We have used a combination of fluorescence anisotropy spectroscopy and fluorescence-based native gel electrophoresis methods to examine the effects of the transcription factor IID-specific subunit TAF130p (TAF145p) upon the TATA box DNA binding properties of TATA box-binding protein (TBP). Purified full-length recombinant TAF130p decreases TBP-TATA DNA complex formation at equilibrium by competing directly with DNA for binding to TBP. Interestingly, we have found that full-length TAF130p is capable of binding multiple molecules of TBP with nanomolar binding affinity. The biological implications of these findings are discussed.

Eukaryotic DNA-dependent RNA polymerase II works in concert with the six general transcription factors (GTFs)1 TFIIA, -B, -D, -E, -F, and -H to catalyze mRNA gene transcription (1). These components act either sequentially (2) or as a part of a holoenzyme complex (3, 4) to form a multicomponent preinitiation complex on promoter DNA. A highly conserved feature of most eukaryotic mRNA promoters is the TATA box element present 25 base pairs upstream from the transcription start site. TFIID, in combination with TFIIA and TFIIIB, can recognize the TATA element and form a platform for subsequent preinitiation complex formation. The TATA box-binding protein (TBP), as its name suggests, is the protein within the 15-subunit TFIID holocomplex (5) that makes primary contact with the TATA element, though several TBP-associated factor (TAF) subunits comprising TFIID contribute to promoter binding (6–8). Binding of TFIID to TATA elements is central to the control of transcription (9, 10).

Recruiting TFIID to the promoter, a process thought to be mediated in part by direct activator-TFIID interactions, is probably a key and universal mechanism of gene activation (9, 10). However, the largest subunit of metazoan TFIID, which in Drosophila (d) or humans (h) exhibits an apparent molecular mass of ~250 kDa, termed d- or hTAF130p, respectively (herein termed d/hTAF130p), also contains a number of intrinsic enzymatic activities including histone acetyltransferase (11), protein kinase (12), and ubiquitin activating/conjugating activity (13). Each of these activities could be targets for transcriptional activators, and mutation of histone acetyltransferase, protein kinase, or ubiquitin activating/conjugating activity domains decreases transcription of subsets of genes in vivo (13–15). The yeast ortholog of d/hTAF130p, TAF130p, is encoded by a single-copy essential gene (TAF130/TAF145; Refs. 16 and 17).

Like its metazoan counterparts, the yeast protein, TAF130p, contains a histone acetyltransferase domain as well as a number of other essential sequences (18, 19) whose exact functions remain to be defined. One highly conserved and important element found in this (family of) TAF are domains capable of directly binding the TBP subunit of TFIID (17, 18, 20–24). Both Drosophila TAF250p and yeast TAF130p appear to contain at least two TBP binding domains (18, 23, 25): a high affinity N-terminal TBP binding domain and a less well defined, apparently lower affinity, C-terminal TBP binding domain(s). The N-terminal domain, termed TAND (for TAF N-terminal domain), has been analyzed in detail and comprises two elements, subdomain I (amino acids (aa) 11–77 in dTAF250p, aa 10–37 in yeast TAF130p and subdomain II (aa 82–156 dTAF250p, aa 46–71 in yeast TAF130p; Refs. 26 and 27). Subdomain I has been proposed to bind to the concave DNA binding surface of TBP and actively dissociate TBP from TATA DNA (23), whereas subdomain II is thought to interact with the convex surface of TBP and contribute to overall TAF-TBP binding affinity.

Surprisingly, reconstituted TBP-TAF complexes containing d/hTAF250p exhibit reduced formation of TBP-DNA complex (8, 28–31) relative to free TBP. The TAF130p TAND domain has been implicated in this down-regulation of DNA binding. Experiments utilizing the isolated N-terminal TAND domains derived from dTAF250p, hTAF130p, or yeast TAF130p show that this TBP binding element decreases TBP-DNA interactions at equilibrium. Consistent with these results, the NMR-derived structure of the heterologous dTAND I (aa 11–77) bound to yeast core TBP (aa 49–240) (32) shows that TAND I comprises three α-helices and a β-hairpin, unstructured when free in solution, but when complexed with TBP becomes structured and displays features mimicking the bent and splayed TATA box DNA in the TBP-TATA DNA binary complex. Clearly, occupancy of the concave surface of the TBP subunit of TFIID by TAF130p sequences would dramatically decrease the...
ability of TFIIID to bind DNA (33). Nonquantitative equilibrium measurements of the interaction of TBP and both dTAF230p and yeast TAF130p as monitored via gel mobility shift, DNase I footprinting, pull-downs, protein blotting, and our own quantitatively time-resolved fluorescence spectroscopy assays are consistent with the idea that both the yeast and metazoan TAFs decrease the binding of TBP to TATA-DNA at equilibrium (18, 20–23, 27, 28, 30). One group has argued (23) that the TAND domain forms a ternary TAND-TBP-DNA complex that actively dissociates TBP from DNA. However, the mechanism by which the TAND domain operates remains to be elucidated. Moreover, the majority of studies on dTAF250p and yeast TAF130p have been performed with just the TAND fragment of the respective proteins. Clearly, modulation of TFIIID-TATA DNA interactions by an integral TFIIID subunit merits detailed investigation.

