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Isolation of cDNA, Chromosome Mapping, and Expression of the Human TBP-Like Protein

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TBP is an essential factor for eukaryotic transcription. In this study, we identified a human cDNA encoding 21-kDa TBP-like protein (TLP). The TLP ORF, carrying 186 amino acids, covered the entire 180 amino acids of the C-terminal conserved domain of human TBP with 39% identity and 76% similarity. FISH determined that human *tlp* gene was located at chromosome 6 region q22.1-22.3. Northern blot analysis demonstrated that TLP mRNAs were expressed in various human tissues ubiquitously. We found that the TLP proteins exist in multiple mammalian cells and chicken cells. Although the *Drosophila* TBP-related factor (TRF) is a neurogenesis-related transcription factor, expression of TLP was nearly constant throughout the neural differentiation of P19 cells. Unlike TRF, TLP did not bind to the TATA-box nor direct transcription initiation *in vitro.* Similarity between TRF and TLP was considerably lower (35 in alignment score) than that between *Drosophila* TBP and human TBP (88 in alignment score). Multiple amino acids critical for the TBP function were deleted or substituted in TLP. We suggest that TLP is not a *bona fide* vertebrate counterpart nor a direct descendant of TRF.

Key Words: **TBP; TLP; TRF; transcription factor; FISH; chromosome mapping.**

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The nucleotide and amino acid sequences reported in this paper will appear in the DDBJ, EMBL, and GeneBank databases with the following accession number: AB020881. T.O., T.K., and Y.M. contributed equally to this work.

Abbreviations used: TBP, TATA-binding protein; TLP, TBP-like protein, FISH, fluorescence *in situ* hybridization; EST, expressed sequence tag; CCD, C-terminal conserved domain of TBP; a.a., amino acid(s); hTBP, human TBP.

The TATA-binding protein (TBP) is one of the general transcription factors, and included in complex general transcription factors such as SL1, TFIID, and TFIIIB for RNA polymerase-I, -II, and -III, respectively (1). Accordingly, TBP is regarded as a universal transcription factor (2). In the case of RNA polymerase-II-driven genes, TBP or TFIID binds to the TATA-box, then gathers other general transcription factors and RNA polymerase-II to form a preinitiation complex (3–6). Thus, TBP is a key molecule in gene regulation, and studies on TBP itself and its related factors are significant to elucidate the gene regulation. TBP can be divided into N-terminal variable domain and C-terminal conserved domain (CCD) with 180 amino acids (a.a.). The CCD is extraordinarily conserved among organisms (1, 7, 8), and most of mutations within the CCD results in loss of TBP function (9).

Initially, eukaryotes were thought to contain no TBP family protein. Indeed, *S. cerevisiae* contains a single *tbp* gene (10). In 1993, a TBP-resembling protein referred as TBP-related factor (TRF) was identified in *Drosophila.* TRF was demonstrated to have TATA-binding and transcriptional activation functions like TBP, and to be involved in neural gene expression (11). However, since then, no other TBP-resembling protein has been documented in higher eukaryotes including vertebrates. In this communication, we describe the isolation of a human cDNA of TBP-like protein (TLP). We determined its chromosomal position and gene expression pattern that is most likely unrelated to neural function. Taken together with its structural feature, we concluded that TLP is not a *bona fide* human homologue of *Drosophila* TRF.

MATERIALS AND METHODS

Cloning and homology search for human TLP. Two DNAs (W26331 and W07871) were found as *tbp*-resembling sequences in

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the human EST cDNA library. Two sets of RACE primers (CTTAAGGAAGATTGCTTTGGAAGG [1st] and GATTGCTTTGGAAGGAGCAAATGT [2nd] for 3′-RACE and ACCTAGTTTCTGCAGACTACGGGC [1st] and GACTACGGGCTAAGCGTCTGGCAC [2nd] for 5′-RACE) were employed to obtain each terminal fragment of *tlp* cDNA. Then, PCR was performed for human retina- and liver-derived RACE libraries (Marathon-Ready cDNA, Clontech) at 94-68 °C. The obtained fragments were used as primers to amplify a longer cDNA fragment of human *tlp* by the use of human retina cDNA library (Quick Clone cDNA, Clontech). Sequence alignment was performed by the CLUSTAL W program (12).

