Genet. Res., Camb. (1999), **74**, *pp.* 139–144. *With* 4 *figures.* Printed in the United Kingdom © 1999 Cambridge University Press

Genetic variants of the tuberous sclerosis 2 tumour suppressor gene in mouse *t* haplotypes

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(*Received 20 January 1999 and in revised form 26 March 1999*)

Summary

The murine *t* complex on chromosome 17 contains a number of homozygous lethal and semi-lethal mutations that disrupt development of the mouse embryo. We recently characterized an embryonic lethality in the rat that results from a germ-line mutation in the tuberous sclerosis 2 (*Tsc-2*) tumour suppressor gene (the Eker mutation). Remarkably, mouse embryos homozygous for t^{w8} mutation display cranial defects reminiscent of those observed in rat embryos homozygous for the Eker mutation. To determine whether the *Tsc-2* gene, which is in the *t* complex, is mutated in t^{w8} or other *t* haplotypes, we characterized this gene in a series of *t* haplotype mice. Four *Tsc-2* polymorphisms were identified: three in the coding region and one intronic that appeared to be common to all *t* haplotypes analysed. No evidence was found to argue that the *Tsc-2* gene is altered in t^{w8} haplotype mice. However, in the t^{w5} haplotype we found a G to T mutation in *Tsc-2* that was present only in this *t* haplotype. In contrast to other polymorphisms within the *Tsc-2* coding region which did not result in amino acid changes in *Tsc-2* gene product tuberin, this mutation substituted a phenylalanine for a conserved cysteine in t^{w5} tuberin. Within the *t* complex, the *Tsc-2* gene and the putative t^{w5} locus appeared to map to different positions, complicating identification of *Tsc-2* as a candidate for the t^{w5} locus and suggesting that the G to T mutation in the *Tsc-2* gene may have arisen independently of the t^{w5} functional mutation.

1. Introduction

Mouse *t* hapolotype mutations occur within the *t* complex, a 15 cM region of the proximal end of mouse chromosome 17, and, with some exceptions, are embryonic lethal in the homozygous (t^x/t^x) condition (Bennet, 1975; Artzt, 1984). Mice bearing semi-lethal mutations have viable homozygous offspring with no other adverse phenotype than male sterility, which was attributed to mutations unrelated to the lethal *t* haplotype mutations. However, the percentage of homozygous pups from semi-lethal *t* haplotype parents is lower than expected from Mendelian inheritance of a single gene (Bennet, 1975). The *t* complex, which was originally identified in a limited (approximately 20%) population of wild mice, consists of four large non-overlapping inversions (Hammer *et al.*, 1989; Hamvas *et al.*, 1996) that almost completely suppress recombination of this region with the normal mouse chromosome 17. The *t* haplotype mutations have been of interest to developmental geneticists for a number of years, as each mutation disrupts development of the mouse embryo at a specific stage (Bennett, 1975; Babiarz, 1983; Artzt, 1984), thus making it possible to identify the network of genes critical for normal embryogenesis. Several putative loci for these mutations have been established by linkage analysis (Howard *et al.*, 1990; Ebersole *et al.*, 1992; Hamvas *et al.*, 1996) but the specific genes responsible for embryonic death of homozygous *t* haplotype mice, except for the recent report of a candidate for t^{w73} (Verhaagh *et al.*, 1998), are not known.

The mouse tuberous sclerosis 2 (*Tsc-2*) tumour suppressor gene was mapped to chromosome 17 within the *t* complex (Olsson *et al.*, 1995; Pilz *et al.*, 1995). In Eker rats (Eker & Mossige, 1961), a germ-

