Inhibition of TATA Binding Protein Dimerization by RNA Polymerase III Transcription Initiation Factor Brf1

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The Brf1 subunit of TFIIIB plays an important role in recruiting the TATA-binding protein (TBP) to the upstream region of genes transcribed by RNA polymerase III. When TBP is not bound to promoters, it sequesters its DNA binding domain through dimerization. Promoter assembly factors therefore might be required to dissociate TBP into productively binding monomers. Here we show that Saccharomyces cerevisiae Brf1 induces TBP dimers to dissociate. The high affinity TBP binding domain of Brf1 is not sufficient to promote TBP dimer dissociation but in addition requires the TFIIIB homology domain of Brf1. A model is proposed to explain how two distinct functional domains of Brf1 work in concert to dissociate TBP into monomers.

Eukaryotic genes are transcribed by one of three RNA polymerases, pol I, II, or III. In higher eukaryotes, there are two main pathways of assembling a pol III transcription complex, which are governed in large part by distinct promoter elements located either upstream or downstream of the transcriptional start site (1–3). In the budding yeast Saccharomyces cerevisiae, one major assembly pathway exists, and it involves the binding of TFIIIC to promoter elements internal to tRNA genes. TFIIIC then recruits TFIIIB to a location immediately upstream of the transcriptional start site (4, 5). TFIIIB then directs pol III to the transcriptional start site. TFIIIC is composed of three subunits: the TATA binding protein (TBP), Brf1, and Bdp1 (6–10).

TBP is important for the expression of essentially all eukaryotic genes. It is a saddle shaped protein having amino- and carboxy-terminal stirrups and a concave/convex surface (11, 12). TBP binds to a TATA box promoter element when it is present. However, most pol III genes with an internal promoter lack a TATA box. TBP is delivered to the upstream region of these promoters by Brf1, and Brf1 is recruited by TFIIIC (13–15).

The amino-terminal half of Brf1 is homologous to the pol II general transcription factor TFII B (16–18). TFII B binds to TBP along its carboxy-terminal stirrup, pinning it to DNA (19). In contrast to TFII B, the TFII B homology domain of Brf1 has not been shown to independently bind TBP. Where or whether it interacts with TBP is largely unknown. Some evidence places it near the carboxy-terminal terminal stirrup of TBP (20, 21). The amino-terminal domain of Brf1 interacts with the TBP subunit of TFIIIC as well as the C34 subunit of pol III (14, 22). This domain also plays an important role in open complex formation (23, 24), the stage at which the template and transcribed strands are separated in preparation for transcription initiation. The carboxy-terminal half of Brf1 is unrelated to TFII B but is conserved among Brf proteins. A portion of the carboxy-terminal half (437–506) of Brf1 binds with high affinity to the convex surface of TBP, snaking along the “top” of TBP and down its amino-terminal stirrup (25).

TBP self-associates into dimers and possibly higher order structures through its concave DNA binding surface and carboxy-terminal stirrup (26–39). Dimers must dissociate into monomers prior to DNA binding. In vivo, TBP dimerization might play an important role in preventing unregulated expression of quiescent pol II genes (30, 37, 39). Mutations in TBP that disrupt TBP dimerization cause increased expression of lowly expressed genes, suggesting that dimer dissociation is at least partly rate-limiting in the expression of these genes. Dimer dissociation does not appear to be rate-limiting at highly expressed genes, presumably because of direct or indirect alleviation of TBP auto-inhibition by transcription factors. Indeed, the pol II transcription factor TFIIB stimulates TBP/TATA interactions in part through dissociation of TBP dimers (29). Because TBP homodimerization is an intrinsic property of TBP, any polymerase system that utilizes TBP is expected to be endowed with an activity that dissociates TBP dimers.

Here we explore the possibility that Brf1, the primary TBP-interacting protein within the pol III transcription system, inhibits TBP dimerization when it engages TBP. We explore the mechanism by which Brf1 affects TBP dimerization by examining which domains of Brf1 are important for this reaction and whether Brf1 can bind TBP dimers and induce their dissociation. Our results suggest that Brf1 binds to TBP dimers and induces their dissociation. The presence of this activity in Brf1 suggests that TBP dimers are a physiological target of the pol III transcription machinery.

