The T transcription factor functions as a dimer and exhibits a common human polymorphism Gly-177-Asp in the conserved DNA-binding domain

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Abstract T is a transcription factor which activates transcription by binding to repeated arrangements of the dodecamer 5′-AGGTGTGAAATT-3′. Using in vitro synthesised T protein, we have demonstrated that T binds to its target DNA as a homodimer and that truncated protein containing only the N-terminal 233 amino-acid residues, which comprise the DNA-binding domain, can form a dimer. We also report a common human polymorphism, Gly-177-Asp, within the DNA-binding domain at a position which is a conserved glycine residue in T homologues from other vertebrates. The proposition that T forms heterodimers with other members of the T-box transcription factor family and the implications for disorders of axial development are discussed.

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Key words: Transcription factor; T gene polymorphism; T-box protein; T dimer; Notochord

1. Introduction

The *T* gene encodes a transcription factor [1–4] which is vital for normal formation of posterior mesoderm and for axial development in vertebrates. Complete deficiency of T in mouse (e.g. *T Brachyury* mutant, T^{Br}) and zebra fish (*ntl* mutant) is embryonic lethal and leads to failure of notochord differentiation and formation [5–8] and abnormal migration of mesoderm cells from the primitive streak; mice heterozygous for mutant alleles exhibit abnormalities of posterior mesoderm development associated with short or absent tails.

A modular structure has been defined for the T protein by deletion analysis in cell transfection studies [2] and by using heterologous expression systems [9]. DNA-binding activity is conferred by the N-terminal half (amino-acid residues 1–229) of the protein while the activation domain lies within the C-terminal half. In the mouse, the activation domain of the T protein can be divided into four regions, consisting of two transcription activators and two repressors [2], while in *Xenopus* and zebra fish only one activator region has thus far been identified [9]. The DNA-binding domain is highly conserved and defines a novel family of transcription factors, the T-box proteins, members of which have been identified in man [10], mouse [11,12] and several invertebrates [13,14]. Some of these genes have also been implicated in developmental abnormalities and like *T*, are haploinsufficient. For example, mutations in the human *TBX5* gene have been associated with the autosomal dominant Holt-Oram syndrome [15,16], characterised by limb malformations and cardiac septation defects, while *Drosophila* deficient for the T-box protein, *omb*, show abnormal distal wing formation [17].

T protein activates transcription by binding to a specific DNA target sequence. The consensus binding site of T protein has been identified by target site selection in vitro; the preferred configurations contain either a palindromic arrangement of the dodecamer 5′-AGGTGTGAAATT-3′ or directly repeated dodecamer sites [1,2]. In this paper, we describe experiments which indicate that T, like many other transcription factors, binds to its target DNA as a homodimer, and that amino-acid residues important for dimerization lie in the amino-terminal part of the protein. We also describe a polymorphism within the human T DNA-binding domain which conceivably could affect T dimer stability in vivo.

2. Materials and methods

2.1. Protein synthesis

RNA was prepared from intervertebral discs, dissected to remove the outer rings of collagen fibres, taken from human 10–13-week gestation fetal samples. First-strand cDNA was generated using random oligonucleotide primers. Templates for the synthesis of mRNA encoding the DNA-binding domain of human *T* [18] were constructed by polymerase chain reaction (PCR) amplification in two stages. In the first stage, cDNA was amplified using a *T*-specific forward primer corresponding to the 5′ UTR sequence 5′-CAGGGAAGGTGGATCTCAGGTAG-3′ and a reverse primer corresponding to sequence from the 3′ end of the DNA-binding domain in exon 5, 5′-CGGGTTCCTCCATCATCTCTTTG-3′ to generate a 769-bp product. The conditions were 15 s at 95°C, 30 s at 58°C, 45 s at 72°C for 35 cycles. In the second stage, the product was re-amplified with a forward primer containing the T7 bacteriophage promoter sequence, a Kozak consensus, the 5′ Met codon (underlined) and 21 bp of 5′ protein-coding sequence (5′-GGATCCTAATACGACTCACTATAGGAACAGACCAC CATGAGCTCCCCTGGCACCGAGAGC-3′). The conditions were 15 s at 95°C, 30 s at 65°C, 45 s at 72°C for 35 cycles and the product size 736 bp. The PCR product was purified after electrophoresis by electro-elution and re-amplified. Diagnostic *Bam*HI digests and PCR amplification using internal primers were used to confirm that the DNA-binding domain (aa 1–233) was correctly amplified.