FISH mapping. Lymphocytes isolated from human blood were used for the assay. Cells in α-MEM (Gibco) with 10% fetal calf serum and phytohemagglutinin were synchronized by BrdU treatment (0.18 mg/ml), then released the block in the normal medium with thymidine (2.5 μg/ml). Cells were harvested and slides were made by using a standard procedure (13). The isolated human *tlp* cDNA was biotinylated with dATP using BioNick Labeling Kit (BRL). The procedure for FISH detection was performed according to Heng *et al.* (13). Chromosome was stained with DAPI. FISH signals and DAPI banding pattern were recorded separately by taking photographs, and the assignment of the FISH mapping was achieved by superimposing FISH signals with DAPI-banded chromosomes.

Northern blotting. Multiple Tissue Northern Blot membrane on which various tissue-derived rat poly(A)$^+$ RNA had been blotted were purchased from Clontech. Each lane included 2 μg of poly(A)$^+$ RNA. This membrane had been confirmed by β-actin probe to be blotted with the same amounts of RNAs. Probe DNA included the whole TLP cDNA sequence. The Northern procedure was performed by using QuikHyb hybridization solution (Stratagene) for 1 hr.

Antibodies, nuclear extracts, and Western blotting. TLP polypeptide was expressed in *E. coli* by T7-expression system as described before (14). Overexpressed TLP carrying an oligo-histidine tag at the N-terminus of the coding sequences was purified with Ni-agarose followed by several kinds of conventional column chromatography. Polyclonal antibody was generated in rabbits and purified with the antigen-conjugated column. HeLa cell nuclear extract was prepared by a standard procedure (15). Rat nuclear extract was prepared from highly purified rat liver nuclei as described previously (16). Cell extracts for P19 were prepared by the method described by Schreiber *et al.* (17). For Western blotting, proteins were separated by 15% SDS-PAGE, electrophoretically transferred to PVDF membranes (Millipore), and detected by the ECL protocol (Amersham). Anti-TBP antibody is described (18).

Culture of P19 cell. P19 mouse embryonal carcinoma cells were grown in α-MEM supplemented with 10% fetal calf serum. For retinoic acid (RA) treatment, all-*trans* RA (0.5 mM) was added to the medium, and the cells were cultured in a non-coated dish for 4 days. The cells were then collected and inoculated into a collagen-coated dish, and grown for an additional 2 days (19). On day 6 from the RA treatment, the cells apparently showed altered morphology characteristic of neurons (see Results).

Indirect immunofluorescence staining. Indirect immunofluorescence staining of paraformaldehyde-fixed P19 cells for detection of neurofilament was performed as described (19), using anti-neurofilament antibody (Sigma).

RESULTS

Identification of a Human Gene for TBP-Like Protein

To detect a gene resembling human *tbp*, we searched an EST database of human cDNA clones, and found two possible clones submitted as W26331 and W07871. Based on sequence data of these clones, we designed PCR primers for RACE and amplified a couple of short DNAs. By using these DNAs as primers, we finally cloned one long cDNA with 1246 nucleotides from a human retina cDNA library (Fig. 1A). One long ORF, which encoded a protein of 186 a.a., was identified, whose calculated molecular mass was 20.8-kDa. We designated this cDNA as TLP (TBP-like protein) according to the reason described below. We aligned the a.a. sequences of human TLP and TBP (Fig. 1A), and found that TLP exhibited a significant sequence similarity (76%) with the 180 a.a. of the CCD of human TBP (hTBP), which is an essential portion in the TBP. TLP covered the entire CCD, and only two a.a. were deleted (Fig. 1A). Recently, several different EST clones with similarity to *tlp* were submitted in databases (data not shown). Sequences of all those clones were coincident with that of the cloned TLP (data not shown), suggesting that we obtained a *bona fide* cDNA sequence for human *tlp*. We further found an analogous cDNA of mouse which can encode a polypeptide identical to hTLP (data not shown).