EXPERIMENTAL PROCEDURES

Construction of Brf1 Deletion Derivatives—Escherichia coli plasmids expressing polyhistidine-tagged Brf1 derivatives were created by PCR amplification of regions of the Brf1 gene encoding 1–596 (full length),

This paper is available on line at http://www.jbc.org
Brf1 Inhibits TBP Dimerization

Previously we employed Brf1 derivatives and polyhistidine-tagged TBP to study the Brf1-TBP interaction (1). Brf1 inhibits TBP dimerization, and these interactions are underlined. The binding afﬁnity of Brf1 for TBP was determined by a Brf1-TBP Interaction Assay. Brf1 derivatives and polyhistidine-tagged TBP were purified as described (37). The methods used for the Brf1-TBP Interaction Assay are as follows. Recombinant E. coli (BL21) cells (1.5 liter) were grown in YT medium (15 g of bactotryptone, 15 g of yeast extract, 7.5 g of NaCl, 3 ml of 1% NaOH) containing 0.2% of ampicillin per liter at 37 °C to an optical density at 595 nm (OD 595) of 0.7 and were induced with 20 mg of isopropyl-β-D-thiogalactoside per liter at 37 °C for 3 h at 37 °C. Additional ampicillin was added upon induction. Cells were harvested by centrifugation, washed, resuspended in 50 mM Tris-HCl, pH 7.5, 200 mM potassium chloride, 12.5 mM magnesium chloride, 10% glycerol, and 0.1 mM phenylmethylsulfonyl ﬂuoride, and quickly frozen in liquid nitrogen. Cells were thawed and mixed with 0.85 mg of lysozyme per ml for 10 min at 4 °C and with 0.2% IGEPA (CS-630) for 5 min. Extracts were sonicated to reduce viscosity and then centrifuged in an SS34 rotor (RC5C centrifuge) at 15,000 rpm for 30 min at 4 °C. Supernatants were removed and pellets were transferred to a type A Dounce homogenizer and resuspended in H35 buffer (20% glycerol, 2 mM magnesium chloride, 20 mM HEPES, pH 8.0, 350 mM potassium chloride, 1% sodium dodecyl sulfate, 0.1% phenylmethylsulfonyl ﬂuoride, 0.1% IGEPA CS-630) then centrifuged in an SS44 rotor (RC5C centrifuge) at 15,000 rpm for 15 min at 4 °C. The supernatant was again removed and the pellet was transferred to a type A Dounce homogenizer, washed with H35 buffer, and centrifuged in an SS44 rotor (RC5C centrifuge) at 15,000 rpm for 15 min at 4 °C. The supernatant was removed and inclusion bodies containing Brf1 were transferred from the pellet with G6 buffer (6 M guanidine hydrochloride, 100 mM Tris-HCl, pH 7.5, 2 M β-mercaptoethanol) and centrifuged in an SS44 rotor (RC5C centrifuge) at 15,000 rpm for 15 min at 4 °C. The supernatant was bound in a batch solution to TALON resin (Clontech) overnight at 4 °C. After incubation, the resin was washed in batch with G6 buffer. The resin was transferred to a column, and the protein was eluted in fractionation buffer (6 M guanidine hydrochloride, 100 mM Tris-HCl, pH 7.5, 10 mM β-mercaptoethanol, 500 mM imidazole, and 250 μM of protease inhibitor mixture (Sigma)). Renaturation of the protein in solution was achieved through dialysis against 20 mM Tris acetate (80% cation), 200 mM potassium chloride, 2 mM magnesium chloride, 20% glycerol, 0.1 mM phenylmethylsulfonyl ﬂuoride, and 0.1% IGEPA CS-630 to lower the guanidine concentration to less than 0.1 M. Brf1 aliquots were frozen in liquid nitrogen and stored at −80 °C.

