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# Genetic mapping of the human homologue (*T*) of mouse *T*(*Brachyury*) and a search for allele association between human *T* and spina bifida

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We describe a genetic analysis of the human homologue (T) of the mouse T (Brachyury) gene; human T was recently cloned in our laboratory. The protein product of the T gene is a transcription factor crucial in vertebrates for the formation of normal mesoderm. T mutant Brachyury mice die in midgestation with severe defects in posterior mesodermal tissues; heterozygous mice are viable but have posterior axial malformations. In addition to its importance in development, T has intrigued geneticists because of its association with the mouse t-haplotype; this haplotype is a variant form of the t-complex and is characterized by transmission ratio distortion, male sterility and recombination suppression. We have identified a common polymorphism of human T by single strand conformation polymorphism (SSCP) and used this in mapping studies and to re-investigate the idea that human T is involved in susceptibility to the multifactorial, neural tube defect, spina bifida. Our mapping data show that human T maps to 6q27 and lies between two other genes of the t-complex, TCP1 and TCP10. These data add to the evidence that in man the genes of the t-complex are split into two main locations on the short and long arms of chromosome 6. We have used an allele association test which is independent of mode of inheritance and penetrance to analyse data from the spina bifida families. Using this test we find evidence for a significant (p = 0.02) association between transmission of the TIVS<sub>7</sub>-2 allele of the human T gene and spina bifida.

#### INTRODUCTION

The protein product of the T gene is crucial in vertebrates for normal axial development. The T mutant Brachyury was one of the earliest developmental loci to be characterized in the mouse (1) and several different Brachyury mutant alleles have been described [reviewed in (2)] each of which affects posterior mesoderm formation and notochord differentiation. Mouse embryos homozygous for mutant alleles die in midgestation and display severe defects in posterior mesodermal tissues, with the notochord, allantois and somitic mesoderm most strongly affected. Heterozygous mice with only a single functional T gene are viable but have shortened or non-existent tails, fusions between gut and neural tube and localized duplications of one or both structures, and occasional malformations of the sacral vertebrae, [reviewed in (3)]. T is expressed abundantly in the tissues most affected in the mutant mice, the primitive streak, the notochord and in the tail bud during axis elongation (4). Post-gastrulation, T expression is confined to notochord cells of the nucleus pulposus. There is now strong evidence to suggest that the T protein is a transcription factor, regulating genes involved in posterior mesoderm formation and notochord differentiation. The protein product accumulates in nuclei and its N-terminal portion binds to a specific DNA target sequence (5-7) but as yet target genes regulated by T have not been identified. There has been some progress in unravelling the mechanisms which regulate the T gene itself, and it has been shown that in *Xenopus T* expression can be induced by factors involved in mesoderm induction such as the morphogen activin A, and basic fibroblast growth factor (FGF) (8,9). T is highly conserved amongst vertebrates, emphasizing its important role in mesoderm development throughout the subphylum. Homologues of T have been found in zebrafish (Zf-t) (5) *Xenopus (Xbra)* (8) chicken (*Ch-T*) (10) and the lower chordate,

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Halocynthia roretzi, (As-T) (11). We have recently cloned and characterized the human Thomologue, human T(12). The pattern of T expression appears to be essentially identical in all vertebrate species studied. In the zebra fish, the early lethal mutation no tail (ntl) has been identified as a mutation of the T homologue. ntl embryos die after hatching and lack a differentiated notochord and their most posterior somites (13).

In addition to its importance as a developmental gene, T has intrigued geneticists because of its association with the mouse t-complex. This is a 12–15 cM region of mouse chromosome 17 which includes the major histocompatability locus (MHC) and a number of other expressed genes. The t-haplotype is a naturally



occurring genetic variant of the t-complex, defined by a series of four non-overlapping inversions, and characterized by transmission ratio distortion, male sterility and recombination suppression. The presence of T mutations in this region expose the t-haplotype, since animals carrying a mutant T allele (on the other chromosome 17) homologue) in combination with a wild-type t-complex have short tails, whereas animals carrying a mutant T allele in combination with the t-haplotype are born tail-less. A number of loci within the t-complex are required for the non-Mendelian transmission which maintains the t-haplotype [t-complex distorter and responder loci – Tcd-1, Tcd-2, Tcr, etc.; reviewed in Silver (14)]. In man, the loci which make up the t-complex in mouse are split into two main locations far apart from each other on the short and long arms of chromosome 6. Human homologues in the proximal region of the mouse t-complex map to 6q26–27, while those in the distal segment map in 6p21.3 (15–17). Since in the mouse T maps proximally, we would predict that human T maps to 6q. Here we show that indeed human T maps to 6q27 distal to TCP1.

