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Transcriptional Functions of a New Mammalian TATA-binding Protein-related Factor*

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A mammalian protein highly homologous to TATA-binding protein (TBP) has been identified and cloned. The recombinant mammalian TBP-related factor binds to the TATA box of the Ad-MLP and forms stable complexes with TFIIB on the promoter DNA. The mammalian TBP-related factor is able to substitute for TBP in supporting transcription by RNA polymerase II in an *in vitro* reconstituted system.

The TATA-binding protein (TBP)[1] is a universal transcription factor that nucleates the assembly of transcription preinitiation complexes on genes transcribed by all three RNA polymerases (1). In cell extracts, TBP is associated with several polypeptides, the so-called TBP-associated factors (TAFs) (2, 3). The main complex of TBP and TAFs (TAFIs) required for transcription by RNA polymerase I is called SL1; however, a different complex (TAFIIs) is required for transcription by RNA polymerase II and is called TFIID. TBP also associates with RNA polymerase III-specific TAFs (TAFIIIs) forming the TFIIIB transcription factor.

TBP is one of the most conserved proteins during the eukaryotic evolution and consists of two direct repeats encompassing the C-terminal two-thirds of the polypeptide which are highly conserved in all known TBPs (4, 5). For long time it was thought that eukaryotic cells contain a single TBP that mediates transcription by all three RNA polymerases. However, a few years ago a *Drosophila* gene product highly homologous to TBP was cloned that has been called TBP-related factor (dTRF) (6). Biochemical analyses have shown that TRF can form a stable complex with TFIIA and TFIIB on a TATA-containing promoter and substitute for TBP in directing transcription *in vitro* by RNA polymerase II (7). In cell extracts TRF is associated with a novel set of TAFs (nTAFs) forming large multiprotein complexes. *In situ* hybridization experiments have revealed that TRF mRNA is expressed in the central nervous system and in primary spermatocytes in adults (6). Immunostaining experiments using anti-TRF antibodies showed that TRF protein is expressed in most of the early embryo (until developmental stage 13); however, during embryonic development becomes restricted to cells of the nervous system and gonads. Immunofluorescence of *Drosophila* polytene chromosomes has shown that TRF protein is associated with a few sites on the chromosome (7).

In an effort to analyze whether mammalian cells contain homologues of TBP, we have identified and cloned a mammalian TBP-related factor (mTRF) from mouse embryos and human cells that shows considerable amino acid sequence similarity to TBP and *Drosophila* TRF. Recombinant mTRF can bind to the TATA box and substitute for TBP in directing transcription by RNA polymerase II.

EXPERIMENTAL PROCEDURES

Cloning of mTRF—To identify homologues to TBP, we searched the expressed sequence tag (EST) data base of GenBank™ by using the TBLASTN program and the amino acid sequence of human TBP and *Schizosaccharomyces pombe* TBP as a probe (8). Several ESTs were identified that encode different portions of a protein with significant homology to the C-terminal portion of human TBP and *Drosophila* TRF. The human ESTs encoding the different portions of the polypeptide homologous to TBP (accession numbers AA281228, W26331, AA448144, and AA412574) were overlapped to obtain a complete open reading frame (ORF). EST AA412574 was obtained from the IMAGE consortium through Research Genetics and sequenced further. The ORF contained in the cDNA was identified using the ORF Finder program at the National Center for Biotechnology Information. ESTs of mouse origin (accession numbers AA798230, AA840611, AA821478, and W89738) encoding a similar polypeptide were overlapped, and the ORF was predicted as described above.