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https://doi.org/10.1017/S0016672399003985 Published online by Cambridge University Press

line mutation in the *Tsc-2* gene that abrogates normal function of the *Tsc-2* gene product tuberin (the Eker mutation) results in embryonic lethality when homozygous (Eker *et al.*, 1981; Rennebeck *et al.*, 1998). Homozygous Eker embryos die before embryonic day 13·5, which corresponds to embryonic day 11·5 in mice, and display cranial defects ranging from papillary overgrowth to dysraphia (Rennebeck *et al.*, 1998). Strikingly, mouse embryos homozygous for the t^{w8} mutation have cranial defects reminiscent of those observed in rat embryos homozygous for the Eker mutation of the *Tsc-2* gene. The t^{w8} haplotype is semi-lethal; some t^{w8}/t^{w8} mice die before embryonic day 11·5, but others grow into morphologically normal adult mice (Bruck, 1967). While the genes responsible for semi-lethality in these mice have not been precisely mapped in the *t* complex, segregation of lethality with the t^{w8} haplotype suggests that at least one of these genes is within the *t* complex.

To determine whether the *Tsc-2* gene is the site of the t^{w8} or other *t* haplotype mutations, we characterized this gene in several T/*t* and *t*/*t* haplotype mice. The dominant T mutation (*Brachyury*, another gene mapped to chromosome 17) affects development of the notochord and is embryonic lethal when homozygous (Bennett & Dunn, 1958). Heterozygous T/*t* mice have a tail-less phenotype that is conventionally used to maintain *t* haplotype mouse colonies. In general, in the progeny of tail-less *t*/*t* parents with the same *t* mutation, there are only tail-less heterozygous T/*t* and no homozygous *t*/*t* offspring, indicating that a particular *t* mutation is embryonic lethal. However, crosses between T/*t* mice that bear semi-lethal *t* mutations produce both tail-less heterozygotes (T/*t*) and normal-tailed homozygotes (*t*/*t*) in the same litter (Bennett, 1975; Artzt, 1984).

In our panel of T/*t* and *t*/*t* haplotype mice we found several novel polymorphisms in the *Tsc-2* gene that may be useful as genetic markers for mice carrying *t* haplotypes. No evidence was obtained by Southern, Single Strand Conformation Polymorphism (SSCP) and Western analysis to suggest that the *Tsc-2* gene is altered in t^{w8} mice, eliminating this gene as a candidate for the t^{w8} gene. However, a mutation in *Tsc-2* was identified which resulted in a amino acid substitution with possible functional consequences, which was only present in t^{w5} haplotype mice.

2. Material and methods

(i) *Tissues*

t Haplotype mice (4–10 months old) were obtained from Dr K. Artzt (The University of Texas, Austin, TX, USA). The kidneys, hearts, spleen and brains were obtained from these mice and snap-frozen in liquid nitrogen for nucleic acid and protein extraction.

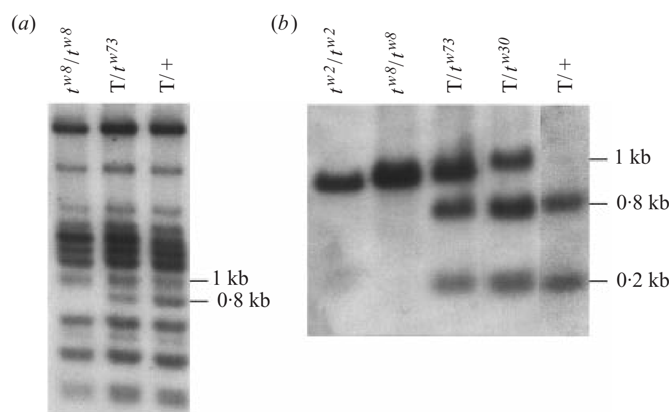

Fig. 1. Southern analysis of the *Tsc-2* gene in *t* haplotypes. T/+, C3H mice heterozygous for the T locus. Genomic DNA obtained from mouse spleen was digested with *Pst*I and hybridized to different fragments of the *Tsc-2* gene. RT-PCR fragments spanning exons 16–37 (*a*) and 29–30 (*b*) were used as probes for hybridization.

(ii) *Southern analysis*

Genomic DNA from livers of C3H and t^{w8} mice was kindly provided by Dr K. Artzt. Also genomic DNA was obtained from spleens of these mice and other *t* haplotype mice by a kit from Qiagen (Chatsworth, CA). Southern analysis with *Eco*RI, *Bgl*II, *Pst*I, *Bam*HI and *Hind*III was performed as previously described (Kleymenova *et al.*, 1998). The RT-PCR products spanning different exons of the mouse *Tsc-2* gene were used as probes for hybridization.