In addition to mapping studies, we have also re-investigated the idea that T might be involved in susceptibility to the neural tube defect (NTD) spina bifida. Genetic associations between spina bifida and T were searched for and not found >10 years ago (18-20). At that time, it was expected that human T would lie close to the MHC locus on chromosome 6p21.3, and linkage between spina bifida and the HLA (class I MHC) gene was tested. The recent cloning and chromosome localization of human T(12) makes it reasonable and possible to look directly for an association between T and spina bifida. Searching for linkage to spina bifida is not straightforward. Heritability has been estimated to be  $\sim 60\%$ , but there is uncertainty about the mode of inheritance and penetrance, and there is evidence for genetic heterogeneity (21). Bearing in mind these problems, we have used an allele association test (22) which does not require prior knowledge of mode of inheritance and is independent of penetrance and family size.

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Figure 1.SSCP analysis of human T intron 7 sequence polymorphism. Samples were amplified from DNA of unrelated members of the CEPH families. Individual homozygous and heterozygous for the  $TIVS_7$ -1,  $TIVS_7$ -2 and  $TIVS_7$ -3 alleles are indicated.

#### RESULTS

Single strand conformation polymorphism (SSCP) of human *T* and mapping by linkage analysis

Table 1. Human T allele frequencies in unaffected unrelated individuals

Population	TIVS7-1	TIVS7-2	TIVS7-3	n	
CEPH Utah	0.756	0.238	0.006	86	
CEPH French	0.813	0.188	0	24	
CEPH total	0.768	0.227	0.005	110	
Dutch/British	0.700	0.300	0	33	
Total	0.752	0.245	0.003	143	

A 268 bp fragment from intron 7 of the human *T* gene was amplified from DNA samples of unrelated CEPH individuals and examined using the Phastgel system for single strand DNA conformation. Variation in banding pattern was detected after electrophoresis at 10°C for 1.5 h, and family studies showed that this was due to the occurrence of three alternative alleles designated  $TIVS_7$ -1,  $TIVS_7$ -2 and  $TIVS_7$ -3 at the human *T* locus (human *T* intron 7 =  $TIVS_7$ ). Typical SSCP analyses are shown in Figure 1. The overall allele frequencies in the CEPH population of 110 individuals were calculated as  $TIVS_7$ -1 allele = 0.768,  $TIVS_7$ -2 allele = 0.227,  $TIVS_7$ -3 allele = 0.005. Allele frequencies for the Utah and French components of the CEPH families are given separately in Table 1 but are not significantly different.

We have used this polymorphism to estimate the linkage between human *T* and other markers on chromosome 6. Twenty five CEPH families were informative. Table 2 shows the results of the CRI-map 'build' multipoint linkage analysis as sex-specific maps giving both recombination fractions and cM. This analysis places human *T* between *D6S186* and *D6S264*, at sex-averaged distances (cM) of *IGF2R*-7.2-*D6S186*-6.2-*T*-3.1-*D6S264*-6.3-*D6S133*. Local support for the map as estimated by flips2 is at least 1000:1 and alternative orders of markers were excluded by flips5, again with odds of at least 1000:1 The largest two-point lod score, z = 36, was obtained with *D6S133* at an approximate recombination fraction of 0.05. These genetic mapping data complement our earlier physical mapping localization of human *T* by fluorescent *in situ* hybridization using a genomic probe (12).

Frequencies are shown for all the CEPH individuals and for the Utah and French subgroups considered separately. Dutch/British are unrelated individuals who have married into families where SB is segregating.



