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Targeted Mutagenesis of a Candidate *t* Complex Responder Gene in Mouse *t* Haplotypes Does Not Eliminate Transmission Ratio Distortion

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ABSTRACT

Transmission ratio distortion (TRD) associated with mouse *t* haplotypes causes +/*t* males to transmit the *t*-bearing chromosome to nearly all their offspring. Of the several genes involved in this phenomenon, the *t* complex responder (*Tcr^t^*) locus is absolutely essential for TRD to occur. A candidate *Tcr^t^* gene called *Tcp10b^t^* was previously cloned from the genetically defined *Tcr^t^* region. Its location, restricted expression in testis, and a unique postmeiotic alternative splicing pattern supported the idea that *Tcp10b^t^* was *Tcr^t^*. To test this hypothesis in a functional assay, ES cells were derived from a viable partial *t* haplotype, and the *Tcp10b^t^* gene was mutated by homologous recombination. Mutant mice were mated to appropriate partial *t* haplotypes to determine whether the targeted chromosome exhibited transmission ratios characteristic of the responder. The results demonstrated that the targeted chromosome retained full responder activity. Hence, *Tcp10b^t^* does not appear to be *Tcr^t^*. These and other observations necessitate a reevaluation of genetic mapping data and the actual nature of the responder.

THE *t* haplotypes are variant forms of the *t* complex, a 20-cM region of DNA in proximal mouse chromosome *17*. Although most *t* haplotypes contain a recessive developmental lethal mutation, and males heterozygous for two complementing *t* haplotypes are sterile, these variant chromosomes propagate to high frequencies in wild mouse populations due to male transmission ratio distortion (TRD). This process causes male mice heterozygous for a *t* haplotype and a wild-type form of the *t* complex (*t*/+) to transmit the *t* chromosome to nearly all of their offspring. Recombination in +/*t* heterozygotes is greatly suppressed due to four relative inversions between *t* and + chromosomes. This region of recombination suppression formally defines the *t* complex and allows *t* haplotypes to propagate as intact genetic units in mouse populations.

TRD is believed to occur through the action of four *trans*-acting distorter (*Tcd*) loci upon a *t* complex responder locus, *Tcr* (LYON 1984; SILVER and REMIS 1987). *Tcr* is the central locus in TRD; if the *t* haplotype form (*Tcr^t^*) is absent, no distortion occurs. A male that carries all four *t* haplotype distorter loci (*Tcd^t^*) and is heterozygous for *Tcr^t^* can transmit the *Tcr^t^*-containing chromosome *17* homologue to >95% of progeny. However, if a male is heterozygous for *Tcr^t^* and no distorters are present, the *Tcr^t^*-containing homologue is transmitted to <15% of offspring. The distorters act in an additive fashion; as doses increase from none to the full complement, *Tcr^t^* transmission increases (LYON 1984).

Genetic evidence indicates that the genes responsible for homozygous male sterility (the *t* complex sterility loci, or *tcs*) are identical to the distorters (LYON 1986). Because all the distorters must be present for high level TRD to occur, the four inversions serve to genetically "lock" these *t* haplotype alleles together. Each of the TRD genes is associated with one of the inversions (HAMMER *et al.* 1989).

Little is known about the biological mechanism of TRD. Unlike the SD system in Drosophila, where one genotype of sperm degenerates due to improper chromatin condensation (TOKUYASU *et al.* 1972), +/*t* males produce equal numbers of + and *t* sperm (SILVER and OLDS-CLARKE 1984). Artificial insemination experiments have shown that *t* sperm are not superior to +, but that the + sperm from +/*t* heterozygotes are functionally defective (OLDS-CLARKE and PEITZ 1985; SEITZ and BENNETT 1985). There is some evidence to indicate that the + sperm in heterozygotes undergo a premature acrosome reaction, although this has not been demonstrated directly (BROWN *et al.* 1989).

*Tcr^t^* has been mapped to an interval of ~40 kb in the center of *t* haplotypes (the *D17Leh66b* locus), as defined by molecular and genetic analyses of recombinant chromosomes called partial *t* haplotypes (FOX *et al.* 1985; ROSEN *et al.* 1990; BULLARD *et al.* 1992). Partial *t* haplotypes are the products of rare recombination events between a *t* haplotype and a wild-type chromosome, and thus contain only a portion of *t* haplotype DNA. Within this 40-kb region is a gene called *Tcp10b^t^*. This gene is expressed exclusively in the germ cells of testis. While transcription begins at the spermatocyte stage, its RNA undergoes an unique alternative splice beginning at the haploid stage of spermatogenesis (SCHI-

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MENTI *et al.* 1988; CEBRA-THOMAS *et al.* 1991). Hence, the close genetic association of this gene to the *Tcr^t* interval, testis-specific expression pattern, and candidate lesion in the form of abberant processing makes *Tcp10b^t* an attractive candidate for *Tcr^t*.

