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Mouse *Brachyury the Second (T2)* Is a Gene Next to Classical *T* and a Candidate Gene for *tct*

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ABSTRACT

The mouse *Brachyury the Second* (*T2*) gene is 15 kb away from classical *Brachyury* (*T*). A mutation in *T2* disrupts notochord development, pointing to the existence of a second *T/t* complex gene involved in axis development. *T2* encodes a novel protein that is disrupted by an insertion in $T2^{Bob}$ mice. Sequence analysis of *T2* from several *t* haplotypes shows that they all share the same changed stop codon, and, thus, *T2* is a candidate gene for the *t* complex tail interaction factor. *T1*, *T2*, and the unlinked *t-int* are distinct and unrelated loci, and mutations in these genes do not complement one another genetically. Either their products interact in the same pathway during the genesis of the embryonic axis, or the *T/t* region itself is truly complex.

THE *Brachyury* gene (*T*) encodes a transcription factor essential for the genesis and maintenance of mesoderm and notochord (Kispert and Herrmann 1993). There has been a long-standing debate about the existence of a second *Brachyury* gene in the proximal region of the mouse *t* complex (reviewed in MacMurray and Shin 1988; Nadeau *et al.* 1989; Rennebeck *et al.* 1995). All *t* haplotypes are derived from a single ancestral event that occurred 4–5 mya (Silver *et al.* 1987), and, consequently, they all carry the *t* complex tail interaction factor (*tct*), a recessive mutation that is genetically inseparable from *T* (Justice and Bode 1988). *T* heterozygous mice have shortened tails; however, *tct* has no phenotype on its own but causes tailless mice when heterozygous with *T* (*T/tct*). *tct* thus acts as an enhancer of *T*. Although the close linkage of these two loci and their failure to complement suggests that they are allelic, no defect in the *T* gene of *t* haplotypes has been found (B. Herrmann, personal communication). Moreover, exogenous transgenic copies of the *T* gene fail to rescue the embryonic lethality caused by homozygosity for the original *Brachyury* deletion (Stott *et al.* 1993), suggesting that other essential embryonic gene(s) map to this 200-kb region.

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We previously showed that *Brachyury the Second* (*T2*) interacts with *tct* such that *T2/tct* mice have tails that are short stumps (Rennebeck *et al.* 1995). Genetic separation between *T* and *tct* has been sought but never documented (M. Justice, personal communication), suggesting that *T* and *tct* are either the same gene or closely linked genes. As a result, the question of allelism between *T* and *tct* has remained open.

Our previous analysis of $T2^{Bob}$ showed it to be an insertional mutation mapping within the original *Brachyury* deletion (*T*). This suggested that $T2^{Bob}$ is either a novel allele of *T* or *tct* or that it acts in the same pathway as these genes because they all interact phenotypically (Rennebeck *et al.* 1995). The homozygous $T2^{Bob}$ syndrome is similar to but less severe than the homozygous *T* phenotype. Whereas *T* mutants do not achieve a placental connection and lack floorplate and the most posterior somites (Chesley 1935), $T2^{Bob}$ homozygous embryos achieve all these milestones. In contrast to *Brachyury* mutants, in which a few notochord precursors can be transiently detected but fail to persist or proliferate (Herrmann 1991; Conlon *et al.* 1995), $T2^{Bob}$ homozygotes contain many notochord cells distributed discontinuously along the axis. Although these cells can induce a visible floorplate, they are only distinguishable from the surrounding mesenchyme because they express the notochord markers *sonic hedgehog* and *Brachyury* (Rennebeck *et al.* 1995). Thus, the primary defect in $T2^{Bob}$ homozygotes appears to be the inability to organize or maintain the notochord as a structure.