^{35}S-methionine-labelled human T DNA-binding domain protein was synthesised by in vitro transcription and translation using a Promega TNT T7-coupled reticulocyte lysate system according to the supplier and using between 200 and 400 ng of purified PCR product. Full-length mouse T protein (436 aa) was also synthesised using as template full-length mouse cDNA cloned downstream of a T7 promoter in the pCR 3 vector. The size and integrity of in vitro translated proteins were checked by SDS-PAGE. The amount of T protein synthesised (cpm ^{35}S-methionine) was quantified in two ways. Firstly, by trichloroacetic acid (TCA) precipitation and scintillation counting according to standard procedures. Secondly, by phosphoimaging of the SDS gel; signals of individual bands were quantified using a phosphoimager (Fujix BAS1000) and MacBAS V2.0 software.

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2.2. Single strand conformation polymorphism (SSCP) analysis and genotype analysis

Primers derived from introns 2 and 3 of the human *T* gene [18] TX3F 5′-TGCCACCAATCCTGTATCTGTCTC-3′ TX3R 5′-AAGCGATCCGCCTCTGTCCTTCTCA-3 were used to amplify exon 3 DNA from unrelated human individuals and gorillas. The PCR conditions were 15 s at 95°C, 30 s at 65°C and 30 s at 72°C for 5 cycles and 15 s at 95°C, 30 s at 63°C and 30 s at 72°C for 30 cycles. PCR product samples were mixed 1 : 1 with loading buffer (95% formamide, 20 mM EDTA, 0.05% bromophenol blue), heated to 97°C for 10 min and snap-cooled on ice. Primers were also designed to amplify part of exon 3 of the mouse gene as follows: mTX3F 5′-CCTTGCATAAGTATGAACCTCGG-3′ mTX3R 5′-TATGAACTGGGTCTCGGGAAAGC-3′ and the PCR conditions were 15 s at 95°C, 30 s at 56°C and 30 s at 72°C for 35 cycles. Electrophoresis was carried out in a 10% polyacrylamide gel (66.7 : 1) in 0.5×TBE (45 mM Tris, 45 mM borate, 1 mM EDTA) at 300 V for 1.75 h at room temperature. The gels were washed/fixed in ethanol/acetic acid and stained using silver nitrate and sodium borohydride as described by Harvey et al. [19].

2.3. Electrophoretic mobility shift assays

The DNA probe used in electrophoretic mobility shift assays was the BS.p-binding site described by Kispert and Herrmann [1] which represents a 24-bp palindromic fragment flanked by *Sma*I half sites. Double-stranded oligonucleotides were chemically synthesised (Oswel, Edinburgh, UK), annealed in 100 mM NaCl and labelled using T4 polynucleotide kinase and [γ^{32}P]ATP (Amersham, UK).

Aliquots of in vitro translated T protein were pre-incubated at 25°C for 20 min with 1 μg poly (dI-dC).poly (dI-dC) (Pharmacia), with or without unlabelled competitor DNA oligonucleotide (100-fold excess). ^{32}P-BS.p (25 000 cpm) was added to the protein and incubated at 25°C for a further 30 min in a final volume of 20 μl of 10% glycerol (v/v), 10 μg ml^{-1} bovine serum albumin, 75 mM NaCl, 25 mM HEPES pH 7.4, 1 mM $MgCl_2$, 0.25 mM EDTA, 0.1% Nonidet P-40 (v/v), 1 mM PMSF, 1 mM DTT, 1 μg ml^{-1} leupeptin, 1 μg ml^{-1} pepstatin. Binding reactions were electrophoresed in a pre-run 5 or 6% polyacrylamide gel in 0.5×TBE buffer at 4°C and 10 V cm^{-1} for 2.3 (5%) or 2.8 (6%) h. Gels were vacuum-dried onto 3MM paper and autoradiographed. In some instances, the dried gel was phosphoimaged and ^{32}P signals from individual bands quantified by phosphoimaging as described above.

