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 TFIIIB 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) 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 (TAFs) required for transcription by RNA polymerase I is called SL1; however, a different complex (TAFIs) is required for transcription by RNA polymerase II and is called TFIIID. 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 TFIIIB on a TATA-containing promoter and substitute for TBP in directing transcription by RNA polymerase II (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 TAF 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 AA12574) 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 AA798250, AA840611, AA821478, and W97973) 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 Nhel restriction site and the one encoding the C terminus contained a BamHI site. Both primers were cloned in-frame into the vector pET15b, Novagen, which adds a His6 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 A600 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 ni-trilotriacetic acid.

Specific Transcription Reactions—Transcription reactions and prod-

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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 MgCl2, 50 mM KCl, 10 mM ammonium sulfate, 12% (v/v) glycerol, 5 mM 2-mercaptoethanol, 2% (v/v) polyethylene glycol 20,000, RNase T1 (2 units), 0.6 mM ATP, 0.6 mM CTP, and 15 μM [α-32P]UTP, and 0.3 μg of supercoiled pMLC2AT 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 TFIIIB (20 ng), recombinant TFIIIF (30 ng), recombinant TFIIIE (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 TRF 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 bromophenol 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 GenBankTM 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 time. Several ORFs were identified in mouse and human encoding a similar 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. 2A shows that a protein of Mr 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 Ni2+ -NTA-agarose chromatography (Fig. 2B, lane 3), and its identity was confirmed by Western blot analysis using antibodies against the His6 tag epitope (Fig. 2C, lane 2). Mammalian TRF Binds to the TATA Box and Forms Stable Complexes with TFIIA—One of the characteristics of TBP is to bind to the TATA boxes and form stable complexes with TFIIA and TFIIIB on the promoter (4, 9, 12–14). To investigate the possibility that mTRF could bind to the TATA box of the promoter, we performed gel retardation experiments using the Ad-MLP TATA box. Fig. 3A (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. 3A, 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. 3A, 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).

To analyze the effect of TFIIA on the binding of mTRF to the TATA box, we carried out the gel retardation experiment described in Fig. 3B. Human TBP in the presence of TFIIA forms a complex (Fig. 3B, lanes 2, 3, and 4); however, TFIIA by itself does not (Fig. 3B, lane 5). As indicated in Fig. 3A, mTRF by itself binds strongly to the TATA box (Fig. 3B, 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. 3B, lanes 7 and 8). However, TFIIA by itself binds strongly to the TATA box in the absence of TFIIA (Fig. 3A, lane 5), 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).
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 direct 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 TFIIA, 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 TFIIIA, 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 (designated 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. 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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