Fundamental insights into the molecular mechanisms of gene control have been derived from the elucidation of the three-dimensional structures of GTFs bound to promoter DNA. The solution of the ternary structure of TFIID-TBP-DNA (34, 35), TFIIB-TBP-DNA (36, 37), and NC2/Dr1-TBP-DNA (38) complexes has brought true molecular level mechanistic insights to the transcription field (39). The structure of the heterologous yeast TBP-Drosophila TAND TAF250p complex adds to this important body of information regarding mechanisms of RNA polymerase II preinitiation complex formation. However, of necessity, such structural information is static and cannot directly report the types of dynamic interactions that must occur among these molecules in living cells. For this reason we wanted to analyze both the equilibrium and kinetic aspects of the interactions between TBP, TATA-DNA, and purified full-length recombinant TAF130p using homologous components. To accomplish this goal, we used fluorescently labeled DNA as previously (18, 40) and also developed a fluorescent variant of TBP. Using these probes, we performed solution cuvette (41) and gel mobility shift assays (42), two complementary methods capable of precisely performing biophysical macromolecular interaction measurements. This is the first report combining these two techniques to study TBP dynamics. We have found that full-length recombinant yeast TAF130p does not form a stable ternary complex with yeast TBP bound to TATA DNA and consequently does not actively dissociate the TBP-DNA complex; rather, full-length TAF130p binds to TBP competitively with respect to DNA. Further, we have found that full-length yeast TAF130p binds to yeast TBP with nanomolar binding affinity and that each molecule of this protein is able to bind multiple molecules of TBP. The biological consequences of these TAF130p-TBP interactions are discussed.

**EXPERIMENTAL PROCEDURES**

**Fluorophores for Protein and DNA Labeling—Rhodamine X-isothiocyanate and tetramethyl rhodamine maleimide were purchased from Molecular Probes Inc. (Eugene, OR). These reactive fluorophores were used to modify the genetically introduced single sulfhydryl group in TBP by reaction with the cysteine residues of TBP to form a covalent disulfide bond (31).**

**Fluorophores for Protein and DNA Labeling—Rhodamine X-isothiocyanate and tetramethyl rhodamine maleimide were purchased from Molecular Probes Inc. (Eugene, OR). These reactive fluorophores were used to modify the genetically introduced single sulfhydryl group in TBP by reaction with the cysteine residues of TBP to form a covalent disulfide bond (31).**

---

**Modulation of TBP DNA Binding by TAF130p**

TAF130p forms a ternary TAND-TBP-DNA complex that is capable of precisely performing biophysical macromolecular interaction measurements. This is the first report combining all three techniques to study TBP dynamics. We have found that full-length recombinant yeast TAF130p does not form a stable ternary complex with yeast TBP bound to TATA DNA and consequently does not actively dissociate the TBP-DNA complex; rather, full-length TAF130p binds to TBP competitively with respect to DNA. Further, we have found that full-length yeast TAF130p binds to yeast TBP with nanomolar binding affinity and that each molecule of this protein is able to bind multiple molecules of TBP. The biological consequences of these TAF130p-TBP interactions are discussed.

**Modulation of TBP DNA Binding by TAF130p**

TAF130p forms a ternary TAND-TBP-DNA complex that is capable of precisely performing biophysical macromolecular interaction measurements. This is the first report combining all three techniques to study TBP dynamics. We have found that full-length recombinant yeast TAF130p does not form a stable ternary complex with yeast TBP bound to TATA DNA and consequently does not actively dissociate the TBP-DNA complex; rather, full-length TAF130p binds to TBP competitively with respect to DNA. Further, we have found that full-length yeast TAF130p binds to yeast TBP with nanomolar binding affinity and that each molecule of this protein is able to bind multiple molecules of TBP. The biological consequences of these TAF130p-TBP interactions are discussed.

**Modulation of TBP DNA Binding by TAF130p**

TAF130p forms a ternary TAND-TBP-DNA complex that is capable of precisely performing biophysical macromolecular interaction measurements. This is the first report combining all three techniques to study TBP dynamics. We have found that full-length recombinant yeast TAF130p does not form a stable ternary complex with yeast TBP bound to TATA DNA and consequently does not actively dissociate the TBP-DNA complex; rather, full-length TAF130p binds to TBP competitively with respect to DNA. Further, we have found that full-length yeast TAF130p binds to yeast TBP with nanomolar binding affinity and that each molecule of this protein is able to bind multiple molecules of TBP. The biological consequences of these TAF130p-TBP interactions are discussed.
RESULTS

We previously used fluorescence anisotropy to examine the interaction of yeast TBP with DNA (40). In these experiments we utilized fluorescence anisotropy spectroscopy binding measurements to probe both kinetic and thermodynamic features of this DNA binding reaction. The fluorophore in these experiments was either the endogenous single tryptophan of TBP (residue 26) or an extrinsic fluorophore, RhX, added to the TATA box DNA target DNA. We encountered two unrelated complications when using the single endogenous tryptophan at amino acid residue 26 in the N terminus of yeast TBP as a fluorescent reporter. The first was that, in the absence of TATA-DNA, the N terminus of TBP containing the tryptophan fluorophore behaved as if it were hydrodynamically uncoupled from the majority of the protein mass. Additionally, upon DNA binding, the hydrodynamic coupling became even less (matching that observed in 6 m guanidine HCl) and the tryptophan fluorescence emission maximum shifted by tens of nanometers, to 353 nm, identical to the unfolded protein. These results indicated that the Trp-26 fluorophore is clearly in a much different environment in the absence and presence of DNA. Second, tryptophan is inherently a very weak fluorophore and thus could only productively be utilized with TBP in the micromolar concentration range. Indeed, it was this lack of sensitivity coupled with the unusual spectral properties of the tryptophan fluorophore that prompted us to develop a site-specific, extrinsically labeled TATA-DNA probe to study the protein-DNA or protein-protein interactions of this important transcription factor.