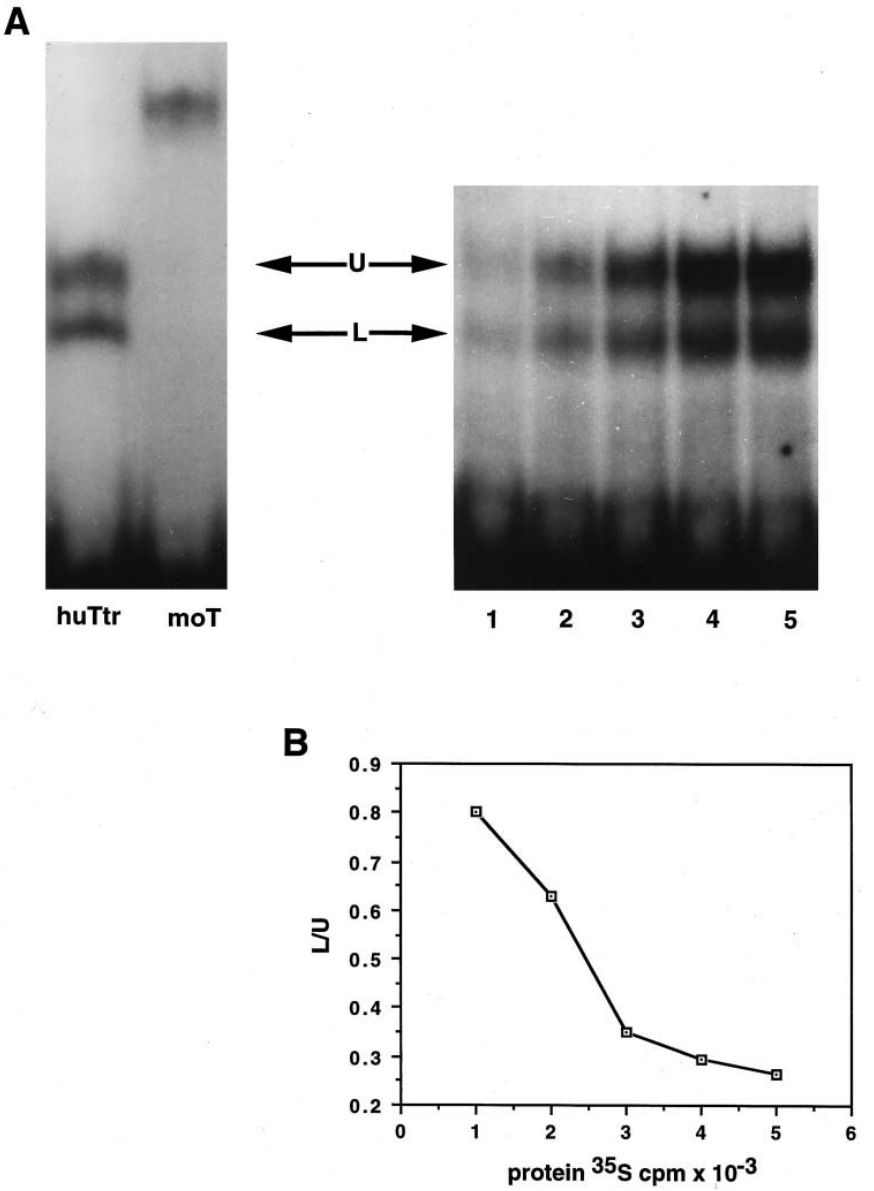

Fig. 1. (A) EMSA of truncated human T protein (huTtr) and full-length mouse T protein (moT) with ^{32}P-BS.p as probe. The right panel illustrates the effect of using increasing amounts (lanes 1–5) of huTtr in the binding assay. Fast- (L) and slow-mobility (U) DNA/huTtr protein complexes are indicated. (B) Graph of L/U plotted against increasing huTtr concentration.

3. Results

3.1. Analysis of the DNA-binding activity of truncated human T protein

Intervertebral discs of 10–13-week human fetal samples were used to isolate human *T* mRNA. We have previously shown that *T* is expressed in the remnant notochord cells of the nucleus pulposus in the intervertebral disc and that this is a moderately accessible source of *T* mRNA [18]. Human T protein for DNA/protein-binding studies was generated by in vitro translation. *T* cDNA encoding the DNA-binding domain, aa 1–233, was amplified, tagged with a T7 polymerase promoter and used as template for in vitro transcription/translation. Studies of mouse T protein [1,2] have shown that the N-terminal 229 amino acids are sufficient for DNA binding. As a positive control, the full-length mouse T protein (436 aa) was also synthesised in vitro.