In two previous efforts to functionally address the question of whether *Tcp10b^t* is *Tcr^t*, transgenic mice containing genomic or cDNA constructs of *Tcp10b^t* were created. Breeding was performed to determine if the transgenes either underwent segregation distortion characteristic of the responder, or if transmission ratios of *t* haplotype or wild-type versions of chromosome *17* were altered. Both studies found some evidence for an effect of the transgenes on segregation ratios, although the results were largely inconclusive (BULLARD *et al.* 1992; SNYDER and SILVER 1992). The possibilities were raised that (1) *Tcp10b^t* might be a part of a more complicated *Tcr^t* locus, (2) *Tcr^t* cannot function properly outside its normal genomic context, or (3) TRD is sensitive to relative expression levels of Tcp10 homologues (of which there are three in both *t* and + chromosomes), and the transgenic experiments could not accurately recapitulate the endogenous balance in *t* haplotypes.

An unequivocal test of a candidate *Tcr^t* gene would be to abolish its activity and determine if this correlates with loss of TRD. We therefore developed *t* haplotype ES cells and mutated the *Tcp10b^t* gene by homologous recombination. The resultant animals still retained complete responder activity. These results appear to rule out *Tcp10b^t* as the responder.

MATERIALS AND METHODS

Generation of *t* haplotype ES cells: Mice of the genotype t^{h49}/t^{h49} in a noninbred background were used to create ES cell lines. The stock was originally obtained from the colony of LEE SILVER at Princeton, then maintained at Case Western Reserve University for 4 years. Females were superovulated and mated, and blastocysts were collected. Following immunosurgery to remove trophectoderm cells, ES cell lines were established on irradiated STO feeder cells (SOLTER and KNOWLES 1975; ROBERTSON 1987).

Construction of targeting vector, gene targeting, and creation of chimeric mice: DNA fragments from a t^{w5} allele of *Tcp10b^t* were isolated from the cosmid clone CW-6 (BULLARD *et al.* 1992). The 5′ half of *Tcp10b^t* in t^{h49} is derived from t^{w5}, although there is a possibility that the 3′ portion of this gene is of t^6 origin (BULLARD *et al.* 1992). A 2.5-kb *Eco*RI/*Spe*I fragment from the 3′ end of *Tcp10b^t*, and a 5.5-kb *Bam*HI fragment were successively ligated to the neomycin resistance (neo) vector, pbAneo, which contains the neo gene under the control of a mouse β-actin promoter (TOMASIEWICZ *et al.* 1993). A thymidine kinase gene driven by the HSV-TK promoter was added for selection against nontargeted integrations (CAPECCHI 1989).

The targeting plasmid was linearized with *Not*I, phenol/chloroform extracted, and ethanol precipitated. Twenty micrograms of linearized DNA was electroporated into 1×10^7 ES cells at 500 μF and 230 mV, then plated onto mitotically inactivated SNL7/67 feeder cells (MCMAHON and BRADLEY 1990). After 24 hr, G418 was added to a concentration of 250 μg/ml, and surviving colonies were picked ~10 days later. Screening for targeted insertions was performed by the method of RAMIREZ-SOLIS *et al.* (1992). ES cell DNAs were digested with *Eco*RI and analyzed by Southern blotting with the probe R/B.2 (see Figure 1).

Fifteen targeted ES cells were injected into the blastocoel cavities of C57BL/6J blastocysts and transferred to pseudopregnant C57BL/6J × DBA/2J foster mothers. The t^{h49} ES cells are homozygous for a wild-type agouti allele, therefore chimeric offspring were detected by coat color.

Molecular genotyping of knockout allele and *t* haplotypes: Detection of the targeted *Tcp10b^t* allele in normal tailed animals was performed by Southern analysis of tail DNA digested with *Eco*RI and hybridized to the probe R/B.2 (see Figure 2). In the case of tailless animals generated when the founder chimera (containing germ cells of the genotype t^{h49}/t^{h49bt-}) was mated to Tt^{h50} or Tt^{s6}, progeny were typed by Southern analysis as above to determine if the parent contained the targeted allele. Although the partial *t* haplotypes Tt^{h50} and Tt^{s6} were detected by the Brachyury (short tail) appearance when in *trans* to a wild-type chromosome and the tailless phenotype when in *trans* to t^{h49}, molecular verification was performed to guard against (1) crossovers between *T* and *t* haplotype DNA, and (2) severe manifestation of the Brachyury phenotype that can cause some *T*/+ animals appear to be tailless. Tt^{h50} and Tt^{s6} were scored in a PCR assay for the *Hba-ps4* locus (SCHIMENTI and HAMMER 1990), and presence of the t^{h49} haplotype was verified by a PCR assay for the *Tcp1^t* locus (MORITA *et al.* 1993).