Analysis of axis development by many laboratories confirms that neural tube defects in mice and, by implication, in humans are a heterogeneous group of malformations caused by numerous factors. In addition to the effects that environmental agents may have, the functions of nonallelic pairs of genes have been tied to axial development. Elevated occurrences of neural tube

defects have been found in mice that are double mutants for the transcription factors *Splotch* and *extra toes* (Copp 1995), as well as *Splotch* and *curly tail* (Estibeiro *et al.* 1993). *undulated-Patch* double-mutant mice also have a phenotype reminiscent of an extreme form of spina bifida occulta in humans (Helwig *et al.* 1995). Another valuable and well-studied model is the combination of mutant alleles of *Brachyury* and *tct* (Park *et al.* 1989) and, more recently, *Brachyury* and *btm* (Fujimoto *et al.* 1994), in which penetrance of spina bifida approaches 100%. Molecular access to multiple genetic components of the *T/tct* model may lead to a better understanding of this frequent birth defect.

We have identified a new gene, *T2*, that maps 15 kb centromeric to *T* (hereafter referred to as *T1*). The two genes are transcribed in the same direction. The $T2^{Bob}$ allele is a 200-kb transgenic insertion with a concomitant deletion of 3 kb containing two of its exons. Despite this disruption, the genomic locus of *T1* and its early phase of expression are apparently not affected in the $T2^{Bob}$ mutant. Although this suggests the presence of a second axis-determining gene in this region, the possibility remained that the insertion disrupted some previously undefined 5′ regulatory element of notochord expression of *T1*. The cloning of a novel gene, *Brachyury the Second (T2)*, bridging the insertion site but not sharing any sequence with *T1* may resolve this issue and explain the *Brother of Brachyury (Bob)* phenotype. Moreover, the sequence of *T2* in three different *t* haplotypes reveals a common change that leads to 12 extra hydrophobic amino acids (aa) at the C terminus of the conceptual protein. Taken together, the map position of the *T2* gene, its mutant phenotype, the interaction of *T2* and *tct*, and the presence of a *t* haplotype-specific change strongly suggest that the *t* haplotype copy of *T2* is a candidate gene for *tct*.

MATERIALS AND METHODS

Nomenclature: Because the phenotypes of *T1* and *T2* are so similar and genetic background is known to affect the penetrance of axis defects, it is possible that some alleles originally classified as *T* may really be mutations in *T2*. To avoid confusion, it becomes necessary to determine whether in fact *T1* or *T2* (or both) are mutated in the more than a dozen known "*T* alleles." In consultation with the mouse nomenclature committee, we propose that the original *Brachyury* deletion and any deletion known to affect both genes retain the designation *T*, and the previously characterized *Brachyury* gene should be called *T1* with a superscript for the allele, (*e.g.*, as in $T1^{Wis}$). Likewise, the *T2* allele *Brother of Brachyury*, described by Rennebeck *et al.* (1995), is $T2^{Bob}$. Finally, if *tct* proves to be an allele of *T2*, it should be designated $T2^{tct}$.

Molecular techniques: The Exon Trapping II System from Bethesda Research Laboratories (BRL, Gaithersburg, MD) was used according to the manufacturer's instructions. *R*andom *a*ccess *r*etrieval of *g*enetic *i*nformation by *P*CR (rargip) screening was done according to Abe (1992). Biotinylation of probe was done with the Bionick kit (BRL). Streptavidin-coated magnetic beads were purchased from Promega Biotech (Madison, WI). Exon trapping and rargip clones were authenticated by analysis of the genomic sequence flanking the two trapped exons. This revealed that the splice donor/acceptor sites of the exons in the rargip clones were identical to those used by the exon trapping system. Subcloning of PCR fragments was done using the pGEM-TA vector (Promega Biotech). Sequencing was done using Sequenase version 2.0 (United States Biochemical, Cleveland, OH) or an ABI automated sequencer (Applied Biosystems, Foster City, CA). Total RNA was isolated by the LiCl/urea method (Geliebter 1987). mRNA selection was done with the poly(A) Tract system (Promega Biotech). Reverse transcription (rt) reactions were done using Superscript II (BRL) according to the manufacturer's recommendations using either oligo$(dT)_{18}$ or gene-specific primers and 1-μg aliquots of RNA; 5′ RACE was done with the BRL 5′ RACE kit. PCR reactions used the Expand PCR system (Boehringer Mannheim, Indianapolis, IN) and an MJR thermal cycler. rtPCR from embryos used total RNA isolated from embryonic day (E)8 and E8.5 embryos using Trizol (BRL). First-strand cDNA was synthesized using oligo(dT) and the Superscript II RNAse H^- Reverse Transcriptase kit from BRL. PCR was performed using primers 107962 (5′ ACT ATG TGT AAG ACA AGG ACG 3′) and 5681CAC (5′ CAC ATT GTT CAC CCA GTA TCG 3′). PCR conditions were 30 cycles of denaturation for 1 min at 94°, annealing for 30 sec at 55°, extension for 1 min at 72°, and final extension at 72° for 10 min. PCR reactions were analyzed by agarose gel using a 100-bp ladder molecular marker from BRL. All radiolabeling, DNA preparation, and library screenings were performed using standard techniques. Sequence analysis was performed using the MacVector software package (IBI/Kodak). Database searches were performed using the BLAST, TBLASTN, and TBLASTX programs on the NIH/NLM World Wide Web server (http://www.ncbi.nlm.nih.gov/blast/).

