Development 122, 3597-3605 (1996)
Printed in Great Britain © The Company of Biologists Limited 1996
DEV3451

The dominant white spotting oncogene allele *Kit*$^{W\text{-}42J}$ exacerbates XY^{DOM} sex reversal

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SUMMARY

The Y chromosome from certain populations of *M. m. domesticus* is incapable of normal testis determination in the B6 inbred strain resulting in XY hermaphrodites or XY females (XY^{DOM} sex reversal). B6 consomic strains have been developed with either transient (B6-Y^{AKR}) or severe (B6-Y^{TIR}) XY^{DOM} sex reversal. We report that a point mutation, the dominant white spotting oncogene allele, *Kit*$^{W\text{-}42J}$, exacerbates XY^{DOM} sex reversal. In B6-Y^{AKR}, penetrance of the trait is low; however, in B6-Y^{TIR}, *Kit*$^{W\text{-}42J}$ exacerbated sex reversal to such an extent that almost all XY progeny developed into females. The exacerbation of sex reversal was not linked to retardation of early fetal growth or reduction of testis size. Furthermore, semiquantitative RT-PCR for the testis-determining gene, *Sry*, suggests that exacerbation of sex reversal in B6-Y^{TIR} is not due to blockade of *Sry* expression, a substantial delay in initiation of *Sry* expression, or exceptionally low levels of *Sry* mRNAs. We propose that *Kit*$^{W\text{-}42J}$ enhances sex reversal by adversely affecting a critical step in testis differentiation that is downstream of *Sry*.

Key words: *Kit*, sex determination, testis differentiation, sex reversal, Y chromosome, angiogenesis, mouse

INTRODUCTION

Whether the mammalian fetal gonad develops into a testis or ovary is determined by the presence or absence of the Y chromosome (Y Chr). The gene on the Y Chr that is responsible for triggering testis determination is the sex-determining region of the Y (*Sry*) (Gubbay et al., 1990; Sinclair et al., 1990). *Sry* encodes a high mobility group (HMG) protein, a class of non-histone, chromosomal proteins comprising three families: HMG-1/HMG-2, HMG I-Y/HMG I-C and HMG-14/HMG-17 (Bustin et al., 1990). SRY belongs to the HMG-1/-2 family, which is characterized by a DNA-binding motif designated the HMG domain (Grosschedl et al., 1994). Only the HMG domain is evolutionarily conserved among SRY proteins. Although many HMG-1/-2 proteins non-specifically bind bent DNA and DNA four-way junctions, some, like SRY, are sequence-specific transcription factors (Grosschedl et al., 1994). SRY recognizes and binds the nucleotide sequence 5′-CATTGTT-3′, inducing the target DNA sequence to bend into an angle of 60°-85° (Giese et al., 1994).

One strategy to decipher the molecular genetics of sex determination is to study models of abnormal sex determination. In the XY^{DOM} sex reversal model, normal testis determination fails when the Y Chr from certain populations of the house mouse subspecies *Mus musculus domesticus* is introduced into the laboratory inbred strain C57BL/6 (B6) (Eicher et al., 1982). Testis determination is normal in B6, *M. m. domesticus* and (B6 ♀ × *M. m. domesticus* ♂) F_1 progeny. However, backcrossing an F_1 male to a B6 female results in a percentage of the XY progeny developing into either hermaphrodites or females (Eicher and Washburn, 1983). XY females are sterile due to the majority of embryos dying by the 2-cell stage (Merchant-Larios et al., 1994). XY hermaphrodites with normal male external genitalia are usually fertile and can function as males.

Y^{DOM} Chrs are classified into three functional classes based on their ability to induce testes in B6 (Biddle and Nishioka, 1988; Nagamine, 1994; Nagamine et al., 1987b). The first is exemplified by the Y^{DOM} Chr found in the FVB/N strain. Backcrossing the FVB/N Y Chr into B6 (strain = B6-Y^{FVB}) has no adverse effect on sex determination (Fig. 1A). Testis differentiation proceeds normally, with testicular cords becoming evident under the dissecting microscope at about 12.5 days post coitum (dpc). The second class, illustrated by the AKR Y^{DOM} Chr, gives transient sex reversal. The tempo of testis differentiation in B6-Y^{AKR} is delayed resulting in ovotestes that are easily observed at 13-14 dpc (Fig. 1B). However, with subsequent fetal development testis differentiation recuperates and normal testes are present at birth. The third class is illustrated by the Y^{DOM} Chr of the strain B6-Y^{TIR}. In B6-Y^{TIR} the pace of testis differentiation is slower still, resulting in severe sex reversal. Often the only evidence of testis differentiation at 13 dpc is a slight swelling in the mid-region of the gonad (Fig. 1C). Gonads in which testicular cords do not form develop into ovaries (Fig. 1D).

B6 is an inbred strain. Therefore, autosomal, pseudoautosomal and X-linked genes are predicted to be identical among the B6-Y^{FVB}, B6-Y^{AKR} and B6-Y^{TIR} consomic strains and the locus (or loci) responsible for the different degrees of testis determination is linked to their Y^{DOM} Chrs. The logical