RESULTS

Creation of *t* haplotype ES cells: Evaluating a candidate *Tcr^t* gene by targeted mutagenesis poses unique problems. Since *Tcr^t* exists only in *t* haplotypes, it is necessary to mutate candidate genes in *t* haplotype-derived ES cells to assay for effects on TRD. Furthermore, the candidate *Tcp10b^t* gene is one member of a gene family with three to four copies in *t* haplotypes. The individual members share at least 98.6% similarity in nucleotide sequence (PILDER *et al.* 1992). Therefore, transfected targeting DNA can potentially recombine with any of the multiple endogenous homologues. Finally, complete *t* haplotypes cause male sterility or lethality when homozygous, necessitating the use of an ES cell genotype that does not affect fertility or viability.

Since no complete or partial *t* haplotype contains *Tcp10b^t* in isolation, the partial *t* haplotype t^{h49} was chosen as the genotype from which to derive ES cells. This contains two Tcp10 genes, *Tcp10a^t* and *Tcp10b^t* (BULLARD and SCHIMENTI 1990), and is fully fertile. ES cells were generated from matings of homozygotes, and three ES cell lines were derived. Two of the three lines were evaluated for ability to generate germline chimeras, and one proved capable of doing so.

Targeted mutagenesis of *Tcp10b^t*: Targeting of *Tcp10b^t* proved to be difficult. Following a failure to generate homologous recombinants in a screen of over 1000 clones with two different targeting constructs, a third (pTKBH4neoE) was successful. The targeting strategy is diagrammed in Figure 1. Targeted clones were recovered at a frequency of about 1 in 50 G418 and gancyclovir-resistant transformants.

