Mapping the Principal Interaction Site of the Brf1 and Bdp1 Subunits of Saccharomyces cerevisiae TFIIIB*

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The Brf1 subunit of the central RNA polymerase (pol) III transcription initiation factor TFIIIB is bipartite; its N-terminal TFIIIB-related half is principally responsible for recruiting pol III to the promoter and for promoter opening near the transcriptional start site, whereas its pol III-specific C-terminal half contributes most of the affinities that hold the three subunits of TFIIIB together. Here, the principal attachment site of Brf1 for the Bdp1 subunit of TFIIIB has been mapped by a combination of structure-informed, site-directed mutagenesis and photochemical protein-DNA cross-linking. A 66-amino acid segment of Brf1 is shown to serve as a two-sided adhesive surface, with the side chains projecting away from its internal deletion proteins has been used to demarcate the interacting Bdp1 domain to a 66-amino acid segment that includes the SANT domain of this subunit and is phylogenetically the most conserved region of Bdp1.

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The RNA polymerase (pol) III transcription apparatus of yeast comprises, in addition to the polymerase itself, three transcription initiation factors, TFIIIA, -B, and -C. The three-subunit TFIIIB is the core initiation factor required and sufficient for recruiting pol III to the promoter and accurately initiating transcription. The large six-subunit TFIIIC places TFIIIB on DNA upstream of the transcriptional start site and protects the generally very small pol III transcription units from encroachment by repressive chromatin (1, 2). TFIIIA is the 5 S RNA gene-specific factor that places TFIIIC on the transcription unit-inner promoter of these genes (reviewed in Refs. 3–5).

The work that is presented here deals with the structure of TFIIIB, which is composed of three subunits: the TATA-binding protein (TBP), Brf1, and Bdp1. TBP and Brf1 bind tightly and co-purify, whereas Bdp1 dissociates during later steps of conventional column chromatography. (The two separated fractions of TFIIIB have been designated B and C, and B double prime has been adopted as the eponym of the Bdp1 suberved region of Bdp1.) The TBP core (amino acids 61–240), and a large C-terminal fragment of Brf1 showed the latter making essentially continuous contact along both lobes of the convex surface of TBP and also interacting with a side and stirrup of the N-terminal TBP lobe (7). The structure also suggested that it might be possible to separate the N- and C-terminal domains of Brf1 and reconnect them through TBP. Indeed, a triple fusion protein that reconfigures TFIIIB topology by placing the TBP core between the N- and C-proximal domains of Brf1 fully replaces both Brf1 and TBP for TFIIIC-dependent and -independent transcription in vitro. Together with Bdp1, this Brf1-TBP fusion protein forms an extremely stable TFIIIB-DNA complex whose footprint is indistinguishable from that of the wild type TFIIIB-DNA complex. The Brf1-TBP triple fusion is also able to replace Brf1 in vivo (8).

Although Bdp1 is an essential yeast protein, removal of large segments from the N- and C-ends is compatible with some level of viability. The strongest conservation of sequence is confined to a single SANT domain located in the C-terminal one-third of Bdp1 (the three-helix SANT domains are deployed as DNA interaction as well as protein interaction modules), and weak conservation extends to either side of this element, over most of the C-terminal half. Not only is the participation of Bdp1 essential for recruitment of pol III to the promoter (presented as double-stranded DNA) by TFIIIB, but it also plays an essential role in initiating promoter opening (9).

In this work, we have exploited the structure of the Brf1(439–596):TBP core(61–240):DNA ternary complex for a systematic site-directed mutagenesis that identifies individual residues constituting the primary Bdp1-binding site of Brf1. The result that emerges from the analysis is that Brf1 homology segment II serves as a two-sided adhesive for fixing Bdp1 to TBP. A large collection of C-terminal, N-terminal, and internal deletion variants of Bdp1 has been used to show that its...
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SANT domain (amino acids 415–472) is the Bdp1 segment that interacts with the C-terminal domain of Brf1.