Studies by others have also examined TBP and TATA-DNA interactions and generated data consistent with many of our results (49–53). Thus, it has been determined that yeast TBP, which is primarily monomeric when free in solution at low (<1–5 μM) concentrations (54, 55), binds and dissociates from DNA relatively slowly (40, 56) particularly at lower temperatures ($k_{diss}$, TBP = 1.66 × 10$^{-3}$ s$^{-1}$; $k_{diss}$, TBP = 4.3 × 10$^{-2}$ min$^{-1}$; dissociation $t_{1/2}$ ≈ 10 min). These analyses set the foundation for future studies, which will be extended to more physiologically relevant multiprotein DNA-TBP interactions. Numerous biochemical studies have shown that a plethora of proteins such as the GTFs TFIIA (57) and TFIIB (58) as well as TAF130p (18, 21) Mot1p (59, 60), Brf1p (61), and NCI2/Drl1 (62, 63) bind TBP and modulate its ability to bind DNA. As a prelude to investigating these multiple interactions in more detail, we carried out a series of fluorescence experiments designed to study the interaction of purified, recombinant full-length TAF130p, the major TBP-binding subunit of the TFIIID complex, with TBP in presence and absence of TATA-DNA. Our experiments utilized both fluorescence anisotropy and fluorescence-based native gel electrophoresis methods. We compared these two complementary analytical approaches to investigate the competitive dynamics among TBP, TATA-DNA, and TAF130p.

Generation of a High Sensitivity Extrinsically Labeled TBP for Fluorescence Binding Assays—As useful as the RhX-labeled TATA-DNA probe was in our previous work (18, 40), this fluorophore only allowed us to examine the behavior of TBP from the perspective of changes in the hydrodynamics of DNA. For this reason we generated a form of TBP suitable for site-specific labeling with exogenous fluorophores. A wide range of bright, sulfhydryl-reactive fluorophores are commercially available and could conceivably be used to label WT TBP. However, yeast TBP contains two endogenous and potentially reactive cysteine residues at amino acid positions 78 and 164. Rather than deal with the complications of determining both the site and stoichiometry of labeling of the wild type protein with such fluorophores, we generated a form of yeast TBP in which the endogenous cysteines at positions 78 and 164 were mutated to generate TBP$^{Cys\rightarrow Gly}_1$—the nonessential, nonconserved N-terminal sequences are indicated by the gray bar; the conserved and essential 180-amino acid-long domain of TBP is shown in black. The location of the three amino acids mutated to generate TBP$^{Cys\rightarrow Gly}_1$ are shown, Ser-61 changed to Cys, Cys-78 changed to Ala, and Cys-164 changed to Ala. Panel B, recombinant TBP$^{Cys\rightarrow Gly}_1$ binds DNA efficiently. Shown is an autoradiogram of a gel mobility shift assay comparing the relative DNA binding activities of WT TBP and TBP$^{Cys\rightarrow Gly}_1$ (0, 1, 2.5, 5, 10, and 100 ng of each form of TBP added). Panel C, TBP$^{Cys\rightarrow Gly}_1$ supports yeast cell growth when present as the sole source of TBP in vivo. Shown is a photograph of a 5-fluoroorotic acid-containing agar plate upon which were streaked five yeast strains expressing either no TBP-encoding gene (VECTOR) or TBP-encoding genes expressing WT TBP, TBP$^{Cys\rightarrow Gly}_1$, a functional truncation variant of TBP ($\Delta$1–57; see panel A and Ref. 44) or a nonfunctional variant of TBP ($\Delta$94–115).

Fig. 1. Properties of TBP$^{Cys\rightarrow Gly}_1$. Panel A, a schematic of the 240-amino acid TBP$^{Cys\rightarrow Gly}_1$. The nonessential, nonconserved N-terminal sequences are indicated by the gray bar; the conserved and essential 180-amino acid-long domain of TBP is shown in black. The location of the three amino acids mutated to generate TBP$^{Cys\rightarrow Gly}_1$ are shown, Ser-61 changed to Cys, Cys-78 changed to Ala, and Cys-164 changed to Ala. Panel B, recombinant TBP$^{Cys\rightarrow Gly}_1$ binds DNA efficiently. Shown is an autoradiogram of a gel mobility shift assay comparing the relative DNA binding activities of WT TBP and TBP$^{Cys\rightarrow Gly}_1$ (0, 1, 2.5, 5, 25, and 100 ng of each form of TBP added). Panel C, TBP$^{Cys\rightarrow Gly}_1$ supports yeast cell growth when present as the sole source of TBP in vivo. Shown is a photograph of a 5-fluoroorotic acid-containing agar plate upon which were streaked five yeast strains expressing either no TBP-encoding gene (VECTOR) or TBP-encoding genes expressing WT TBP, TBP$^{Cys\rightarrow Gly}_1$, a functional truncation variant of TBP ($\Delta$1–57; see panel A and Ref. 44) or a nonfunctional variant of TBP ($\Delta$94–115).
indicating that these mutations had no significant effect upon the ability of TBP to productively bind TATA DNA (see also below).

