Vol. 193, No. 1, 1993 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 28, 1993 Pages 453-459

BINDING ACTIVITY OF THE HUMAN TRANSCRIPTION FACTOR TFIID

Christine ICARD-LIEPKALNS

Laboratoire de Génétique Moléculaire de la Neurotransmission, C.N.R.S.,
91198 Gif-sur-Yvette, France

Received April 20, 1993

SUMMARY: In order to investigate the conformational state of the human TFIID, we studied the structure of the TATA-box binding protein (TBP) which is the DNA-binding subunit of the transcription factor TFIID required for transcriptional initiation by RNA polymerase II.
We showed that TBP was able to form dimers and tetramers by chemical crosslinking, subunit exchange, ultracentrifugation and gel shift experiment.
These findings indicate that the TBP homodimers could be the inactive binding form of TFIID and therefore could explain the lack of Gal4-activated transcriptional activity of the *E. coli*-expressed human TBP.

The general transcription initiator factor TFIID plays a primary part in activation of eukaryotic genes transcribed by RNA polymerase II. Binding of TFIID to the TATA box initiates the assembly of other general transcription factors as well as RNA polymerase II at the promoter, resulting in a preinitiation complex capable of accurate transcription initiation *in vitro*. Functional or structural homologs of TFIID were cloned from *Schizosaccharomyces pombe, Drosophila,* humans and *Arabidopsis* (reviewed in Ref. 1). The genes all encoded a core region, a C-terminal segment of 180 amino-acids, which contains the DNA-binding region of TFIID and is highly conserved between species.

Although the yeast TATA binding protein (TBP) has been shown to bind the TATA sequence as a monomer by mobility shift assay (2), and the monomeric structure of the *Arabidopsis thaliana* TBP has now been determined by X-ray crystallography (3), nothing is known yet about the role of the various amino-terminal extensions in transcriptional regulation. Substitution of the TFIID fraction partially purified from HeLa cells, with pure, cloned, *E. coli*-expressed TBP does not restore SP1-activated transcription from a TATA box (4).

In this paper, we present evidence that human TBP can form homodimers, and that this kind of interaction could be used as a transcriptional regulatory mechanism.

MATERIALS AND METHODS

Protein purification

HeLa cells nuclear extract and S100 fraction were prepared according to Dignam et al. (5). TFIID was partially purified from HeLa cells nuclear extracts essentially as described elsewhere (6,7).

0006-291X/93 $4.00

Gal4 (1-147) + AH protein was expressed and purified according to Lin et al. (8). TBP and CREB fusion proteins were prepared and purified as described elsewhere (9). TBP was obtained by thrombin cleavage of the glutathione-S-transferase (GST)-TBP fusion protein and further purified by chromatography on a phosphocellulose column followed by a DEAE-sepharose column. Phosphorylation of TBP was done according to Zamore and Green (10).

Western protein hybridization and immunoprecipitation
The identification of human TBP and TFIID by Western blots was performed as follows. The protein fractions were analysed on a 10 % SDS-PAGE, blotted onto an immobilon PVDF transfer membran (Millipore), and reacted with antibodies directed against recombinant human TBP then with donkey antirabbit conjugate. The immunological reaction with the antisera was carried out in 0.5 M NaCl, 0.05 M Tris-HCl pH 8, 0.1 % Tween 20, 15 % skim milk. The reactions were visualized by ECL reagents (Amersham).
Immunoprecipitation with the anti GST antibody was performed using pansorbin cells (Calbiochem). Four hundred ng of protein were incubated for 60 min on ice with anti-GST polyclonal antibody then precipitated with 50 µl of pansorbin cell suspension (10%), 60 min at 4°C. Cells were washed 3 times with RIPA buffer, incubated 10 min at 30°C and pelleted. The clear supernatant supplemented with Laemmli sample buffer was analysed on a 10 % SDS-PAGE.