**Chemical Cross-linking Assay**—Reactions were performed in buffer A (20 mM Tris acetate, pH 7.5, 4 mM magnesium chloride, 4 mM spermidine, 0.1 mM EDTA, 75 mM potassium glutamate, 50 μg/ml heparin, 0.025% IGEPA CS-630, and 5% glycerol) in a volume of 10 μl at 30 °C.

**RESULTS**

**Brf1 Inhibits TBP Dimerization**—Previously we employed two distinct assays to measure TBP dimerization, chemical and densitometric scans of autoradiographs. Local background was subtracted, and the data were normalized to total TBP recovered. Data from quadruplicate assays were plotted as a function of time and globally fit to the equation $S_b = \Delta S_{brf1} + S_{glob}$, using Kaleidagraph software. $S_b$ is the normalized data. $\Delta S$ is the computer-ﬁtted change in signal during the reaction timecourse, and $S_{glob}$ is the computer-ﬁtted background at equilibrium. The data were converted to a reaction coordinate by the algebraic equation: reaction coordinate $= 2 - (S_b - S_{glob})/\Delta S$.**
cross-linking and a GST pull-down assay (28, 31, 37). As shown in Fig. 1A (lane 1), purified TBP cross-links into dimers in the presence of the cysteine-specific homobifunctional cross-linker BMH. However, increasing concentrations of purified recombinant Brf1 inhibited the cross-linking of yeast TBP dimers (lanes 2–4). Inhibition was observed at roughly equimolar concentrations of purified Brf1 and TBP. Non-specific proteins failed to inhibit the cross-linking (not shown and Ref. 30), indicating that Brf1 was not simply titrating out the cross-linker. In addition, we did not detect any cross-linking of TBP to Brf1, possibly reflecting that cysteines on Brf1 were at sub-optimal distances from cysteines located on TBP.

To address whether the inhibition was caused by Brf1 rather than some contaminant in the preparation, Brf1 was depleted with either Brf1 antibodies or mock depleted with non-specific antibodies (Fig. 1B). As shown in lanes 5–7 of Fig. 1A, Brf1 depletion abolished the inhibitory effect, whereas the control depletion (lanes 8–10) gave the same level of inhibition as untreated Brf1. As a further control, TBP was titrated back into the inhibited reaction, restoring TBP dimerization (lanes 11–13), suggesting that Brf1 inhibition of TBP dimerization involved stoichiometric interactions.

As a second measure of TBP dimerization, we used a GST pull-down assay in which the conserved DNA binding/dimerization core of TBP was fused to GST. Because we have found that human TBP tends to form more stable dimers in vitro than yeast TBP (37), the assay was performed with both the human (Fig. 1C) and yeast (Fig. 1D) core. In both cases, little or no full-length TBP was retained on the resin in the absence of the core TBP dimerization domain (Fig. 1, C and D) lane 1 versus 2). In both experiments, titration of purified Brf1 inhibited the pull-down of TBP (Fig. 1, C and D, lanes 3 and 4), confirming that Brf1 inhibits TBP dimerization.

Both the TFII B Homology Domain and the Carboxy-terminal Domain of Brf1 Are Required to Inhibit TBP Dimerization—To address the specificity of dimer inhibition by Brf1 and determine what structural domains on Brf1 are important, we constructed several previously described deletion mutants of Brf1 (Fig. 2A) (22). Region 1–262 corresponds to the TFII B-homology domain, and region 263–596 corresponds to the TBP-binding carboxy-terminal domain. A smaller region (263–431) with diminished affinity for TBP has also been described (22). To verify their binding affinity for TBP, the Brf1 derivatives were immobilized on nickel-agarose resin and incubated with TBP. As shown in Fig. 2C, 263–596 had the highest affinity for TBP.