As a rigorous physiological test of the effect of generating the S61C/C78A/C164A mutant form of TBP, we separately introduced several control plasmids or a plasmid carrying the gene encoding TBP<sup>Cys<sup>r</sup>-r</sup> into yeast cells and, using the 5-fluorescent acid-mediated plasmid shuffle technique (48), exchanged these genes for the WT allele. The resulting yeast strains had but a single plasmid. As shown in Fig. 1C, the WT TBP, Δ1–57 TBP, and the Cys<sup>r</sup>-r TBP-encoding genes supported vigorous growth. As expected, the gene encoding TBP scored as essential (44) because exchanging WT TBP with either no second TBP-encoding gene (i.e. empty plasmid vector, see plate quadrant labeled VECTOR, Fig. 1C) or a known defective form (44) of TBP (quadrant labeled Δ94–115, Fig. 1C) prevented growth of the resultant yeast strains. As viability and growth critically sample TBP function, the fact that TBP<sup>Cys<sup>r</sup>-r</sup> supports cell growth strongly argues that these three mutations in TBP did not compromise any critical function of the molecule and thus the altered protein would serve well as a reporter in our biophysical and biochemical assays of TBP function. This conclusion is further supported by the data presented below (see Figs. 4–8).

**Fluorescence Anisotropy Readily Monitors Dissociation of TBP Bound to TATA DNA by Full-length Recombinant TAF130p**—Anisotropy describes the average rotational motion of a fluorophore that occurs during the lifetime of the excited state (−ns). Protein association and dissociation reactions alter the average hydrodynamic properties of the fluorophore-protein (or -DNA) complex and is reflected in a change in rotational motion and hence anisotropy, abbreviated r. We used site-specifically labeled DNA for our first anisotropy-based assays of TBP protein-DNA or protein-protein interactions. An extremely useful aspect of the anisotropy assays is that they monitor both equilibrium and kinetic association/dissociation reactions in real time because separation of reactants and products is not needed to monitor anisotropy. To begin to study the biophysics of TBP protein-protein interactions we, chose TAF130p for our initial studies. The TFIID-specific subunit, TAF130p, is the TAFp within the holocomplex that can directly interact with TBP, as demonstrated by various blotting and pull-down assays, and has been proposed to play a key “scaffold” role in TFIID organization or assembly (20, 22, 23, 25, 65). We therefore wanted to examine the influence of full-length recombinant TAF130 protein upon the ability of TBP to interact with DNA.

The fluorescence anisotropy version of the TBP, DNA, TAF130p interaction experiment is presented in Fig. 2. In this experiment, rhodamine X-labeled 14-mer TATA DNA (Ad2 MLP TATA box DNA) was first mixed with increasing concentrations of TBP (50 nM DNA and 0–50, 0–100, and 0–250 nM TBP, Fig. 2A, circle, triangle, and square, respectively), allowed to reach equilibrium, and then the anisotropy of the DNA signal measured. As expected (18, 40), anisotropy increased as a function of TBP concentration and plateaued at an r value of ~0.19 when the DNA was all driven into binary TBP-DNA complex. Our previous study (40) utilized stoichiometric binding conditions (i.e. micromolar protein and DNA concentrations) and established a 1:1 binding stoichiometry. As a reference point, the slope of the initial part of this binding isotherm (Fig. 2A) extrapolates to a breakpoint consistent with a 1:1 complex. The three binding reactions of Fig. 2A were carried out to differing final mole ratios of TBP to DNA: 1:1, 2:1, and 5:1. Importantly, full-length TAF130p protein does not bind to DNA. Anisotropy is constant at r ~ 0.15 across the complete TAF130p titration range when TAF130p is added directly to labeled TATA DNA (Fig. 2C). However, when TAF130p was titrated into the three reactions containing TBP-DNA binary complex (i.e. 50, 100, or 250 nM TBP final), we observed a gradual, TAF130p-induced, dose-dependent decrease in anisotropy (Fig. 2B). As we will show more directly below, this drop in anisotropy resulted from a decrease in the equilibrium levels of the TBP-DNA complex.