The in vitro translated proteins were examined for their ability to bind specifically to the synthetic target DNA sequence, BS.p [1], using electrophoretic mobility shift assays (EMSA). Both the full-length mouse protein (moT) and the truncated human protein (huTtr) bound to DNA (Fig. 1A). moT gave rise to a single slow-moving DNA/protein complex, while two complexes of differing mobility were detected using huTtr (L, fast-mobility band; U, slow-mobility band). The relative proportion of the two huTtr complexes was dependent upon protein concentration and this is clearly demonstrated by an experiment where the amount of protein added to the binding mix was varied > 5-fold (Fig. 1A). In Fig. 1B, these data are presented graphically as a ratio of L to U; as the amount of protein increases, the relative proportion of the upper, slow band increases and L/U decreases.

One explanation for this observation is that the slower-mobility band represents huTtr protein binding to DNA as a dimer and that the faster band is huTtr binding as a monomer. To test this idea, advantage was taken of the differences in size between the moT and huTtr polypeptides; DNA/protein-binding reactions were carried out using both proteins together and amounts of huTtr translation product which favour the formation of the putative dimer. Under these conditions, a novel DNA/protein complex, consistent with the formation of a heterodimer between huTtr and moT was seen, with mobility intermediate to the complexes formed by huTtr and moT alone (Fig. 2). The complexes formed in the moT/huTtr-binding reaction were specific to the BS.p T-binding site as judged by competition assays in which excess unlabelled BS.p competed for the formation of complexes, when added to the binding mix. Competition was not seen using an unrelated oligonucleotide probe designated MCK (Fig. 2).

This experiment strongly suggests that T protein normally binds to its target DNA site as a dimer. We therefore suppose that the single DNA/protein complex seen using moT (full-

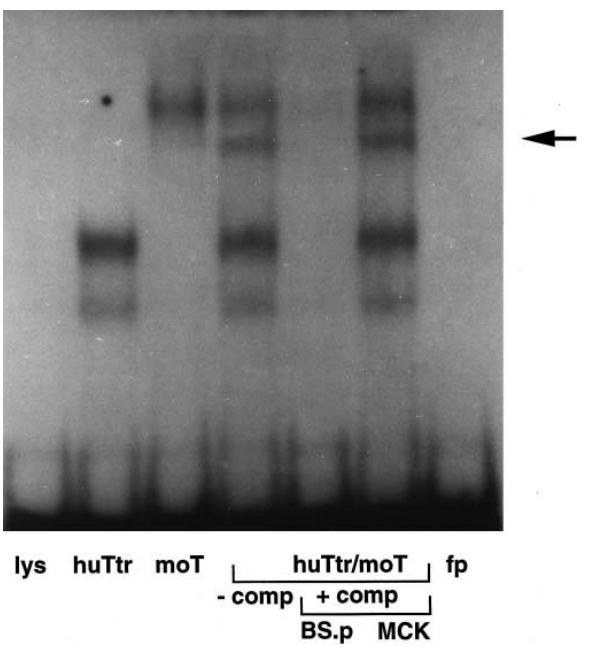

Fig. 2. EMSA of truncated human T protein (huTtr) and full-length mouse T protein (moT) either seperately or together (huTtr/moT) with ^{32}P-BS.p as probe. Unlabelled competitor (comp) DNA (BS.p or MCK) was included in the binding reaction as indicated. fp, probe without protein; lys, unprogrammed reticulocyte lysate. Arrow indicates the heterodimer complex.