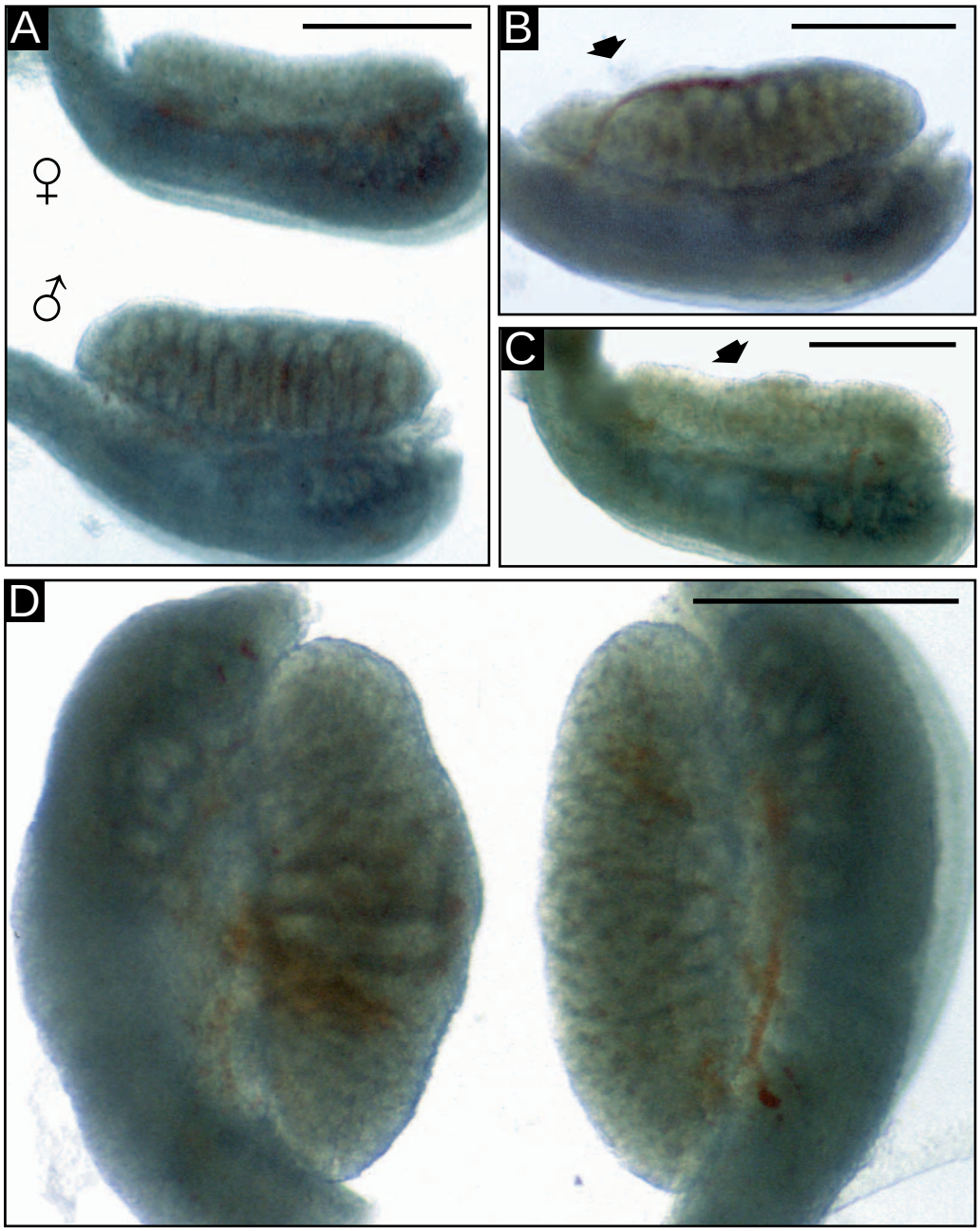

Fig. 1. Whole mounts of fetal gonads. (A) B6-Y^{FVB} normal 13 dpc ovary and testis. (B) B6-Y^{AKR} 13 dpc XY ovotestis. Note developing capsular artery (arrow) associated with testicular but not ovarian regions of the ovotestis. (C) B6-Y^{TIR} 13 dpc XY gonad with swelling (arrow) in the mid-region of the gonad as the only sign of testis differentiation. (D) Pair of B6-Y^{TIR} 14 dpc XY gonads obtained from a single fetus. The left gonad has only three testicular cords and is developing into an ovotestis. The right gonad lacks testicular cords and is differentiating into an ovary. Bar, 0.5 mm.

candidate is the testis-determining gene *Sry* and the simplest explanation is that these Y^{DOM} Chrs have *Sry* alleles that result either in structural or regulatory variants.

DNA sequence analysis confirmed the existence of Sry^{DOM} alleles that encode structural variants. The SRY protein isoforms vary only in the size of a glutamine repeat (Coward et al., 1994). However, a more detailed analysis revealed no correlation between the size of the glutamine repeat and the ability of a Y^{DOM} Chr to induce testes in B6. It was concluded that the SRY^{DOM} protein isoforms are functionally equivalent (Carlisle et al., 1996). If Sry^{DOM} alleles are the underlying cause of XY^{DOM} sex reversal, the allelic differences must be linked to variations in *Sry* transcription or translation. Alternatively, another Y Chr locus (or loci) is responsible for XY^{DOM} sex reversal.

A molecular genetic analysis of how XY^{DOM} sex reversal is exacerbated (or rescued) may identify genes that impinge, either directly or indirectly, on sex determination. The $Kit^{W\text{-}19H}$ (= W^{19H}), $Kit^{W\text{-}e}$ (= W^{e}) and $Mgf^{Sl\text{-}d}$ (= Sl^{d}) mutations exacerbate XY^{DOM} sex reversal (Burgoyne and Palmer, 1991; Cattanach et al., 1988). *Kit* (= *W*, dominant white spotting) is an oncogene that encodes a cell surface receptor tyrosine kinase of the *Pdgfr*/*Csf-1r* family (Chabot et al., 1988; Geissler et al., 1988b). Mast cell growth factor (= KIT ligand, steel factor, stem cell factor) is encoded at the steel locus (*Sl*) and is the ligand of KIT (Huang et al., 1990; Williams et al., 1990; Zsebo et al., 1990). *Kit* and *Mgf* are semidominant mutations with pleiotropic effects on the survival, proliferation and differentiation of cells in three unrelated developmental systems: gametogenesis, hematopoiesis and melanogenesis.

Exacerbation of XY^{DOM} sex reversal is most severe with *Kit*$^{W\text{-}19H}$. Introducing *Kit*$^{W\text{-}19H}$ into B6-Y^{AKR} transforms the transient sex reversal to complete sex reversal analogous to that seen in B6-Y^{TIR} (Cattanach et al., 1988). *Kit*$^{W\text{-}19H}$ is a chromosomal deletion of ≥3 cM (Geissler et al., 1988a). Growth retardation (= runting) is characteristic of many autosomal deletions, including *Kit*$^{W\text{-}19H}$. Cattanach (1995) proposed that *Kit*$^{W\text{-}19H}$ aggravates sex reversal by nonspecifically retarding early fetal or gonadal development. This delays initiation of testis differentiation and allows ovary differentiation to occur in the XY gonad. In support of this hypothesis a variety of autosomal deletions (*T*^{hp}, *T*^{orl}, *Wwt*, *Spt*, *Swf*) are reported to exacerbate sex reversal in B6-Y^{AKR} (Cattanach et al., 1995; Washburn et al., 1990). In addition, *Kit*$^{W\text{-}e}$ is reported to be a deletion (Burgoyne and Palmer, 1991) and *Mgf*$^{Sl\text{-}d}$ results from a 4-kb intragenic deletion (Brannan et al., 1991; Flanagan et al., 1991). However, evidence demonstrating growth retardation of the fetus or gonads at the time of sex determination are lacking. Furthermore, it is intriguing that mutations at both *Kit* and *Mgf* enhance sex reversal raising the possibility that this effect is specifically related to the MGF-KIT signal transduction pathway.