MATERIALS AND METHODS

DNA Templates and Probes—The linear 366-bp transcription template based on pU6b.boxB was prepared as described (10). Three photochemical cross-linking probes placing a photoactive nucleotide in the nontranscribed strand at bp −39/−38, −28, and −13/−12, respectively, were prepared by primer extension with 5′-[N⁴-(p-azidobenzoyl)-3-aminonapthy]dUTP essentially as described previously (11) with the transcribed strand of the 57-bp SNR6 electrophoretic mobility shift assay probe (bp −56 to +1) described in Ref. 8 serving as template. The nontranscribed strand sequence of this DNA from bp −40 to −10 is

\[ \text{TATACGTTTTTTTTTTTUG \text{ where } U \text{ represents sites for placement of } [N⁴-(p-azidobenzoyl)-3-aminonapthy]dUTP, \] and lowercase letters indicate the adjacent labeled nucleotides of the respective probe.

Proteins—RNA polymerase III was purified as described (12) (mono Q fraction) and is expressed as fmol of enzyme active for specific transcription. All other proteins are expressed as fmol of protein. The TBP(61–240) core domain (designated TBPc) was generously provided by Z. S. Juo (Stanford University). Bdp1, Bdp1(138–594), Bdp1(263–594), and the Bdp1 internal deletion mutants (all C-terminally His6-tagged) were constructed (each C-terminally His6-tagged and with an additional C-terminal FLAG tag for Brf1n-TBPc) were expressed as fmol of enzyme active for specific transcription. All other proteins are expressed as fmol of protein. The TBPc fraction) and is expressed as fmol of enzyme active for specific transcription. All other proteins are expressed as fmol of protein.

Protein-DNA Photochemical Cross-linking—Protein-DNA complexes were formed at 20 °C for 60 min in 20 μl of the Reaction Buffer specified above (without bovine serum albumin and with 2-mercaptoethanol in place of dithiothreitol) and 50–70 mM NaCl. Reaction mixtures contained 5 fmol each of the −39/−38 and −13/−12 probes (Figs. 2A, 3, 4A, 6 (C and D), and Table 1) or 3 fmol each of the −39/−38, −28, and −13/−12 probes (Fig. 6, A and B) and the proteins specified in the figure legends. Following the incubation, 150 ng of poly(dA-dT)poly(dA-dT) was added, and each sample was UV-irradiated for 7 min. Nuclease treatment prior to SDS-PAGE followed (17).

Cross-linking efficiencies of unaltered and mutant TBPC-Brf1c proteins were derived from the composite mean of all cross-linking experiments regardless of Bdp1 and TBPC-Brf1c concentrations, since the concentration of TBPC-Brf1c was saturating, and titrations of Bdp1 indicated negligible competition with TBPC-Brf1c for cross-linking. The phosphor image intensity for the DNA adduct of each mutant TBPC-Brf1c variant (E_i) was initially normalized to that of reference type TBPC-Brf1c (E_o) in the individual experiment (r_i = E_i/E_o). The following method was employed in order to remove the compounding effect of variation in E_o, the cross-linking signal for reference type TBPC-Brf1c: 1) the mean values of the normalized cross-linking efficiency for the reference type protein (E_o) and each variant (E_i) was determined from all experiments; 2) the ratio of each TBPC-Brf1c variant and reference type value in individual experiments to the cross-experiment mean (P_i = E_i/E_o; P_o = E_o/E_o) was determined; and 3) the average, \( \overline{P} \), of all of the P_i and P_o values for an individual experiment, taken together, was then used to renormalize r_i for each individual experiment (i.e. R_i = r_i/\overline{P}i) and averages of R_i (i.e. R) were taken. The same procedure was used to renormalize Bdp1/TBPC-Brf1c cross-linking ratios between experiments for each variant protein (Q_i). Finally, the Bdp1 cross-linking efficiency of each variant protein was calculated as the product of its renormalized Bdp1/TBPC-Brf1c cross-linking ratio (Q_i) multiplied by its mean renormalized TBPC-Brf1c cross-linking efficiency (\( \overline{R} \)).

Nomenclature—Protein fusions as well as photochemically generated protein-DNA cross-links are designated by a hyphen (e.g. TBPC-Brf1c); noncovalent association between proteins or between proteins and DNA is designated by a dot (e.g. the TBP-Brf1-DNA complex).