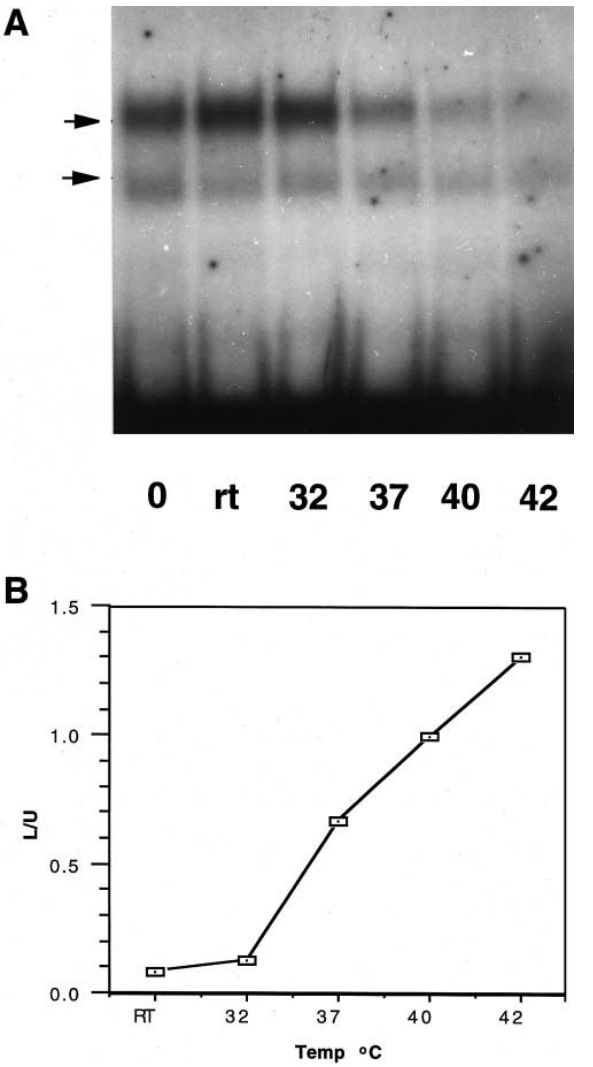

Fig. 3. (A) Thermostability assay of BS.p/huTtr complex. Post-binding incubations were carried out for 10 min at the temperatures indicated, rt to 42°C; (rt), room temperature. 0, a binding reaction carried out entirely at 0°C. Fast- (L) and slow-mobility (U) DNA/huTtr protein complexes are indicated by arrows. (B) Graph of L/U plotted against post-binding incubation temperature.

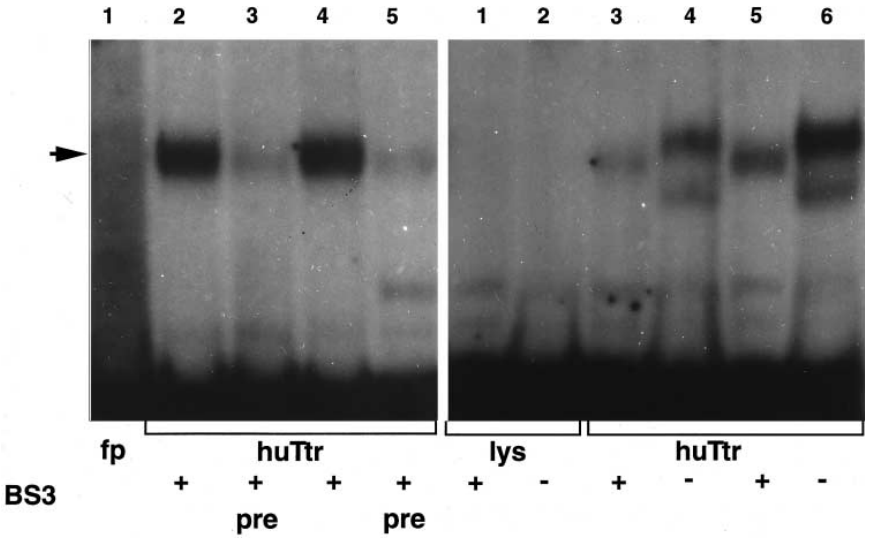

Fig. 4. EMSA of huTtr cross-linked with a 10-fold molar excess of Bis(sulphosuccinimidyl)suberate (BS3). BS3 was added to the binding reaction (+) either simultaneously with the BS.p probe (pre, lanes 3 and 5, left panel) or after completion of the binding reaction (lanes 2 and 4, left panel; lanes 1, 3 and 5, right panel). −, without cross-linker; fp, probe without protein. Arrow indicates the single complex formed in presence of cross-linker.

length mouse T protein) contains T as a dimer, and that the detection of both monomer- and dimer-containing complexes using huTtr alone is due to the truncation of the human T polypeptide and consequent dimer instability. Thermostability studies in which the DNA/protein-binding reaction was incubated, post-binding, at various temperatures show that the DNA/huTtr dimer complex is relatively unstable between 32 and 37°C (Fig. 3A,B). It seemed reasonable to suppose that covalent cross-linking of the huTtr subunits would stabilise the dimeric structure. This was tested using an ε amine-specific, bifunctional reagent [BS3 Bis(sulfosuccinimidyl)suberate] to cross-link the huTtr protein after binding to BS.p DNA. Cross-linking led to the formation of a single complex with mobility very similar to that of the huTtr slow-mobility complex, the dimeric form (Fig. 4, right hand panel). The slight shift in mobility of the cross-linked dimer compared to the non-cross-linked form can be attributed to the blocking of positively charged lysine amino groups by the cross-linker. It is of interest to note that addition of cross-linker prior to binding of huTtr to the DNA target inhibited DNA binding (Fig. 4, left hand panel), pointing to the involvement of huTtr lysine residues in the DNA/protein interaction.