Several features of these competition experiments are notable. First, stable TAF130p-TBP-DNA ternary complexes do not appear to be formed because there is no increase in anisotropy upon TAF130p addition to the TBP-DNA binary complex. Second, even at substoichiometric molar ratios of TAF130p to TBP, TAF130p dramatically reduced the amount of TBP-DNA complex (TAF130p alone did not bind DNA) and anisotropy eventually reached the point where no TBP-DNA complex was observed. Third, there was no lag in DNA anisotropy decrease upon increasing TAF130p concentration in these binding com-
petition reactions. Moreover, even when there was a mole excess of TBP relative to DNA, this free non-DNA bound TBP, did not appear to affect the competitive behavior of TAF130p. Finally, both the 100 nM TBP and 250 nM TBP reactions are “left-shifted” ~3–4 fold relative to the behavior expected if TAF130p interacted with DNA-bound TBP and free TBP equivalently at a 1:1 molar ratio (i.e. 50% competition at [TAF130p] < [TBP-DNA] or [TBP]total). There are at least two explanations for the unusual competitive behavior of TAF130p toward TBP (TBP-DNA) displayed here. TAF130p could either have a significantly greater affinity toward the TBP-DNA binary complex than for free TBP, or, alternatively, each TAF130p may bind multiple TBP molecules. This question is explored directly below.

Kinetics of Interaction of TBP with DNA in the Absence and Presence of Purified Full-length Recombinant TAF130p—To complement our equilibrium TAF130p competition assays, we performed a series of kinetic experiments designed to examine the interactions of TBP with TATA target DNA in the presence and absence of varying concentrations of full-length TAF130p.

We were interested in testing, if TAF130p affected either the rate of association or dissociation of TBP with DNA, either step(s) in TBP-DNA interaction could be a possible target(s) for decreased binding at equilibrium. We were particularly intrigued with performing these kinetic studies, as these experiments might allow us to determine whether TAF130p actively dissociated TBP-DNA complexes by forming a ternary TAF130p-TBP-DNA complex as suggested previously by others (23). Active dissociation would proceed at a rate greater than the intrinsic TBP-DNA dissociation rate observable in the presence of an excess of unlabelled DNA.

We examined the kinetics of association (Fig. 3A) and dissociation (Fig. 3B) of TBP to RhX-DNA in the presence and absence of full-length yeast TAF130p using stopped-flow methods. In typical stopped-flow association experiments, reactions were performed by mixing 100 μl of RhX-DNA from syringe 1 with 100 μl of either free TBP or TBP pre-bound with increasing concentrations of TAF130p from syringe 2. In the control experiment, 100 nM RhX-DNA from syringe 1 was mixed with 150 nM TAF130p from syringe 2; no increase in RhX-DNA anisotropy was observed (data not shown). As might have been predicted from the experimental data presented immediately above in Fig. 2B, no significant amount of a stable TAF130p-TBP-DNA ternary complex formed in these association or dissociation reactions. Anisotropy values never increased to a value greater than that of the binary TBP-DNA complex (cf. green trace in Fig. 3A). Increasing amounts of TAF130p decreased both the apparent rate and extent of TBP-DNA binary complex formation. This result is consistent with our previously published work (18) and the work of others (21) on the interaction of N-terminal fragments of TAF130p with TBP. Absorption of TBP-DNA complex, full-length TAF130p and DNA compete for binding to TBP.

In the dissociation experiments (Fig. 3B), reactions were performed by mixing 100 μl of pre-bound RhX-DNA-TBP complex from syringe 1 with 100 μl of increasing concentrations of TAF130p from syringe 2. As a control, to measure the intrinsic dissociation rate of TBP-DNA complexes, we set up a separate reaction where a large molar excess (2000 nM) of unlabeled TATA box DNA (Fig. 3B, red trace) alone was added in syringe 2. Analysis of these data indicated that, within error, the dissociation rate of TBP-DNA binary complexes in the presence of full-length TAF130p is apparently no faster than the intrinsic, DNA-mediated (i.e. 2000 nM TATA DNA chase curve) competition derived off rate. Again, no significant amount of a TAF130p-TBP-DNA ternary complex was detected as anisotropy (r) never exceeded −0.19, the r value of the TBP-DNA binary complex. These data argue that, rather than actively dissociating TBP-DNA complex through a ternary TAF130p-TBP-DNA complex, full-length TAF130p and DNA compete for binding to TBP.

Spectroscopic Measurement of the Direct Interaction between Labeled TBP and Full-length Recombinant TAF130p in the Absence of DNA—We used our TMR-labeled TBPcol3–304 (TMR-TBP) to directly examine the interaction of TBP with TAF130p in the absence of DNA. Previous Far Western and (GST-)TBP pull-down experiments (17, 21, 28, 30) demonstrated, in a nonquantitative fashion, that such direct interactions occur. As shown in Fig. 4, TAF130p did indeed interact directly and with high affinity with TBP. Additional titration experiments performed using varying concentrations of reactants indicated that these molecules interact with a KD of ~0.6 nM (data not shown). Under the stoichiometric binding conditions specified here, we calculate that 1 mol of TAF130p bound ~4 mol of TBP. Interestingly, this binding stoichiometry is entirely consistent with that calculated from the left shift of the TAF130p concentration required for efficient competition in the anisotropy experiment presented in Fig. 2B. Neither the exact stoichiometry of TBP nor the stoichiometry of TAF130p in the TFIIID holo-complex has been reported (see also below).
TAF130p—To perform quantitative EMSA assays, we needed to include carrier protein (BSA) to minimize nonspecific absorption of the dilute solutions of TBP, DNA, and TAF130p to reaction vessels and components. Although the addition of BSA to our binding buffer precluded silver stain visualization of proteins after electrophoresis, it significantly increased the precision and accuracy of our analyses. Our first quantitative EMSA assays (Fig. 6, A and B) represent the gel shift equivalent of the anisotropy study shown in Fig. 2. For these assays we utilized vertical gel electrophoresis because both free RX-DNA and TBP-DNA complexes migrated toward the anode (cf. Fig. 5). Binary TBP-DNA complex readily formed as the concentration of TBP was raised from 0 to 150 nM (Fig. 6A, gel scan inset). Maximal TBP represented a 5:1 mole excess of TBP to DNA. It should be noted that under these conditions the RhX signal is slightly quenched upon binding to TBP. The TBP-DNA complex was sensitive to TAF130p competition (Fig. 6B) as observed above in the cuvette anisotropy experiment of Fig. 2B. Importantly, this TAF130p competition curve is again left-shifted such that substoichiometric amounts of TAF130p (i.e. ~30 nM) competed away 50% of the binary TBP-DNA complex. As observed in the experiments shown in Figs. 2 and 3, TAF130p alone does not bind DNA (Fig. 6B, lane 15).