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## Human T and spina bifida: association analysis

DNA from 31 Dutch families and 19 British families was examined for allele association between the human T and spina bifida (SB) using the transmission disequilibrium test (TDT) described by Spielman et al. (22). In this test, the frequency of transmission of a marker allele from a heterozygous parent to an affected offspring is evaluated. Under the hypothesis of no linkage, an equal number of transmissions of TNS7-1 and TWS<sub>7</sub>-2 is expected. Because the number of heterozygous parents is small, data from the Dutch and British groups were combined (this test is not affected by population differences in allele frequency and there was no significant difference in allele frequency between the two groups). In the Dutch families there were 31 parents heterozygous for TNS<sub>7</sub>-7 and TNS<sub>7</sub>-2 and for the British families there were eight heterozygous parents who transmitted alleles to affected offspring (34 and eight transmissions respectively). These parents transmitted 42 alleles to their SB offspring in a manner which was significantly different from the no linkage hypothesis, 13 TIVS<sub>7</sub>-1 and 29 TIVS<sub>7</sub>-2 alleles; with  $\chi_{ld} = 6.09$  and  $\rho = 0.02$ , (1 degree of freedom). Spielman *et al.* (22) pointed out that it was important to bear in mind that differences detected by TDT might not reflect allele association but could be an artefact brought about by meiotic segregation distortion. This process would apply both to affected and unaffected offspring. Therefore, we compared these two classes of offspring with respect to the transmission of  $TVS_7$ -1 and TIVS<sub>7</sub>-2 alleles and the figures are shown in Table 3. Among the affected offspring, 69% of alleles received from the heterozygous parents were  $T/S_{7}$ -2 (29 of 42 of alleles transmitted), while the corresponding figure for unaffected offspring was 48.2% (27) of 56 of alleles transmitted) close to the 50.50 distribution expected. Amongst the CEPH families, the 32 heterozygous parents transmitted 49% TNS<sub>7</sub>-7 alleles and 51% TNS<sub>7</sub>-2alleles (128 77/S<sub>7</sub>-7 alleles and 133 77/S<sub>7</sub>-2 alleles) to their offspring in a manner close to that expected.



**Figure 2.** Sequence of human 7 intron 7 DNA amplified from individuals homozygous for the *TIVS*<sub>7</sub>-7 and *TIVS*<sub>7</sub>-2 alleles. The region encompassing the mutation is indicated in upper case lettering.

Table 2. Sex-specific map showing the position of human 7 relative to other 6q markers with distances expressed as recombination fraction ( $\theta$ ) and Kosambi centimorgans (cM)

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**Table 3.** Comparison of alleles *TIVS*<sub>7</sub>-*I* and *TIVS*<sub>7</sub>-*2* transmitted from heterozygous parents to SB-affected offspring (affected), unaffected sibs and unaffected CEPH family offspring

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## Sequence analysis of the human TTIVS, polymorphism

Sequence analysis of DNA amplified from individuals homozygous for the  $T/VS_{7}$ -1 and  $T/VS_{7}$ -2 alleles has demonstrated that this polymorphism is due to a single base change T to C, at a position 79 bp downstream from the 5' end of intron 7 (Fig. 2). This base change creates a *Hae*III site, thus it is possible to analyse the polymorphism directly by PCR and restriction enzyme digestion. **Table 4.** Lod scores between human 7 and spina bifida under dominant (D) and recessive (R) models with two different values of disease allele frequency (daf)

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The apparent association between SB and the  $TIVS_7$ -2 allele amongst familial NTD cases revealed by TDT analysis was not reflected in an increased frequency of the  $TIVS_7$ -2 allele amongst the 89 samples from sporadic cases of SB where frequencies were  $TIVS_7$ -1 0.713,  $TIVS_7$ -2 0.275 and  $TIVS_7$ -3 0.011 (n = 89). Amongst the sporadic cases, there were only five instances where DNA was available from parents and where one parent was heterozygous. These five parents transmitted two  $TIVS_7$ -1 alleles and three  $TIVS_7$ -2 alleles to their affected offspring.

Linkage analysis



In addition to allele association studies, conventional linkage analysis was carried out between human *T* and SB. The population frequencies of  $TIVS_7$ -1 = 0.752,  $TIVS_7$ -2 = 0.245 and  $TIVS_7$ -3 = 0.003 (derived by combining data from 110 CEPH and 33 unrelated Dutch and British individuals; Table 1) were used in this analysis. Sex-pooled two point lod scores were calculated using both dominant and recessive models, and the results are summarized in Table 4. Results using two different levels of disease allele frequency showed no evidence for linkage between familial SB and the *T* gene. It is not clear whether these results can be relied upon given that the genetic component of SB is likely to be complex and linkage analysis may be confounded by genetic heterogeneity.

