Self-association of the Amino-terminal Domain of the Yeast TATA-binding Protein*

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The amino-terminal domain of yeast TATA-binding protein has been proposed to play a crucial role in the self-association mechanism(s) of the full-length protein. Here we tested the ability of this domain to self-associate under a variety of solution conditions. Escherichia coli two-hybrid assays, in vitro pull-down assays, and in vitro cross-linking provided qualitative evidence for a limited and specific self-association. Sedimentation equilibrium analysis using purified protein was consistent with a monomer-dimer equilibrium with an apparent dissociation constant of ~8.4 μM. Higher stoichiometry associations remain possible but could not be detected by any of these methods. These results demonstrate that the minimal structure necessary for amino-terminal domain self-association must be present even in the absence of carboxyl-terminal domain structures. On the basis of these results we propose that amino-terminal domain structures contribute to the oligomerization interface of the full-length yeast TATA-binding protein.

The TATA-binding protein (TBP)† is required for transcription initiation in eukaryotes and Archaea (1, 2). Together with TBP-associated protein factors (TAFs) (3, 4), it forms multisubunit complexes required for transcription by RNA polymerases I, II, and III (2, 5–7). TBP is a two-domain protein with a strongly conserved carboxyl-terminal domain (~80% sequence identity among eukaryotes, 8, 9) and an amino-terminal domain highly divergent in both length (18–173 residues) and sequence (8, 10–12). The carboxyl-terminal domain is required for DNA binding and interactions with TAFs, general transcription factors (2, 13, 14), and self-association reactions (15, 16). Less is known about the function of the amino-terminal domain, although it has been shown to be necessary for association to at least the dimer level in HeLa and yeast cells (16, 36, 37). On the basis of these results it has been proposed that monomer-oligorimer equilibria affect the concentration of TBP monomer that is available for transport into the nucleus for transcription-regulatory functions or for degradation (15, 16, 32–34, 38, 39).

Little is known about the mechanism(s) of TBP self-association. Crystal structures of the carboxyl-terminal domain of yeast TBP reveal a saddle-shaped molecule of ~180 amino acid residues with a concave DNA-binding face and a convex TAF- and transcription factor-binding face (9, 21, 23, 30, 31). In the absence of DNA, this crystallizes as a dimer, stabilized by extensive contacts between the concave surfaces (9, 30, 31). Light scattering and more recent sedimentation equilibrium results indicate that dimers of the carboxyl-terminal domain are also present in solution (30). On the other hand, full-length yeast TBP has a monomer-tetramer-octamer pattern of self-association over a wide range of solution conditions (24, 33, 34, 40). These contrasting self-association mechanisms suggest that the amino-terminal domain plays an important role in the association mechanism. Evidence of this involvement comes from significant changes in the fluorescence spectrum and anisotropy of Trp-26 (roughly the middle of the amino-terminal domain) that accompany TBP self-association (34, 40).

Based on these observations, we have proposed that the amino-terminal domain contributes structures necessary for the monomer-tetramer-octamer association pattern (34). This might occur if some or all of an interaction surface were contained in the amino-terminal domain. If this were the case, isolated amino-terminal domains might be expected to self-associate. In the experiments described below we tested this prediction and present the first evidence, to our knowledge, of a self-association activity for the amino-terminal domain.

MATERIALS AND METHODS

Reagents—Agar base was obtained from Difco. Restriction endonucleases and DNA ligase were from Promega. Taq DNA polymerase, buffers, and dNTPs for PCR were provided in kit form by Qiagen. Protein cross-linking reagents DFDNB (1,5-difluoro-2,4-dinitrobenzene) and ANB-NOS (N-5-azido-2-nitrobenzoyloxyxycinnimide) were obtained from Pierce. All other biochemicals were from Sigma.