RESULTS

Analysis of 30 kb around the site of the $T2^{Bob}$ transgenic insertion using the conventional techniques for gene searching failed to turn up definitive evidence of a gene. Therefore, we extended the search using two complimentary approaches: exon trapping and a cDNA direct selection technique (rargip, Abe 1992). Whereas the candidate exons from trapping yield genomic fragments with functional splice donor/acceptor sites, rargip allows the retrieval of very rare cDNA sequences from complex pools of uncloned cDNAs.

Exon trapping using *Sac*I genomic fragments from around the insertion site yielded two potential exons of 107 and 228 bp. rargip selection was done using a 15-kb genomic clone containing the insertion site as a probe against E9.5 and E10.5 cDNA pools. rargip clones were screened with the two trapped exons as probes. Clones positive with both exons were isolated and analyzed. Although the two trapped exons map 5 kb apart in the genome, they were 80 bp apart in the cDNA clones. The largest clone (1119 bp) was used to screen six embryonic cDNA libraries from stages E7.5–E11.5. The fact that we retrieved only two positive clones from a total of 12×10^6 independent recombinants attests to the rarity of the *T2* message. The two positive clones, isolated from E10.5 and E11.5 libraries, were contiguous

```
   1 GGTGTTCACAGCAAGGACCATCCAGGGAGAGAACAGGAAGTTGTGGAGGATGGCCCGGAG
  61 AAGGAGAGGATAAAACAGGGCCCGCACCAACCTGGCTTGGATCCACCCAAGCATCGAGGG
 121 GACACAGCGAGTCTTCTCGCTCGGCTCACGGAATGGAGAAAAAGATGCCAACAGCATGGA
 181 GCTGCCCAGAGGCTGCATCGAGGACCTGAGGAGAGCAAGACTATGTGTAAGACAAGGACG
 241 GATTCCCAAAGCAAGATGTATCTTAGCCGGTTGTATCGGATGTATTCCACCTCCCTTGCT
 301 AACATGGAATTCTCCAGAAAACTGCTGGAAAGAGATGGCCGCTTTGCAGACATGGATGCT
         M  E  F  S  R  K  L  L  E  R  D  G  R  F  A  D  M  D  A    19
 361 GAGCACAGGGCCCGAAGCCTGCTGGATTACATGGTCCCAAGAGAACGTACACAGGCAGAT
      E  H  R  A  R  S  L  L  D  Y  M  V  P  R  E  R  T  Q  A  D    39
 421 GTTCCACCAAGAGAACGTACACAGGCAGATGTTCCACCAAGGGAACTAGAGGAGGAGGAC
      V  P  P  R  E  R  T  Q  A  D  V  P  P  R  E  L  E  E  E  D    59
 481 TCCCCAACCCCTGAGCAGCAGCCTCAAGCAGGACCTGCCCTCCGATTTCTGAAGTGCCAG
      S  P  T  P  E  Q  Q  P  Q  A  G  P  A  L  R  F  L  K  C  Q    79
 541 AGCCCAGAAAGTCCACAGAGACGTTCCCACCTGAAGACAGGAAGACACCAAGTGACCCTG
      S  P  E  S  P  Q  R  R  S  H  L  K  T  G  R  H  Q  V  T  L    99
 601 TGTGGCACGAGCAAGCTCTCCGAACACGAGAGGGGCACACTTGCTCCTGCCAGGAGGACC
      C  G  T  S  K  L  S  E  H  E  R  G  T  L  A  P  A  R  R  T   119
 661 ACAGATCCCAGACCTACAGCACCCCTGACCCTTGCACACGTGATCCAGACCCATCCTGTG
      T  D  P  R  P  T  A  P  L  T  L  A  H  V  I  Q  T  H  P  V   139
 721 GTGGAGGCCAAGACAGCAAGTCGATACTGGGTGAACAATGTGGATGAAGAATAAGTTTTT
      V  E  A  K  T  A  S  R  Y  W  V  N  N  V  D  E  E  *         159
 781 TTTTTGTCTTTTTTTTTTTTTAAACAGTCATTAGATAAAGTCCAAACAGGATATGACAGCT
 841 TTCTTTTTCTTTGAGTCTGCATTATGATAAGCAGCGAGGTGACACCATCATGCAGAGCCC