**Fig. 1. Brf1 inhibits TBP dimerization.** A, purified recombinant yeast TBP (55 nM) was incubated in the absence (lane 1) or presence of increasing concentrations of Brf1 (12, 36, 120 nM) of either purified recombinant Brf1 (lanes 2–4), immunodepleted Brf1 (lanes 5–7), using volumes equivalent to 12, 36, 120 nm Brf1, or mock dephospho debrf1 (lanes 8–10) for 10 min at 30 °C. The reaction shown in lanes 11–13 contained Brf1 (110 nM) and TBP (55, 550, and 1100 nM, respectively). An immunoblot of yeast TBP is shown. The migration of TBP dimers is denoted by XL and uncross-linked TBP by un-XL. B, silver-stained polyacrylamide gel of purified recombinant Brf1 (lane 2), immunodepleted Brf1 (lane 3), and mock dephospho debrf1 (lane 4). Brf1 denotes a polyhistidine tag. Zn F denotes zinc finger, the arrows reflect a structural repeat, and I, II, and III denote conserved domains in the carboxy terminus. B, silver-stained polyacrylamide gel of purified recombinant Brf1 deletion derivatives. C, 110 nM TBP was incubated for 1 h at 0 °C with nickel-Sepharose either in the absence (lane 2) or presence of 37.5 nM full-length Brf1 (lane 3) or Brf1 deletion mutants (lanes 4–6), which were retained on the resin via their polyhistidine tag. The resins were then washed and TBP was analyzed by immunoblotting. 10% of the input TBP is shown in lane 1.

**Fig. 2. Brf1 deletion constructs.** A, schematic of the deletion derivatives used in this study, which are based upon similar constructs described previously (22). His denotes a polyhistidine tag, Zn F denotes zinc finger, the arrows reflect a structural repeat, and I, II, and III denote conserved domains in the carboxy terminus. B, silver-stained polyacrylamide gel of purified recombinant Brf1 deletion derivatives. C, 110 nM TBP was incubated for 1 h at 0 °C with nickel-Sepharose either in the absence (lane 2) or presence of 37.5 nM full-length Brf1 (lane 3) or Brf1 deletion mutants (lanes 4–6), which were retained on the resin via their polyhistidine tag. The resins were then washed and TBP was analyzed by immunoblotting. 10% of the input TBP is shown in lane 1.
followed by full-length Brf1 and the other derivatives. This hierarchy of affinities is essentially identical to that described earlier (22).

Next we addressed whether the various deletion derivatives of Brf1 could inhibit TBP dimerization. As shown in Fig. 3 using the human or yeast TBP core pull-down assay, we found that although full-length Brf1 inhibited TBP dimerization, none of the truncation mutants were inhibitory. Therefore, both the TFIIIB-homology domain and the carboxyl-terminal TBP binding domain of Brf1 were required to inhibit TBP dimerization. We also mixed derivatives 1–262 and 263–596 and found that they did not inhibit TBP dimerization (data not shown). Thus, despite Brf1 (263–596) having a high affinity for TBP, it must be physically connected to the amino-terminal TFIIIB-homology domain to inhibit TBP dimerization. The fact that individual or combined fragments of Brf1 failed to inhibit TBP dimerization indicated that the inhibitory effect of the full-length protein is unlikely to be caused by nonspecific binding of Brf1 to TBP.

**Brf1 binds TBP dimers**—To explore the mechanism by which Brf1 might inhibit TBP dimerization, we first examined whether Brf1 could stably bind to TBP dimers or whether Brf1 binding and TBP dimerization were competitive events. Stabilization of TBP dimers was achieved by incubating full-length wild type Brf1 (0, 5, 10, 20 nM) or with increasing concentrations of Brf1 truncation mutants (5, 10, 20 nM), as indicated. After incubation at 4 °C for 1 h, the resins were washed and TBP content was analyzed by immunoblotting with TBP antibodies. B, same as described for A except that 20 nM yeast GST-180C, 45 nM yeast TBP, and 30, 90, and 300 nM Brf1 derivatives were used. The small amount of inhibition observed in lane 10 was not reproducible.