Glycerol gradient ultracentrifugation
Ten µg protein of TBP (DEAE fraction) was sedimented through a 2 ml gradient from 15-40 % glycerol in 12 mM Tris pH 7.9, 40 mM Hepès pH 8.4, 60 mM KCl, 5 mM $MgCl_2$, 1 mM PMSF, for 16 hrs at 55 000 rpm in a Beckman TLS55 rotor for a table top TL100 centrifuge. Two drop fractions were collected from the bottom of the tube and analysed by Western blot or counted by Cherenkov determination. One hundred µg of each protein standards: carbonic anhydrase, bovine serum albumin and alcohol deshydrogenase were run in a parallel glycerol gradient and their sedimentation determined by coomassie blue staining of the SDS-PAGE analysis of the fractions.

***In vitro* transcription**
Transcription was analysed by primer extension using an E4 primer: 5'AAAAAACACCACTCG ACA 3', and the plasmid DNA pG5E4T bearing five Gal4 binding sites (8).

Chemical crosslinking
Two hundred ng of TBP and 20 µl of partially purified TFIID were treated with 1 mM 1,6-Bis maleimidohexane (BMH) from Pierce in buffer D (20 nM Hepès pH 8, 100 mM KCl, 20 % glycerol, 0.2 mM EDTA, 1 mM DTT, 1 mM PMSF) for 1 hr at room temperature. The reactions were stopped by adding Laemmli buffer and 8 M urea and analysed by electrophoresis on a 10 % PAGE followed by Western blot with the anti-TBP antibody which was revealed by the Amersham ECL system.

Mobility shift assay
Electrophoretic mobility shift assays were performed as described elsewhere (2) except that the complexes formed were resolved by native gel electrophoresis in 0.25 X TBE at room temperature. Oligonucleotide sequences of the double stranded synthetic DNA used in the binding reaction were as follows:

with a TATA element used for the binding of TBP
5'TGAGGATCCGCTATAAACTCGAGTG 3'
with a CRE element used for the binding of CREB
5'GAGGGGCTTTGACGTCAGCCTGGCCT 3'.

RESULTS

TBP is absent from the S100 fraction of HeLa cells and does not support Gal4-activated transcription *in vitro*.

TBP appears to be present only in the nuclear extract (NE) and not in the S100 fraction of the HeLa cells as shown by Western blot (Fig. 1). There is no size differences between the natural TBP component of TFIID present in NE and the recombinant, *E. coli*-expressed, TBP.

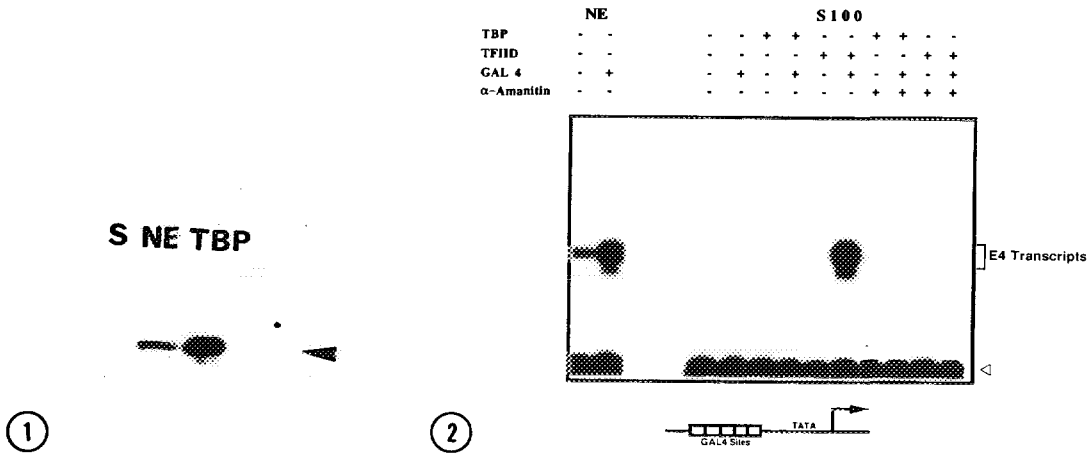

Figure 1. Absence of TBP from the S100 fraction.
Twenty µg of total proteins from the S100 fraction and the nuclear extract (NE) and 100 ng of purified, *E. coli*-expressed TBP were analysed on a 10% SDS-PAGE followed by a Western blot using a polyclonal anti-GST-TBP antibody.