Chromosome Mapping of Human Tlp Gene

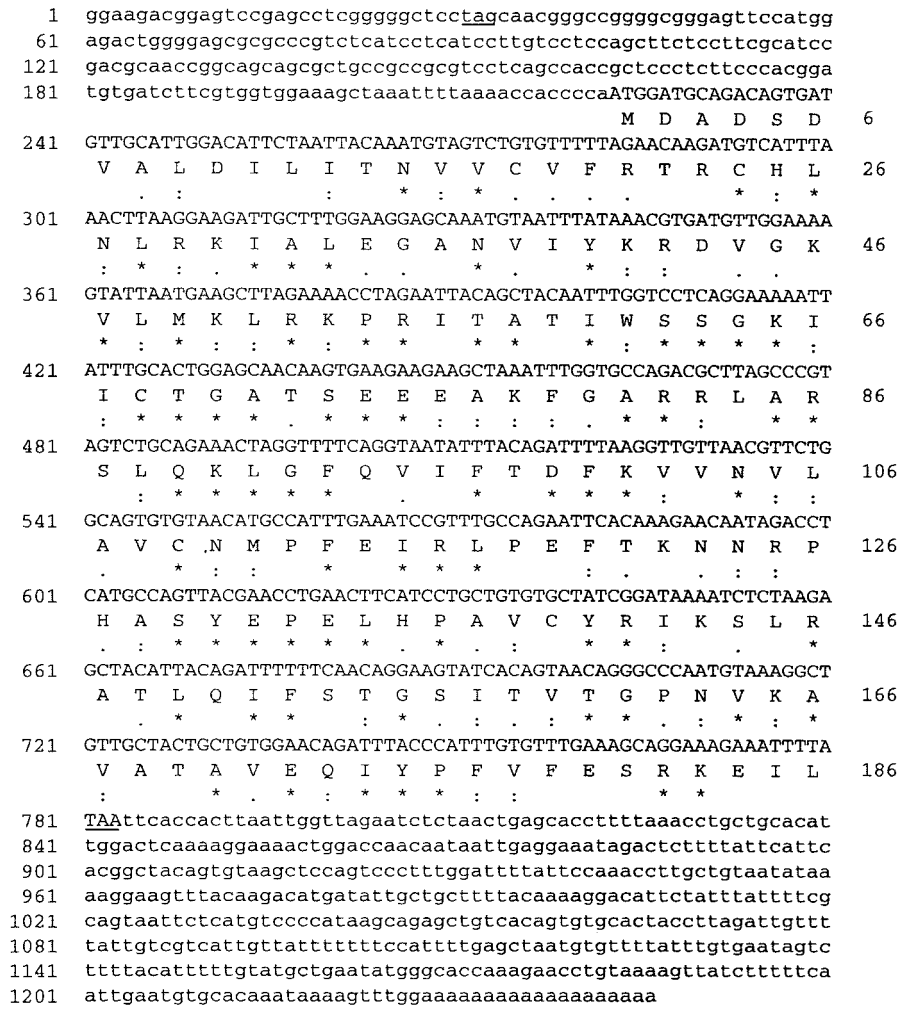
We determined chromosomal position of human TLP by FISH detection. Normal human lymphocytes were analyzed with TLP cDNA probe. In the hybridization condition used here, *tlp* does not cross-react with neither human *tbp* nor *Drosophila trf*. Under the condition used, the hybridization efficiency showed approximately 63% for this probe (data not shown). The FISH revealed a pair of positive signals on one of the chromosomes (Fig. 2A and B). From the FISH detection and DAPI-banding pattern of 10 independent photos, the location of the probe sequence was assigned at long arm of the chromosome 6 region q22.1-22.3 (Fig. 2C). We defined that *tlp* gene is located in this region.