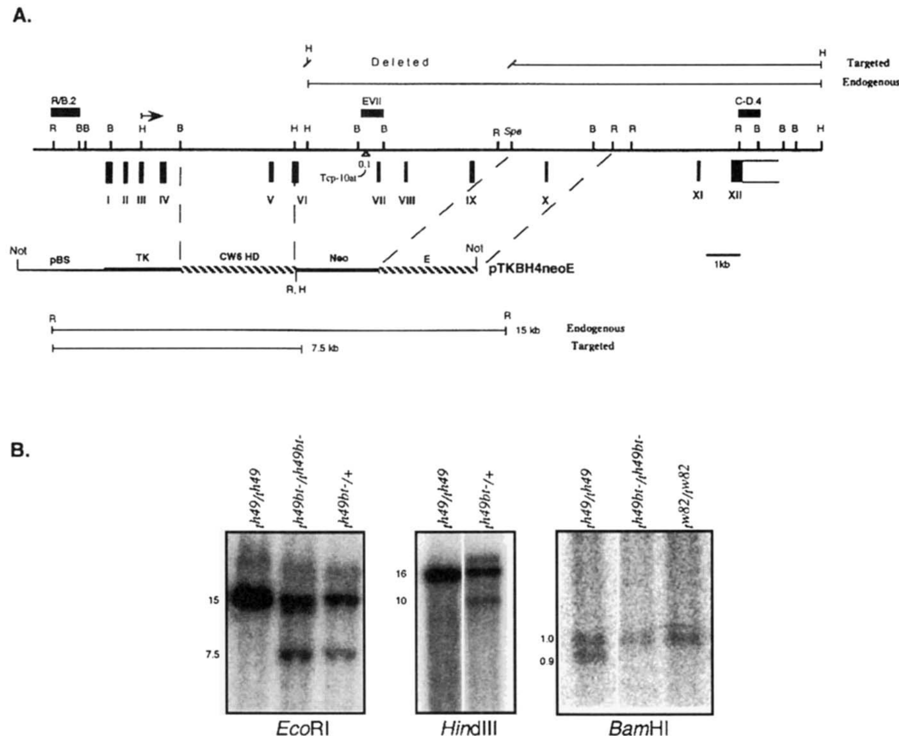

FIGURE 1.—Targeted mutagenesis of the *Tcp10b^t* gene. (A) The genomic structure of *Tcp10b^t* is diagrammed to scale. The location of exons (vertical black rectangles numbered with roman numerals) and most of the restriction sites is adapted from previous reports (BULLARD and SCHIMENTI 1990, 1991). The transcriptional start site is indicated by a horizonatal arrow above the exon map. Probes used in Northern and Southern hybridizations are indicated as dark boxes above the restriction map. The targeting vector is shown below the genomic map. The transcriptional orientation of the neo gene is the same as Tcp10. Regions of homology to the gene contained in the vector are indicated by striped lines. Predicted *Eco*RI and *Hin*dIII fragments for endogenous and targeted alleles are diagrammed at the top and bottom. Other *Spe*I sites exist in addition to the one shown. Scale in kb is indicated in the lower right. The location of a 100-bp insertion in *Tcp10a^t* relative to *Tcp10b^t* is indicated under the EVII probe. (B) Southern blots of DNA samples digested and hybridized with the indicated enzymes and probes are shown. Sizes of endogenous and targeted fragments are indicated in kb. For the *Eco*RI and *Hin*dIII digests, band sizes can be correlated with the diagram in A.

Tcp10 transcripts first appear at the pachytene stage of male meiosis. Although Tcp10 homologues in both *t* and + chromosomes possess highly similar amino acid-coding sequences, the *Tcp10b^t* gene switches to a unique postmeiotic alternative splicing pattern that dramatically alters the carboxy portion of the predicted protein product (CEBRA-THOMAS *et al.* 1991). Because this processing anomaly is the only major difference between *Tcp10b^t* and other Tcp10 homologues in terms of predicted amino acid coding sequence, it was postulated that this alternatively spliced product represents the responder activity (CEBRA-THOMAS *et al.* 1991). The mutagenesis strategy outlined below provides a direct test of this.

The targeting construct was designed to delete most of exon VI and all of exons VII–IX. This spans the region containing the two unique alternative postmeiosis splicing events in *Tcp10b^t*: (1) an in-frame skipping of exon VIII and (2) splicing from a cryptic donor inside exon IX to the acceptor of exon X. The cryptic exon IX donor alters the reading frame of *Tcp10b^t* transcripts by eliminating 145 amino acids from the carboxy terminus and replacing it with coding potential for an entirely different stretch of 45 amino acids (CEBRA-THOMAS *et al.* 1991).

Southern blot analysis of one targeted clone is shown in Figure 1B. In untargeted t^{h49} DNA, both the *Tcp10a^t* and *Tcp10b^t* genes yield a 15-kb *Eco*RI fragment with the R/B.2 probe, and a 16-kb *Hin*dIII fragment with the C-D.4 probe. The targeted clone has truncated bands of 7.5 and 10 kb, respectively, as predicted for an appropriate homologous integration (Figure 1). Following blastocyst injection of this clone to create a germline chimera, analysis of *Bam*HI digests with the EVII probe was performed to determine which of the two Tcp10 homologues was mutated. This probe hybridizes to a 1.0-kb *Bam*HI fragment in *Tcp10a^t* and a 0.9 fragment in *Tcp10b^t* (BULLARD and SCHIMENTI 1990). As shown in Figure 1B, untargeted t^{h49} DNA yields bands corresponding to both genes, whereas the t^{w82}/t^{w82} partial *t* haplotype, which retains only the *Tcp10a^t* gene (HOWARD *et al.* 1990; J. SCHIMENTI, unpublished observations), contains only the 1.0-kb fragment. Mice homozygous for the targeted insertion display only the *Tcp10a^t* 1.0-kb fragment. Therefore, mice created with this targeted ES cell line contain the designed mutation in

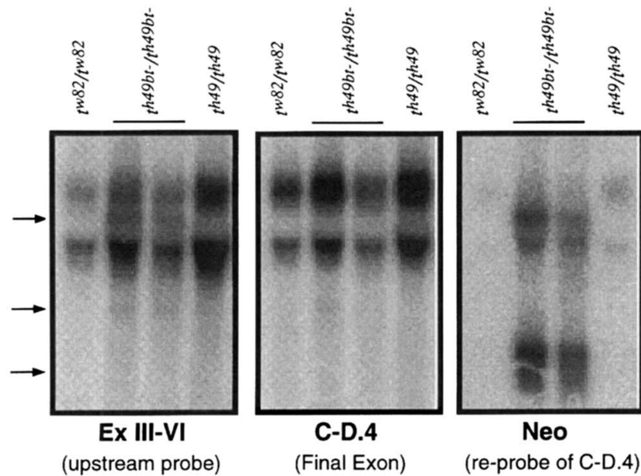

FIGURE 2.—Northern blot analysis of testicular RNAs. Ten micrograms of total testicular RNA was run in each lane of a 1.2% gel. The genotypes of the samples are indicated, as are the probes hybridized to each filter (see Figure 1). The neo hybridization was performed on the filter previously probed with C-D.4, and remaining weak hybridization signals serve as reference points. The two *th49b*$^{t-}$ RNA samples were derived from different mice. The arrows refer to bands mentioned in the text.

the candidate *Tcr*^{t} gene *Tcp10b*^{t}. The mutant allele is designated *Tcp10b*$^{t-}$, and the corresponding haplotype, *th49b*$^{t-}$.