Strains, Plasmids, and Growth Conditions—Plasmid pKA9-TBP, encoding full-length yeast TBP was the kind gift of Dr. Michael Brenowitz, Albert Einstein University. The Escherichia coli strain used as a "re-

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‡ The abbreviations used are: TBP, TATA-binding protein; TAF, TBP-associated protein factor; DFDNB, 1,5-difluoro-2,4-dinitrobenzene; ANB-NOS, N-5-azido-2-nitrobenzoyloxyxycinnimide; IPTG, isopropyl-1-thio-β-D-galactopyranoside.

§ Since these surfaces are not available for interaction with DNA, this is one way that dimerization might modulate DNA binding.

¶ M. Fried and M. Daugherty, unpublished results.

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porter" for two-hybrid experiments was Bacteriophage™, a restriction-minus, Kan^R derivative of XL1-Blue MR obtained from Stratagene. Strain XL1-Blue MR (Stratagene) was used for routine propagation of plasmids. Expression plasmids were propagated in E. coli BL21 Star™ cells (Invitrogen). Cells containing the two-hybrid bait plasmid pBT (3.2 kb, Cam^R, from Stratagene) and prey plasmid pTRG (4.4 kb, Tet^R, from Stratagene) were maintained on LB agar containing chloramphenicol (50 µg/ml) and tetracycline (15 µg/ml), respectively. Plasmids pBt-LGF2 and pTRG-Gal11p were supplied by Stratagene.

The expression vectors used for protein purification and pull-down assays were pPROTet.E (2.2 kb, Cam^R, from Clontech) encoding an amino-terminal (His-Asn)_6 affinity tag for in-frame fusion with target genes and pFLAG.mac (5 kb, Amp^R from Sigma) expressing an amino-terminal FLAG epitope tag (Asp-Tyr-Lys-Asp-Asp-Asp-Lys) for in-frame fusion with target genes. These were maintained on LB agar containing 34 µg/ml chloramphenicol and 50 µg/ml ampicillin, respectively.

The LB selection agar for two-hybrid experiments contained 30 µg/ml carbenicillin, 15 µg/ml tetracycline, 34 µg/ml chloramphenicol, and 50 µg/ml kanamycin (CTCΔK). Primers P3 and P4 were verified by PCR analysis and DNA sequencing. Primers P3 and P4 (Table I) were used for PCR amplification of TBP amino-terminal domain from plasmid pKA9-TBP using primers P1 and P3 (Table I). Reaction products were digested with XhoI and BglII and ligated into pFLAG.mac (linearized with the same enzymes). The product was designated pBTN1 with primers P8 and P9 were digested with XhoI and BglII and ligated into pPROTet.E (linearized with the same enzymes). The product was designated pBTN1 and pBTN2. Primer P2 also encodes a flexible (Gly4Ser)3 linker connecting the plasmid-encoded TBP amino-terminal (His-Asn)_6-tagged TBP amino-terminal domain was purified by absorption on cobalt affinity resin (Talon, Clontech) followed by elution with 50 mM sodium phosphate (pH 7.0 at 20 °C), 300 mM NaCl and then eluted with 5 M ammonium acetate. Likewise, the plasmid-encoded AREP repressor protein to the TBP amino-terminal (bait) domain.

 Constructs for protein purification and pull-down assays were prepared as follows. Primers P7 and P8 (Table I) were used for PCR amplification of TBP amino-terminal sequences from pTRG-N1. After cleavage with SalI and NotI, reaction products were ligated into pPROTet.E and transformed with the plasmid pPROTet.E (empty) or with pPROTet.E-N1 (encoding the TBP amino-terminal domain) and grown with agitation at 30 °C. Protein expression was induced with anhydratedotetracycline (final concentration, 0.4 µg/ml) when cultures reached A600 = 0.3. After incubation for 8 h at 30 °C, cells were collected by centrifugation and lysed at 4 °C with lysozyme (0.75 mg/ml) followed by sonication (3 × 30 s with a pause for 30 s on ice between each burst). The TBP amino-terminal domain was purified by absorption on cobalt affinity resin (Talon, Clontech) followed by elution with 50 mM sodium acetate, 300 mM NaCl, pH 5.0 as specified by the manufacturer. Samples were concentrated using Centricon YM3000 filter devices (Millipore), and pH was adjusted to 7.0 with sodium phosphate buffer.