 901 GAACCGTGAATTTGGATGATGGAGACAGTCAGGCAGGCAGCACAGTGGGCAGCAGCGCTG
 961 AGCAGACTCAGACATGAGGCTCCAGCCCATGGCTAGTCACTTCTTCTGACTCATGGTCCT
1021 TCTGCCTGGTGCTCTCTTTGCAGCATCTTAAATAAAATAAGCTTGTTTTCTTTCTTAAAA
1081 AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
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Figure 1.—Sequence of *T2* cDNA and deduced ORF. Dashed underline at aa 32–54: overlapping 12-aa repeat. Solid underline at nt 1051–1056: polyadenylation consensus sequence. Bolded methionines: possible initiation codons. Open boxes at positions 298–307 and 346–355: Kozak consensus sequences. Intron-exon boundaries are represented by Y's.

with our largest rargip clones, but they did not extend as far 5′ as those clones. Sequence analysis reveals an open reading frame whose beginning is coincident with the 5′ end of the sequence (Figure 1). Because there are no stop codons before the first AUG at position 223, it is possible that an ATG might lie 5′ to the start of the known sequence. Two lines of evidence argue against this. First, the only clones we retrieved from 5′ RACE terminate downstream of the start of the known cDNA sequence (data not shown). Second, there is an in-frame stop codon in the genomic sequence 20 bp upstream of the 5′-most nucleotide in exon 1 with no obvious splice consensus sequence between it and the start of the cDNA sequence. There are Kozak consensus translational start sequences (boxed) centered around the ATGs at nucleotides (nt) 304–306 and 352–354. The stop codon is at nt 772, with a polyadenylation consensus sequence at nt 1051. Thus, translation may produce a protein with a molecular weight of 17.8 kD and another protein with a molecular weight of 14.3 kD. A curious feature of either protein is a 13-aa overlapping repeat (dashed underline in Figure 1). A search of currently available databases, including expressed sequence tags, revealed no significant similarity to previously reported sequences.

To position the *T2* gene relative to *Brachyury*, two contigs were constructed consisting of one cosmid and four lambda clones and covering a total of 80 kb. There is a 5-kb gap between the *T1*-containing cosmid and the closest lambda clone, as determined by pulsed-field gel electrophoresis (PFGE; Figure 2 and data not shown). The insertion in the *T2* gene occurred 15 kb centromeric to the *T1* gene (Figure 2). Thus, the classic *T* mutation, which is a 160- to 200-kb deletion with the *T1* gene located in the approximate center (Herrmann *et al.* 1990), includes both *T1* and *T2.*

T2 contains nine exons spanning 30 kb and is in the same transcriptional orientation as *T1.* As diagrammed in Figure 2, the $T2^{Bob}$ insertion has exons 5 and 6 deleted and exons 7–9 displaced, leaving coding potential intact for only the first 14 aa. Because the presumed 5′ promoter region is intact, some aberrant transcripts may be made in the mutant.