To determine the effects of the mutation, Northern blot analysis was performed with probes corresponding to the 5′ and 3′ ends of the Tcp10 transcripts. Since the targeted deletion would not cause a frameshift if the primary transcript were to splice over the neo insertion, fusing exons V–X (deleting amino acids 160–309; SCHIMENTI *et al.* 1988), it is possible that an altered mRNA product could be produced that would be shortened by 248 amino acids or 744 nucleotides. Testicular RNA from t^{h49}/t^{h49} mice produce two major bands on Northern blots of ~2.2 and 1.8 kb, corresponding to products of both *Tcp10a*^{t} and *Tcp10b*^{t}. The two species are generated by a combination of alternative splicing and polyadenylation patterns in both genes (SCHIMENTI *et al.* 1988; BULLARD and SCHIMENTI 1991; CEBRA-THOMAS *et al.* 1991). The Northern blot patterns produced by a probe corresponding to the final exon (C-D.4) were identical between targeted and untargeted testicular RNA samples (Figure 2), except for a very faint novel band in the homozygous mutant samples ~400 bp smaller than the shortest normal transcript (middle arrow, Figure 2). It was also detected by the 5′ probe Ex III-VI (Figures 1 and 2), but the exact nature of this species is not clear. It represents a very minor portion of Tcp10 RNA in the cells. The absence of a 744-nucleotide truncated band indicates that the mutant allele does not splice over the neo insertion in the exact manner described above.

An identical Northern blot probed with an upstream fragment (Ex III-VI) produced a novel RNA species in the knockout mice that was intermediate in size between the two major bands. The upper 2.2-kb band, which displays a stronger hybridization signal when probed with the C-D.4, appears to correspond to transcripts containing the further downstream of two major alternative polyA sites (BULLARD and SCHIMENTI 1991). Since the aberrant 2.0-kb transcript in the knockout was larger than expected for a species that does not include terminal Tcp10 coding sequences, it is likely to constitute a fusion between the 5′ exons of *Tcp10b*^{t} and neo, whose promoter contains the first exon, first intron, and the 5′ end of exon 2 of the mouse β-actin gene. When this same Northern blot was subsequently probed with a radiolabeled neo fragment, the aberrant band hybridized strongly (Figure 2). Hence, the targeted disruption results in the production of a fusion transcript containing the 5′ end of *Tcp10b*^{t} and neo. The neo probe also detected a novel smaller species in the targeted mice. Since this smaller species does not hybridize to Tcp10 sequences, it is likely the product of the neo gene itself. Finally, neo hybridized to a species identical in size to the lower band seen with the two Tcp-10 probes. This, along with the 2.0-kb abberant band described above, are probably products of alternative splicing of the Tcp-10 5′ untranslated exons.

Homozygous mutants proved to be fertile as expected, since they retain a functional copy of *Tcp10a*^{t}. The mutant chromosome therefore resembles t^{w82} in terms of intact Tcp10 constitution. Whether there is an evolutionary advantage for this functional redundancy is unknown, but the Tcp10 duplications were part of larger duplication blocks that contain the responder (ROSEN *et al.* 1990). Hence, Tcp10 redundancy may be entirely incidental to duplication events that could have been required for the evolution of the responder.

The fusion product could potentially encode the first 160 amino acids of *Tcp10b*^{t} from exons III–V, assuming that the hybrid transcript splices from exon V of *Tcp10b*^{t} to the second exon of the bactin-neo fusion gene. It is conceivable that responder function is completely encoded in these 160 amino acids, even as a fusion. However, this seems unlikely; the only unique amino acid difference present in all *Tcp10b*^{t} alleles, relative to the other expressed Tcp10 genes in both *t* and + haplotypes, is a highly conservative change from arginine to lysine at residue 120, located in exon V (PILDER *et al.* 1992). The most distinguishing characteristic of the *Tcp10b*^{t} gene, the postmeiotic alternative splicing event described above, was obliterated by the targeting. Additionally, the targeting event eliminates the most evolutionarily conserved portion of the gene between mice and humans, exons XI–XII (ISLAM *et al.* 1991). There is 78% amino acid identity between human and mouse genes over 102 amino acids in this region, which is comprised entirely of a unique nonamer repeat unit (SCHIMENTI *et al.* 1988). Interestingly, whereas exon V