Expression and Purification of Recombinant mTRF—The ORF of mTRF was amplified by polymerase chain reaction using oligonucleotides designed from the sequence obtained from the data base. The oligonucleotide primer encoding the N terminus of the protein contained a *Nde*I restriction site and the one encoding the C terminus contained a *Bam*HI site. The human ORF was amplified from cDNA purified from a HeLa cDNA library in Lambda ZAP (Stratagene), and the mouse ORF was amplified from embryo cDNA (Marathon ready cDNA, CLONTECH). The amplified fragment was digested with *Nde*I and *Bam*HI restriction enzymes and cloned in-frame into the *Nde*I and *Bam*HI sites of a bacterial expression vector (pET15b, Novagen), which adds a His_6 tag at the N terminus of the polypeptide. Positive clones were sequenced using the Sequenase version 2 kit (U. S. Biochemical Corp.). All our attempts to express human TRF in bacteria were negative, and only mouse TRF could be expressed.

Mouse TRF in pET15b was expressed in BL21(DE3). Bacteria were grown in Luria-Bertoni medium at 37 °C to an A_{600} of 0.6, and the production of recombinant protein was induced with 0.5 mM IPTG and grown for an additional period of 4 h. Bacteria were harvested by centrifugation and lysed by mild sonication at 4 °C in 20 mM HEPES (pH 7.9), 500 mM KCl, 0.1% (v/v) Nonidet P-40, 10% (v/v) glycerol, 2 mM imidazole, and 0.1 mM phenylmethylsulfonyl fluoride. The lysate was cleared by centrifugation and loaded onto a 1-ml column of nitriloacetic-acid-agarose containing immobilized Ni^{2+} ions (Ni^{2+}-NTA-agarose, Qiagen). The column was washed with 10 column volumes of lysis buffer supplemented with 20 mM imidazole and eluted with 10-ml linear gradient of imidazole (0.02–0.3 M) in lysis buffer. Fractions containing recombinant mouse TRF were detected by Western blot with monoclonal antibodies against the His_6 tag (CLONTECH), pooled, and dialyzed against 20 mM HEPES (pH 7.9), 100 mM KCl, 10% (v/v) glycerol, 1 mM dithiothreitol, 0.1 M EDTA, and 0.1 mM phenylmethylsulfonyl fluoride.

Specific Transcription Reactions—Transcription reactions and prod-

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[1] The abbreviations used are: TBP, TATA-binding protein; TAF, TBP-associated factor; TRF, TBP-related factor; dTRF, *Drosophila* TRF; mTRF, mammalian TRF; EST, expressed sequence tag; ORF, open reading frame; IPTG, isopropyl-1-thio-β-D-galactopyranoside; NTA, nitrilotriacetic acid.

uct analyses were performed as described previously (9). Reactions mixtures (40 μl) were incubated at 30 °C for 45 min and contained 20 mM HEPES-KOH (pH 7.9), 8 mM $MgCl_2$, 50 mM KCl, 10 mM ammonium sulfate, 12% (v/v) glycerol, 5 mM 2-mercaptoethanol, 2% (w/v) polyethylene glycol 20,000, RNase T1 (2 units), 0.6 mM ATP, 0.6 mM CTP, and 15 μM [α-^{32}P]UTP, and 0.3 μg of supercoiled pML(C2AT) template DNA, which contains the MLP promoter fused to a G-less cassette (10). Transcription factors added to the reactions were: recombinant TFIIA (30 ng), recombinant TFIIB (20 ng), recombinant TFIIF (30 ng), recombinant TFIIE (20 ng), TFIIH purified from HeLa nuclear extract, and affinity purified RNA polymerase II (50 ng). The transcription factors were purified as described (11). Mouse TRF or recombinant TBP was added as indicated in the figure legends. The reaction products were separated on a 4% polyacrylamide/urea gel. Gels were dried and exposed overnight to Kodak MS x-ray films.

DNA Binding Assays—DNA binding assays were performed as described (9). The protein components, as indicated in the figure legends, were incubated with 0.1–1 ng (approximately 5000 cpm) of labeled DNA probe containing the Ad-MLP sequences from −40 to +20 for 30–45 min at 30 °C. The complexes formed were separated by electrophoresis through a 4% polyacrylamide gel containing Tris borate-EDTA buffer (1× TBE; pH 8.2, 40 mM Tris, 40 mM boric acid, 1 mM EDTA) supplemented with 4% (v/v) of glycerol. Electrophoresis was performed at 100 V until the bromphenol blue dye reached the bottom of the gel. The gels were dried and exposed overnight to x-ray films.