(iii) *SSCP analysis*

Total RNA from frozen mouse tissues was isolated by standard CsCl centrifugation. Reverse transcription (RT) was performed as previously described (Kleymenova *et al.*, 1997) and 1 μl of this reaction was used for polymerase chain reaction (PCR) to amplify products of approximately 400 bp. As the mouse *Tsc-2* sequence had not been published at the beginning of this study, the primers to amplify 17 PCR fragments covering the entire coding region of the mouse *Tsc-2* gene (Fig. 1*a*) were generated from the highly homologous human and rat *Tsc-2* sequences retrieved from the GenBank database (accession nos. X75621, U24150) and their sequences and PCR conditions are available upon request. Two microlitres of ^{32}P-labelled PCR product was mixed with 8 μl of loading buffer (95% formamide, 0·5% EDTA, 0·05% bromophenol blue, 0·05% xylene cyanol and 20 mM-NaOH), denatured at 95 °C for 3 min, chilled on ice, and electrophoresed at 5W overnight in a 1× mutation detection enhancement gel (ATBiochem, Malvern, PA). The dried gels were exposed for 4–8 h to X-ray film with an intensifying screen.

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(iv) *Western analysis*

Cell lysates were obtained from T/C3H and *t* haplotype mice brains pulverized in liquid nitrogen. Western analysis using anti-tuberin serum #5063 d.50 which was raised against amino acids 1387–1784 of human tuberin, was performed as previously described (Wienecke *et al*., 1995). Mouse fibroblast cell line NIH 3T3 was used as a positive control.

3. Results

Gross alterations or loss of heterozygocity (LOH) of the *Tsc-2* locus in t^{w8} or other *t* haplotypes, including t^{w2}, t^{w5}, t^{w32}, t^{w35}, t^{w73}, t^{w75}, t^{w120} and t^{w130}, was assayed by Southern analysis. As only the t^{w2} haplotype is semi-lethal and has viable homozygotes, heterozygous T/t^{w5}, T/t^{w32}, T/t^{w35}, T/t^{w73}, T/t^{w75}, T/t^{w120} and T/t^{w130} mice were used. Intensity of single copy bands from DNA digestion with *Eco*RI, *Bgl*II, *Pst*I, *Bam*HI or *Hind*III was the same in C3H mice used as a control and homozygous or heterozygous *t* haplotypes, indicating that all *t* alleles studied retained the *Tsc-2* gene. Southern analysis revealed a *Tsc-2* polymorphism, recognized as different *Pst*I digestion patterns in C3H and *t* haplotypes (Fig. 1*a*). Using ^{32}P-labelled probes that hybridized to different exons of the murine *Tsc-2* gene, we mapped this polymorphism to intron 29, where all *t* haplotypes examined lacked the *Pst*I site (Fig. 1*b*). To confirm the results obtained by Southern analysis, we designed primers specific for exons 29 and 30 of the murine *Tsc-2* gene that yielded an approximately 1 kb PCR fragment from genomic DNA. The wild-type PCR product exhibited a normal *Pst*I digestion pattern, yielding two fragments of 800 and 200 bp, whereas in homozygous t^{w8} and t^{w2} mice only a single 1 kb fragment was observed after *Pst*I digestion. Heterozygous *t* haplotype mice exhibited both *t* haplotype and normal *Pst*I restriction fragments (1 kb, 800 bp and 200 bp; data not shown).