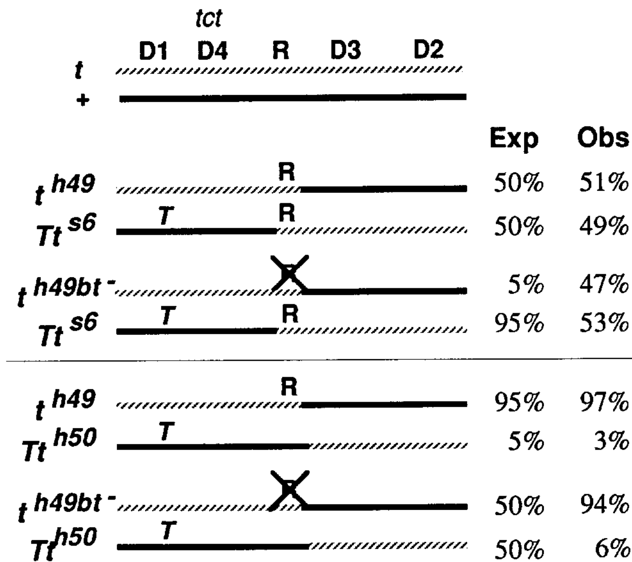

FIGURE 3.—Genetic crosses to assay responder activity. Horizontal black lines and striped lines represent + and *t* chromatin, respectively. At the top of the figure is a diagram of a mouse heterozygous for a complete *t* haplotype. The locations of the distorter genes (D1–D4), the *t* complex responder responder (R), and *t complex tail* (*tct*) locus in *t* haplotypes are indicated. The four crosses performed in this study are shown, with the expected and observed transmission frequencies of each chromosome in a doubly heterozygous male. The structure of Tt^{h50} is shown to contain the D3 distorter, based upon observations reported in the text. In the $Tcp10b^t$ knockouts, the expected percentages assume that $Tcp10b^t$ is the responder (hence, the crossed out R).

TABLE 1

Transmission ratios of mutant and control chromosomes

Knockouts			Nonknockouts		
Male	Br	nt	Male	Br	nt
	Tt^{s6}/t^{h49bt-}			Tt^{s6}/t^{h49}	
1289	35	20	1160	24	23
1207	20	18	1159	22	18
1420	12	15	1288	12	19
1425	15	16	1194	17	18
1427	21	22			
1462	18	12			
1426	12	13			
Total	133 (53.4%)	116 (46.6%)	Total	75 (49%)	78 (51%)
	T/t^{h49bt-}			T/t^{h49}	
1193	47 (77%)	14 (23%)	1287	62 (76.5%)	19 (23.5%)
	Tt^{h50}/t^{h49bt-}			Tt^{h50}/t^{h49}	
UKE#2	4	69	UKE#4	3	62
UKE#7	2	21	UKE#5	0	51
1421	2	29	A-50-1	0	19
1423	1	21	A-50-2	2	28
1427	3	34	A-50-3	2	28
1464	2	33	A-50-4	0	36
Total	14 (6.3%)	207 (93.7%)	Total	7 (3%)	224 (97%)

Br, Brachy mice; nt, normal tail.

sequences are weakly conserved, the human gene contains a lysine at the position corresponding to residue 120 in mouse Tcp10, which, as indicated above, was the only unique residue present in the $Tcp10b^{t-}$ allele relative to the other expressed Tcp10 genes (ISLAM *et al.* 1991). This argues that the arginine to lysine alteration does not affect Tcp10 function in a way that would cause the dramatic phenotype of TRD.

Genetic tests for TRD: Two breeding schemes were used to evaluate the $Tcp10b^{t-}$ allele for responder activity (Figure 3). In both genetic tests, transmission of $th49b^{t-}$ was expected to decrease drastically if $Tcp10b^t$ was in fact Tcr^t. However, in neither case did the mutant mice differ from the controls. These experiments are described in detail below.

The t^{h49} chromosome contains two distorters, *Tcd1* and *Tcd4,* plus the responder. When in *trans* to the partial *t* haplotype Tt^{s6}, which contains Tcr^t, the distorters *Tcd2* and *Tcd3,* and a Brachyury mutation (*T*) to cause taillessness [t^{h49} retains the *t complex tail* (*tct*) locus, which interacts with *T* to cause taillessness], such males have been reported to transmit each chromosome equally (LYON 1984; LYON and ZENTHON 1987; SILVER and REMIS 1987). This is because each chromosome has a copy of Tcr^t, and TRD does not occur unless this gene is heterozygous. The segregation of each chromosome is monitored by crossing to +/+ animals: progeny with short tails ("brachy") have inherited Tt^{s6}, while normal tailed (nt) pups have inherited t^{h49}. In accordance with these predictions, control t^{h49}/Tt^{s6} animals, in which the t^{h49} chromosome was derived from the untargeted allele of the founder germline chimera, demonstrated essentially 50% transmission of t^{h49} (Table 1; Figure 3).