Figure 2. *E. coli*-expressed TBP does not support activated transcription mediate by Gal4 in the S100 fraction.
Transcription was performed with 50 µg of total proteins incubated with 200 ng of DNA template composed of five Gal4 binding sites present upstream of the E4 promoter TATA box.

In vitro transcription performed with NE factors shows classically the basal and Gal4-activated transcriptions (Fig. 2). When proteins from the S100 fraction were used in the same system, neither transcriptions were observed. Addition of cloned TBP did not restore any detectable transcription. But we obtained E4 transcripts by adding partially purified TFIID from HeLa cells to the S100 fraction, showing that all the nuclear factors which belong to the general transcription machinery are present in the S100 fraction except for TFIID which must bind very tightly to DNA, and not leak through the nuclear membrane during the fractionation procedure. The transcripts detected were specifically synthesized by the RNA polymerase II, since the assay was α-amanitin sensitive.

Identification of multimeric TBP subunits by chemical crosslinking, immunoprecipitation and sedimentation.

Treatment of TBP with the homobifunctional sulfhydryl crosslinking agent (BMH) resulted in the formation of stable TBP dimers and tetramers (Fig. 3). High mol. wt. proteins were obtained following BMH treatment with 3 independent preparations of TBP (lanes 5,6,9). This chemical crosslinking with the partially purified TFIID resulted in a unique band of about 100 kDa.

Immunoprecipitation of the GST-TBP fusion protein by the anti-GST antibody shows that we were able to co-precipitate TBP as well as TFIID when they were added to the reaction mix. The anti GST antibody did not immunoprecipitate TBP nor TFIID alone (Fig. 4). Sedimentation analysis with glycerol gradients indicated that recombinant TBP exists in solution as a mixture of

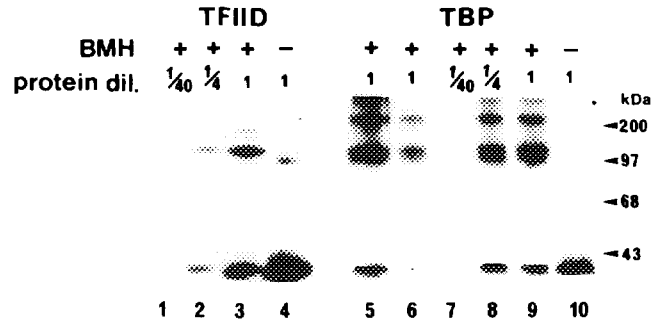

Figure 3. Chemical crosslinking of TBP and TFIID by BMH.
Two hundred ng of TBP and 20 µl (2 mg/ml proteins) of partially purified TFIID were incubated for 1 hr at room temperature with 1 mM 1,6-Bis maleimidohexane (BMH), then analysed by Western blot with an anti TBP polyclonal antibody.

monomers, dimers and tetramers (Fig. 5A). When sedimented in a binding reaction, the nucleoprotein complexes sedimented as a narrow peak at the level of the alcohol deshydrogenase, the smaller molecular weight sedimented like the free probe (Fig. 5B).