To examine t^{w8} haplotype mice for small deletions, insertions or point mutations in the *Tsc-2* gene, RT-PCR and SSCP analysis were performed using total RNA from brain to amplify different exons of this gene. Seventeen overlapping approximately 400 bp RT-PCR products covering the entire *Tsc-2* coding region (Fig. 2*a*) were of predicted sizes in t^{w8} mice (data not shown) and were analysed by SSCP analysis. Mobility shifts between t^{w8} mice and C3H mice, which lack the *t* complex and have the wild-type *Tsc-2* gene, were detected for fragments 4, 7 and 8 of the murine *Tsc-2* gene (Fig. 2*b–d*). Direct sequencing of a sense strand of these fragments revealed A to G, G to A and C to T substitutions in t^{w8} mice at positions 1299 (fragment 4), 2190 (fragment 7) and 2390 (fragment 8) of the reported mouse *Tsc-2* cDNA respectively that was confirmed by sequencing of an antisense strand of independent RT-PCR products (data not shown). None of the substitutions resulted in amino acid changes in mouse tuberin.

To determine whether the *Tsc-2* substitutions found in t^{w8} mice occurred in other *t* haplotypes, RT-PCR and SSCP analysis of fragments 4, 7 and 8 was performed on the same panel of *t* haplotypes as above. T/C3H mice were used to ensure that animals with and without the T locus have the same *Tsc-2* sequence. All three RT-PCR products from all *t* haplotypes analysed were of expected sizes (data not shown). All three fragments from a homozygous t^{w2} mouse had the same SSCP migration pattern as t^{w8} fragments (Fig. 2*b–d*). All heterozygous *t* haplotypes analysed, except T/t^{w5} and T/t^{w73}, had both wild-type bands and bands with t^{w8} mobility (Fig. 2b–d). However, the T/t^{w73} mouse had only the wild-type fragment 4 (Fig. 2*b*), based on its migration pattern, although its fragments 7 and 8 exhibited both wild-type and *t* haplotype-specific bands. That all three *Tsc-2* mutations found in t^{w8} mice were also present in mice with other *t* haplotypes indicated that these substitutions were genetic polymorphisms.

To confirm that the *Tsc-2* gene was not the site of the t^{w8} mutation, we sequenced the coding region of *Tsc-2* from t^{w8} (GenBank accession no. AF132986) and C3H mice and compared it with previously reported *Mus musculus Tsc-2* (Kim *et al*., 1995). No nucleotide changes other than the polymorphisms described above were found in the Tsc-2 gene of the t^{w8} mouse, consistent with the results of the SSCP analysis. Sequencing of RT-PCR fragments 4, 7 and 8 in T/t^{w32} revealed both wild-type and *t*-haplotype specific sequences at positions 1299, 2190 and 2390, respectively. In addition to the *t* haplotype polymorphisms that were identified in our study, other nucleotide differences between the previously reported *Tsc-2 Mus musculus* sequence and our *Tsc-2* sequences (C3H and t^{w8}) were noted. In both t^{w8} and C3H, we found C at position 1293, C at position 3240 and G at position 3303, whereas T, T and A, respectively, were reported previously at these positions (Kim *et al*., 1995).

Mice have three splicing isoforms of the *Tsc-2* gene expressed in a tissue-specific manner (Hu *et al*., 1995; Kim *et al*., 1995). These isoforms differ by exclusion of exons 25 or 31. By RT-PCR we analysed the splicing pattern of the *Tsc-2* gene in the brain (which contains all three isoforms), kidney and heart from t^{w8}, t^{w2}, *T*/t^{w5}, *T*/t^{w32}, *T*/t^{w35}, *T*/t^{w73}, *T*/t^{w75}, *T*/t^{w120}, *T*/t^{w130} and C3H mice. No differences in splicing were observed among these mice (data not shown). Expression of tuberin in the brains of *t* haplotype and T/C3H mice was assessed by Western analysis with anti-tuberin serum #5063 d.50 which was raised against carboxyl terminus of human tuberin. A