RESULTS

The recently determined structure of the ternary complex formed by Brf1c, TBPC, and DNA, in which Brf1 residues 437–506 were resolved (7), provides a framework for using site-directed mutagenesis to identify

Xhol fragment replacement of the original clone. The C-terminal truncation clones were sequenced and transferred as a SacII-Xhol fragment to replace Brf1c in the original pET21d-TBPC-Brf1c construct. Mutagenic primer sequences are available upon request. The resulting Brf1c and TBPC-Brf1c mutant proteins were purified as described (8).

Transcription—Protein-DNA complexes were formed at 20 °C for 60 min in 20 μl of Reaction Buffer (40 mM Tris-Cl, pH 8.0, 7 mM MgCl₂, 3 mM dithiothreitol, 100 μg/ml bovine serum albumin, 6–8% (v/v) glycerol, 5 μg/ml poly(dG-dC)/poly(dG-dC)), and 50 mM (Fig. 2B) or 70 mM (Fig. 5) NaCl with 60 fmol of the 366-bp U6b.boxB DNA, 10 fmol of pol III (added after a 40-min incubation for Fig. 2B, and the quantities of Bdp1, TBPC-Brf1c, and Brf1n or Brf1(1–365) specified in the figure legends. Five μl of a nucleotide mixture (1 mM ATP, GTP, and CTP and 125 μM [α-32P]UTP (10 cpm/fmol)) in Reaction Buffer was added for 30 min of multiple round transcription. Reactions were stopped, and samples were processed for denaturing gel electrophoresis as previously described (16).

Protein-DNA Chemical Cross-linking—Protein-DNA complexes were formed at 20 °C for 60 min in 20 μl of the Reaction Buffer specified above (without bovine serum albumin and with 2-mercaptoethanol in place of dithiothreitol) and 50–70 mM NaCl. Reaction mixtures contained 5 fmol each of the −39/−38 and −13/−12 probes (Figs. 2A, 3, 4A, 6 (C and D), and Table 1) or 3 fmol each of the −39/−38, −28, and −13/−12 probes (Fig. 6, A and B) and the proteins specified in the figure legends. Following the incubation, 150 ng of poly(dA-dT)poly(dA-dT) was added, and each sample was UV-irradiated for 7 min. Nuclease treatment prior to SDS-PAGE followed (17).

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Brf1c residues that are important for Bdp1 interaction. We anticipated one possible complication in interpreting the outcome of this systematic amino acid substitution screen: the Brf1c amino acid 439–506 segment interface with TBP is notably extensive and spread out (Fig. 1A), and the stability of at least some secondary structure features of Brf1c (e.g. helix H22) may depend on interaction with TBP (7). Thus, diminished Bdp1 binding by Brf1 mutants may not be solely due to the loss of direct Brf1-Bdp1 contacts but can also arise because of effects on the proper folding and/or alignment of Brf1c along its extensive TBP interface.

To circumvent this possible complication, we have tethered Brf1c to TBPC with the flexible 12-amino acid connector (GS)₆, taking advantage of the fact that the C terminus of TBPC (M240; space filled in cyan in Fig. 1A) lies within ~20 Å of the N terminus of Brf1c (P439; space filled in green in Fig. 1A). TBPC-Brf1c, with a hypothetical orientation of the connector (in orange) is shown in Fig. 1A. The high local concentration

FIGURE 1. Model of Brf1(439–596) linked to TBPC(61–240) (TBPC-Brf1c) and of the mutations generated within the resolved segment of Brf1. A, a model of the TBPC-Brf1c fusion. TBPC (cyan ribbon), the resolved Brf1(439–506) segment (light green ribbon) and DNA (sticks) are from Ref. 7. A (GS)₆ linker (a possible conformation is shown in orange) was used to connect TBPC residue Met240 (space-filled, cyan) to Brf1 residue Pro439 (space-filled, light green). The view displays the transcriptional start site-proximal face of this complex. B, Brf1 residues in the resolved segment that were mutagenized are space-filled and color-coded as in C. Views of the convex surface and N-terminal side of TBP are shown. Rotations from the view shown in A are indicated at the left. C, sequence of the resolved segment of Brf1. Amino acids whose side chains contact TBP are in lowercase. Mutagenized residues are colored (Leu and Ile (yellow), Asn and His (green), Thr and Ser (orange), Asp and Glu (red), and Lys and Arg (blue)), with the substituted amino acid shown below the sequence, and the location of Brf1 α helices H21 to H25 is indicated.
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afforded by this tether and its flexibility should diminish the sensitivity of Brf1c-Bdp1 binding to adventitious effects of mutations on Brf1c-TBPc binding. A similar approach was used to rescue the TFIIA binding defect of a TBP mutant protein in yeast (18). The ability of TBPc-Brf1c to assemble Bdp1 onto DNA and to function for transcription has been documented (8) and is further analyzed below.