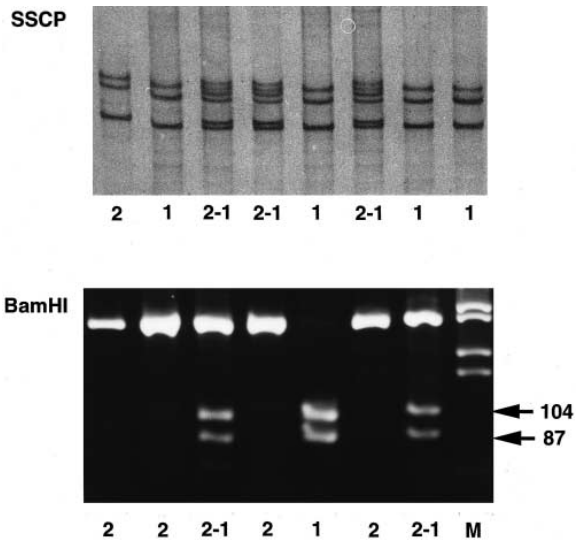

Fig. 5. Upper panel: SSCP analysis showing human *T* exon 3 sequence polymorphism in DNA from 8 unrelated individuals. Lower panel: *Bam*HI digestion patterns of human *T* exon 3 sequence from individuals of different phenotype. The size of the digested fragments (104 and 87 bp) is indicated.

3.2. *A common human polymorphism Gly-177-Asp affects dimer stability*

As part of an investigation of *T* as a candidate gene for congenital disorders of axial development, we have searched for mutations in the human gene. Analysis by SSCP of exon 3 amplified from DNA of unrelated individuals has identified a commonly occurring structural variant (Fig. 5). Family studies show that this can be attributed to the occurrence of two alternative alleles at the human *T* locus and sequence analysis has identified a G to A change at bp 530 in the human *T*-coding sequence [18]. This transition leads to the gain of a *Bam*HI site (Fig. 5) and an amino-acid substitution Gly to Asp at aa 177. The frequency of the Asp-177 allele in the British population is 0.51 and varies between 0.33 and 0.59 amongst different populations (Papapetrou et al., 1997, personal communication).

Amino-acid residue 177 lies in the DNA-binding domain within the highly conserved T-box. In *T* sequences of different chordate species, Gly occurs at this position (mouse [20], zebra fish [3], *Xenopus* [21], chick [22], *Amphioxus* [23], ascidian tunicate [24]). Analysis of *T* genomic DNA by PCR and restriction enzyme digestion, from six different strains of mice (C57BL, DBA/2, CBA, P12 M. spretus and Lcells) and from two gorillas, showed Gly at this position in all samples. In mouse, Gly-177 is encoded by GGC (instead of GGT in humans) and forms an *Apa*I site which was digested in all mouse strains tested. Both gorilla DNAs were digested with *Ava*II, which digests the Gly-177 sequence but not Asp-177 in humans, whereas *Mbo*I, which digests Asp-177 sequence but not Gly-177 in humans, did not digest either gorilla sample.