Among the *T2* rtPCR products analyzed, we identified two alternatively processed forms of the transcript. One of these uses an alternative polyadenylation site and results in a 200-bp truncation of the 3′ UTR. The other isoform has exons 6 and 7 spliced out and codes for a 55-aa protein that lacks the 13-aa overlapping repeat. This is in fact an isoform that is similar to the genomic structure we have found in *Mus molossinus*, where the repeat is missing (see below).

The *T2* message is extremely low in abundance. Northern blots and both whole-mount *in situs* from E8.5–10.5 and ^{35}S *in situ* to sections at E10.5 were negative. However, the message is readily detectable by rtPCR from E7.5 to E11.5, the period in which notochord differentiation and development occurs. Thus, the expression of *T2* is notably lower than that of *T1*, which is easily detected by Northern hybridization and local-

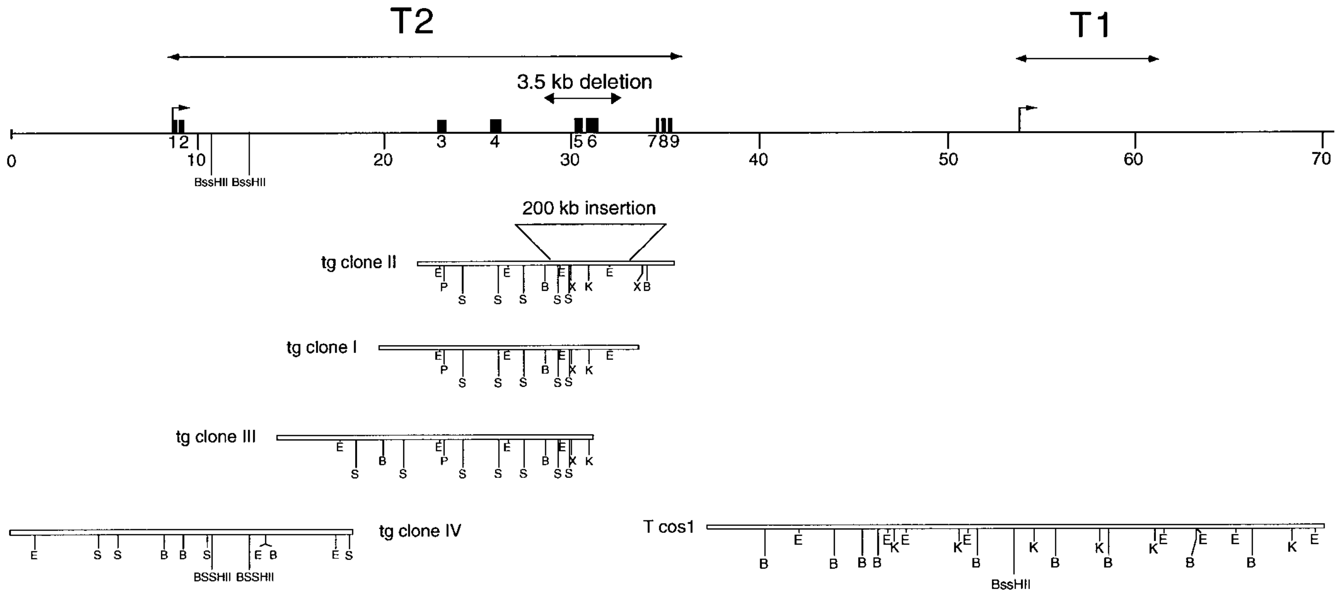

Figure 2.—Structure of the *T2* gene and orientation relative to *T1.* Black boxes indicate size and position of *T2* exons. Exons 4 and 6 were found by exon trapping. Exons 5 and 6 are deleted by the transgenic insertion. CpG island containing two *Bss*HII restriction sites is located between exons 2 and 3. Arrows indicate transcriptional orientation. Represented restriction sites: *Sac*I (S), *Bam*HI (B), *Eco*RI (E), *Xba*I (X), *Pme*I (P), and *Kpn*I (K).