Gene Expression for TLP

We examined the gene expression of TLP in various human tissues by Northern blotting (Fig. 3A). We detected a specific signal with 1.5-kb that is roughly agreed with the length of the obtained cDNA (1.25-kb) if we considered the short poly(A) tail (Fig. 1A). We found *tlp* gene was ubiquitously expressed. The placenta contained small amounts of the transcript. It was noteworthy that, different from neurogenesis-specific TRF, the brain did not contain plenty of the transcripts.

We investigated the existence of TLP protein in nuclear extracts of HeLa cell and rat liver by Western blotting (Fig. 3B). We prepared oligo-histidine-tagged recombinant TLP in *E. coli*, purified it (Fig. 3B, lane 1), and used it to generate antibody in rabbits. Specificity of the purified anti-TLP antibody to TLP has been confirmed because the antibody did not detect even a 60-fold excess amount of TBP (data not shown). Con-

A



B

	dTBP	hTBP	dTRF
hTLP	35	39	35
dTRF	58	60	
hTBP	88		

FIG. 1. Sequence of human TLP. (**A**) Isolation of a cDNA of human TBP-like protein (TLP). Nucleotide and predicted amino acid sequences of TLP are shown. Amino acids (a.a.) are numbered from the expected translation initiation codon at nucleotide position 223. In-frame stop codons are denoted by underlines. Identical, highly similar, and weakly similar a.a. in the CCD with 180 a.a. of human TBP (hTBP) are shown by asterisks, double dots, and single dots, respectively. The initial a.a. (serine at 159) of CCD of hTBP was aligned with valine at 7 of TLP. Two single-a.a. deletions in TLP were observed between 41K and 42R, and 166A and 167V when aligned with hTBP by the CLUSTAL W program. (**B**) Comparison of a.a. sequences of TBP-resembling factors. Similarity among the CCD of hTBP, human TLP (hTLP), *Drosophila* TRF (dTRF), and *Drosophila* TBP (dTBP) was assessed by the CLUSTAL W program (12), and calculated aligned scores are presented.

sistently, the antibody did not crossreact with TBP of 37.7-kDa (Fig. 3B, lanes 2 and 3). The anti-TLP antibody detected a protein with an apparent molecular size of 24-kDa in HeLa and rat liver nuclear extracts (Fig. 3B, lanes 3 and 2), whose apparent molecular mass on SDS-PAGE was similar to that of the calculated one (20.8-kDa) and that of recombinant TLP. In lane 3 of Fig. 3B, we observed 22- and 32-kDa bands. These may reflect post-translational modification and non-specific reaction, respectively. The highly purified nuclei were shown not to contain such non-specific bands (Fig. 3B, lane 2), assuming that some contami-

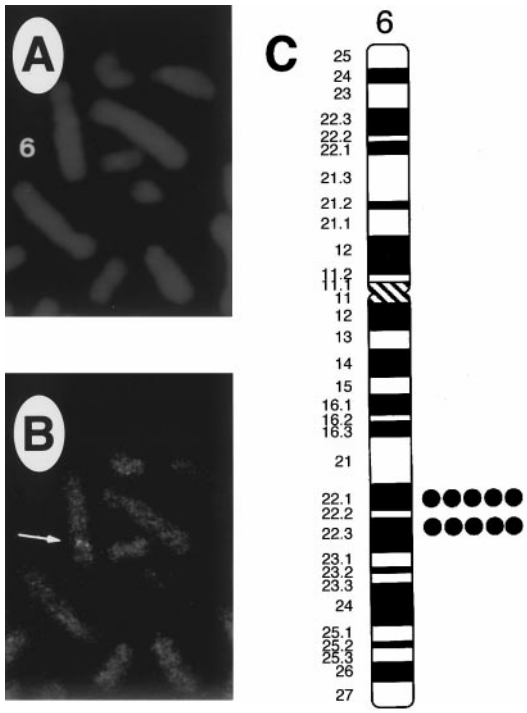


FIG. 2. Chromosome mapping of TLP. Human chromosomes were analyzed by FISH detection by using human *tlp* sequence probe. (**A**) DAPI-staining of the chromosomes and identification of chromosome 6. (**B**) FISH observation for identical chromosomes presented in panel A. An arrow indicates the position of the specific signal. (**C**) Schematic representation of human chromosome 6, and summary of FISH detection of 10 independent samples. Position of the signals are indicated with dots.