In order to investigate whether this substitution of an un-

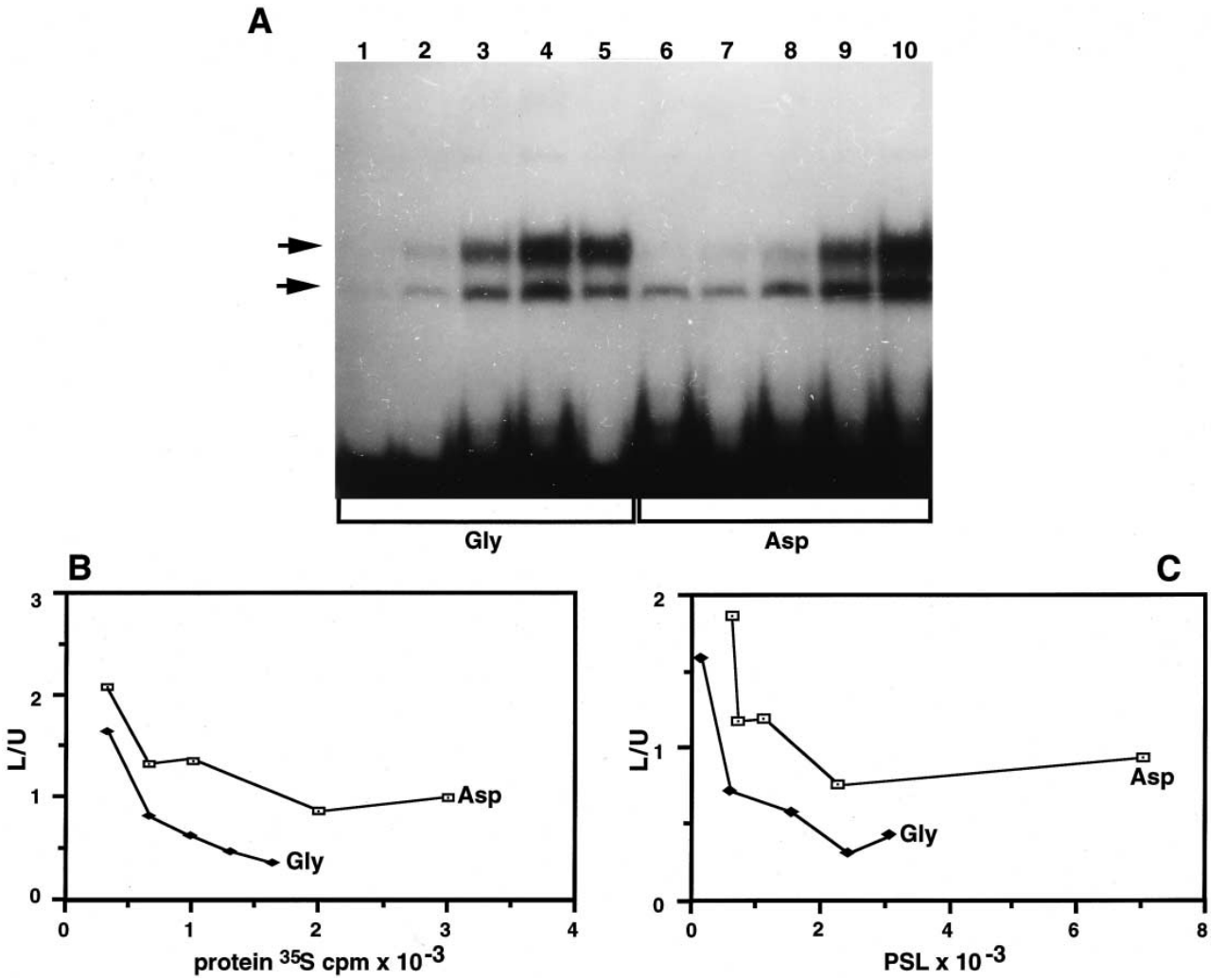

Fig. 6. (A) Comparison of the DNA-binding properties of huTtr Asp-177 and huTtr Gly-177, each at five different protein concentrations. DNA/huTtr protein fast- (L) and slow-mobility (U) complexes are indicated by arrows. (B) Graph of L/U formed by each type of protein (Asp-177 and Gly-177) plotted against the amount of protein (^{35}S cpm) added to the binding mix or (C) against the total amount of DNA/protein complex formed (PSL).

charged amino acid by an acidic residue, in a crucial, conserved region of the DNA-binding domain, affects the DNA-binding characteristics of the T protein, we have carried out EMSAs using huTtr with either Gly or Asp at position 177. mRNA was prepared from fetal intervertebral discs from 8 individuals and cDNA synthesised by RT-PCR. Samples from individuals homozygous for Gly-177 and Asp-177 were selected by typing cDNA for the exon 3 polymorphism by *Bam*-HI digestion and these samples were used for in vitro transcription/translation.

The newly synthesised Gly-177 and Asp-177 huTtr proteins were used in DNA/protein-binding studies. Both types of huTtr bind to BS.p and both form BS.p/huTtr monomer and dimer complexes, with higher amounts of dimer formation at increased protein concentrations. However, the two types of huTtr showed a difference in the relative amounts of the dimer and monomer BS.p/huTtr complexes they formed. Fig. 6 shows a typical experiment and summarises the data graphically, in two different ways. When the data are expressed as L/U for different amounts of protein (measured as ^{35}S after TCA precipitation) added to the binding reaction, it can be seen that relatively more of the Gly-177 huTtr is present as BS.p/dimer complex than for Asp-177 huTtr at the same protein level (Fig. 6B). Similarly, when the results are presented as L/U for different amounts of total BS.p bound (measured as ^{32}P by phosphoimaging of the EMSA bands), there is relatively more BS.p/dimer complex for Gly-177 huTtr than is found for Asp-177 huTtr at any level of BS.p complexed (Fig. 6C). So, for example, if lanes 2 and 4 of the Gly-177 EMSA are compared with lanes 7 and 9 of the Asp-177 EMSA, it can be seen that the Gly-177 samples show higher amounts of the slower-mobility, dimer complex. These results show that Gly-177 huTtr and Asp-177 huTtr differ in dimer/DNA complex stability.