As suggested by the data presented in Fig. 5, labeled TBP can also be used to monitor TBP-DNA interactions. However, in this case horizontal gel electrophoresis was utilized so that complexed and free TBP were monitored. As shown in Fig. 7A, TBP-DNA binary complex formation was efficient under these stoichiometric binding conditions. TMR-TBP forms a 1:1 complex with TATA DNA, and this binary complex is sensitive to TAF130p competition (Fig. 7B). We observed that, at the expense of TBP-DNA complex, TMR-TBP was driven into TBP-TAF130p complexes. As observed with the RhX-labeled DNA, the TMR-TBP fluorescence signal is somewhat quenched upon DNA binding. In contrast TMR-TBP fluorescence was enhanced upon binding to TAF130p. The reason(s) for these behaviors are unknown at present. Once more the efficacy of the TAF130p competition reaction suggested that ~4-5 mol of TBP/mol of TAF130p were bound. To independently investigate this question, binding stoichiometry was once more measured by titrating free TMR-TBP with TAF130p and separating TAF130p-bound TBP from free TBP by horizontal EMSA. As shown by the gel scan and corresponding quantitation of this experiment (Fig. 8), we again observed an ~4:1 TBP:TAF130p binding stoichiometry.

**DISCUSSION**

In this study we have utilized a combination of sensitive spectroscopic and biochemical methodologies to examine the molecular details of the interaction of TBP with target TATA-DNA in the presence of purified full-length TAF130p, the major structural or scaffold subunit of the TFIIID complex. This study is the first step in understanding the behavior of TBP in a more native, physiological context while still allowing for detailed biochemical and biophysical measurements. TBP is a fascinating molecule, one that displays tremendous versatility in its molecular interactions by playing an essential role in mediating all intracellular gene transcription. This versatility is manifest through the interaction of TBP with a wide array of proteins, and each of these interaction partners imbues unique properties upon the protein. Thus, TBP participates as an integral subunit of at least three distinct multisubunit initiation factor complexes (SL1, TFIIID, and TFIIIB), each of which comprise distinct sets of TAF proteins bound to TBP. SL1 directs RNA polymerase I-mediated transcription of rRNA genes, TFIIID drives RNA polymerase II-mediated transcription of mRNA encoding genes, and TFIIIB participates in RNA
polymerase III-mediated transcription of tRNA genes. To fully understand the complex activities of TBP within each of these multisubunit complexes, it is crucial that we understand the molecular details of the interaction of TBP with the key subunits of each of these initiation factors. Knowledge of these interactions is crucial, given the fact that the multiple TAFs compete for interaction with TBP in vitro (66) and in vivo (67). The experiments outlined in this report are a first important step toward this goal.

TBP clearly interacts with the TATA box promoter element of RNA Polymerase II transcribed mRNA encoding genes. However, exactly which proteins escort TBP to these promoters in vivo is debated, because promoter bound TFIID-specific TAFs are postulated to compete directly with TATA DNA for binding to TBP and that the TAF-TBP interaction could well be a necessary step toward this goal. TBP clearly interacts with the TATA box promoter element of RNA Polymerase II transcribed mRNA encoding genes. However, exactly which proteins escort TBP to these promoters in vivo is debated, because promoter bound TFIID-specific TAFs are postulated to compete directly with TATA DNA for binding to TBP and that the TAF-TBP interaction could well be a necessary step toward this goal. Therefore, we have demonstrated that full-length recombinant TAF130p behaves in a fashion similar to the isolated TAND domains. We have found that full-length TAF130p competes directly with TATA DNA for binding to TBP and that the TBP-TAF130p protein-protein interaction is quite tight. TAF130p binds TBP with an affinity stronger than 0.5 nM, ~8-fold stronger than the affinity we measured previously for TBP and the TAND I fragment of Drosophila TAF250p (32). In this structure the TAF fragment, a random coil when free in solution, assumes a TATA-DNA mimetic structure when bound to TBP. These data, in combination with other biochemical equilibrium assays previously published led to the idea that TAF130p might tonically repress TATA box DNA binding by TBP and that this protein-protein interaction could well be a target for trans-regulation. Indeed, Nakatani and colleagues (27) found that a fragment of dTAF250p competed with the VP16 activation domain for interaction with specific amino acid sequences on the concave surface of TBP. These data are consistent with their hypothesis that the TAF130p TAND domain and transactivators compete with DNA for interactions on TBP's DNA binding surface (79). This continuous negative action of the TAND may be antagonized, or derepressed, by the action of TFIIA (Refs. 21 and 30; see, however, Ref. 28). Although the dynamics of these processes remain to be examined, the methodologies described in this report will certainly prove useful in dissecting these complex interactions.