E. coli BL21 cultures transformed with pFLAG.mac (empty) or with pFLAG.mac.N1 (encoding the TBP amino-terminal domain) were grown at 30 °C to A600 = 0.5, and protein expression was induced with 1 mM IPTG. Growth was continued at 30 °C for an additional 8 h. Cells were harvested by centrifugation and lysed by incubation with lysozyme followed by sonication as described above.

Samples were prepared by mixing 20 µg of purified (His-Asn)_6-tagged amino-terminal protein with crude cell lysate proteins (1 mg) containing FLAG-tagged amino-terminal domain in a reaction volume of 800 µl. The incubation buffer was 94 mM sodium phosphate (pH 7.0 at 20 °C), 6.8 mM sodium acetate, and 222 mM NaCl. Samples were incubated at 4 °C for 3 h. Cobalt affinity resin was equilibrated with 50 mM sodium phosphate (pH 7.0 at 20 °C), 300 mM NaCl. Samples were incubated (batchwise for 1 h at 4 °C) with cobalt affinity resin (1-ml packed volume). The resin was washed with 5 volumes of 50 mM sodium phosphate (pH 7.0 at 20 °C), 300 mM NaCl and then eluted with 5 volumes of the same buffer adjusted to pH 5.0. Eluates were concentrated using Centricon YM3000 concentrators and analyzed by SDS-PAGE with immunoblot detection using anti-FLAG and anti-TBP antibodies.

**Self-association of the Amino-terminal Domain of Yeast TBP**

**Table I**

| Name                  | Sequence (5' to 3') | Comments                                    |
|-----------------------|---------------------|---------------------------------------------|
| pBT                | TGACTGTAGTCTCCATGGCCGTCGAGGGAACTTTAAAGGA   | Forward primer for pTRG cloning             |
| pPROTet              | TGACTGTAGTCTCCATGGCCGTCGAGGGAACTTTAAAGGA   | Forward primer for pBT cloning              |
| pTRG                | TGACTGTAGTCTCCATGGCCGTCGAGGGAACTTTAAAGGA   | Reverse primer for pBT and pTRG cloning     |
| pTRG-N1             | TGACTGTAGTCTCCATGGCCGTCGAGGGAACTTTAAAGGA   | pTRG 5′ sequence primer                    |
| pTRG-Gal11p         | TGACTGTAGTCTCCATGGCCGTCGAGGGAACTTTAAAGGA   | Reverse primer for pPROTet.E subcloning    |
| pBTN1               | TGACTGTAGTCTCCATGGCCGTCGAGGGAACTTTAAAGGA   | Reverse primer for pFLAG.mac subcloning    |
| pBTN2               | TGACTGTAGTCTCCATGGCCGTCGAGGGAACTTTAAAGGA   | Reverse primer for pFLAG.mac subcloning    |
| pBTN1:pTRG-N1       | TGACTGTAGTCTCCATGGCCGTCGAGGGAACTTTAAAGGA   | pPROTet.E 5′ sequence primer               |
| pBTN2:pTRG-N1       | TGACTGTAGTCTCCATGGCCGTCGAGGGAACTTTAAAGGA   | pFLAG.mac 5′ sequence primer               |

**Table II**

To avoid cloning artifacts three independent TBP amino-terminal domain subclones were used. They are designated N1, N2, and N5.

**Table II**

| Challenge pairs                     | Negative control pairs | Positive control pairs |
|-------------------------------------|------------------------|------------------------|
| pBTN1:pTRG-N1                      | pBT:pTRG               | pBT-LGF2:pTRG-Gal11p   |
| pBTN2:pTRG-N1                      | pBT:pTRG               | pBT-LGF2:pTRG-Gal11p   |
| pBTN1:pTRG-Gal11p                  | pBT:pTRG               | pBT-LGF2:pTRG-Gal11p   |