FIG. 3. Individual Brf1 domains do not inhibit TBP dimerization. A, 300 nM of human GST-180C was incubated with 6 nM yeast TBP preincubated with increasing concentrations of wild type Brf1 (0, 5, 10 nM) or with increasing concentrations of Brf1 truncation mutants (5, 10, 20 nM), as indicated. After incubation at 4 °C for 1 h, the resins were washed and TBP content was analyzed by immunoblotting with TBP antibodies. B, same as described for A except that 20 nM yeast GST-181C, 45 nM yeast TBP, and 30, 90, and 300 nM Brf1 derivatives were used. The small amount of inhibition observed in lane 10 was not reproducible.

In contrast, highly active promoters are transcribed at a low basal level is stimulated by TBP dimer/Pol II promoter lacking its upstream activating sequence and SNR6 that lacked one of its DNA mutants (30). In contrast, highly active promoters are inhibited by these mutants. The data suggest that TBP homodimerization contributes to transcriptional repression by blocking access of TBP to promoters. This inhibitory interaction is expected to be eliminated under conditions of activated transcription. A new rate-limiting step such as TBP/TATA stability might dictate transcriptional output, and this would be susceptible to mutations along the DNA binding surface of TBP, as was observed.

To test whether TBP dimer dissociation is potentially relevant to pol III transcription in vivo, we examined the effect of a dimer/DNA defective mutant TBP(V161R) on transcription of the pol III-transcribed SNR6 gene. Like most pol III genes, SNR6 is highly transcribed in growing yeast cells, and so it is not expected to be stimulated by TBP(V161R). In an effort to mimic the basal pol II system that was stimulated by TBP(V161R), we used a variant of SNR6 that lacked one of its
critical control elements, the B box (41, 45). snr6(ΔBB) is transcribed at 1-10% of the level of SNR6 and thus is significantly impaired in transcription. This allele does not support cell viability and thus must be accompanied by a wild type SNR6.

The wild type SNR6 gene is modified (referred to as ψ-WT) making it distinguishable in reverse transcriptase-PCR reactions from snr6(ΔBB) (41, 45). As shown in Fig. 5, when transcript levels are normalized to ψ-WT, the presence of TBP(V161R) caused a modest but reproducible increase in expression of snr6(ΔBB). This level of increase was not reproduced in the presence of a different TBP mutant V161E, which shows defects in DNA binding but is less defective in dimerization than V161R (30). The use of V161E helps control for effects caused by defects in DNA binding. This observation is consistent with the notion that although TBP dimer dissociation might not be rate-limiting for activated pol III transcription, potentially because of the activity of Brf1, it can be at least partially rate-limiting at pol III promoters when they are not activated.

DISCUSSION

Through interactions with promoter-bound TFIIIC, Brf1 plays an important role in loading TBP onto the promoters of TATA-less tRNA and other pol III-transcribed small RNA-encoding genes. Inasmuch as TBP has a relatively high affinity for non-specific DNA (46) and in this capacity can assemble pol II transcription complexes, the cell is likely to employ a number of mechanisms to prevent this nonproductive and potentially detrimental assembly. Our previous studies (26, 30, 37, 39, 40) suggest that TBP dimerization represents a physiologically important auto-inhibitory mechanism by which unregulated binding of TBP to DNA is prevented. If so, then there are likely to be mechanisms that dissociate TBP dimers as a prelude to productive binding. In the pol II system, we have found that TFIIA
The Brf1 carboxyl-terminal domain binds to TBP dimers along the exposed convex surface of TBP dimers. The TFIIB homology region is then proposed to dissociate dimers into monomers, possibly through interactions with one or the other stuffer of TBP. Brf1 is denoted as B.

Fig. 7. Model for how Brf1 might dissociate TBP dimers. In this model, the Brf1 carboxyl-terminal domain binds to TBP dimers along the exposed convex surface of TBP dimers. The TFIIB homology region is then proposed to dissociate dimers into monomers, possibly through interactions with one or the other stuffer of TBP. Brf1 is denoted as B.

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