nating cytoplasmic fractions in the extract could yield these bands. We found chicken cells also contained an equivalent protein (data not shown). Above results demonstrated that TLP polypeptide actually exists in human and other mammalian cells, and probably in the vertebrates.

Functional Incompetency of TLP In Vitro

Functions of TBP are binding to TATA-box and activation of basal transcription from TATA-containing promoter. In the case of *Drosophila* TRF, it has an equivalent activity as the authentic TBP does (11). We purified TLP overexpressed in *E. coli* (Fig. 3B, lane 1). We examined DNA-binding ability by gel shift assay using TATA-box of the adenovirus major late (AdML) promoter (18, 20). Moreover, we examined transcriptional activation function by the use of the adenovirus E4 and AdML promoters in the reconstituted *in vitro* transcription system which includes recombinant TBP, TFIIB, TFIIF, and purified RNA polymerase-II (21, 22). However, in both assays, we were not able to detect positive effects of TLP (data not shown). These results suggest that TLP does not have an intrinsic activity *in vitro* neither for DNA-binding nor activation of transcription.

TLP during In Vitro Neurogenesis

Drosophila TRF is expressed predominantly in the nervous system and involved in neurogenesis (11, 23). We addressed how TLP's level alters along with neural differentiation. We determined TLP protein in mouse P19 cells which are subjected to the neural differentiation by retinoic acid (RA) treatment. P19 cell extended neurites like a native neuron on 6 days upon RA treatment (Fig. 4A-b). Neurofilament, a representative neuronal marker was detected in the transformed cells by indirect immunostaining (Fig. 4A-d). We then determined TLP by Western blotting, and found that the level of TLP in P19 cells was nearly constant regardless of their differentiation status (Fig. 4B-a). Expression of TBP in P19 was also roughly constant throughout the differentiation process (Fig. 4B-b). It is thus suggested that TBP is not a critical factor for mammalian neurogenesis at least *in vitro.*

DISCUSSION

The eukaryotic haploid genome was initially thought to contain a single *tbp*-related gene. However, Crowley *et al.* (23) reported the existence of a TRF in *Drosophila*. TRF considerably resembles TBP (60 [human] and 58 [*Drosophila*] in alignment scores), and it binds to the AdML TATA-box and activates transcription (11). Since TRF was discovered in the mutant fly "shaker" (23), which has a defect in its nervous system, and was abundantly expressed in the nervous system, TRF is believed to be involved in neural gene transcription. In this study, we found a *tbp*-like sequence in human EST

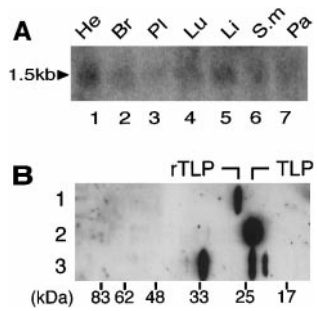


FIG. 3. Expression of *Tip* gene. (**A**) Expression of TLP mRNA in human tissues. Gene expression was determined by Northern blotting using human Multiple Tissue Northern Blot (Clontech). Arrowhead: specific signal of 1.5 kb TLP transcript. He, heart; Br, brain; Pl, placenta; Lu, lung; Li, liver; S.m, skeletal muscle; Pa, pancreas. (**B**) Detection of TLP protein by immunoblotting. One nanogram of the purified recombinant TLP carrying a histidine tag (rTLP, lane 1), 20 μg of rat liver nuclear extract (lane 2), and 20 μg of HeLa nuclear extract (lane 3) were analyzed with the anti-TLP antibody. Positions of rTLP and native TLP are indicated.