**E. coli Two-hybrid Assays**—The method used was based on the work of Dove et al. (42, 43) and is shown schematically in Fig. 1. Aliquots of challenge bait plasmids (pBTN1 and pBTN2), challenge prey plasmids (pTRG-N1 and pTRG-N5), and control vectors pBT (with no insert), pTRG (with no insert), pBT-LGF2, and pTRG-Gal11p were co-transformed as pairs (Table II) into freshly prepared competent cells. Transformations were carried out in triplicate by the CaCl2/heat shock method (41). Following heat shock, samples were incubated on ice for 10 min and diluted into YEGM medium (41), and aliquots of each transformation mixture were plated in duplicate on CTCK and CTCK + IPTG agar media. Colonies were counted after growth at 30 °C for 48 h.

**Protein Pull-down Interaction Assay—E. coli BL21 Star cells** were transformed with the plasmid pPROTet.E (empty) or with pPROTet.E-N1 (encoding the TBP amino-terminal domain) and grown with agitation at 30 °C. Protein expression was induced with anhydratedotetracycline (final concentration, 0.4 µg/ml) when cultures reached A600 = 0.3. After incubation for 8 h at 30 °C, cells were collected by centrifugation and lysed at 4 °C with lysozyme (0.75 mg/ml) followed by sonication (3 × 30 s with a pause for 30 s on ice between each burst). The TBP amino-terminal domain was purified by absorption on cobalt affinity resin (Talon, Clontech) followed by elution with 50 mM sodium acetate, 300 mM NaCl, pH 5.0 as specified by the manufacturer. Samples were concentrated using Centricon YM3000 filter devices (Millipore), and pH was adjusted to 7.0 with sodium phosphate buffer.

E. coli BL21 cultures transformed with pFLAG.mac (empty) or with pFLAG.mac.N1 (encoding the TBP amino-terminal domain) were grown at 30 °C to A600 = 0.5, and protein expression was induced with 1 mM IPTG. Growth was continued at 30 °C for an additional 8 h. Cells were harvested by centrifugation and lysed by incubation with lysozyme followed by sonication as described above.

Samples were prepared by mixing 20 µg of purified (His-Asn)_6-tagged amino-terminal protein with crude cell lysate proteins (1 mg) containing FLAG-tagged amino-terminal domain in a reaction volume of 800 µl. The incubation buffer was 94 mM sodium phosphate (pH 7.0 at 20 °C), 6.8 mM sodium acetate, and 222 mM NaCl. Samples were incubated at 4 °C for 3 h. Cobalt affinity resin was equilibrated with 50 mM sodium phosphate (pH 7.0 at 20 °C), 300 mM NaCl. Samples were incubated (batchwise for 1 h at 4 °C) with cobalt affinity resin (1-ml packed volume). The resin was washed with 5 volumes of 50 mM sodium phosphate (pH 7.0 at 20 °C), 300 mM NaCl and then eluted with 5 volumes of the same buffer adjusted to pH 5.0. Eluates were concentrated using Centricon YM3000 concentrators and analyzed by SDS-PAGE with immunoblot detection using anti-FLAG and anti-TBP antibodies.

**Protein Cross-linking—Extracts were prepared from E. coli BL21 Star EBM cells expressing (His-Asn)_6-tagged TBP amino-terminal domain pROFET.E.N1 or from cells containing the empty control vector.**
pPROTetE by the lysozyme-sonication method. (His-Asn)_6-tagged proteins were partially purified by cobalt affinity chromatography as described above. The cross-linkers used were DFFDNB (spacer length, ~3 Å) and ANB-NOS (spacer length, ~7.7 Å). Cross-linking reactions (30 μl) contained 0.3 mg/ml protein and 5 mM cross-linker in buffer consisting of 110 mM sodium phosphate, 10 mM sodium acetate, and 115 mM NaCl, pH 7.0 at 20 °C. Samples were incubated at room temperature in the dark for 30 min. Samples containing ANB-NOS were then exposed to light from a short wavelength UV light box (Fotodyne) for 1 min. Proteins were resolved by SDS-PAGE, and TBP amino-terminal domain peptides were detected by immunoblotting using anti-TBP polyclonal antibody.