RESULTS

Cloning and Expression of mTRF—To identify mammalian genes encoding proteins homologous to TBP, we used the BLAST server at the National Center for Biotechnology Information to screen the nonredundant GenBank™ EST data base by querying with the amino acid sequence of human TBP. Several ESTs from mouse and human encoding different portions a protein with homology to TBP were found. The nucleotide sequence of the ESTs contained a ORF of 558 base pairs that encodes a polypeptide of 186 amino acids with a calculated molecular mass of 20.5 kDa. The polypeptide shows significant homology to the amino acid sequence of the C-terminal domain of all the TBP proteins that have been cloned at the present (64% similar and 41% identical) and to *Drosophila* TRF (60% similar and 38% identical; Fig. 1). Both ORFs from mouse and human encode a identical protein (Fig. 1, compare mTRF and hTRF).

To test the biochemical activities of mTRF, we inserted the amplified mouse ORF into an expression vector, and the recombinant protein was produced in bacteria. Fig. 2*A* shows that a protein of M_r 25,000 is produced upon induction with IPTG (*lanes 2* and *3*). The molecular mass of the recombinant protein is slightly higher than the predicted size. This protein was purified by Ni^{2+}-NTA-agarose chromatography (Fig. 2*B*, *lane 3*), and its identity was confirmed by Western blot analysis using antibodies against the His_6 tag epitope (Fig. 2*C*, *lane 2*).

Mammalian TRF Binds to the TATA Box and Forms Stable Complexes with TFIIB—One of the characteristics of TBP is to bind to the TATA boxes and form stable complexes with TFIIA and TFIIB on the promoter (4, 9, 12–14). To investigate the possibility that mTRF could bind the TATA box of the promoter, we performed gel retardation experiments using the Ad-MLP TATA box. Fig. 3*A* (*lanes 2* and *3*) shows that yeast or human TBP form a stable complex on the TATA box in the presence of recombinant human TFIIA. As reported earlier (9) human TBP (Fig. 3*A*, *lane 5*) or yeast TBP (data not shown) does not form a stable complex in TBE gels in the absence of TFIIA. However, in contrast to TBP, mTRF binds strongly to the TATA box in the absence of TFIIA (Fig. 3*A*, *lane 4*), and its binding is not enhanced further by TFIIA (*lanes 7* and *8*). The binding of TRF is specific for the TATA box, because it can be competed by an excess of unlabeled oligonucleotide containing the TATA box (data not shown).