ized by whole-mount *in situ* hybridization to the primitive streak and notochord (Herrmann 1991).

When the second gene in the *T* deletion affecting mesoderm development was identified, an obvious question was whether it was an allele of *tct* or possibly a third gene. To examine the *T2* gene in *t* haplotypes, rtPCR was used to amplify the *T2* transcript from total RNA isolated from individual T/t^{w5} E10.5 embryos. Because *T* is a deletion, these embryos are hemizygous, containing only the *t* copy of *T2.* Analysis of six clones from t^{w5} revealed a change in the sequence of *T2* at the third position of the putative stop codon. As expected from their common ancestry (Silver *et al.* 1987), two other *t* haplotypes (t^{w32} and t^{wpa1}) contain the identical change, even though these particular *t* haplotypes come from three very separate wild populations: New York, Montana, and France (Guenet *et al.* 1980), respectively. This A → C transition changes the stop codon to a tyrosine. These extra bases, combined with the mutated stop codon, cause the conceptual *T2* protein of *t* haplotypes to contain an additional 12 aa at the C terminus (Figure 3B). Eight of these residues are phenylalanine, creating a very hydrophobic tail. This molecular lesion seems reasonable because *tct* homozygotes have no visible phenotype. It should be kept in mind, however, that there is currently no way to distinguish whether this change is functionally significant or merely a *t*-to-wild-type polymorphism.

The allelism of *T2* and *tct* was not confirmed using the only known independent *tct* mutant, tct^k, induced by ethylnitrosourea mutagenesis in a wild-type chromosome (Bode 1984). Sequence analysis of coding regions from genomic PCR products amplified from tct^k and wild-type controls revealed no tct^k-specific modification. It remains possible that the mutation in tct^k has a difficult-to-detect point mutation in a noncoding region. Expression analysis of *T2* is not possible because these mice are extinct.

In an attempt to find other alleles of *T2,* we sequenced several candidates. Among them was *btm,* a mutation derived from *M. molossinus* that is recessive and causes a short tail, but in compound heterozygotes with *T,* causes spina bifida (Fujimoto *et al.* 1995). Interestingly both *btm* and its *molossinus* parent were polymorphic for a deletion of 10 aa in the repeat, very much like the alternate spliced form. No significant changes were found in *btm* or other haplotypes sequenced. A list of the various polymorphisms is presented in Table 1.

The most relevant genetic test of "the two *T*s" would be to look for complementation of $T2^{Bob}$ with a null allele of only the *T1* gene; however, none were known to exist. The *T* mutations described are large deletions, an ENU mutation in a *t* haplotype already carrying the *tct* mutation, or gain-of-function alleles such as $T1^C$. $T1^{LAF}$ is a new spontaneous allele of *Brachyury.* Its phenotype resembles the standard *Brachyury* null. Southern analysis of $T1^{LAF}$ homozygotes shows an insertion in the 5′ end of the *T1* gene that disrupts the T box (G. Rennebeck, L. Flaherty and K. Artzt, unpublished results). Nine of 31 embryos dissected from crosses of $T1^{LAF}/+ \times T2^{Bob}/+$ at E9.5–E10.5 had grossly abnormal posterior ends similar to the $T1^{LAF}$ phenotype. Thus, given the genomic structure of *T2* and the available mutations, *T1* and *T2* appear to be noncomplementing, nonallelic genes.