If the targeted mutation were to abolish Tcr^t activity, then >90% of offspring from t^{h49bt-}/Tt^{s6} males should be brachy since all distorters will be present in one copy, and only Tt^{s6} would have an active responder. This prediction is based on a large body of genetic tests of similar genetic configuration, see (LYON 1984) for example. However, males of this genotype showed a transmission pattern similar to controls, indicating that $th49b^{t-}$ retains responder activity.

In the course of generating these animals, two tailless males, one presumably Tt^{s6}/t^{h49} (#1287) and the other Tt^{s6}/t^{h49bt-} (#1193), sired a disproportionately high number of brachy progeny. However, upon molecular analysis, they both proved to be homozygous for the wild-type allele at the distal *Hbaps4* locus, which maps in the vicinity of *Tcd2* (Figure 3). Hence, these animals had inherited recombinant chromosomes from their $Tt^{s6}/+$ parents, whereby crossover had occurred be-

tween T and the beginning of t haplotype DNA, effectively rendering them $t^{h49}/+$ and $t^{h49bt-}/+$, respectively. $t^{h49}/+$ males display low responder-dependent transmission of the t^{h49} chromosome (LYON 1984). Hence, the low transmission of t^{h49bt-} by male 1193 is additional evidence that the knockout did not inactivate *Tcr^t*.

The second genetic test to measure responder activity was performed by placing the knockout chromosome against the distal partial t haplotype Tt^{h50} (Figure 3). Like Tt^{s6}, it also carries a linked T mutation, hence scoring of progeny is done by tail length. Since it lacks a t responder, compound heterozygotes (t^{h49}/Tt^{h50}) have a complete set of distorters (see below), and a single t responder. Males of this genotype were expected to transmit t^{h49} at frequencies of ~95%, similar to complete t haplotypes (LYON 1984). Chimeric mice derived from untargeted t^{h49} ES cells were mated to Tt^{h50}, and four tailless males (A-50-1 through A-50-4) were mated to wild type to examine transmission ratios (Table 1). Two hundred twenty-four of 231 pups had normal tails, thus demonstrating that 97% inherited t^{h49}. It was previously suggested that Tt^{h50} contained all distorters except *Tcd3* (LYON 1984; SILVER and REMIS 1987), but such males would have been expected to transmit t^{h49} to only ~80% of progeny, as in t^{h49}/t^{h18} males (SILVER and REMIS 1987). These data here suggest that Tt^{h50} actually extends further proximal and probably contains *Tcd3*. Two additional t^{h49}/Tt^{h50} control males, whose t^{h49} chromosome was derived from the founder chimera harboring the t^{h49bt-} allele, cumulatively transmitted t^{h49} to 113 of 116 (97.4%) of offspring (Table 1; summarized in Figure 3).

If the targeted mutation had eliminated responder activity, neither chromosome would have *Tcr^t*, and equal transmission of each would be expected (LYON 1984). The transmission ratios of six t^{h49bt-}/Tt^{h50} males showed extremely high transmission of t^{h49bt-}, cumulatively at a rate of 93.7%, similar to the controls. These data clearly demonstrate that mutation of *Tcp10b^t* did not alter *Tcr^t*-dependent segregation distortion of t^{h49}.

DISCUSSION

These experiments provide strong evidence that the *Tcp10b^t* gene is not involved in transmission ratio distortion of mouse t haplotypes and is therefore not *Tcr^t*. While it is possible that the induced mutation left intact the portion of Tcp10 that mediates TRD, the Tcp10/neo fusion contains no amino acids that are absolutely unique among mouse and human homologues. It is conceivable that the precise amino acid sequence is not important, but that the truncated amino terminal polypeptide encodes responder activity. This is generated in t haplotypes by the postmeiotic alternative splice, and the targeting event might essentially mimic this by abolishing the normal carboxy terminus that contains the conserved exons XI and XII. Exons III and IV, which remain in the *Tcp10b^{t-}* allele, encode leucine zipper motifs that are also present in humans (ISLAM *et al.* 1991). Perhaps these domains effect *Tcr^t* activity only when in isolation. However, expression of the alternatively spliced species from transgenes failed to produce the expected *Tcr^t* phenotype (BULLARD *et al.* 1992). Although there were some indications that these transgenes altered transmission weakly, we now conclude that those data reflected minor statistical variation, and in combination with the results here demonstrate that *Tcp10b^t* is not involved in TRD.