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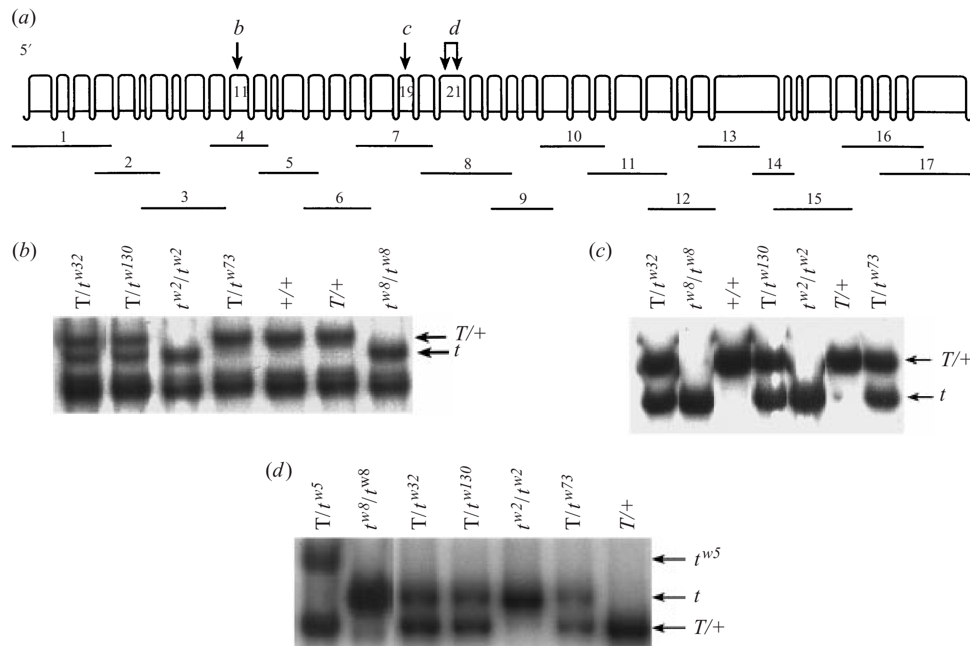

Fig. 2. SSCP analysis. (*a*) Schematic representation of the mouse *Tsc-2* coding sequence. Continuous lines represent overlapping RT-PCR fragments examined for mutations by SSCP analysis. Vertical arrows indicate positions of polymorphisms in fragments 4, 7 and 8 (*b*), (*c*) and (*d*). Representative samples of SSCP analysis of the fragments 4, 7 and 8, respectively, in mouse *t* haplotypes t^{w8}/t^{w8}, t^{w2}/t^{w2}, T/t^{w5}, T/t^{w32}, T/t^{w73} and T/t^{w130}. +/+, C3H mice; T/+, C3H mice heterozygous for the T locus.

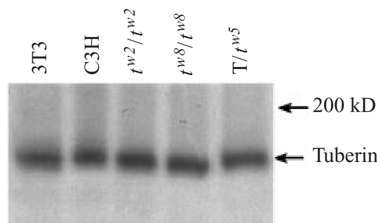

Fig. 3. Western analysis of tuberin in *t* haplotype mice. 3T3, mouse fibroblast cell line (positive control); T/+, C3H heterozygous for the T locus.

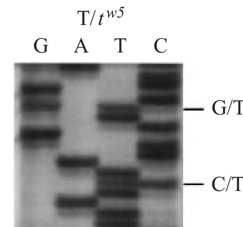

Fig. 4. Sequence alteration associated with t^{w5} mutation in the mouse *Tsc-2* gene. C/T, *t* haplotype-specific polymorphism; G/T, t^{w5} mutation.

180 kDa single band was detected in all samples analysed; there were no differences in tuberin expression in t^{w8} mice compared with other *t* haplotype or wild-type mice (Fig. 3).

Finally, in contrast to the other *t* haplotype mice, an additional *Tsc-2* alteration was found in the T/t^{w5} mouse which had a RT-PCR fragment 8 that migrated differently from the fragment 8 of mice with other *t* haplotypes and C3H mice (Fig. 1 *d*). Direct sequencing of the sense strand of this fragment, amplified from T/t^{w5} brain RNA, revealed T and G at position 2398 of reported murine Tsc-2 sequence, where both T/C3H and t^{w8} mice have a T (Fig. 4). Sequencing of the antisense strand of independent RT-PCR product confirmed this finding (data not shown). While none of the polymorphisms found in t^{w8} and other *t* haplotypes changed amino acids in mouse tuberin, the t^{w5}-specific mutation in the *Tsc-2* gene substituted a phenylalanine for a conserved cysteine at position 799 in mouse tuberin, suggesting that in contrast to other *t* haplotype polymorphisms, this alteration could have functional consequences.