In this report we have demonstrated that full-length recombinant TAF130p behaves in a fashion similar to the isolated TAND domains. We have found that full-length TAF130p competes directly with TATA DNA for binding to TBP and that the TBP-TAF130p protein-protein interaction is quite tight. TAF130p binds TBP with an affinity stronger than 0.5 nM, ~8-fold stronger than the affinity we measured previously for the TBP-TATA DNA interaction (40). The affinity of yeast TAF130p for yeast TBP is significantly higher than that of the only other TBP binding TAFp that has been characterized to date, Brf1p (TFIIIB70). Librizzi et al. (78) have measured the affinity of Brf1p for the TBP-Ad2 MLP TATA complex and estimate that the Kd describing the interaction between these two proteins is ~45 nM. If TAF130p and Brf1p, which are present within the yeast cell at roughly equivalent concentrations, were to compete for binding to TBP, the TAF130p-TBP complex would form preferentially.

Our data showing that full-length TAF130p tightly binds TBP and prevents subsequent TBP-TATA DNA interactions, as well as the published work of others (see above), illustrates an intriguing conundrum. TAF130p, an integral subunit of the TFIIID complex, appears to act negatively to inhibit DNA binding by TBP (18, 21). These observations were grounded in structural biology with the publication of the NMR structure of the heterologous complex formed between yeast TBP and the TAND I fragment of Drosophila TAF250p (32). In this structure the TAF fragment, a random coil when free in solution, assumes a TATA-DNA mimetic structure when bound to TBP. These data, in combination with other biochemical equilibrium assays previously published led to the idea that TAF130p might tonically repress TATA box DNA binding by TBP and that this protein-protein interaction could well be a target for trans-regulation. Indeed, Nakatani and colleagues (27) found that a fragment of dTAF250p competed with the VP16 activation domain for interaction with specific amino acid sequences on the concave surface of TBP. These data are consistent with their hypothesis that the TAF130p TAND domain and transactivators compete with DNA for interactions on TBP's DNA binding surface (79). This continuous negative action of the TAND may be antagonized, or derepressed, by the action of TFIIA (Refs. 21 and 30; see, however, Ref. 28). Although the dynamics of these processes remain to be examined, the methodologies described in this report will certainly prove useful in dissecting these complex interactions.

We determined that the stoichiometry of interaction between TAF130p and TBP was greater than 1 to 1. Interestingly, using a variety of different methods, we observed that 1 mol of TAF130p bound 4 mol of TBP. This result was surprising, although the stoichiometry of TBP within any of the known initiation factor TBP-TAF complexes (i.e. SL1, TFIIID, or TFIIIB) has yet to be reported. As noted above, it had been hypothesized that there are at least two independent TBP binding domains within TAF130p/TAF250p and it is possible that we are detecting these two apparently independent TBP binding domains in our assays. Potentially complicating the interpretation of our analyses is the fact that TBP has been reported to dimerize (26, 80). Thus, it is possible that dimeric TBP, binding to these two TAF130p/TBP binding domains, was...
Modulation of TBP DNA Binding by TAF130p

Fig. 6. Effect of full-length recombinant TAF130p on TBP-DNA binary complexes as monitored by quantitative EMSA using labeled TATA DNA as reporter. Panel A, formation of TBP-DNA binary complexes. 32 nM RhX-labeled TATA DNA was incubated without TBP or in the presence of increasing concentrations of TBP (15–150 nM) allowed to reach equilibrium (20 min) and then fractionated for 10 min at 400 V at 4 °C on a vertical 6% polyacrylamide gel cast and run in TGM buffer. The gel was scanned using a Bio-Rad fluorescence FX imager and the resulting image processed and quantitated (quantitation shown; n = 3) using Bio-Rad Quantity One software (gel scan, inset). T, free TBP; D, free DNA; T-D, TBP-DNA binary complex. Panel B, effect of TAF130p addition on TBP-DNA binary complex. 32 nM RhX-labeled TATA box 14-mer DNA prebound to excess TBP (300 nM) was incubated for 20 min at room temperature with increasing concentrations of TAF130p (0–225 nM), and the reactions were then fractionated by electrophoresis for 10 min at 400 V at 4 °C on a vertical 6% polyacrylamide gel cast and run in TGM buffer. The gel was scanned using a Bio-Rad fluorescence FX imager and the resulting image processed and quantitated (quantitation shown; n = 3) as described above (gel scan, inset). T, free TBP; D, free DNA; T-D, TBP-DNA binary complex.