To determine the molecular and cellular mechanisms by which XY^{DOM} sex reversal is exacerbated, we studied the effects of the *Kit*$^{W\text{-}42J}$ allele on testis differentiation in XY^{DOM} strains. We focused on *Kit*$^{W\text{-}42J}$ for three reasons. First, *Kit*$^{W\text{-}42J}$ is one of the most severe alleles among the ≥60 *Kit* mutations reported (Geissler et al., 1981). Therefore, if *Kit* plays a role in testis differentiation, its effect would be more easily observed. Second, the molecular basis for the *Kit*$^{W\text{-}42J}$ mutation is well characterized and results from a missense mutation in the kinase domain that abolishes tyrosine kinase activity (Tan et al., 1990). Third, *Kit*$^{W\text{-}42J}$ arose spontaneously in B6/J and is maintained on the B6 background (Geissler et al., 1981). Therefore, one can introduce this allele into XY^{DOM} sex reversed strains without introducing non-B6 genes that could confound the interpretation of the results.

We show that, although *Kit*$^{W\text{-}42J}$ has no effect on testis determination in B6, it exacerbates sex reversal in B6-Y^{AKR} and B6-Y^{TIR}. In B6-Y^{AKR}, *Kit*$^{W\text{-}42J}$ heterozygosity had a weak effect in aggravating sex reversal and *Kit*$^{W\text{-}42J}$ homozygosity did not guarantee complete sex reversal. In contrast, in B6-Y^{TIR}, *Kit*$^{W\text{-}42J}$ heterozygosity resulted in >95% of XY progeny developing a female phenotype, demonstrating an exacerbation of sex reversal analogous to that obtained with the deletion *Kit*$^{W\text{-}19H}$. We show that exacerbation of sex reversal is not linked to retardation of early fetal growth or testis size or to abnormal expression of *Sry*, the testis determining gene. We propose that *Kit*$^{W\text{-}42J}$ enhances sex reversal by adversely affecting a critical step in testis differentiation that is downstream of *Sry*.

MATERIALS AND METHODS

Mice

B6-Y^{AKR} and B6-Y^{TIR} were derived by backcrossing to B6/J for >N$_{30}$ generations the Y^{DOM} Chrs of AKR and Posch-1, respectively (Nagamine et al., 1987b). B6-Y^{TIR}, formerly B6.Y^{Dom}, was renamed to reflect the geographical origin of its Y Chr (Tirano, Italy) (Carlisle et al., 1996). B6/J and B6-*Kit*$^{W\text{-}42J}$ mice were purchased from Jackson Laboratories (Bar Harbor, ME).

Mice were categorized into one of three phenotypes based on the gross anatomy of their gonads and accessory ducts: male, female or overt hermaphrodite. Male phenotypes have bilateral testicular gonads with associated epididymides and vasa deferentia. Female phenotypes have bilateral ovaries with associated oviducts and uteri. Overt hermaphrodites have a testicular and contralateral ovarian gonad or one or both gonads associated with both Müllerian and Wolffian duct derivatives (putative ovotestis) (Nagamine et al., 1987a,b). Testis lengths were measured under the dissecting microscope using an eyepiece micrometer.

Fetuses were obtained from timed pregnant matings [day of vaginal plug = 0.5 days post coitum (dpc)]. Gonadal primordia, mesonephros and associated mesonephric (Wolffian) ducts from a single fetus were placed in 5 μl phosphate-buffered saline in a 0.5 ml microcentrifuge tube, frozen on dry ice and stored at –85°C.

Determination of chromosomal sex

Chromosomal sex (XX or XY) was determined either by probing genomic DNAs on dot blots for the presence of a Y Chr using the Y-specific repetitive sequence Y353/B (Bishop et al., 1985) and/or by identifying the presence of the Y Chr gene zinc finger-Y using the polymerase chain reaction (PCR), as previously described (Nagamine et al., 1990).

Determination of *Kit*$^{W\text{-}42J}$ genotype

Melanogenesis in *Kit*$^{W\text{-}42J}$ heterozygotes is severely compromised resulting in almost completely white coats (Geissler et al., 1988a). Identifying newborn *Kit*$^{W\text{-}42J}$ heterozygotes among wild-type (+/+) littermates was performed by scoring for the absence of pigmented hair follicles in the dermis (Fig. 2A).

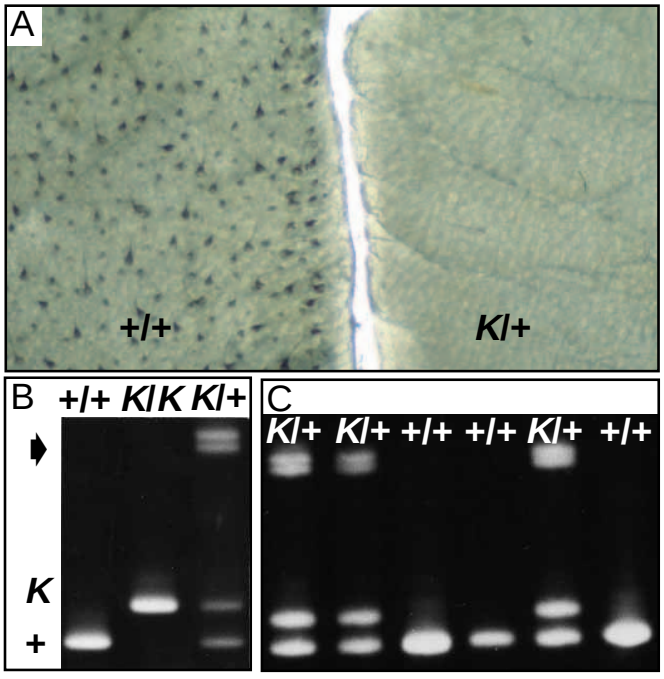

Fig. 2. *Kit*$^{W\text{-}42J}$ genotyping. (A) Dorsal skin of wild-type (+/+) and *Kit*$^{W\text{-}42J}$/+ (*K*/+) newborns demonstrating the absence of pigmented hair follicles in the latter. (B) Denaturing gradient gel electrophoresis of *Kit* PCR amplified products derived from wild-type (+/+), *Kit*$^{W\text{-}42J}$ homozygous (*K*/*K*) and *Kit*$^{W\text{-}42J}$ heterozygous (*K*/+) samples. The wild-type (+), *Kit*$^{W\text{-}42J}$ (*K*) and heteroduplex (◗) bands are noted. (C) Representative DGGE genotyping of (B6-*Kit*$^{W\text{-}42J}$/+ ♀ × B6-Y^{TIR} ♂) F$_1$ 11.5 dpc fetuses identifying three *Kit*$^{W\text{-}42J}$ heterozygous and three +/+ samples.