Sedimentation Equilibrium Analysis—The (His-Asn)_6-tagged amino-terminal domain was purified to homogeneity (by SDS-PAGE criteria) by immobilized metal and ion-exchange chromatography. Aliquots were transferred into 10 mM Tris (pH 7.0), 100 mM KCl buffer using a centrifugal concentrator (Centricon YM3000). Samples were brought to sedimentation equilibrium at 4 °C in a Beckman XL-A analytical ultracentrifuge equipped with an AN-60 rotor. Absorbance values were measured at 280 nm as functions of radial position. Five scans were averaged for each sample at each rotor speed. The approach to equilibrium was considered to be complete when replicate scans separated by ≥6 h were indistinguishable. Solvent densities were calculated using the formula and tables published by McMorris and Voelker (44).

At sedimentation equilibrium, the absorbance at a specified wavelength and position in the solution column is given by Equation 1 (44, 45).

\[ A(r) = \sum_{n} a_n \exp(-r^2/2 \sigma_n^2) \] (Eq. 1)

Here \( A(r) \) is the absorbance at radial position \( r \); the summation is over all species, \( n \); \( a_n \) is the absorbance of the \( n \)th species at the reference position \( r = 0 \); \( \sigma_n \) is the partial specific volume (0.699 for TBP amino-terminal domain), \( \rho \) is the solution density, \( \omega \) is the rotor angular velocity, \( R \) is the gas constant, and \( T \) is the absolute temperature. The base-line offset term \( \zeta \) compensates for slight position-independent differences in the optical properties of different cell assemblies. Global analysis of data obtained at different rotor speeds was performed with the program NONLIN.4

RESULTS

Yeast TBP Amino-terminal Domains Interact in Living E. coli Cells—The output signal of the classical yeast two-hybrid assay (cell survival and growth) results from enhanced transcription of one or more indicator genes (49). Interactions between yeast transcription factors may be difficult to study by this assay because two-hybrid constructs containing these proteins may be capable of activating yeast transcription in the absence of the protein-protein interaction of interest (false positive results). In addition, other yeast transcription factors may compete with or inhibit the interaction of interest, giving rise to false negative results. To minimize the potential for these complications we have used an E. coli two-hybrid assay (Refs. 42 and 43, summarized schematically in Fig. 1). While this strategy does not prevent all false positive and false negative results it has the advantage that the TBP amino-terminal domain is an exogenous protein so any interactions that it may have with the E. coli transcription machinery are likely to be adventitious.

In the two-hybrid method that we have used, the proteins of interest are expressed as carboxyl-terminal fusions with the \( \lambda \) cl protein (bait constructs in plasmid pBT) or with the \( \alpha \) subunit of RNA polymerase (prey constructs in plasmid pTRG). Thus, appropriate controls for the absence of interaction are plasmid pairs in which bait and prey contain only \( \lambda \) cl protein and \( \alpha \) subunit of RNA polymerase, respectively (since no additional sequences are added, we refer to these as “empty” constructs). The two-hybrid data shown in Fig. 2 are normalized to

4 NONLIN for the Macintosh was obtained from www.cauma.edu/software.

FIG. 2. Two-hybrid analysis of self-association of the amino-terminal domain. Three challenge pairs representing different isolates of TBP amino-terminal domain were tested: pBTN1 and pTRGN1 (N1:N1), pBTN2 and pTRGN1 (N2:N1), and pBTN1 and pTRGNS5 (N1:N5). Triplicate transformations were grown on CTCK and CTCK + IPTG media (columns labeled “+I”). Negative control pairs, pBTN1 with empty pTRG (N1:E) and empty pBT with pTRGNS1 (E:N1), represent background levels of growth with unpaired expression of amino-terminal domain bait (pBTN1) and prey (pTRGNS1) constructs. All values are the average number of colonies on three test plates normalized to the average obtained with control cells carrying two empty vectors (pBT:pTRG). The error bars represent S.D. in colony numbers propagated through this normalization.