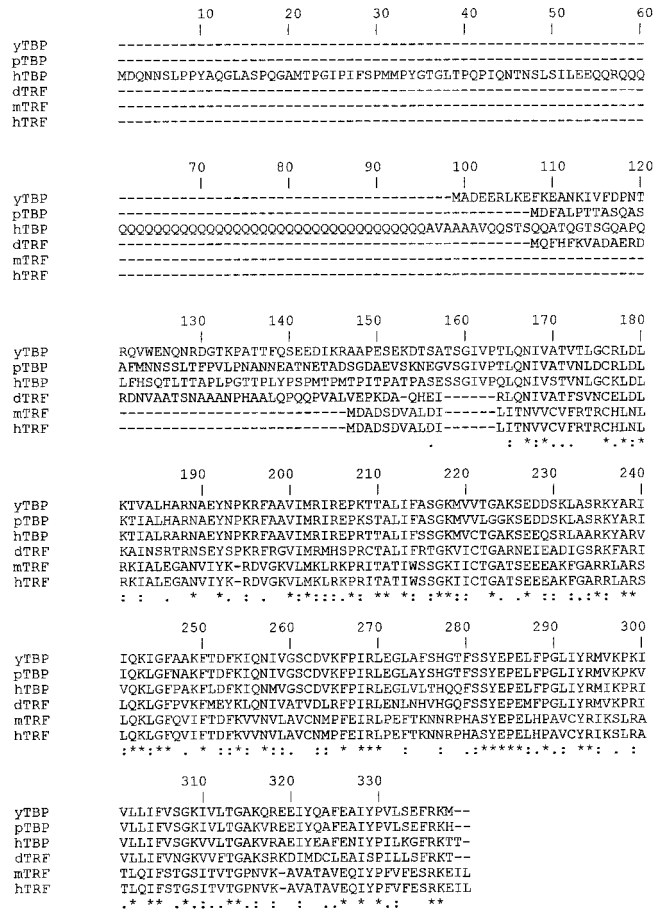

FIG. 1. **Sequence alignments of mTRF, dTRF, and human and yeast TBP.** The alignment was performed using the Clustal W program at the PBIL in Lyon, France. *Asterisks* indicate identical amino acids, and *double dots* indicate conserved amino acids. The *numbers* indicate the amino acid position of human TBP. The *dashes* represent spaces or no amino acid.

To analyze the effect of TFIIB on the binding of mTRF to the TATA box, we carried out the gel retardation experiment described in Fig. 3*B*. Human TBP in the presence of TFIIB forms a complex (Fig. 3*B*, *lanes 2*, *3,* and *4*); however, TFIIB by itself does not (Fig. 3*B*, *lane 5*). As indicated in Fig. 3*A*, mTRF by itself binds strongly to the TATA box (Fig. 3*B*, *lane 6*), forming a high molecular weight complex. The complex was dependent on the amount of mTRF, since a smaller amount of mTRF does not form a complex (Fig. 3*B*, compare *lanes 6* and *9*). Mammalian TRF in the presence of TFIIB also forms complexes (*lanes 7* and *8*), but in contrast to human TBP, it forms at least two different complexes. Those complexes are dependent on the presence of TFIIB, because an equal amount of mTRF alone does not produce a complex (*lane 9*).

Mammalian TRF Substitute for TBP in a Specific in Vitro Transcription Assay—It is known that the C-terminal domain of TBP is sufficient for TATA binding and basal transcription initiation by RNA polymerase II (4, 5). The great similarity between TBP and mTRF and the ability of mTRF to bind to the TATA box and to form complexes with TFIIB on the promoter DNA suggest that mTRF can substitute for TBP in *in vitro* transcription. To study this possibility, we carried out transcription reactions reconstituted with affinity purified RNA polymerase II, recombinant transcription factors, and either TBP or mTRF using as a template the Ad-MLP. Fig. 4 shows that our reconstituted system in the absence of TBP gave no detectable transcription (*lane 1*); however, as expected, human or yeast TBP (*lanes 2* and *5*) can direct transcription from the