4. Discussion

The cranial abnormalities in t^{w8} embryos, which are strikingly similar to the brain defects observed in Eker rat embryos homozygous for *Tsc-2* mutation, led us to examine whether *Tsc-2* alterations are responsible for the developmental defect observed in t^{w8} mice. In the brain of viable homozygous t^{w8} mice, which undergo normal development, tuberin was expressed, and we did not detect LOH or mutations in the *Tsc-2* gene. These findings indicate that this gene appears not to be a site for t^{w8} mutation. In the present study four

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Tsc-2 gene polymorphisms, three in the coding region and one in an intron, were mapped on mouse *t* alleles. These polymorphisms appeared to be *t*-haplotype-specific with respect to C3H and *Mus musculus* mice. All four *Tsc-2* polymorphisms are novel and have not been previously described. These polymorphisms could be useful as genetic markers in linkage analysis; in particular, the intronic *Pst*I polymorphism may be useful for identifying the *Tsc-2* locus in *t* haplotypes by direct PCR. In addition to the *Tcp* 1 gene polymorphisms (Willison *et al.*, 1986), the *Tsc-2* polymorphisms are located precisely within the *t* complex, making it possible to follow the *Tsc-2* locus in linkage studies.

Direct sequencing of the coding region of the *Tsc-2* gene in C3H and t^{w8} mice revealed some differences between a previously reported mouse *Tsc-2* sequence (Kim *et al.*, 1995) and our sequence. Similar to *t* haplotype polymorphisms, these nucleotide changes would not translate into amino acid changes in mouse tuberin. That the *Tsc-2* polymorphisms were conserved between *t* haplotypes confirms the limited genetic variability in these mice due to suppressed recombination of *t* chromatin, which is generally believed to originate from a common ancestor (Silver *et al.*, 1987).

In contrast to the *Tsc-2* polymorphisms that were common in all *t* haplotypes studied, the G to T mutation at position 2398 appeared to be specific for the t^{w5} haplotype. While polymorphic changes in the *Tsc-2* gene reported here did not cause amino acid substitutions, the t^{w5} mutation results in substitution of a phenylalanine for a cysteine that is conserved between human, rat, mouse and even *Fugu rubripes*. How this mutation contributes to t^{w5} haplotype lethality is not clear at present. The H-2 haplotype of t^{w5} is different from other *t* haplotypes (Artzt *et al.*, 1985) and the t^{w5} anonymous DNA sequences located within the *t* complex are the most divergent from other *t* haplotypes (Yeom *et al.*, 1992). On the normal mouse chromosome 17 the *Tsc-2* gene is mapped near D17Lon1 (Olsson *et al.*, 1995; Pilz *et al.*, 1995) and the putative locus for the t^{w5} lethal gene was found to be inseparable from H-2K (Ebersole *et al.*, 1992), implying that the *Tsc-2* gene is different from the t^{w5} locus. Though the *Tsc-2* mutation appears to have no effect on *Tsc-2* splicing or level of tuberin expression in t^{w5} mice, the conserved cysteine that was replaced in these mice by phenylalanine is within a domain of tuberin highly conserved between species. Cysteine may participate in the formation of bonds that stabilize the functional conformation of tuberin and substitution of bulky phenylalanine for relatively small cysteine may affect tuberin activity. Functional studies of the impact the t^{w5} mutation are necessary to shed light on the role of this *Tsc-2* mutation in t^{w5} haplotype lethality.

We thank Dr K. Artzt for reagents and helpful discussions, and Dr J. Schimenti for critically reviewing the manuscript. The technical assistance of K. Sowell and D. Trono is appreciated. This work was supported by NIH grant CA 63613 to C. L. W.

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