Kit$^{W\text{-}42J}$ genotyping of fetuses and newborn pups was performed by parallel denaturing gradient gel electrophoresis (DGGE) (Myers et al., 1990). 1-2 μg of total RNA, prepared from liver, brain, spleen or gonadal tissues, were reverse transcribed with MMTV-RT (GIBCO/BRL) in a final volume of 20 μl for 1 hour at 45°C using 2.5 pmoles of a 46-mer oligo $(dT)_{17}$ primer modified to include restriction enzyme sites at its 5′ end. 1 μl of the resulting cDNAs was used to amplify a 191-base pair (bp) *Kit*$^{W\text{-}42J}$ fragment using primers (sense primer = nucleotide (nt) 2271-2291, antisense = nt 2399-2421) that flank the *Kit*$^{W\text{-}42J}$ point mutation (Qiu et al., 1988). The sense primer was designed with a 40-nt GC-clamp (Sheffield et al., 1989) at its 5′ end. 10 μl of the PCR amplified products were size fractionated on a 7% acrylamide gel containing a 35-65% denaturant gradient (100% denaturant = 7 M urea/40% (vol/vol) formamide). Electrophoresis was performed at 60°C in DGGE buffer (40 mM Tris, 20 mM sodium acetate, 1 mM EDTA, pH 7.4) using a commercial apparatus (C.B.S. Scientific Company, Inc.). The *Kit*$^{W\text{-}42J}$ homoduplex fragment migrates slower relative to the +/+ fragment (Fig. 2B). Heterozygotes have both homoduplex fragments plus two heteroduplexes that are generated during the PCR (Myers et al., 1990).

Semiquantitative RT-PCR

Fetuses at 11.5 dpc were staged by tail somite number, counting from the base of the genital tubercle (Hacker et al., 1995). For *Sry* and *Lim1* RT-PCR, 13 *Kit*$^{W\text{-}42J}$/+ XY fetuses (tail somites = 11, 12, 12, 13, 15, 15, 15, 16, 17, 17, 17, 18, 19) and 4 +/+ XY fetuses (tail somites = 14, 15, 15, 17) obtained from B6-*Kit*$^{W\text{-}42J}$/+ ♀ × B6-Y^{TIR} ♂ crosses were studied. In addition, 2 (B6-*Kit*$^{W\text{-}42J}$/+ ♀ × B6-Y^{TIR} ♂) F_1 XX fetuses (tail somites = 13, 15) and 3 (B6 ♀ × B6-Y^{TIR} ♂) F_1 XY fetuses (tail somites = 14, 15, 15) were examined.

Total RNA was prepared from individual pairs of urogenital ridges using RNeasy (Qiagen, Inc.). RNA samples were treated with RQ1 DNase (Promega Corp.) (37°C, 30 minutes), phenol extracted, ethanol precipitated, then resuspended in 11 μl of diethyl pyrocarbonate-treated water containing 1.0 U/μl of rRNAsin (Promega Corp.) and 1.5 mM dithiothreitol.

To confirm the absence of genomic DNA, 1 μl of the RNA preparation was PCR amplified using primers specific for either Müllerian inhibiting substance or *Sry*. The remaining 10 μl of RNA were reverse transcribed as above but using Superscript II (GIBCO/BRL). 1 μl of the cDNA was used for PCR.

Murine *Sry* transcripts are of two types: a conventional linear transcript and a circular transcript that lacks a cap and poly(A) tail (Capel et al., 1993). To ensure amplification of only linear *Sry* cDNAs, the sense primer (nt 8054-8080) was designed from a site that is upstream of the splice acceptor (nt 8200) that is used to generate the circular transcript. The antisense primer (nt 8644-8665) was from a region 3′ of the HMG box. PCR conditions were as previously described and results in a 612-bp fragment (Carlisle et al., 1996).

Semiquantification of the *Sry* PCR assay was performed by adding 5 μCi ^{33}P-dATP to each tube. RT-PCR for *Lim1* was used as an internal control (sense primer = nt 1030-1054, antisense = nt 1588-1611, numbering according to Barnes et al. (1994); cDNA amplified product = 582-bp). Within the urogenital ridge, *Lim1* mRNAs are found primarily in the mesonephric tubules and ducts, and metanephros (Barnes et al., 1994; Fujii et al., 1994). Preliminary experiments showed 30 PCR cycles to be within the exponential phase of the amplification reaction for both *Sry* and *Lim1*. 5 or 10 μl of the PCR amplified products were fractionated on 4% polyacrylamide gels and the gels dried. Radioactivity in the *Sry* and *Lim1* bands were quantified on the PhosphorImager (Molecular Dynamics, Inc.) using ImageQuant 3.3 software with volume integration and local background correction, and the *Sry*/*Lim1* ratios calculated. Each cDNA sample was tested twice in separate assays.

Two controls were routinely performed. First, each sample was amplified for the presence of *Hprt* cDNAs as previously described (Nagamine et al., 1990) to check for RNA/cDNA degradation and/or suboptimal cDNA synthesis. Second, a negative control in which water was substituted for the cDNA sample was run with each assay to check for contaminating PCR amplified products.

Statistical analysis

The χ^2 test was used to test the null hypothesis that there is no difference between the observed chromosomal sex ratios (XX:XY) or *Kit* phenotype ratios (+/+, *Kit*/+, *Kit/Kit*) and the theoretical Mendelian ratios. The null hypothesis that there is no difference in average newborn weights among genotypes was tested by determining if the difference of their means was significantly different from the Student's *t* distribution. Rejection of the null hypothesis for both tests was set at $P \leq 0.05$ (Snedecor and Cochran, 1976).

RESULTS

Kit$^{W\text{-}42J}$ does not delay testis differentiation in B6

Whether *Kit*$^{W\text{-}42J}$ adversely affects sex determination in normal strains has never been studied. To ascertain if *Kit*$^{W\text{-}42J}$ has a deleterious effect on testis differentiation, (B6-*Kit*$^{W\text{-}42J}$/+ ♀ × B6-*Kit*$^{W\text{-}42J}$/+ ♂)F_1 XY fetuses were examined at 12.75 dpc for the presence of testicular cords. Testicular cords are first visible under the dissecting microscope at about 12.5 dpc. Examining the fetal gonads at 12.75 dpc ensured that most fetal testes would have initiated testis differentiation. Of 100 fetuses examined, 43 had gonads with incipient testicular cords. It is reasonable to assume that *Kit*/+ or *Kit*/*Kit* genotypes would be absent or rare among the 43 fetuses if *Kit*$^{W\text{-}42J}$ substantially delays testis differentiation. DGGE genotyping identified 8 +/+, 24 *Kit*/+ and 11 *Kit*/*Kit* fetuses, a ratio that is not statistically different from the expected Mendelian ratio of 1:2:1 (χ^2=1.023, P=0.613). We conclude that *Kit*$^{W\text{-}42J}$ heterozygosity or homozygosity does not significantly delay testis differentiation on the B6 genomic background.