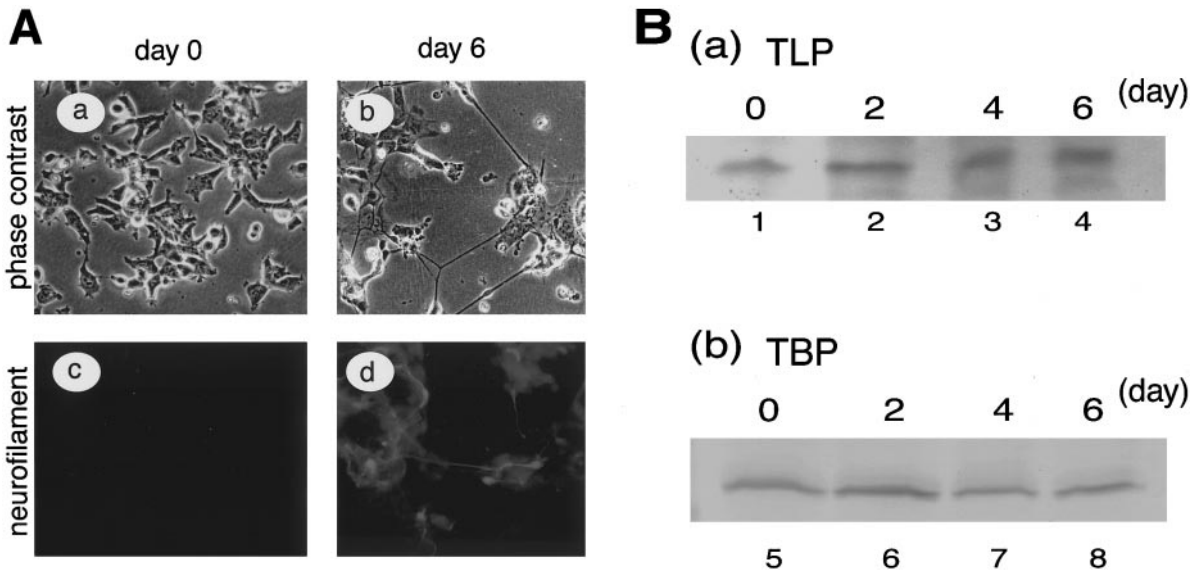


FIG. 4. TLP and TBP are expressed constantly in P19 cell during neural differentiation. Mouse P19 cells were allowed to undergo neural differentiation caused by retinoic acid (19). (**A**) Neural differentiation of P19 cell. P19 cells at day 0: native cells (panels a and c) and day 6: differentiating cells (panels b and d) were examined. Panels a and b: phase-contrast microscopic observation. Panels c and d: indirect immunostaining for neurofilament in native and differentiating cells. (**B**) Amounts of TLP (a; lanes 1–4) and TBP (b; lanes 5–8) in P19 cells. These two proteins in differentiating P19 cells were determined by immunoblotting samples taken every two days from day 0 to day 6 by immunoblotting. TLP and TBP were detected with each specific antibody. Whole cell extracts (10 μg) were used for each assay.

clones, and finally identified one cDNA (Fig. 1A). This cloned gene was designated as *tlp*. We did not refer to it as *trf* because of its drastic divergence from *Drosophila trf* and its functional incompetency. Since TLP is highly homologous to hTBP (76% similarity), and covers the entire 180 a.a. of TBP's CCD (Fig. 1B), we denoted TLP as one of the human TBP-resembling proteins. The FISH detection of human *tlp* gene assigned chromosome 6 region q22.1-22.3. It is interesting because the human *tbp* gene is also mapped on the chromosome 6 (*i.e.*, 6q27) (24).