A remarkable 29 of the 67 amino acid residues of Brf1c that are resolved in the ternary Brf1-TBP-DNA complex have side chains that hydrogen-bond to or form van der Waals contacts with residues of TBP (sequence in Fig. 1C, lowercase residues) (7)). Twenty-six of the 38 amino acids whose side chains are not in contact with TBP were substituted, along with Asp498 and Glu484, which make limited van der Waals contacts with TBP, and Asp560 and Asn473, which form hydrogen bonds with TBP in at least two of the four crystallographically independent complexes (mutated residues are highlighted in Fig. 1C).

The large aliphatic side chains of Leu and Ile (yellow in Fig. 1B) and large polar side chain of Asn and His (dark green) were replaced with the small aliphatic side chain of Ala; small polar residues Thr and Ser (orange) were replaced with Leu; acidic residues Asp and Glu (red) were replaced with Lys; basic residues Lys and Arg (blue) were replaced with Glu (Asp for Arg485). The effect of mutations on the Bdp1-Brf1 interaction was examined by photochemical protein-DNA cross-linking. The same method has been used previously to identify radical sequence substitutions in TBP that interfere with binding to the TFIIIB-related N-terminal half of Brf1 in a DNA complex (19).

We first examined the potential contribution to Bdp1 binding of the amino acid 507–596 segment of Brf1c, which is not resolved in the ternary complex structure. The 51 C-terminal Brf1 residues (546–596) have been shown not to provide a major Bdp1-binding site (6). Two additional C-terminal truncations of Brf1c were generated in the TBPc-Brf1c fusion protein, one ending at amino acid 511 and the other at amino acid 497, within the Brf1 helix H25 (Fig. 1C).

DNA complexes were formed with Bdp1 and TBPc-Brf1c (or its derivatives) and a mixture of two probes covering the SNR6 (U6 snRNA) gene promoter region (bp −56 to bp +1, the start site of transcription), one with the photoactive thymidylate analogue 5-[N′-(p-azidobenzooyl)]-3-aminoallyl UMP (20) at bp −39 and −38 and with [α-32P]-dCMP at bp −37 on the nontranscribed strand to monitor the presence of Bdp1, and the other with [N′′- (p-azidobenzooyl)]-3-aminoallyl UMP at bp −13 and −12 and [α-32P]-dCMP at bp −11 to monitor the presence of TBPc-Brf1c. Fig. 2A shows a photochemical cross-linking experiment with Bdp1 and “full-length” TBPc-Brf1c (i.e. extending to the C terminus of Brf1) or truncated at Brf1 residue 511 or 497. The concentration of TBPc-Brf1c (20 nM) was saturating for DNA cross-linking, but Bdp1 (20 nM) was limiting for assembly onto the TBPc-Brf1c-DNA complex. Truncation of TBPc-Brf1c to amino acid 511 or 497 did not appreciably impair the assembly of Bdp1 (A; quantified below each lane), even at a 2-fold lower Bdp1 concentration (data not shown). Truncation to amino acid 511 or 497 decreased the DNA cross-linking efficiency of TBPc-Brf1c (lanes 3 and 4) but not to the extent observed in the absence of TBPc-Brf1c tethering (data not shown). As noted previously (6, 21), TBPc alone was capable of assembling Bdp1 to a small extent (lane 1).