Exacerbation of sex reversal in B6-Y^{AKR}

B6-Y^{AKR} has a delay in testis differentiation resulting in the transient development of ovotestes (Nagamine et al., 1987b). To determine if *Kit*$^{W\text{-}42J}$ exacerbates this delay in testis differentiation, (B6-*Kit*$^{W\text{-}42J}$/+ ♀ × B6-Y^{AKR} ♂) F_1 newborns were examined for evidence of XY sex reversal. Of 107 pups examined, 27 were XY (Table 1A), one of which was a hermaphrodite with a left testicular gonad and a contralateral putative ovotestis that was associated with both Müllerian and Wolffian duct derivatives (Fig. 3A,B). No XY females were identified. Neither the XY:XX (45:62; χ^2 = 2.7, P=0.101) nor +/+:*Kit*/+ (47:60; χ^2 = 1.58, P=0.222) ratios were significantly different from the theoretical 1:1 ratio suggesting that sex reversed *Kit*/+ fetuses were not lost in utero.

To determine if the low percentage of sex reversal was due to female imprinting, we transferred *Kit*$^{W\text{-}42J}$ through the male. No hermaphrodites were identified among 33 XY *Kit*/+ offspring (Table 1B). Again the XY:XX (53:52; χ^2 = 0.01, P>0.90) and +/+:*Kit*/+ (43:62; χ^2 = 3.438, P=0.068) ratios were not significantly different from the theoretical 1:1 ratio.

In both crosses, a slight but insignificant increase in number of *Kit*/+ genotypes was observed. The increase in *Kit*/+ genotypes becomes statistically significant when the results of the two crosses are combined (90:122, χ^2 = 4.83, P=0.029) (Table 1C). This is unexpected and additional experiments are needed to confirm if this is the result of sampling error or the

Table 1. Phenotypes of B6-Y^{AKR} +/+ and *Kit*/+ newborns

A. (*Kit*/+ ♀ × B6-Y^{AKR} ♂) F_1 (*n*=107)

+/+				*Kit*/+			
♂ XY	⚥ XY	♀ XY	♀ XX	♂ XY	⚥ XY	♀ XY	♀ XX
18	0	0	29	26	1	0	33

B. (B6 ♀ × B6-Y^{AKR} *Kit*/+ ♂) F_1 (*n*=105)

+/+				*Kit*/+			
♂ XY	⚥ XY	♀ XY	♀ XX	♂ XY	⚥ XY	♀ XY	♀ XX
20	0	0	23	33	0	0	29

C. Total (*n*=212)

+/+				*Kit*/+			
♂ XY	⚥ XY	♀ XY	♀ XX	♂ XY	⚥ XY	♀ XY	♀ XX
38	0	0	52	59	1	0	62

Table 2. Average testis length of B6-Y^{AKR} +/+ and *Kit*/+ newborns

A. *Kit*/+ ♀ × B6-Y^{AKR} ♂

	+/+	*Kit*/+
n	34	42
$\overline{X}$	1.09*	0.97*
s	0.09	0.06

B. B6 ♀ × B6-Y^{AKR} *Kit*/+ ♂

	+/+	*Kit*/+
n	36	50
$\overline{X}$	1.20*	1.03*
s	0.10	0.09

n = sample size, $\overline{X}$ = mean, s = standard deviation,
* = statistically significant.

B6-Y^{AKR} genotype. An increase in *Kit*/+ genotypes was not observed with B6-Y^{TIR} (see below).

Because only a single B6-Y^{AKR} *Kit*/+ hermaphrodite was identified, it raised the question of whether this unique sample indeed carried a Y^{AKR} Chr or was the result of a breeding error. B6-Y^{AKR} and B6-Y^{TIR} give diagnostic Y Chr hybridization patterns when Southern blots are probed with the Y-repetitive sequence Y353/B (Nagamine, 1994). Southern analysis confirmed that the hermaphrodite had a Y^{AKR} Chr (data not shown). We conclude that *Kit*$^{W\text{-}42J}$ exacerbates XY sex reversal in B6-Y^{AKR} but the penetrance of this trait is low.

Kit$^{W\text{-}42J}$ homozygosity does not guarantee sex reversal

(B6-*Kit*$^{W\text{-}42J}$/+ ♀ × B6-Y^{AKR} *Kit*$^{W\text{-}42J}$/+ ♂) F_1 newborns were screened for sex reversal to determine if *Kit*$^{W\text{-}42J}$ homozygosity further exacerbates sex reversal. Although most *Kit*$^{W\text{-}42J}$ homozygous fetuses die from complications of macrocytic anemia, rare homozygotes can be found at birth (Geissler et al., 1981). From 13 litters, 54 pups were obtained of which the XY:XX ratio was 27:27; none were sex reversed. DGGE genotyping of the XY samples identified 11 +/+, 15 *Kit*/+ and 1 *Kit*/*Kit*. As expected, nearly all *Kit*/*Kit* fetuses died in utero. Significantly, the single homozygous newborn had a male phenotype with bilateral testes (Fig. 4). Minor abnormalities in its internal genitalia were observed: its right testis (0.75 mm) was smaller relative to the left (0.9 mm), its right epididymis' cauda was not completely formed (Fig. 4) and the average lengths of its testes were smaller relative to its heterozygous and +/+ littermates (0.83 mm versus 0.98 mm versus 1.2 mm, respectively).

The combined data demonstrate that *Kit*$^{W\text{-}42J}$ can exacerbate sex reversal in B6-Y^{AKR} but its penetrance is low (1/76 XY *Kit*/+ and *Kit*/*Kit* = 1.3%). Because the majority of homozygous fetuses died in utero, the data do not permit us to determine if sex reversal increases in *Kit*$^{W\text{-}42J}$ homozygotes relative to heterozygotes. However, the identification of a homozygous male, albeit not completely normal, demonstrates *Kit*$^{W\text{-}42J}$ homozygosity does not guarantee sex reversal.

Kit$^{W\text{-}42J}$ decreases B6-Y^{AKR} testis size

The homozygous male had testes that were smaller relative to testes from its littermates. The length of the testes from (B6-*Kit*$^{W\text{-}42J}$/+ ♀ × B6-Y^{AKR} ♂) and (B6 ♀ × B6-Y^{AKR} *Kit*$^{W\text{-}42J}$/+ ♂) F_1 newborns were measured to ascertain if *Kit*$^{W\text{-}42J}$ decreases testis size.

In both crosses, males had right testes that averaged slightly smaller than the left. However, the differences were not statistically significant so right and left measurements were pooled.

Compared to +/+ testes, testes of *Kit*/+ males were significantly smaller in both crosses (Table 2A, t=2.31, P<0.025; Table 2B, t=8.10, P<0.001). We conclude that *Kit*$^{W\text{-}42J}$ decreases testis size.