Tcp10b^t had been an extremely strong candidate for *Tcr^t* not only for its testis-specific expression pattern and alternative postmeiotic splice that made it unique among the Tcp10 genes, but for compelling physical and genetic mapping data. The most reliable and useful mapping reagents are partial t haplotypes. Phenotypically, the genetic test for a responder is very robust, provided the sample size is sufficient. A case in point is the second genetic test described herein, whereby a proximal partial t haplotype such as t^{h49} that contains *Tcr^t* will show dramatic distortion in the presence of distal distorters. The breakpoints of all the recombinant partial t chromosomes analyzed to date unequivocally place *Tcr^t* in a genetic interval (*D17Leh66b*) of between 40 and 155 kb (ROSEN *et al.* 1990; BULLARD *et al.* 1992). Data on the existence of possible alleles of *Tcr^t* with different activities (LYON and ZENTHON 1987) led us to localize *Tcr^t* to a region of ~40 kb, within which *Tcp10b^t* resides (BULLARD *et al.* 1992). Assuming now that the region occupied by *Tcp10b^t* does not contain the responder, *Tcr^t* presumably resides in a region of ~20 kb just downstream of *Tcp10b^t*. We have found that cosmid clones from this region are very unstable. While it is difficult to draw any conclusions based on this, it raises the possibility that the responder is not a gene *per se* but a structural anomaly. In the segregation distortion system in *Drosophila melanogaster*, the responder is in a region of centromeric heterochromatin, and the "sensitive" and "insensitive" forms are actually determined by the number of repeated elements (WU *et al.* 1988). This affects chromatin condensation and spermiogenesis. *Tcr^t* in mice, however, is not located in centromeric heterochromatin.

The *Tcr^t* region has a number of unusual structural and evolutionary features. The 220-kb *D17Leh66b* interval lies between two major inversions that distinguish wild-type and t haplotypes. The great majority of rare crossovers in $+/t$ heterozygotes occur at the junctions of this interval, and many breakpoints are clustered in a region of 10–20 kb (SCHIMENTI *et al.* 1987; BULLARD and SCHIMENTI 1990). Contained within are large duplicated tracts of DNA called T66 elements, ranging from 100 to 220 kb, that have been highly unstable in recent evolutionary history (SCHIMENTI *et al.* 1987; ROSEN *et al.* 1990). The proximal t complex inversion had a breakpoint within the T66 family of elements, leading

to the current day "wild-type" chromosomes, and that contributed to genetic isolation of *t* haplotypes (HAMMER *et al.* 1989). Finally, there is evidence that T66 elements in wild-type chromosomes undergo frequent rearrangements, since different inbred strains and *t* haplotypes have been found to have variant orientations and copy numbers of these elements (SCHIMENTI *et al.* 1987; NADEAU *et al.* 1989; HOWARD *et al.* 1990).

While these phenomena may be coincidental, it is possible that the unusual DNA structures may be responsible for the responder activity. If the responder "gene" was actually a variant chromatin structure, this would explain its *cis* active effects. This is an attractive possibility, considering current evidence indicating that products of expressed genes appear to be shared among meiotic partners through cytoplasmic bridges (BRAUN *et al.* 1989; CALDWELL and HANDEL 1991). If chromatin structure underlies the distortion phenotype, it is possible that the phenomenon is related to meiotic chromosome pairing. However, experiments with tertiary trisomy of chromosome *17* suggest that pairing is not responsible (AGULNIK *et al.* 1991). If Tcr^t is in fact a chromatin anomaly, it may manifest itself by functionally affecting spermatozoan function in a mechanical way that involves the *trans*-active distorter products.

A final possibility is that Tcr^t is a loss-of function mutation, which appears to be the case for *Tcd1* (LYON 1992). However, this would imply that different partial *t* haplotypes with Tcr^t activity would have to lack a common piece of wild-type chromatin. There are several contradictions of this requirement. For example, the partials t^{h53} and t^{w82} both lack Tcr^t activity and have breakpoints into wild-type chromatin more distal than t^{h2}, which does have Tcr^t (BULLARD and SCHIMENTI 1990; J. SCHIMENTI, unpublished observations). The only common denominator with Tcr^t-bearing chromosomes is that they must have the *D17Leh66b* region of DNA from *t* haplotypes.

Since there is no certainty as to the nature of Tcr^t at this point, further attempts to identify it might best be conducted by systematically mutating the remaining genetic interval in which it must reside. The t^{h49} ES cells should be a useful reagent for such experiments.

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