#### DISCUSSION

We report the genetic mapping and detailed localization of the human homologue, human *T*, of the mouse *T* gene to human chromosome 6 close to the 6q26-27 border, between *D6S186* and *D6S264*. There have *T* gene has a distribute the second s



MAS 6q24-27 THBS2 6q27 TCTE3 6q27

D6S264. Thus, human T can be added to the segment of 6q26-27 which is conserved between man and mouse and is representative of those genes which lie in the proximal region of the mouse t-complex. The genetic mapping data allow us to determine the approximate position of human T relative to other homologues of the mouse t-complex, TCP1, TCP10, PLG, IGF2R, MAS, THBS2 and TCTE3, which map to 6q26–27. By combining our data with published maps and linkage analyses (24–26) and averaging genetic distances we can construct the consensus map shown in Figure 3. This shows that human T lies between TCP1/PLG (plasminogen) and TCP10 at ~4.3 cM from TCP1/PLG and 10.4 cM from TCP10. Mouse T and Tcp1 are a similar distance apart to their human counterparts. The relative position of TCP10 in man and mouse is more difficult to compare; in the mouse wild-type haplotype, the subregions of the *Tcp10* gene family are split into two loci which flank T, while in man the TCP10 homologues lie at one locus (15) distal to human T. It has been suggested previously that this arrangement of the human TCP10 homologues more closely resembles that of the mouse t-haplotype than the wild-type (17), but our studies show that human Tis at a greater distance to TCP10 than in either of the alternative arrangements of the mouse genome, indicating that other rearrangements of the DNA have occurred. We have also investigated whether there is any association between human T and the neural tube defect (NTD) spina bifida. Multifactorial inheritance of NTD is generally accepted, and a number of contributing maternal environmental influences including folate status, diabetes, epilepsy and hyperthermia have been recognized [for review see (27)]. Good candidates for the genes that interact to determine genetic predisposition to NTD

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**Figure 3.** Consensus map showing the position of human *T* in 6q27 relative to homologues of other genes which comprise the t-complex on mouse chromosome 17. Data from published maps and linkage analyses were combined (24–26) and genetic distance averaged.

have also begun to emerge from studies both in man and mouse. For example, in mouse the *curly-tail* (*ct*) mutant is a useful animal model of human NTD, and involves a major gene mapping to mouse chromosome 4 and at least three modifier genes mapping to other chromosomes (28). It is of relevance to this present study that Letts et al. (29) have mapped one of the curly-tail modifier loci, mct1, to mouse chromosome 17. mct1 contributes significantly to the variation in penetrance of NTD and while this gene maps into the t-complex it appears to be too distal to be co-identical with T. Another mouse mutant which has been associated with spina bifida is the Splotch variant of the Pax3 gene. Splotch is the mouse model of human Waardenburg syndrome type I (WSI) and although spina bifida is not a common feature of WSI there are several reports of patients with WSI and spina bifida. A search by linkage analysis failed to find evidence for linkage between PAX3 and spina bifida (30); however, a very recent report suggests that a variant PAX3 allele which differs from the common allele by a silent base change in a paired box domain codon, occurs with a considerably higher frequency in sporadic spina bifida cases than in controls (31). In man, the relationship between folate metabolism and NTD prevention is well recognized (32) and has been explored at the molecular genetic level. Very recently a mutation in the methylene tetrahydrofolate reductase (MTHFR) gene associated with decreased MTHFR activity was identified as a risk factor for spinal bifida, for both mothers and offspring (33). This finding suggests part of the mechanism whereby folate exerts a protective role in the aetiology of spina bifida.

Here we add human T to the list of possible candidate genes involved in the manifestation of NTD by reporting an association between human T and spina bifida. In mouse, a deletion of T(Brachyury) leads to defective primitive streak and notochord morphogenesis. Spina bifida is not a common feature of T(Brachyury) although, in mice homozygous for this mutation, the neural tube is often severely kinked in the caudal region and the surface ectoderm tends to form large fluid-filled blisters [reviewed in (2)]. However, amongst a series of chimeric mice generated by introducing embryonic stem cells homozygous for T(Brachyury) into wild-type host embryos, those most severely affected showed a failure to close the posterior neural folds, as well as gross disorganization of the other posterior tissues and allantois (3). Abnormalities of the neural tube in T(Brachyury) mice would not be unexpected, given that the patterning of the neural tube is determined by the notochord, which is apparently absent in homozygotes. Neural tube closure occurs later in T(Brachyury) mice than in wild-type litter mates (34) and closure of the posterior neuropore is delayed in heterozygotes (35). In normal mice, the neural tube and notochord are physically closely associated, and in heterozygous T(Brachyury) mice, fusions between neural tube and notochord are not uncommon [reviewed] in (2)]. It will be important to test the strength of the association between human T and SB which we have reported, by increasing the number of families tested. It will also be sensible to explore ways in which genetic and environmental heterogeneity can be reduced; for example by separating the folate responsive and unresponsive families. Since the  $TIVS_7$  variation lies in a non-coding sequence we plan to look elsewhere in the human Tgene for variation which acts directly to influence susceptibility to SB. An obvious target for such a search is the conserved region encoding amino acids 1–229, which is known to confer DNA binding activity on mouse T (6). The C-terminal half of the T protein is an equally important target since it contains paired activation and repression domains (7); truncation of these sequences in the mouse  $T^c$ ,  $T^{wis}$  and Xenopus XbraB304 mutants leads to loss of normal T function despite the presence of an intact DNA binding domain (2,36).