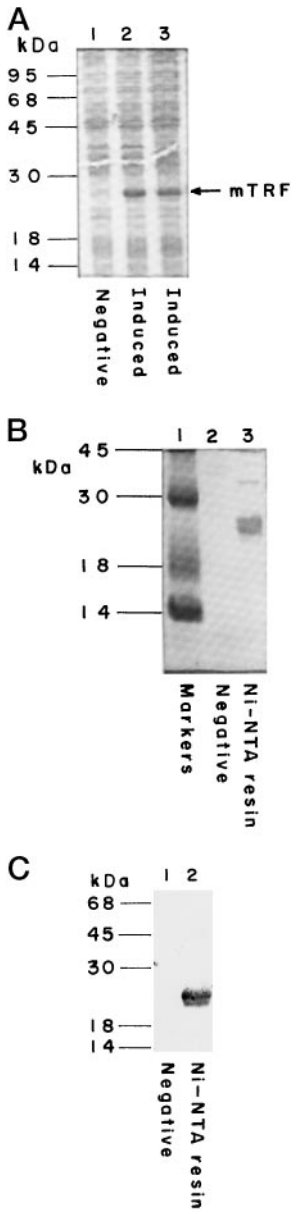

FIG. 2. **Expression and purification of mTRF.** The mouse ORF encoding TRF was cloned in-frame into the bacterial expression vector pET15b, and the protein was induced and purified from BL21 (DE3) bacteria. *A*, a 12% Coomassie Blue-stained SDS-polyacrylamide gel of bacterial extracts (5 μg) prepared after induction with IPTG (*lanes 2* and *3*). The lane labeled negative is from a control extract prepared from cells that contain pET15b without the insert (*lane 1*). *B*, a 15% Coomassie Blue-stained SDS-polyacrylamide gel showing mTRF (1 μg) purified by Ni^{2+}-NTA-agarose chromatography (*lane 2*). The lane labeled *Negative* (*lane 1*) is a fraction prepared from cells that contain pET15b without the insert. *C*, Western blot analysis of the fractions showed in *B*. The *blot* was incubated with anti-His_6 tag monoclonal antibody and detected using anti-mouse IgG conjugated to alkaline phosphatase.

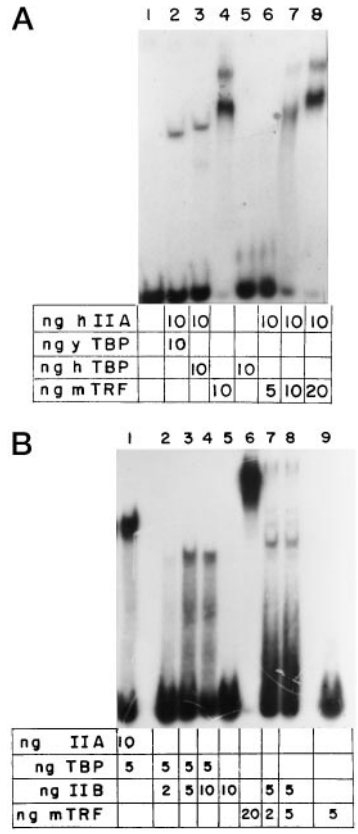

FIG. 3. **Analysis of binding activity of mTRF to the TATA box.** Labeled promoter DNA containing the Ad-MLP TATA box was incubated with different combinations of proteins, as indicated at the *bottom* of each panel. Recombinant human TFIIA, human TFIIB, yeast TBP, and human TBP and mTRF were expressed in bacteria and purified to homogeneity. The products of the binding reaction were analyzed on 5% acrylamide gels that contained 0.5× TBE buffer. *A*, analysis of the binding activity of mTRF to the TATA box. The labeled DNA probe was incubated with different amounts and combinations of factors, as indicated at the *bottom*. In *lane 1*, the labeled probe was incubated with no protein. *B*, analysis of the binding activity of mTRF to the TATA box in the presence of TFIIB. Human TBP was used as a positive control (*lanes 2–4*). Different amounts of mTRF, in either the presence or the absence of TFIIB, were added in *lanes 6–9*.

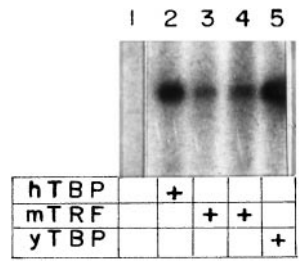

FIG. 4. ***In vitro* transcriptional activity of TBP and mTRF.** *In vitro* transcription reactions were performed using the Ad-MLP and purified recombinant TFIIA, TFIIB, TFIIE, and TFIIF. RNA polymerase II was affinity purified, and TFIIH was purified from HeLa cell nuclear extracts. In *lane 1* no TBP was added. *Lanes 2* and *5* contain 10 ng of human and yeast recombinant TBP, respectively. *Lanes 3* and *4* contain 5 and 10 ng of mTRF.