In addition to the direct effect of *Kit*$^{W\text{-}42J}$ on testis size, a *Kit*$^{W\text{-}42J}$ maternal effect was found that further decreased testis size. The maternal effect was present irrespective of whether males were +/+ (t=4.78, P<0.001) or *Kit*/+ (t=3.7, P<0.001) (Table 2A,B). The net result is that *Kit*/+ sons of *Kit*/+ females had the smallest testes. The data suggest that the uterine environment of *Kit*$^{W\text{-}42J}$/+ females is not optimal for testis differentiation.

Kit$^{W\text{-}42J}$ exacerbation of sex reversal in B6-Y^{TIR} is severe

Introduction of *Kit*$^{W\text{-}42J}$ into B6-Y^{TIR} severely exacerbated sex reversal. Among (B6-*Kit*$^{W\text{-}42J}$/+ ♀ × B6-Y^{TIR} ♂) F_1 +/+ newborns, XY offspring were equally divided among male phenotypes with bilateral testicular gonads, overt hermaphrodites and female phenotypes with bilateral ovaries (Table 3). In contrast, *Kit*/+ XY littermates were almost exclusively of female phenotype. No male phenotypes were obtained and only two overt hermaphrodites were identified. The XY:XX

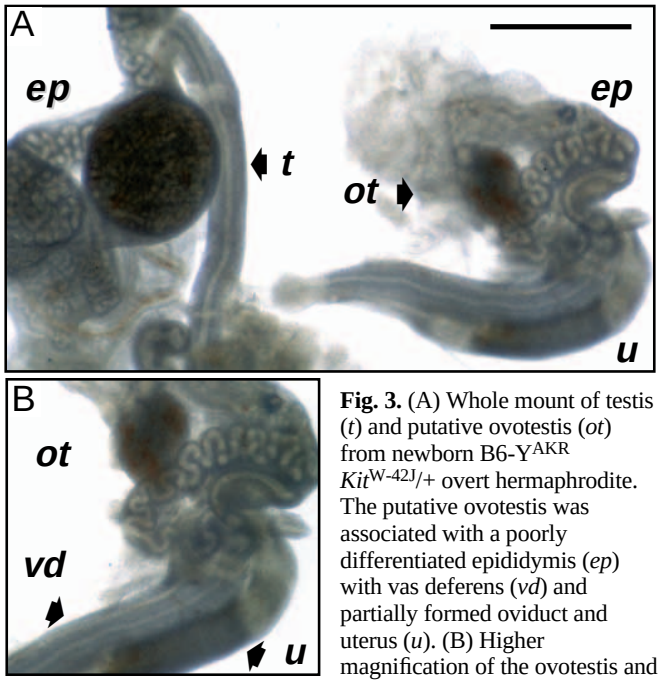

Fig. 3. (A) Whole mount of testis (*t*) and putative ovotestis (*ot*) from newborn B6-Y^{AKR} $Kit^{W\text{-}42J}$/+ overt hermaphrodite. The putative ovotestis was associated with a poorly differentiated epididymis (*ep*) with vas deferens (*vd*) and partially formed oviduct and uterus (*u*). (B) Higher magnification of the ovotestis and associated ducts. Bar, 1.0 mm

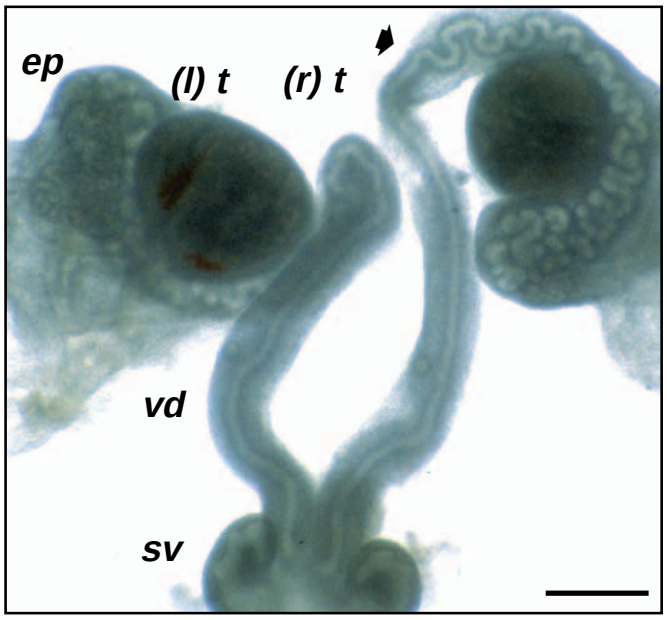

Fig. 4. Internal genitalia from B6-Y^{AKR} $Kit^{W\text{-}42J}$/$Kit^{W\text{-}42J}$ newborn male phenotype. The right testis [(*r*) *t*] is obviously smaller than the left [(*l*) *t*)] and is associated with an epididymis (*ep*) in which its cauda (◗) is incompletely formed. No other abnormalities were observed and the vasa deferentia (*vd*) and seminal vesicles (*sv*) appear normal. Bar, 0.5 mm.

(88:74; χ^2 = 1.21, *P*=0.345) and +/+:*Kit*/+ (87:75; χ^2 = 0.889, *P*=0.374) ratios were not significantly different from the theoretical Mendelian ratio of 1:1. This suggests that the absence of *Kit*/+ male phenotypes and the paucity of *Kit*/+ hermaphroditic phenotypes are not the result of the loss of these phenotypes in utero.

Kit$^{W\text{-}42J}$ maternal effect does not exacerbate sex reversal in B6-Y^{TIR}

It has been reported that a reduction in testis size presages sex reversal (Cattanach et al., 1995). We identified a $Kit^{W\text{-}42J}$ maternal effect that caused B6-Y^{AKR} testis size to be reduced. If the $Kit^{W\text{-}42J}$ maternal effect is deleterious for testis differentiation, an increase in XY females is predicted among (B6-$Kit^{W\text{-}42J}$/+ ♀ × B6-Y^{TIR} ♂) F_1 +/+ offspring. In B6-Y^{TIR}, about 35% of XY offspring develop into XY females (Nagamine et al., 1987b). An increase in percentage of XY females among the +/+ offspring was not observed (Table 3). Indeed, the percentage of XY females among +/+ offspring was only 27% (12/44) (Table 3). This suggests that the maternal effect functions after testis differentiation is committed. We conclude that the $Kit^{W\text{-}42J}$ maternal effect on testis size is not sufficient by itself to exacerbate sex reversal in B6-Y^{TIR}.