(20%), washed twice in ethanol/acetic acid and stained using silver nitrate and sodium thiosulphate exactly as described in the Phastsystem manual.

#### DNA samples from CEPH families and linkage analysis

Genomic DNA was obtained directly from Centre d'Etude du Polymorphisme Humain (CEPH) (37) through the Eurogem initiative; we genotyped DNA samples from 110 unrelated CEPH parents to calculate the allele frequencies of the human Tpolymorphism. To map human T we employed 40 large sibship families from CEPH and carried out the analysis using the Eurogem map of chromosome 6 (24) and the CRI-MAP programme 'build' option at odds of 1000:1. The data were tested with both the 'flips2' and 'flips5' options, also at 1000:1.

#### DNA from spina bifida families; linkage and allele association studies

DNA from 31 Dutch families and 19 British families were examined. Families were ascertained as described previously (30) by referrals from spina bifida clinics and by responses to patients newsletters. Syndromic or chromosome abnormality cases were excluded. Families were defined as a related kindred with at least two affected individuals. Diagnosis was based on clinical information from interviews and from review of the medical records. Four families had cases in three generations, 30 families had cases in two generations, 16 families had cases in a single generation but in eight of these the cases appeared in more than one sibship. The DNA samples from the Dutch families included 38 affected SB individuals, three with SB occulta (SBO) and one with encephalocele. The samples from the British families included DNA from 23 affected individuals of which five had SBO and 18 had SB. In addition, 69 Dutch and 20 British SB sporadics were analysed. The incidence of NTD is 1/1000 in the Netherlands (30) and 3/1000 for SB in UK/Ireland (21). Penetrance was calculated amongst offspring (excluding the probands) as the proportion of individuals estimated to carry the disease genotype who showed the disease phenotype (SB), as described by Ott (23). The penetrance estimate for SB under the dominant model was 0.27 and under the recessive model was 0.52. Disease allele fequencies were calculated from disease incidence and penetrances, and under the dominant assumption were estimated as 0.0018 (Netherlands) and 0.0056 (UK/Ireland) and under the recessive assumption as 0.044 (Netherlands) and 0.076 (UK/Ireland). Linkage analysis was carried out using the M-link programme from the Fastlink package (version 2.1p). Because of the unclear mode of inheritance, we performed analysis under the assumption of either a dominant or a recessive model using the estimated values for penetrance and both values of disease allele frequency. In addition allele association between human T and SB was looked for, using the transmission test for linkage disequilibrium (TDT) described by Spielman et al. (22).

#### MATERIALS AND METHODS

#### Single strand conformation polymorphism analysis

Primers derived from intron 7 of human T were used to amplify DNA from unrelated individuals. The primers were as follows: HuTF1 5'-TGCCTGTGACTTCACGCTGTTTCC-3'; and HuTR1 5'-GAACCAGTAAGTGGCAGAACCAGG-3' and the PCR conditions were 15 s at 95°C, 30 s at 62°C and 30 s at 72°C for 35 cycles. PCR product samples were mixed 1:1 with loading buffer (95% formamide, 20 mM EDTA, 0.05% bromophenol blue), heated to 95°C for 5 min and snap-cooled on ice. Electrophoresis was carried out using the Pharmacia Phastsystem. Samples (0.3 µl) were loaded on a Phast gel Homogeneous 20 (20% polyacrylamide) and separated at 400 V for 90 min at 10°C. The gels were fixed in TCA

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