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nated N1:E). A similar control for the interaction of the TBP amino-terminal domain with λcl protein alone is the pairing of an empty bait construct with the TBP amino-terminal domain RNA polymerase α subunit construct (designated E:N1). Both of these controls gave levels of carbenicillin resistance that were within error the same as that found with the negative control in which both vectors are empty. This indicates that the expression of either amino-terminal bait or amino-terminal prey protein alone is not sufficient to give enhanced carbenicillin resistance. It also indicates the absence of endogenous E. coli proteins that combine strongly with bait or prey proteins to activate transcription from the test promoter.

Greater numbers of carbenicillin-resistant colonies were obtained when both bait and prey plasmids encoded TBP amino-terminal domain protein. Although there was some variance, growth of cells containing amino-terminal domain bait and amino-terminal domain prey constructs in the absence of IPTG gave an average 240% increase in the number of surviving colonies, while growth in the presence of IPTG increased the average number of survivors by 310%. This IPTG effect supports the interpretation that the enhanced carbenicillin resistance of challenge cells reflects enhanced expression of bait and prey gene products.

As a positive control, we examined the carbenicillin resistance of cells containing the plasmids pBT-LGF2 and pTRG-Gal11p. LGF2 is the dimerization domain of the yeast Gal4 transcription activator, while Gal11p is a domain of the yeast Gal11 protein carrying a point mutation that enables it to activate transcription from the test promoter.

Together these results are consistent with an interaction between yeast TBP amino-terminal domain bait and prey proteins (result not shown). Association of TBP Amino-terminal Domains Detected by Pull-down Assay—Pull-down interaction assays (46) were carried out to further test the self-association of TBP amino-terminal domains. FLAG- and (His-Asn)₆-tagged amino-terminal proteins were prepared from E. coli transformed with pFLAG.mac.N1 and pPROTet.E133.N1. (His-Asn)₆-tagged TBP amino-terminal domain (partially purified by cobalt affinity chromatography) was incubated with E. coli cell extract containing FLAG-tagged TBP amino-terminal protein at 4 °C as described under “Materials and Methods.” The sample was divided, and one part was subjected to cobalt affinity chromatography, then to PAGE with immunoblot detection using anti-FLAG monoclonal antibody (Fig. 3, lane b). A band with apparent molecular weight equal to that of FLAG-tagged amino-terminal domain is evidence that this protein is retained by the cobalt affinity resin in the presence of (His-Asn)₆-tagged TBP amino-terminal domain. FLAG-tagged amino-terminal domain was not retained by the resin when applied alone (Fig. 3, lane c). As a second control, an extract from cells containing pPROTet.E (empty vector) was combined with one from cells containing pFLAG.mac.N1 (expressing FLAG-tagged amino-terminal domain). No FLAG-tagged amino-terminal domain was detectable in the eluate of the cobalt affinity column to which this mixture was applied (result not shown). Together these results are consistent with the interpretation that FLAG-tagged TBP amino-terminal domain forms stable complexes with (His-Asn)₆-tagged TBP amino-terminal domain in the presence of components of E. coli cell extract under our buffer conditions.

**TBP Amino-terminal Domains Can Be Cross-linked in Vitro**—Chemical cross-linking was performed to confirm the in vitro interaction of amino-terminal domains and to obtain an estimate of the degree of oligomerization that these molecules experience. Two chemically distinct cross-linkers (DFDNB and ANB-NOS) were tested. Samples of cell extract from E. coli BL21 Star cells expressing (His-Asn)₆-tagged TBP amino-terminal domain (0.3 mg/ml total protein) were incubated with cross-linkers as described under “Materials and Methods” and resolved by SDS-PAGE (Fig. 4). Reaction with cross-linkers resulted in a shift of the amino-terminal domain to a gel mobility corresponding to an Mᵋ = 17,000, slightly less than twice the molecular weight expected for dimeric amino-terminal domain (Mᵋ = 17,800). Higher molecular weight species were undetectable despite nearly quantitative conversion of the monomer into the cross-linked form. Thus, if higher oligomers are formed, they must be present at low concentration relative to the dimer, or they must be inefficiently cross-linked by the reagents that we tested.