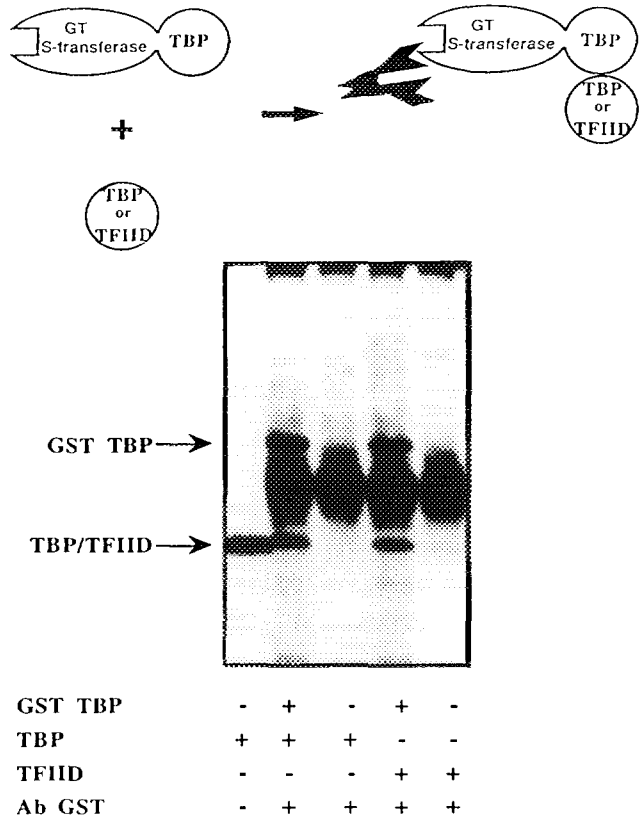

Figure 4. Immunoprecipitation of TBP by sybunit exchange.
Proteins were immunoprecipitated by the anti GST polyclonal antibody, then analysed by Western blot using the anti TBP polyclonal antibody.

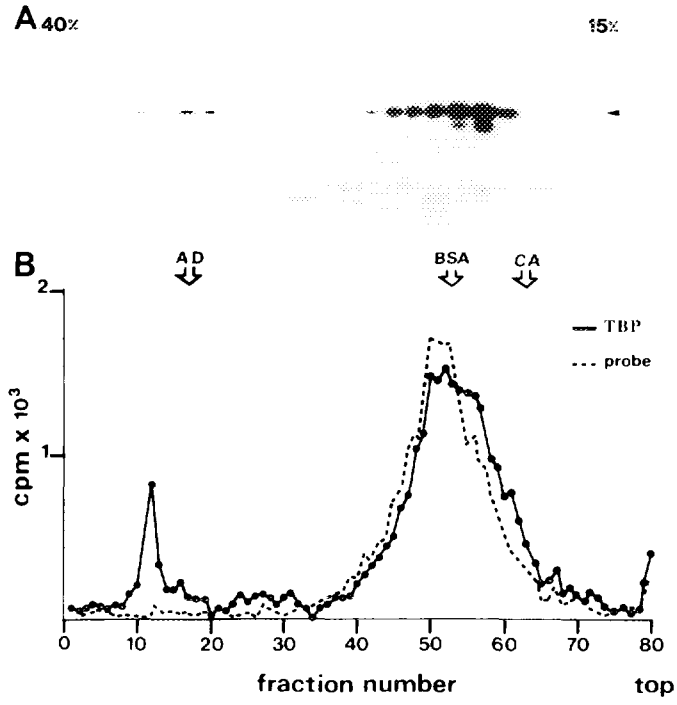

Figure 5. Glycerol gradient centrifugation.
TBP (10 µg) was sedimented on a 15-40% glycerol gradient at 55 000 rpm in a TLS55 rotor using a TL100 ultracentrifuge for 16 hrs at 4°C, as described under Materials and Methods. **A:** Two drop fractions were assayed by Western blot with the anti-TBP antibody. **B:** TBP and a 32P-labeled 25mer double-stranded oligonucleotide bearing a TATA box motif were sedimented in a binding reaction, the fractions were collected and counted. The positions of protein standards run in a parallel glycerol gradient are indicated: AD for alcohol deshydrogenase (150 kDa), BSA for bovine serum albumin (66 kDa), CA for carbonic anhydrase (29 kDa).

Comparison of the DNA binding activities of TBP vs. two protein dimers.

We tested the binding specificity of TBP by gel shift assays (Fig. 6A). Competition by a 50 fold molar excess of cold DNA probe abolished the signal (Fig. 6A, lane 2). The band corresponding to the TBP-DNA complex was not affected by the presence of anti-GST Ab, indicating that the TBP preparation was free of any GST-TBP fusion proteins (Fig. 6A, lane 3), but this binding was specific for the TBP proteins since we observed a supershift when the reaction was done in presence of anti TBP Ab (Fig. 6A, lane 4). Mobility comparison with the CREB protein which has a similar molecular weight as TBP (35 kDa vs. 38 kDa) and the Gal4 protein of 20 kDa mol. wt., indicates that TBP could bind the TATA box as a polymer, considering that both reference proteins have been shown to bind their respective DNA sites as homodimers (11). The CREB protein preparation was contaminated by fusion GST-CREB protein which is responsible for the slow migrating band seen figure 6A, lane 6. This extra band was missing when the binding reaction was performed in presence of antiGST antibody (Fig. 6A, lane 5).