Brf1(1–509) has been shown to be severely defective in TFIIIC-dependent and -independent transcription and, to a lesser extent, in TFIIIB-TFIIIC-DNA complex formation (22). In contrast, Brf1(1–545) displayed at most a 2-fold defect in TFIIIC-independent transcription of SNR6 as supercoiled DNA (6). Since TFIIIC-independent transcription of supercoiled templates by pol III is substantially independent of the C-terminal half of Brf1 (6), we examined the effect of C-terminal truncations of TBPc-Brf1c on transcription of an SNR6-derived template presented as linear DNA. This transcription requires both the N-terminal (TFIIIB-related) half of Brf1 and TBPc-Brf1c (8). The ability of TBPc-Brf1c truncated to Brf1 residue 564, 544, 511, or 497 to support TFIIIC-independent transcription is shown in Fig. 2B (the quantification of two experiments is shown below each lane). Truncation of Brf1c to residue 564 or 544, removing Brf1 homology domain III (amino acids 570–595) (23) diminished transcription 4–5-fold (lanes 1–3; note that TBP binds to the SNR6 TATA box in either orientation, yielding divergent transcripts r-U6 and l-U6). Cross-linking experiments similar to Fig. 2A verified that TBPc-Brf1c(439–596) was competent to assemble Bdp1 (data not shown) and not inadvertently inactive. Further truncation of TBPc-Brf1c to residue 511 or 497 reduced transcription to barely detectable levels (lanes 4 and 5). Thus, the 100 C-terminal amino acids of Brf1 (which include homology region III) do not contribute significantly to Bdp1 assembly, but removing them progressively impairs transcription of linear DNA.

We next examined the effect of amino acid substitution between Brf1 residues 441 and 508 on Bdp1 assembly using a set of 10 TBPc-Brf1c variants containing locally clustered mutations. As with the C-terminal...
TBPC-Brf1c truncations in Fig. 2, the ability of these TBPC-Brf1c cluster mutant proteins to assemble Bdp1 onto DNA was assessed by cross-linking with limiting concentrations of Bdp1 (5 and 10 nM) and saturating concentrations of each mutant TBPC-Brf1c variant (≥20 nM) for both TBPC-Brf1c-DNA and Bdp1-DNA cross-linking. A sample cross-linking analysis is shown in Fig. 3A. Two TBPC-Brf1c cluster mutant proteins (K481E/L482A/R485E (lane 8) and L482A/I486A/L490A (lane 9)) were seen to be clearly defective in assembling Bdp1 when compared with unmutated ("reference") TBPC-Brf1c (lane 2).

The effects of these clustered mutations on Bdp1 assembly were examined more closely by quantifying and normalizing the results of multiple cross-linking experiments with 5 or 10 nM Bdp1 (Fig. 3B). The cross-linking efficiency of Bdp1 was normalized to that of TBPC-Brf1c in the same reaction mixture in order to compensate for differences in phosphor image exposure and probe-specific activity between experiments. These values were then compared with the Bdp1/TBPC-Brf1c cross-linking ratio of the reference TBPC-Brf1c protein (Fig. 3B, lower plot). Although the concentration of each mutant TBPC-Brf1c variant was saturating for cross-linking to DNA, as already specified, the efficiencies of cross-linking consistently differed from that of the reference protein in the same experiment (Fig. 3B, upper plot), with the mutant TBPC-Brf1c proteins generally cross-linking more efficiently. The mean Bdp1 cross-linking efficiencies shown in Fig. 3C compensate for the different cross-linking efficiencies of TBPC-Brf1c variants (as specified under "Materials and Methods").

The quantitative analysis confirmed that the K481E/L482A/R485E and L482A/I486A/L490A mutants were the most defective in binding Bdp1, with relative efficiencies of DNA cross-linking that were at most one-fourth that of the reference protein (Fig. 3C). Of the remaining cluster mutant proteins, N441A/H443A/L444A cross-linked at less than 50% efficiency relative to the reference protein, and so did mutant protein E468K/N471A in the presence of 5 nM Bdp1. The charge reversal mutations (Fig. 1) may disrupt helix H25. Alternatively, the highly acidic or basic patches that are generated by these clustered charge reversal mutations (Fig. 1C) may disrupt helix H25.