TABLE 1

Polymorphisms in T2 gene

Exon no.	Base pair no.	Nucleotide changes	Amino acid	Common to t and +	t haplotype specific	btm specific	Ethylnitrosourea mutation specific	molossinus	Randomly bred strain
1	No change								
2	41	GTT→GCT	Precoding	+				+	
2	61	AAG→GAG	Precoding	+				+	
2	71	ATA→AAA	Precoding	+				+	
2	116	CGA→CGG	Precoding		+				
2	126	ACA→ACG	Precoding			+		+	
3	No change								
4	No change								
5	320	AAA→AGA	Lys→Arg			+	+	+	+
6	1094–1123		Delete 1 repeat			+		+	
6	436	CGT→TGT	Arg→Cys			+		+	
6	487	ACC→GCC	Thr→Ala			+		+	
6	494	GAG→GGG	Glu→Gly			+		+	
6	585	AGA→AGG	Arg→Arg			+		+	
6	590	CAA→CGA	Gln→Arg			+		+	
7	No change								
8 has TAA	789–800		One less T			+		+	
9	No change								

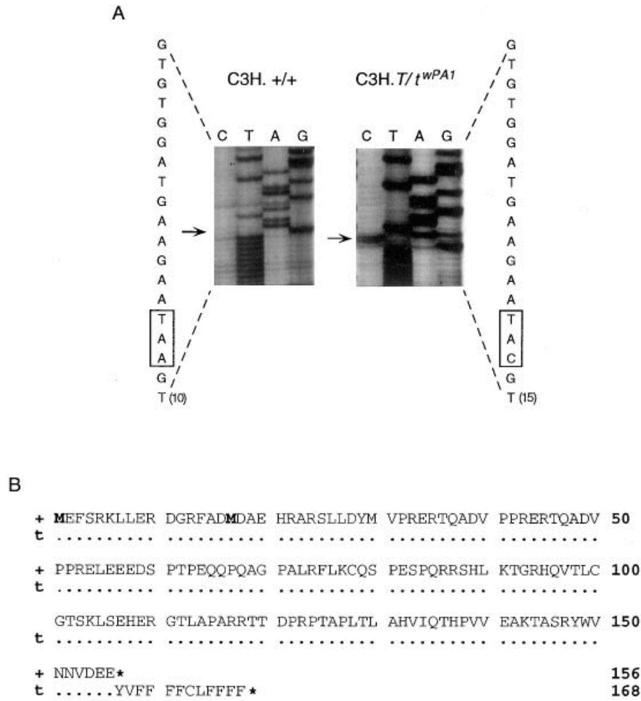

Figure 3.—(A) Sequencing gel of *t* haplotype-specific mutation in the *T2* gene. A comparison of the nt sequence in and around the *t*-specific mutation. Arrow indicates A → C transition in stop codon. (B) Deduced aa sequence of wild-type and *t* copy of *T2* protein. Dots indicate identity. Asterisks indicate stop codons. The *t* copy is identical to normal, except for 12 additional residues on the carboxy tail.

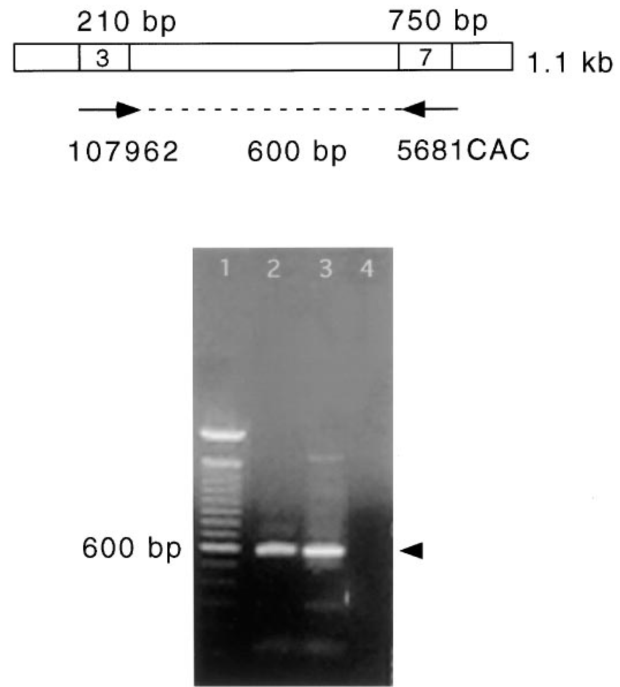

Figure 4.—T2 mRNA is detected as early as E8. Primers in exon 2 at position 210 bp (107962) and in exon 7 at position 750 bp (5681CAC) of the T2 cDNA (2-1) were used to amplify a 600-bp fragment from a pool of cDNAs from E8–E8.5 (lanes 2 and 3). BRL 100-bp ladder molecular markers (lane 1) and dH_2O control (lane 4).