We investigated the role of phosphorylation on TBP binding activity, since it is well established that most bacteria-produced proteins are not phosphorylated (12). A mobility shift assay was done

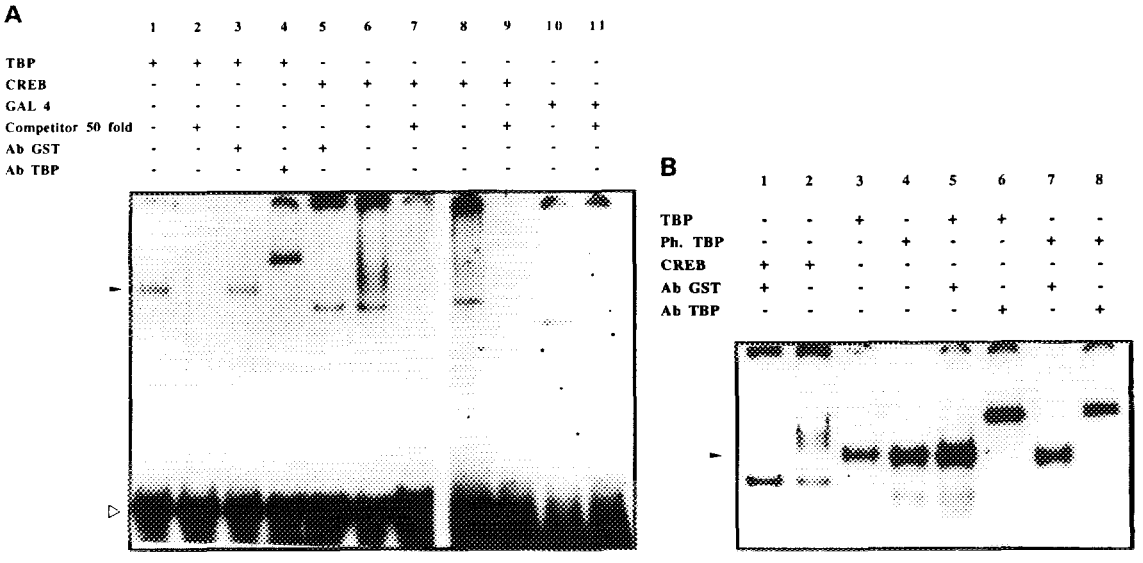

Figure 6. Comparison of TBP binding activity with other protein dimers by mobility shift assay.
A: 50 ng of each protein were incubated in a binding reaction with their respective labeled DNA probe (see Materials and Methods) and analysed in a non denaturing low ionic strength PAGE. **B:** TBP was phosphorylated by the cAMP protein kinase prior to its incubation in the binding reaction. Filled arrow indicates the specific TBP binding. Empty arrow indicates the free probe.

with phosphorylated TBP to compare the relative affinities of the phosphorylated form versus the non phosphorylated form of TBP (Fig. 6B). TBP could be efficiently phosphorylated by cAMP protein kinase (data not shown), but, apart from a slightly enhanced DNA binding affinity, phosphorylation of TBP did not affect its oligomerization state.

DISCUSSION

We have shown by *in vitro* transcription that the S100 fraction provides unique material to study transcription by complementation experiments with TFIID. Since the S100 fraction seems to be specifically depleted in TFIID (Fig. 1,2).

We were able to show by four independent and different methods that TBP was polymeric before and after DNA binding. Direct chemical crosslinking with BMH yield high mol. wt. both with the TBP, *E. coli*-expressed, and the partially purified TFIID from HeLa cells, although the migration pattern between the two preparations was different (Fig. 3). Since TBP had been purified, the high mol. wt. proteins could only be polymers (dimers and tetramers) of the 38 kDa subunit. The presence of a high mol. wt. band in the partially purified TFIID fraction after crosslinking could also be due to an homodimer of the TBP subunit of TFIID or to an heterodimer of TBP with a TBP-associated factor (TAF) part of the TFIID multiprotein complex.