The clearly defective Bdp1 assembly by the two cluster mutant TBPC-Brf1c proteins altering side chains of residues 481–490 encouraged us to define the Brf1 site for Bdp1 binding more closely by screening a large collection of proteins with single amino acid substitutions. A cross-linking experiment with limiting (10 nM) Bdp1 is shown in Fig. 4A. (The concentration of each TBPC-Brf1c single mutation variant, ≥20 nM, was saturating for DNA cross-linking; data not shown.) Variants L482A and I486A were clearly the most defective in DNA-Bdp1 cross-linking, consistent with the Bdp1-binding region defined by cluster mutants K481E/L482A/R485E and L482A/I486A/L490A. Experiments similar to panel A were performed with 5, 10, and 20 nM Bdp1 (generally with 3–6 replicates at each concentration) and quantified. As also observed with the TBPC-Brf1c cluster variants, the cross-linking efficiencies of the single mutation proteins exceeded that of TBPC-Brf1c (supplemental Fig. S1A). These values were used to derive Bdp1 cross-linking efficiencies from Bdp1/TBPC-Brf1c ratios (supplemental Fig. S1B) that are summarized in Table 1. The error margins of the derived Bdp1 cross-linking efficiencies were such that only differences between a single mutant variant and the reference TBPC-Brf1c exceeding a factor...
of 2 were taken to be significant. In the presence of 20 nM Bdp1, only TBPc-Brf1c variants L482A and I486A were clearly defective in Bdp1 assembly according to this criterion; at lower concentrations of Bdp1, TBPc-Brf1c variants L482A, K485E, I486A, I488A, and L490A were also impaired. It is noteworthy that Leu482, Ile486, and Leu490 lie on one exposed side of Brf1 helix H24 (Fig. 4B) on the same face as and proximal to TBP Glu93 (~8 Å apart), for which the radical substitution mutation E93R was found to impair Bdp1 assembly (25).

These results indicate that Bdp1 binds to at least two separate segments of Brf1c, accounting for the fact that none of the TBPc-Brf1c cluster mutant variants reduced Bdp1 assembly to the TBPc-alone base line (Fig. 3A). Among the remaining TBPc-Brf1c single mutation variants, D449K, E463K, E468K, L495A, K504E, and E506K interfered less severely with Bdp1 assembly when Bdp1 was present at 5 and 10 nM. Although the mean Bdp1 cross-linking efficiencies of 9 of the other 12 single mutation TBPc-Brf1c variants (K454E, D460K, N471A, E477K, E478K, K481E, E499K, K501E, and R502D) did not deviate from the reference TBPc-Brf1c sufficiently to be individually significant, they yielded mean Bdp1 cross-linking efficiencies that almost uniformly ranged between 40 and 80% of the reference TBPc-Brf1c efficiency at all Bdp1 concentrations (Table 1). The consistency of these independent observations (involving nine variant proteins, each examined at three Bdp1 concentrations) specifies that additional sites in the extended stretch of Brf1 between the amino acid 441–443 and 482–490 clusters (Fig. 4B) make dispersed and smaller contributions to Bdp1 assembly.

We also examined the effects of the Brf1c cluster mutations on transcription of linear DNA. Since TFIIIB complexes formed with Bdp1, TBP, and only the N-terminal half of Brf1 are competent for transcription of supercoiled SNR6-derived templates (6) and since the transcription of linear DNA requires Brf1 residues C-terminal to amino acid 511 that have little effect on Bdp1 assembly (Fig. 2), it was not clear whether defects in Bdp1 assembly of clustered TBPc-Brf1c mutations would be reflected in transcription. The N441A/H443A/L444A mutations had no effect on transcription (Fig. 5), but an ~2-fold decrease in transcription was generated by K481E/L482A/R485E and L482A/I486A/L490A, and an ~4-fold reduction was generated by K501E/R502D/K504E and E506K/D508K. The last two TBPc-Brf1c variants are at most modestly affected in Bdp1 assembly (Fig. 4B), suggesting that these changes interfere with pol III assembly into the preinitiation complex or at a subsequent step of the transcription cycle.