Table 3. Phenotypes of (*Kit*/+ ♀ × B6-Y^{TIR} ♂) F_1 newborns (*n*=162)

+/+				*Kit*/+			
♂ XY	♀ XY	♀ XY	♀ XX	♂ XY	♀ XY	♀ XY	♀ XX
15	17	12	43	0	2	42	31

Exacerbation of XY^{DOM} sex reversal is not due to early growth retardation of the fetus

One hypothesis for the exacerbation of XY^{DOM} sex reversal is that retardation of fetal growth delays testis development (Cattanach et al., 1995). To ascertain if B6-Y^{TIR} $Kit^{W\text{-}42J}$/+ fetuses were developmentally delayed, we examined (B6-$Kit^{W\text{-}42J}$/+ ♀× B6-Y^{TIR} ♂) F_1 fetuses at 11.5 dpc, the age at which expression of the testis-determining gene *Sry* is maximum and at which any delay in fetal development is predicted to have maximum effect on testis differentiation. Tail somite number is an objective measurement of the developmental age of mouse fetuses from 10-13 dpc (Hacker et al., 1995). Number of tail somites, counting from the genital tubercle, was determined for 34 11.5 dpc fetuses. The fetuses were subsequently genotyped as to XX or XY and the 23 XY fetuses that were identified were genotyped for $Kit^{W\text{-}42J}$ by DGGE (Fig. 2C). 7 XY fetuses were +/+ and had an average number of 14.3 ± 2.5 tail somites (range=11-17). The 16 *Kit*/+ fetuses had an average number of 14.1±3.4 tail somites (range=7-19). The means were not significantly different (*t*=0.138, *P*>0.5). We conclude that the B6-Y^{TIR} $Kit^{W\text{-}42J}$/+ 11.5 dpc fetuses were not developmentally delayed relative to +/+.

Sry expression in B6-Y^{TIR} $Kit^{W\text{-}42J}$/+ urogenital ridges

In the mouse, expression of *Sry* initiates in fetuses with 11-12 tail somites and reaches maximum levels in fetuses with 18 tail somites (Hacker et al., 1995). Semiquantitative *Sry* RT-PCR was performed to determine if exacerbation of sex reversal in B6-Y^{TIR} $Kit^{W\text{-}42J}$/+ fetuses is the result of *Sry* expression being blocked, being delayed in its initiation or being expressed at lower levels relative to +/+ littermates.

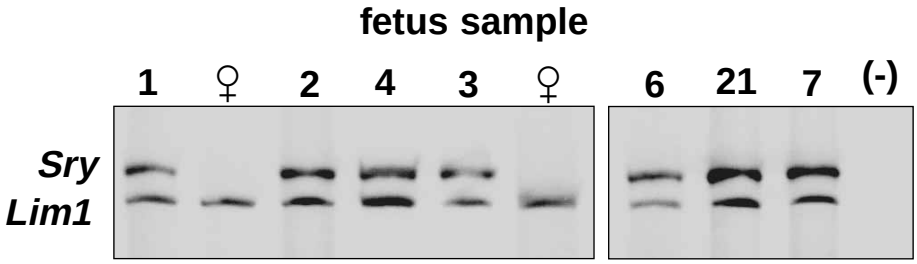

Fig. 5. PhosphorImager autoradiograph for semiquantitative *Sry* and *Lim1* RT-PCR. Samples were 11.5 dpc urogenital ridges from *Kit*$^{W\text{-}42J}$/+ (nos. 4, 6, 21, 7) and +/+ (nos. 1-3) fetuses. *Sry* and *Lim1* PCR assays were performed separately; 10 μl of each reaction were size fractionated in the same well of the gel. Specificity of the *Sry* assay is demonstrated by the absence of *Sry* amplified products in XX (♀) samples. (–), negative control. *Sry* and *Lim1* values for fetus samples 1-4, 6 and 7 are listed in Table 4.

Table 4. Semiquantitative *Sry* and *Lim1* RT-PCR of urogenital ridges from (B6-*Kit*/+ ♀ × B6-Y^{TIR} ♂) F_1 (samples 1-7) and (B6 ♀ × B6-Y^{TIR} ♂)F_1 (samples 8-10) 11.5 dpc fetuses

	fetus	t.s.	Sry	Lim1	Sry/Lim1
+/+	1	14	512,714	437,774	1.17
	2	15	739,614	662,083	1.12
	3	15	629,375	450,784	1.40
Kit/+	4	15	863,386	1,162,779	0.74
	5	15	1,090,529	659,856	1.65
	6	15	478,845	315,175	1.52
	7	16	1,154,591	743,210	1.55
+/+	8	14	7,351	6,084	1.21
	9	15	25,203	14,552	1.73
	10	15	18,053	12,704	1.42

Samples 8-10 were assayed in a separate experiment.
t.s. = tail somite number.

A positive *Sry* signal was amplified in all 11.5 dpc *Kit*$^{W\text{-}42J}$/+ samples studied demonstrating that *Sry* expression was not blocked (*n*=13, tail somites=11-19; Fig. 5). It is noteworthy that the youngest *Kit*$^{W\text{-}42J}$/+ sample was also positive suggesting that initiation of *Sry* expression is not substantially delayed. A direct comparison of *Kit*$^{W\text{-}42J}$/+ and +/+ *Sry* levels was possible only for fetuses of 14-16 tail somites due to insufficient numbers of +/+ samples. *Sry* values and *Sry*/*Lim1* ratios suggest that *Sry* mRNA levels in *Kit*$^{W\text{-}42J}$/ + samples are not significantly reduced relative to +/+ (Table 4). The RT-PCR data suggest that exacerbation of sex reversal in B6-Y^{TIR} *Kit*$^{W\text{-}42J}$/+ XY fetuses is not due to *Sry* blockade, a substantial delay in initiation of *Sry* expression, or exceptionally low levels of *Sry* mRNAs.

DISCUSSION

The data show that *Kit*$^{W\text{-}42J}$ heterozygosity exacerbated sex reversal in B6-Y^{TIR} resulting in >95% of XY *Kit*$^{W\text{-}42J}$/+ progeny developing female phenotypes. This is equivalent to the exacerbation of sex reversal by chromosomal deletions. In B6-Y^{AKR}, *Kit*$^{W\text{-}42J}$ heterozygosity also exacerbated XY sex reversal but the penetrance of the trait was low. The data do not allow us to determine if *Kit*$^{W\text{-}42J}$ homozygosity further exacerbates sex reversal in B6-Y^{AKR}. However, the identification of a *Kit*$^{W\text{-}42J}$ homozygous male phenotype demonstrates that homozygosity does not guarantee sex reversal. Furthermore, because B6-Y^{AKR}, B6-Y^{TIR} and *Kit*$^{W\text{-}42J}$ are on the same B6 genetic background, the exacerbation of XY^{DOM} sex reversal can be attributed specifically to *Kit*$^{W\text{-}42J}$.