We also describe here a novel way to investigate dimerization mechanism by using the GST part of the fusion protein as a "tag" to immunoprecipitate heterodimers of the fusion GST-TBP and the TBP alone which could be formed by subunit exchange (Fig. 4).

By sedimentation experiments on glycerol gradients, we showed the presence of tetramers of TBP before and after binding of the protein to DNA and that the tetrameric form was biologically active since it could bind to the TATA box (Fig. 5A,B). By gel shift experiments, we showed that homodimers of TBP were able to bind DNA (Fig. 6A), and that homodimerization does not seem to occur upon phosphorylation of TBP at least by the cAMP protein kinase (Fig. 6B). The different gel mobilities we observed cannot be the consequence of DNA conformations alone in the nucleo-protein complexes. Wu and Crothers (13) have identified the size of the bending locus for the catabolite gene activating protein (CAP), which is a dimer of 2 X 22.5 kDa, to be between 88-90 bp. In all the binding reactions we used short probes (25 bp), and furthermore, the binding site was always in a central position. Consequently, the relative mobility of TBP, CREB and Gal4 by gel shift assays should reflect only protein size differences.

It is important to note that TBP contains portions of myc-related Helix-Loop-Helix (H-L-H) domains within the direct repeat regions (14). H-L-H domains are known to potentiate homomeric and heteromeric protein-protein interactions between various regulatory factors. Activator proteins might compete with homologous TBP subunits for binding to the surface of the TBP that is bound to the TATA box. Since it has been shown recently (3) that, on binding to DNA, the convex surface of the TBP saddle protein would be presented for interaction with TBP-associated factors, transcription initiation factors and other regulatory proteins. The competition for interaction sites may influence transcription and therefore, we have described here a fine control of TBP homodimerization that could act as negative regulatory mechanism for RNA polymerase II transcription.

ACKNOWLEDGMENTS

This work was supported by the Centre National de la Recherche Scientifique and the National Science Foundation. I thank Drs. M.R. Green and J. Mallet for providing materials used in this study and for critical reading of the manuscript.

REFERENCES

1. Lewin, B. (1990) Cell 61, 1161-1164.
2. Horikoshi, M., Yamamoto, T., Ohkuma, Y., Weil, P.A. and Roeder, R.G. (1990) Cell 61, 1171-1178.
3. Nikolov, D.B., HU, S-H., Lin, J., Gasch, A., Hoffmann, A., Horikoshi, M., Chua, N-H., Roeder, R.G. and Burley, S.K. (1992) Nature 360, 40-46.
4. Pugh, B.F. and Tjian, R. (1990) Cell 61, 1187-1197.
5. Dignam, J.D., Lebovitz, R.M. and Roeder, R.G. (1983) Nucleic Ac. Res. 11, 1475-1489.
6. Reinberg, D., Horikoshi, M. and Roeder, R.G. (1987) J. Biol. Chem. 262, 3322-3330.
7. Reinberg, D. and Roeder, R.G. (1987) J. Biol. Chem. 262, 3310-3321.
8. Lin, Y.S., Carey, M.F., Ptashne, M. and Green, M.R. (1988) Cell 54, 659-664.
9. Smith, D.B. and Johnson, K.S. (1988) Gene 67, 31-40.
10. Zamore, P.D. and Green, M.R. (1989) Proc. Natl. Acad. Sci. USA 86, 9243-9247.
11. Yamamoto, K.K., Gonzalez, G.A., Biggs III, W.H. and Montminy, M.R. (1988) Nature 334, 494-498.
12. Le Bourdellès, B., Horellou, P., Le Caer, J.-P., Denèfle, P., Latta, M., Haavik, J., Guibert, B., Mayaux, J-F. and Mallet, J. (1991) J. Biol. Chem. 266, 17124-17130.
13. WU, H.M. and Crothers, D.M. (1984) Nature 308, 509-513.
14. Gasch, A., Hoffmann, A., Horikoshi, M., Roeder, R.G. and Chua, N-H. (1990) Nature 356, 390-394.