4. Discussion

Most transcription factors, including the basic leucine-zipper proteins, helix-loop-helix proteins, zinc finger proteins, the steroid hormone receptor super family and the CCAAT/enhancer-binding protein family C/EBP, bind to DNA as homodimers. Amongst these, two dimerization structures are relatively well-understood; the leucine zipper characterized by heptad repeats of leucine, which adopt a coiled coil structure

during dimerization, and the helix-loop-helix structure, two short amphipathic helices containing hydrophobic residues at every third or fourth position ([25] and references therein). Neither of these structures is a feature of the T protein and the dimerization domain has yet to be clearly identified. The studies presented here suggest that at least part of this domain lies within the N-terminal half of the protein, but the relative instability of the dimer formed by the truncated T protein indicates that sequences in the C-terminal half are also required.

The most efficient DNA-binding site for T, consists of an inverted repeat of the dodecameric half site separated by a 24-bp spacer [2]. In vitro experiments have shown that two half sites are required for binding and that their spacing is critical for transactivation [2]. These observations fit with our finding that T binds to DNA as a dimer, each subunit contacting one half site. A further enhancement of activation is seen when two palindromic T sites are present and this seems to indicate that two protein dimers might bind to neighbouring sites.

It is now well-recognised that members of a family of transcription factors bind DNA either as homodimers or as heterodimers with other members of the same family [25]. If the members of the family have distinct regulatory and functional properties, this offers enormous scope for diversity of gene regulation. The *T* gene is a member of a family of T-box genes defined by their highly conserved DNA-binding domain [11,14] and it is possible that T forms and functions as a heterodimer with other members of the T-box family. Given that *T* is only expressed in early development and is restricted to primitive streak, pre-notochordal mesoderm, notochord and tail bud, the current best candidate for a dimerization partner in vivo is Tbx6 [12]. *Tbx6* is first detected during gastrulation in the primitive streak and paraxial mesoderm and then in the tail bud [26] and thus overlaps expression of *T*. Interaction between these two T-box proteins in the primitive streak and tail bud may be essential to the normal differentiation of mesoderm in these sites. The proposition that T acts as a dimer and in particular forms heterodimers with Tbx6 offers an attractive explanation for the antimorphic nature of some *T* mutant alleles (T^{c}, T^{wis}, T^{c-2H}) [27,28]. For example, it has been difficult to explain how the T^{c}/T^{Br} mouse can show a more severe phenotype than the T^{Br}/T^{Br} null (T^{Br}, *Brachyury*, is a full deletion of the *T* gene). Interaction of T^{c} and Tbx6 subunits to form inactive complexes would provide a mechanism to explain this phenomenon.

The finding in the human population of a common polymorphism Gly-177-Asp within the T-box DNA-binding domain is of some interest, particularly since this residue is highly conserved in *T* homologues of all vertebrate species sequenced thus far, and does not show common genetic variation in the mouse. This high level of conservation implies a significant role for Gly-177 in normal T function. Modification of this residue to Asp-177 in the human T variant isoform, does not prevent T binding to its target DNA site but appears to reduce the stability of the T dimer formed with truncated protein. This genetic variation may be meaningful in the context of human disorders of axial development. For example, we have recently described an association between an allelic variation in intron 7 of the human *T* gene and susceptibility to spina bifida [29] and are now investigating the distribution of the Gly-177 and Asp-177 alleles amongst our neural tube defect patient samples. It may be relevant that Gly-177 is specific for the T gene and does not occur in other members of the broader T-box family. Asp-177 occurs quite frequently in other T-box genes, for example in mouse *Tbx2* and *Tbx3*, *Drosophila omb* and human *TBX2*, but in human *TBX5* and *TBR1* and mouse *Tbx4*, *Tbx5* and *Tbr1* this codon is Glu-177 and in mouse *Tbx6* is Gln-177 [12,16].

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