Northern analysis of RNAs from various human tissues detected a transcript of 1.5-kb (Fig. 3A) whose size was similar to that of the isolated cDNA (1.25-kb) that carried a short poly(A) tail, suggesting that the band represented TLP mRNA. *Tlp* is thus suggested to be a ubiquitous gene. Western blotting demonstrated the presence of a polypeptide which cross-reacted with anti-TLP antibody, and whose size was coincident with that of the recombinant TLP (Fig. 3B). Since the antibody was specific for TLP, we suggest that the Western signal represents the native TLP protein. The antibody also detected rat TLP (Fig. 3B, lane 2). Because we found TLP equivalent proteins in various mouse tissues and chicken cells (data not shown), we would claim that vertebrates commonly have a *tlp* gene and express its mRNA and protein.

The critical sequences in TBP (*i.e.*, CCD) were highly conserved in TRF but not in TLP. Any deletions within the CCD are known to abolish the TBP function (9). These observations lead to an assumption that TLP has no intrinsic capability for TATA-binding and transcriptional activation. Actually, we have no positive evidence for functions of TLP (data not shown). TLP has two internal deletions when aligned with TBP (Fig. 1A, a.a. positions 194 and 320 of hTBP). At the present time, we do not know the role of TLP in the cell. As TLP was found in nuclear extracts, this protein is supposed to play some roles in nuclear events. It may function in transcription without binding to DNA or with distinct DNA-binding ability. We notice that TLP significantly stimulates TBP-directed *in vitro* transcription when added to the reaction mixture (data not shown). Therefore, TLP may function *in vivo* through an association with some basal transcriptional machineries. Based on data in Figs. 3B and 4, although TRF is involved in the nervous system of *Drosophila* (11, 23), TLP may not have a major role in neurogenesis nor in neural gene expression albeit it participates in transcriptional regulation.

As described above, *Drosophila* harbors the *trf* gene in addition to *tbp* gene (11, 23). Although we initially thought to find out a human *trf*, now we would conclude that TLP is not a *bona fide* human homologue of TRF according to homology search data (Fig. 1) as well as the gene expression pattern (Figs. 3 and 4) and function assay (data not shown). We precisely compared the a.a. sequences of various TBPs, *Drosophila* TRF, and human TLP (Fig. 1B). We noticed that TLP and TRF exhibited an alignment score of only 35, whereas the identity of dTBP and hTBP was considerably high (88 in alignment score) (Fig. 1B). These data

suggest that TLP is not close to neither TBP nor TRF. To elucidate the structural relationship among TBP, TRF, and TLP more clearly, we focused on TFIIA- and DNA-binding regions (25) within the CCD of TBP. TRF and TLP exhibited alignment scores of 60 and 37, respectively, to the TFIIA-binding region of TBP (data not shown). Alignment scores of four DNA-binding regions between TRF and TBP were 100-65, whereas those between TLP and TBP were 64-25 (data not shown). These results are consistent with the fact that TRF binds to the TATA-box (11, 23) but TLP does not (data not shown). Phylogenetically, it is improbable that *tlp* originated from *trf* or its ancestoral gene.

The above assumption brings us to the question as to how many kinds of TBP-resembling proteins do the higher eukaryotes have. Although, the genome of *S. cerevisiae* was proved to have only one *tbp* gene (10), the copy number for *tbp*-related genes in multicellular organisms is yet questionable. To our knowledge, *Drosophila*, human, and higher plants (26, 27) contain at least two *tbp*-related genes. Since a human *tbp* cDNA was cloned in 1993 (23), no other *tbp*-related gene has been identified in the vertebrates. In the mammalian EST databases, we found only *tbp* or *tlp* sequence (data not shown). Higher eukaryotes may contain only two *tbp*-related genes. Perhaps, a single *tbp* ancestor gene might duplicate when multicellular organisms originated, and one copy of the *tbp* gene was strictly conserved through evolution whereas the other copy evolved to *trf* in *Drosophila*, to *tlp* in higher animals, and diverged little in plants. However, it may be phylogenetically more plausible that the ancestor *tbp* gene diverged into *tbp* and *tlp*, then *trf* evoluted from *tbp*. More investigations for seeking out *tbp*-resembling genes in various eukaryotes are required to clarify these issues.

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