![Fig. 3. Visualization of pull-down assay results by SDS-PAGE.](http://www.jbc.org/)
Asn)₆-tagged TBP amino-terminal domain was partially purified by
phosphate, 10 mM sodium acetate, and 115 mM NaCl, pH 7.0 at 20°C
(lane c) subjected to reactions with cross-linkers ANB-NOS (lane a)
or DFNB (lane b). Samples contained 0.3 mg/ml protein in 110 mM sodium phosphate,
10 mM sodium acetate, and 115 mM NaCl, pH 7.0 at 20°C. Following SDS-PAGE, products containing the amino-terminal domain
were detected by immunoblotting with anti-TBP antibody. The electro-
phoretic mobility of the unreacted protein is consistent with a molecular
weight of ~8,900, while those of proteins treated with cross-linkers are
~17,000.

The TBP Amino-terminal Domain Sediments as a Mixture of
Monomers and Dimers—Samples of the (His-Asn)₆-tagged ami-
no-terminal domain were brought to sedimentation equilibrium at
40,000 rpm and 4 °C as described under “Materials and Methods.”
Absorbance measurements were made at 280 nm. The smooth curve
represents the global fit of the monomer-dimer version of Equation 1 to
data sets obtained at 20,000, 25,000, 30,000, and 40,000 rpm. This
analysis returned a monomer molecular weight of 9,650 ± 1,050 in good
agreement with the value predicted from the sequence. The symmetric
residuals demonstrate the compatibility of this model with the data.

This yields an apparent association constant \( K \) scaled in
absorbance units. To convert this to the familiar molar scale we
used the equation,

\[
K_{\text{molar}} = K \left( \frac{e}{d} \right)^2
\]

where \( e \) is the molar extinction coefficient and \( d \) is the optical
path length of the centrifuge cell. The molar extinction coeffi-
cient was estimated to be 5,500 on the basis of amino acid content (48). This analysis returned a value of the apparent
equilibrium constant \( K_{\text{molar}} = 1.20 ± 0.23 \times 10^5 \text{ M}^{-1} \) (equiva-
ently \( K_d = 8.36 ± 1.99 \times 10^{-6} \text{ M} \)). This binding affinity is
sufficient to ensure at least partial self-association of amino-
terminal domains if the bulk concentration of TBP in yeast
nuclei is ~6 μM as estimated previously (33, 34). In addition, it
places the midpoint of the assembly reaction at ~10⁻⁶ M amino-
terminal domain; previous analytical ultracentrifugation stud-
ies have shown that full-length TBP self-associates to form
tetramers and octamers over a similar range of [protein] under
comparable solution conditions (33, 34). However these com-
parisons come with important caveats, which are discussed
below.

**DISCUSSION**

Here we have tested the ability of the amino-terminal do-
main of yeast TATA-binding protein to self-associate. Self-
association might be expected if this domain contributed sur-
faces to the oligomerization interface(s) of the full-length
TATA-binding protein. The four assays that we used test for
interaction under different but representative sets of condi-
tions. The two-hybrid assay seeks interactions in the ionic
conditions and highly crowded environment of the E. coli
cytoplasm. The pull-down assay tests for interaction under condi-
tions of lower macromolecular concentrations but allows the
ionic and small molecule compositions of the reaction mixture
to be controlled by the experimenter (50 mM sodium phosphate
(pH 7.0 at 20 °C), 300 mM NaCl in our experiments). The
cross-linking assay provides independent evidence of interac-
tions that might be detected in the pull-down assay. In addi-
tion, it has the potential to detect interactions that are too
labile to be observed by the pull-down assay or that involve
partners that would not be detected by immunoblot with anti-
FLAG antiserum. Finally sedimentation equilibrium analysis
(carrying out in 10 mM Tris (pH 7.0), 100 mM KCl) shows that
the minimum model for this self-association is monomer-dimer.
While the interaction conditions that these assays test are
unlikely to be identical to those found in yeast nuclei, together
they span a wide range in total macromolecule concentration