The *Kit*$^{W\text{-}e}$ and *Mgf*$^{Sl\text{-}d}$ mutations have also been reported to exacerbate sex reversal in B6-Y^{POS}, a strain analogous to B6-Y^{TIR} (Burgoyne and Palmer, 1991). The effect was less severe in that male phenotypes were apparently obtained with both crosses. The effect of these mutations on B6-Y^{AKR} is not known. Based on the 'timing mismatch' hypothesis, Burgoyne and Palmer (1991) posited that *Kit*$^{W\text{-}e}$ and *Mgf*$^{Sl\text{-}d}$ mutations enhance XY^{DOM} sex reversal by causing ovary determination to initiate earlier relative to testis determination. No evidence was presented to support the premise that initiation of ovary differentiation varies among mouse strains or mutations.

Cattanach et al. (1988, 1995) proposed that exacerbation of XY^{DOM} sex reversal is due to nonspecific retardation of early fetal or gonadal development. With regard to retardation of fetal development, examination of B6-Y^{TIR} 11.5 dpc fetuses revealed no evidence that *Kit*$^{W\text{-}42J}$/+ genotypes, the majority of which are destined to develop into XY females, were retarded in development relative to +/+ littermates. We conclude that, if *Kit*$^{W\text{-}42J}$ retards fetal development, its effect is occurring after 11.5 dpc and therefore after testis determination has initiated.

The present data on retardation of testis development are conflicting making it difficult to separate correlative from causative effects. We documented that *Kit*$^{W\text{-}42J}$ has two effects on testis size. First, there is a direct effect resulting in *Kit*$^{W\text{-}42J}$/+ newborn testes being significantly smaller than testes from +/+ littermates (Table 2A,B). Second, there is an indirect *Kit*$^{W\text{-}42J}$ maternal effect that further reduces testis size. However, the hypothesis that *Kit*$^{W\text{-}42J}$ exacerbates sex reversal by reducing testis size is compromised by the following observations. First, if *Kit*$^{W\text{-}42J}$ retards testis development, one would expect to see a similar but weaker effect in normal strains. We found no evidence that *Kit*$^{W\text{-}42J}$ retards testis differentiation in the normal B6 strain. Second, and more importantly, if a reduction in testis size aggravates XY^{DOM} sex reversal then the *Kit*$^{W\text{-}42J}$ maternal effect is predicted to exacerbate sex reversal in (B6-*Kit*$^{W\text{-}42J}$/+ ♀ × B6-Y^{TIR} ♂) F_1 +/+ offspring. This was not observed (Table 3). We surmise that the factors contributing to

the reduction of testis size act after testis differentiation is already committed.

Sry transcripts are first found in the urogenital ridge of fetuses with 11-12 tail somites, reaching peak levels at about 18 tail somites (Hacker et al., 1995). RT-PCR revealed *Sry* to be present in B6-Y^{TIR} *Kit*$^{W-42J}$/+ XY fetuses from 11-19 tail somites. Semiquantification of the RT-PCR assay failed to reveal any evidence that *Sry* expression in *Kit*$^{W-42J}$/+ urogenital ridges was lower relative to +/+ samples. The RT-PCR data suggest that exacerbation of sex reversal by *Kit*$^{W-42J}$ is not due to blockade of *Sry* expression, a substantial delay in initiation of *Sry* transcription, or exceptionally low levels of *Sry* transcripts at 11.5 dpc. This is in striking contrast to XY sex reversal resulting from deletion of Y Chr sequences where expression of *Sry* is absent or significantly reduced (Laval et al., 1995).

The RT-PCR data suggest that *Kit*$^{W-42J}$'s enhancement of XY^{DOM} sex reversal occurs downstream of *Sry*. KIT plays a key role in the migration and proliferation of primordial germ cells such that homozygotes of most *Kit* alleles have few germ cells (Buehr et al., 1993b; Geissler et al., 1981; Mintz and Russell, 1957). Germ cell number can also be reduced in heterozygotes of severe *Kit* alleles, albeit not to the same extent as seen in homozygotes (Buehr et al., 1993b). For *Kit*$^{W-42J}$, heterozygote females have fewer follicles (Geissler et al., 1981) and heterozygote males have smaller testes (Table 2) suggesting that germ cell number may also be reduced. One hypothesis for the exacerbation of sex reversal in B6-Y^{TIR} *Kit*$^{W-42J}$/+ fetuses is that the tempo of testis differentiation is enhanced by germ cell-preSertoli cell interaction. In normal testes, germ cell number is usually irrelevant since testis differentiation proceeds rapidly. However, in B6-Y^{TIR}, where testis differentiation is severely compromised, germ cell-preSertoli cell interaction may play a decisive role as to whether gonadal tissues follow the testicular or ovarian pathway. Introducing germ-cell-deficient mutations, for example, Hertwig's anemia (Russell et al., 1985) or germ cell deficient (Pellas et al., 1991), into B6-Y^{TIR} would test this hypothesis.

An alternative hypothesis is that KIT and MGF may function during testis angiogenesis. The testis vasculature is derived from endothelial cells and blood vessels that enter from the mesonephros (Buehr et al., 1993a; Merchant-Larios et al., 1993). Three lines of evidence suggest that *Mgf* and *Kit* may function during testis angiogenesis. First, *Mgf* is expressed at high levels in 11.5 dpc fetal gonads and Sertoli cells are known to express *Mgf* (Matsui et al., 1990; Tajima et al., 1991). Second, both MGF and KIT proteins occur on the cell surface of human endothelial cells (Broudy et al., 1994; Miyamoto et al., 1994). Third, a close relationship exist between angiogenesis and developing testicular cords. This is often observed in XY^{DOM} ovotestes where a prominent blood vessel, the capsular artery, develops under the tunica albuginea in the mid-line of the testis and is found closely associated with testicular tissues, often demarcating the boundary between ovarian and testicular regions (Fig. 1B). According to this model, *Kit* or *Mgf* mutations aggravate sex reversal in XY^{DOM} mice by delaying angiogenesis. This further delays testis differentiation allowing ovary differentiation to dominate.

How other mutations, for example, T^{hp}, T^{orl}, *Wwt*, *Spt* and *Swf*, exacerbate XY^{DOM} sex reversal remains to be determined. The present work lays the foundation for these future studies.

It is clear that KIT is not essential for testis differentiation because testes form normally in *Kit* homozygotes on most strain backgrounds. The XY^{DOM} sex reversal model unmasks a possible role for the MGF-KIT signal transduction pathway in testis differentiation because sex determination is already compromised and subsequent set-backs in testis differentiation are easily identified. The XY^{DOM} sex reversal model, with its well-defined genetics and its ready availability of hermaphroditic phenotypes, will prove useful in identifying the role of other genes in sex determination and differentiation.

We thank Ms Katie Manning and Dora Mayse for their expert technical assistance. This research was supported by NICHD HD28976.

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(Accepted 26 July 1996)