.800	0.927	1	
	| Δ rcf |	0.041	0.110	0.019	0.040	0	

Critical D for $\alpha = 0.01$ is 0.3031 for $n_1 = 61$ and $n_2 = 55$

Dmax = 0.110 Not Significant.

Table 17. Kolmogorov-Smirnov goodness-of-fit test of the observed distributions of gonadal phenotypes in the *Splotch delayed* study.

Strain		XY embryos					
		O+O	O+OT	OT+OT	OT+T	T+T	Σ
Sp^d (+/+)		27	10	19	4	3	63
	rcN	27	37	56	60	63	
	rcf	0.428	0.587	0.889	0.952	1	
Sp^d/+		18	13	17	5	3	56
	rcN	18	31	48	53	56	
	rcf	0.321	0.554	0.857	0.946	1	
	| Δ rcf |	0.107	0.033	0.032	0.006	0	

Critical D for $\alpha = 0.01$ is 0.299 for $n_1 = 63$ and $n_2 = 56$

Dmax = 0.107 Not Significant.

Table 18. Kolmogorov-Smirnov goodness-of-fit test of the observed distributions of gonadal phenotypes in the *Splotch* study.

Strain		XY embryos					
		O+O	O+OT	OT+OT	OT+T	T+T	Σ
Splotch (+/+)		23	26	27	20	6	102
	rcN	23	49	76	96	102	
	rcf	0.225	0.480	0.745	0.941	1	
Sp/+		9	19	32	30	10	100
	rcN	9	28	60	90	100	
	rcf	0.090	0.280	0.600	0.900	1	
	$\lvert \Delta \text{rcf} \rvert$	0.135	0.200	0.145	0.041	0	

Critical D for $\alpha = 0.01$ is 0.229 for $n_1 = 102$ and $n_2 = 100$

Dmax = 0.200 Not Significant.

Table 19. A goodness-of-fit test to the 1:1 chromosomal segregation ratio (XX:XY) in the *Splotch* mutation study.

Strain	Live Progeny			df	G	P	
	XX	XY	Total				
Retarded							
(+/+)	65	61	126	1	0.127	>0.1	NS
(Sp^r/+)	62	55	117	1	0.419	>0.1	NS
Delayed							
(+/+)	69	63	132	1	0.273	>0.1	NS
(Sp^d/+)	71	56	127	1	1.776	>0.1	NS
Splotch							
(+/+)	105	102	207	1	0.043	>0.1	NS
(Sp/+)	104	100	204	1	0.078	>0.1	NS
Total G				6	2.716	>0.1	NS
Pooled G	476	437	913	1	1.666	>0.1	NS
G het				5	1.050	>0.1	NS

NS = Not significant for $\alpha = 0.01$.

Table 20. Kolmogorov-Smirnov goodness-of-fit test of the observed distribution of gonadal phenotypes in BC_1 embryos (BALB × BALB.D2.Y-POS F_1) to a single gene, recessive model.

Generation	Frequency of XY Gonadal Phenotypes				
	O+O	O+OT	OT+OT	OT+T	T+T
P BALB.Y-POS[2] estimated (observed)	0.0127	0.0042	0.1392	0.2532	0.591
F_1 BALB × D2.Y-POS[2] (observed)	0	0	0	0	1
BC_1 BALB × BALB.D2.Y-POS F_1 (observed)	0	0	0.0104	0.0104	0.9792
BALB × BALB.D2.Y-POS F_1 (expected)	0.00635	0.0021	0.0696	0.1266	0.7954

[2] Data from Eales et al., 1996

Critical | Dmax | for $\alpha = 0.01$ (2-tailed probability) is 0.0957 for a sample size n = 289.

Observed | Dmax | is 0.1838, reject hypothesis of a single gene difference.

Table 21. Kolmogorov-Smirnov goodness-of-fit test of the observed distribution of gonadal phenotypes in BC_1 embryos (WC × WC.D2.Y-POS F_1) to a single gene, recessive model.

Generation	Frequency of XY Gonadal Phenotypes				
	O+O	O+OT	OT+OT	OT+T	T+T
P WC.Y-POS[2] estimated (observed)	0.031	0.022	0.336	0.199	0.412
F_1 WC × D2.Y-POS[2] (observed)	0	0	0	0	1
BC_1 WC × WC.D2.Y-POS F_1 (observed)	0	0.0054	0.0376	0.1505	0.8065
WC × WC.D2.Y-POS F_1 (expected)	0.0155	0.011	0.168	0.0995	0.706

[2] Data from Eales et al., 1996.

Critical | Dmax | for $\alpha = 0.01$ (2-tailed probability) is 0.1193 for a sample size n = 186.

Observed | D max | is 0.1515; reject hypothesis of a single gene difference.

Table 22. Goodness-of-fit test to the 1:1 chromosomal segregation ratio (XX:XY) in the BALB × BALB.D2.Y-POS F_1 study.

BALB.D2.Y-POS F_1 Sire No.	Live Progeny XX	XY	Total	df	G	P	
1	51	69	120	1	2.710	>0.05	NS
2	59	56	115	1	0.078	>0.1	NS
3	52	56	108	1	0.148	>0.1	NS
4	53	59	112	1	0.322	>0.1	NS
5	45	49	94	1	0.170	>0.1	NS
Total G				5	3.428	>0.1	NS
Pooled G	260	289	549	1	1.533	>0.1	NS
G het				4	1.895	>0.1	NS

NS = Not significant for $\alpha = 0.01$.

Table 23. Goodness-of-fit test to the 1:1 chromosomal segregation ratio (XX:XY) in the WC × WC.D2.Y-POS F_1 study.

WC.D2.Y-POS F_1 Sire No.	Live Progeny XX	XY	Total	df	G	P	
1	50	38	88	1	1.641	>0.1	NS
2	40	32	72	1	0.891	>0.1	NS
3	39	40	79	1	0.013	>0.1	NS
4	38	39	77	1	0.013	>0.1	NS
5	42	37	79	1	0.317	>0.1	NS
Total G				5	2.875	>0.1	NS
Pooled G	209	186	395	1	1.339	>0.1	NS
G het				4	1.536	>0.1	NS

NS = Not significant for $\alpha = 0.01$.

Table 24. G-test of heterogeneity of resorption rate in test matings among the five WC.D2.Y-POS F_1 sires.

Sire	Resorption		
	Live	Resorbed	Total
1	88	15	103
2	72	19	91
3	79	20	99
4	77	17	94
5	79	34	113
Total	395	105	500

G het = 8.487 4 df $P > 0.05$

Not Significant for $\alpha = 0.01$