Ad-MLP. When mTRF was added in place of TBP (Fig. 4, *lanes 3* and *4*), we obtained a good level of transcription, although slightly lower than that obtained with TBP. Those results strongly suggest that mTRF can replace TBP in directing transcription by RNA polymerase II from the Ad-MLP.

It is well known that TBP can support basal transcription initiation by RNA polymerase II; however, it cannot support activated transcription in the absence of TAFs and coactivators in an assay reconstituted with recombinant or highly purified transcription factors and RNA polymerase II. We have also examined the possibility that mTRF could support activated transcription in the absence of TAFs. However, in our system mTRF is not able to support activated transcription using recombinant transcription factors, highly purified TFIIH, affinity purified RNA polymerase II, and the acidic activator Gal4-VP16 (data not shown). This analysis indicates that mTRF can support basal transcription initiation, but it cannot support activated transcription in the absence of TAFs or additional cofactors.

DISCUSSION

In this study, we report the cloning and expression of a protein homologous to TBP that we called mTRF. Our results allow us to conclude that mTRF binds to the TATA box, forms complexes with TFIIB, and is able to substitute for TBP in directing basal transcription by RNA polymerase II. Mammalian TRF is highly homologous to the C-terminal domain of TBP and also shows the two-directed repeated domain structure, and the high degree of homology suggests that it can fold into the same structure as TBP (15, 16).

Mammalian TRF, like TBP, is unable to support activated transcription by Gal4-VP16 in our reconstituted transcription system, which lacks TAFs and coactivators. However, we cannot rule out the possibility that mTRF could support activated transcription in the presence of TAFs, coactivators, or mediator-like activities similar to those described in the yeast and human RNA polymerase II holoenzyme (17–19)

Although it was thought that eukaryotic cells contain a single TBP, it has recently been reported that *Drosophila* cells contain a TBP-like molecule TRF (6). *Drosophila* TRF is highly homologous to TBP, and based in amino acid sequence comparison it has been proposed that both proteins most likely bind similar TATA sequences on the promoter DNA (7, 16). Biochemical analyses of *Drosophila* TRF have revealed that it interacts with both TFIIA and TFIIB, binds to the TATA box of several promoters, and is able to support transcription by RNA polymerase II in place of TBP (7). *In vivo*, *Drosophila* TRF localizes in the central nervous system and male reproductive organs (6). In polytene chromosomes TRF is located in a small number of chromosomal sites (7). In cell extracts TRF is complexed with its own set of novel TAFs (designed nTAFs) (6, 7). Based on those results it has been postulated that *Drosophila* TRF is a cell-specific transcription factor that directs the transcription of a subset of neuron-specific genes (7, 20).

The function of mTRF could be similar to that of *Drosophila* TRF in directing transcription from a small subset of genes in a cell- or tissue-specific fashion. Alternatively, it is also possible that mTRF may play a more general role in the expression of cellular genes. A scan from the available EST data base shows that mTRF is expressed in tissues such as testis, brain, retina, mammary gland, placenta, liver, spleen, and lung. It is also expressed in cells such macrophage, B-cells, and HeLa cells. These data suggest that mTRF may have a more general pattern of expression than dTRF. Mammalian TRF may also be complexed with its own set of TAFs that can confer to mTRF-specific promoter or activator functions, as it has been postulated for *Drosophila* TRF. Our preliminary observations suggest that mTRF in HeLa cell extracts is part of a multiprotein complex, because it elutes from gel filtration columns with an apparent molecular mass of greater than 200 kDa, as detected by Western blot analysis.[2] The molecular cloning of mTRF will allow us to determine its biochemical composition and functions. It will also allow us to determine whether it is expressed in a cell- or tissue-specific fashion.

We do not rule out the possibility that mTRF, like TBP, may also play role in RNA polymerase I and III transcription, or it could use a novel set of basal factors for directing transcription from selected promoters. Because homologues of TBP have been found, it seems plausible that there could be homologues of the other general transcription factors as well.

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