DISCUSSION

Brachyury the Second (*T2*) is a novel gene that partially fulfills the criteria of being the second gene in the *T/t* complex involved in axis development. *T2* maps within the *T* deletion, ~15 kb upstream of the classical *Brachyury* gene. *T2* consists of nine exons. None of its sequence shares homology with the available databases. rtPCR experiments show that the *T2* mRNA is present as early as E8 (Figure 4), consistent with the prediction that *T2* is involved in notochord development (Rennebeck *et al.* 1995). However, inability to detect *T2* expression in the embryo suggests that its product is extremely low in abundance, or that *T2*, like *nodal*, has a short window of expression that was missed in the *in situ* hybridization experiments (Zhou *et al.* 1993a; Collignon *et al.* 1996). Both our rtPCR data and phenotypic analysis (Rennebeck *et al.* 1995) suggest that *T2* expression is probably restricted to the latter phase of *T1* expression, in the notochord.

The close linkage to the classic *Brachyury* gene positions *T2* as a candidate gene for *tct.* Sequence analysis of the *T2* gene in *t* haplotypes shows that the stop codon is altered in all *t* haplotypes, and this change generates a longer T2 protein with a hydrophobic carboxy tail. It is not possible to know if this change is significant because the function of T2 is unknown. However, this relatively minor modification may explain why *tct* has no phenotype by itself.

There is a third component of nonallelic, noncomplementing genes in the *T* system. It is the specific interaction of an unlinked recessive mutation *t-int* with *T1*, *T2*, and *tct.* The *t-int* mutation, like *tct,* has no phenotype on its own, but is an enhancer of tail phenotype in all mice heterozygous for *T1*, $T2^{Bob}$ or *tct* (Artzt *et al.* 1987; Rennebeck *et al.* 1995). Because the interaction of *t-int* with *T1* and *T2* closely resembles that of *tct,* it is likely that the mutation in *t-int* is a similarly minor modification to a protein that may have a major role in axial development.

$T2^{Bob}$ is probably a dominant-negative mutation. Homozygotes of the $T1^{C}$ mutant, which makes a protein with a modified C terminus, have a more severe phenotype than the *T1* null alleles, suggesting that the mutant protein interferes with the function of other protein(s) (Searle 1966; Herrmann *et al.* 1990). There is compelling evidence that $T2^{Bob}$ is also a gain-of-function mutation. First, although + $T1^{LAF}/T2^{Bob}$ + embryos have the same complement of *T1* and *T2* mutations as a *T*/+ deletion heterozygote, namely one good copy of each, the latter has a short tail and the former is an embryonic lethal. Thus, the two genes have a worse phenotype in *trans* than the nulls do in *cis.* Second, the molecular lesion in $T2^{Bob}$ predicts a truncated message and possibly a mutant protein missing the C-terminal 150 aa.

The sequence of *T2* suggests that the close proximity of *T1* and *T2* is not the result of a gene duplication event, nor do they appear to be distantly related. While their proximity might be simply fortuitous, an alternative is that the coordinate regulation of *T1* and *T2* during development is controlled by shared or intertwined regulatory elements. There is precedent for this with *RAG-1* and *RAG-2*, which both function in V(D)J recombination and are only 8 kb apart (Oettinger *et al.* 1990). The same is true for the *Tap1:Tap2:Lmp2:Lmp7* gene complex in the major histocompatibility complex (Zanelli *et al.* 1993; Zhou *et al.* 1993b). Whether the tight linkage of *T1* and *T2* is by accident or necessity can be resolved by their linkage analysis in other chordates.

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