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5 The solution conditions used in previous studies were performed at
4 °C in 20 mM HEPES/KOH (pH 7.9), 1 mM EDTA, 1 mM dithiothreitol,
120 mM KCl; the present studies were carried out at 4 °C in 20 mM Tris
(pH 7.0), 100 mM NaCl.
Self-association of the Amino-terminal Domain of Yeast TBP

The ability of amino-terminal domains to interact in the two-hybrid assay despite the availability of a high concentration of cytoplasmic protein competitors suggests that the interaction of amino-terminal domains is quite specific. This notion is supported by the pull-down assay results that indicate that any competing interactions are not of sufficient strength to displace the FLAG-tagged amino-terminal domain from its (His-Asn)6-tagged partner. Fluorescence and protein footprinting data obtained with full-length yeast TBP are consistent with models in which the amino-terminal domain adopts specific secondary structures and participates in specific tertiary and/or quaternary interactions (24, 34, 40, 47). Our results support this view. The specific self-association of amino-terminal domains in the presence of high concentrations of competing proteins (as seen in the two-hybrid and pull-down assays) is evidence of a functional complementarity that is not likely to be present in an unstructured domain. That these interactions can take place between isolated amino-terminal domains indicates that tertiary contact with the carboxyl-terminal domain is not required for maintenance of conformation(s) that allows this self-association.

The sedimentation equilibrium results are consistent with a homon-mer-dimer pattern of self-association over the protein concentration range 0.5 μM ≤ [amino-terminal domain] ≤ 18 μM. However, limitations in the amount of amino-terminal domain protein currently available have prevented us from reaching greater protein concentrations where higher order associations might be detectable. In the absence of a more stringent test, we regard the monomer-dimer pattern as a minimal model of the self-association mechanism. The cross-linking results, performed at total protein concentrations similar to those of the sedimentation experiment, are consistent with this view. The detection of cross-linked dimers, but not higher oligomers, indicates that if higher order structures are formed they are either present at concentrations too low for detection by our Western blot procedure or that they do not offer the juxtaposition of reactive groups necessary for cross-linking.

The dimer dissociation constant estimated from sedimentation equilibrium data (Kd = 8.36 ± 1.99 × 10−8 M) indicates that amino-terminal domains have the potential to be at least partially associated in vivo if the bulk TBP concentration within yeast nuclei is ~6 μM as predicted (33, 34). This conclusion comes with a number of significant caveats. It does not take into account the currently unknown effects of the TBP carboxyl-terminal domain on association of the amino-terminal domain. It does not take into account the possible effects of competing interactions with other proteins. Finally it does not take into account the crowding effect exerted by the high concentrations of macromolecules present in the nuclei. Each of these effects has the potential to bias the dissociation constant by orders of magnitude. Thus, while the numerical value of Kd indicates that some degree of self-association is possible, it does not provide enough information to allow prediction of the association state of the amino-terminal domains of TBP in vivo.

At present we do not know whether the interactions of isolated amino-terminal domains resemble those that take place in full-length TBP. The recent protein footprinting results of Brenowitz and colleagues (47) suggest that the amino-terminal domain makes extensive contact with the convex face of the carboxyl-terminal domain. These interactions have the potential to compete with the self-association of amino-terminal domains. On the other hand, several TAFs and transcription factors interact with the convex face of the carboxyl-terminal domain near surfaces that may be occupied by the amino-terminal domain. These interactions may be modulated by the interaction between amino- and carboxyl-terminal domains. In this context, the self-association of amino-terminal domains may compete with amino-terminal-to-carboxyl-terminal domain interaction and allow transcription factors and TAFs access to the surface of the carboxyl-terminal domain. A large body of circumstantial evidence (outlined in the Introduction) implicates the amino-terminal domain in the oligomerization mechanism of the full-length TATA-binding protein. The results described here raise the possibility that the amino-terminal domain may form part of the oligomerization interface itself.

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