Fig. 7. Effect of full-length recombinant TAF130p on TBP-DNA binary complexes as monitored by quantitative EMSA using labeled TBP as reporter. Panel A, formation of TBP-DNA binary complexes. 255 nM TMR-TBP (lane 1) was titrated with no, or increasing concentrations of, unlabeled TATA 14-mer DNA (25–750 nM), allowed to reach equilibrium by incubation at room temperature (20 min), and thenfractionated by electrophoresis for 90 min at 4 °C on a horizontal 6% polyacrylamide gel cast and run in TGM buffer. The gel was scanned using a Bio-Rad fluorescence FX imager and the resulting image processed (gel scan, inset) and quantitated (quantitation shown; n = 3) using Bio-Rad Quantity One software. T, free TBP; T-D, TBP-DNA binary complex. Panel B, effect of TAF130p addition on TBP-DNA binary complex. 255 nM TMR-TBP (lane 1) alone or 255 nM TMR-TBP (lanes 2–15) plus 450 nM unlabeled 14-mer TATA DNA was preincubated (20 min) to form binary TBP-DNA complexes. To these reactions increasing amounts of full-length recombinant TAF130p was added (12.5–350 nM). After a 20-min room temperature incubation, the reactions were fractionated by electrophoresis for 90 min at 4 °C on a horizontal 6% polyacrylamide gel cast and run in TGM buffer. The gel was scanned using a Bio-Rad fluorescence FX imager and the resulting image processed (gel scan, inset) and quantitated (quantitation shown; n = 3) using Bio-Rad Quantity One software. T, free TBP; T-D, TBP-DNA binary complex; T-TAF, binary TBP-TAF130p complex.

scored in our experiments. However, as the concentration of TBP used in our experiments never exceeded 300 nM, this seems unlikely; the reported TBP dimerization $K_d$ is 2–50 μM (54, 55). Indeed, the most current analysis of the oligomerization state of full-length TBP suggests that even in the yeast nucleus, if all 30,000–50,000 molecules of TBP were free rather than TAFp-bound (∼10 μM final TBP nuclear concentration; but see Ref. 81), most if not all the TBP would be monomeric (54). These considerations suggest that we measured the interaction of monomeric and not dimeric TBP with TAF130p in our analyses. It is noteworthy that in one recently published study examining the effects of purified full-length human TAF250p on the binding of TBP to the Ad2 MLP TATA, hTAF250p functionally bound at least 2 mol of TBP/mol of hTAF250p (28). Unfortunately, other relevant studies either fail to report the details of their experimental approach sufficiently to allow for calculation of TBP/TAFp molar ratios or only perform TBP-TAFp interaction studies using excess TAFp in the various binding assays. Thus, ultimately, independent analyses will be needed to assess the 4:1 TBP:TAF130p mole ratio we have observed in our experiments. It is interesting, however, that these data could help in part to explain the apparent discrepancy between TBP-open reading frame DNA versus TAF-open reading frame DNA cross-linking in the chromatin immunoprecipitation experiments cited above (68, 69).

We do not yet know whether TAFp behavior similar to that which we observed for TBP and TAF130p occurs in these other systems though protein modulated DNA binding by transcription factors has been observed previously. Among the first such examples of this behavior were bacterial $\sigma$ factors (82), the 74-kDa subunit of the metazoan GTF TFIIH, RAP74 (83), and TFIIIS (84). These proteins all have DNA binding domains whose activities are regulated. In the case of the prokaryotic GTF $\sigma$ factor family, intramolecular sequences mask and control the ability of $\sigma$ to productively interact with DNA. Sente-
nac and co-workers (85) demonstrated that the Brf1p subunit of the RNA polymerase III-specific GTF TFIIIB contains a cryptic DNA binding domain that can be unmasked by partial proteolysis. Similarly Hernandez and colleagues (86) demonstrated that the C terminus of the largest subunit of SNAPC, the SNAP190 subunit, represses specific proximal sequence element DNA binding by the SNAPC (sub)complex and that this repression can be overcome by the SNAP45 subunit. Finally, a solution containing 255 nM TAF130p.

rect binding of TBP to full-length TAF130p binary complex.

Fig. 8. EMSA measurement of direct binding of TBP to full-length TAF130p. A solution containing 255 nM TMR-labeled TBP was mixed with the indicated increasing final concentrations of full-length recombinant TAF130p (12.5–600 nM), allowed to reach equilibrium, and reactions fractionated as detailed in the legends of Figs. 8 and 7. The resulting gel scan (inset) and quantitation (n = 3) are shown. T, free TBP; T-TAF, TBP-TAF130p binary complex.

Acknowledgments—We thank Drs. Yu Bai and Steven Sanders for construction of the baculovirus expressing TAF130p and help in virus propagation and protein production. We also thank all of our laboratory colleagues for generous sharing of reagents, advice, and constructive criticism throughout the course of these studies. Finally, we thank Drs. Al Beth, Jay Kirchner, and Orlando Gumbs for thoughtful comments on the manuscript.

REFERENCES


Modulation of TBP DNA Binding by TAF130p

Fluorescence-based Analyses of the Effects of Full-length Recombinant TAF130p on the Interaction of TATA Box-binding Protein with TATA Box DNA

Utpal Banik, Joseph M. Beechem, Edward Klebanow, Stephanie Schroeder and P. Anthony Weil

_J. Biol. Chem. 2001, 276:49100-49109._
doi: 10.1074/jbc.M109246200 originally published online October 24, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M109246200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 89 references, 51 of which can be accessed free at http://www.jbc.org/content/276/52/49100.full.html#ref-list-1