The SANT Domain of Bdp1 Is Responsible for Brf1c Interaction— Bdp1 can be split at amino acid 352. Bdp1(1–352) (Bdp1n) and Bdp1(352–594) (Bdp1c) individually support TFIIIC-independent transcription of supercoiled DNA in complexes formed with intact Brf1 and TBP (14). Both halves together functionally replace intact Bdp1 in vivo (26). We used split Bdp1 in combination with TBPc-Brf1c and Brf1n-
TBPC (a fusion protein linking Brf1(1–382) directly to the N terminus of TBP core) to determine whether the interactions between Bdp1 and Brf1 can be partitioned to their N- and C-terminal halves by means of protein-DNA cross-linking. A detailed rationalization for using the same cross-linking assay in this context is provided in the supplemental material.

In this experiment (Fig. 6A), the triple fusion protein Brf1n-TBPC-

Brf1c (8) served as the reference. Brf1n-TBPC-Brf1c assembled Bdp1n and Bdp1c separately (lanes 3 and 4, respectively) and together (lane 2) with comparable efficiency. Only a weak background of Bdp1n cross-linking was detected when Brf1n-TBPC-Brf1c was replaced with TBP core (lane 1); this may signify that TBP-Bdp1 interaction is primarily mediated by the N-terminal half of Bdp1. TBPc-Brf1c, on the other hand, was competent in assembling Bdp1c into the DNA complex (lane 7), whereas the level of cross-linking of Bdp1n (lane 6) was indistinguishable from that of TBPc alone (lane 1). No increase in Bdp1n and Bdp1c cross-linking was observed when both halves were present (lane 5). Adding Brf1n to the reaction mixture containing TBPc-Brf1c had no effect on Bdp1c cross-linking (lane 9) but did increase Bdp1n cross-linking (lane 8). Brf1n-TBPC was able to assemble Bdp1n into the DNA complex (lane 11), but it did not secure cross-linking by Bdp1c (lane 12). Inclusion of Brf1c in the reaction with Brf1n-TBPC enabled the assembly of Bdp1c (lane 14). Brf1n-TBPC was also competent in assembling Bdp1c when both Bdp1n and Bdp1c were present (lane 10), indicating the existence of a binding interface between Bdp1n and Bdp1c and/or the unmasking of a Bdp1c binding domain in Brf1n upon binding Bdp1n. We conclude that Brf1n primarily interacts with the N-terminal half of Bdp1 and that Brf1c primarily interacts with the C-terminal half of Bdp1.

A collection of internal deletions as well as a series of N- and C-terminal truncations of Bdp1 were used to further define the region of Bdp1 that interacts with Brf1c. Bdp1 variants with 12–22-amino acid deletions between Bdp1 residues 253 and 449 were examined for the ability to be recruited to the DNA complex by Brf1n-TBPC or TBPC-Brf1c (Fig. 6B). No deletion within this segment of Bdp1 prevented assembly by Brf1n-TBPC (Fig. 6B, top). In contrast, three deletions covering Bdp1 residues 409–449 effectively eliminated binding to the TBPC-Brf1c-DNA complex (Fig. 6B, bottom). As expected from the abil-
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ity of TBPc-Brf1c to recruit the C-terminal half of Bdp1 (Fig. 6A), Bdp1(263–594) was also assembled into a DNA complex by TBPc-Brf1c (Fig. 6C). In contrast, Bdp1 C-terminally truncated to amino acid 465 was not recruited by TBPc-Brf1c, and the ability of TBPc-Brf1c to recruit Bdp1 truncated to amino acid 477 was significantly impaired (Fig. 6D, bottom; compare with Bdp1 assembly with wild type Brf1 in the top panel). The ability of TBPc-Brf1c to assemble Bdp1 C-terminally truncated to amino acid 493 or 506 into the DNA complex was substantially retained. These results specify that the entire segment of Bdp1 spanning amino acids 410 – 476 is essential for Brf1c binding, with an additional contribution provided by amino acids 477 – 492. This region largely overlaps the SANT domain (amino acid residues 415 – 472) (27) that is the most highly conserved Bdp1 sequence motif.

DISCUSSION

Bdp1 is essential for transcription by pol III in vitro, but no single region of Bdp1 is required for TFIIC-independent transcription of SNR6 as supercoiled DNA (13). Much of Bdp1 can be deleted with retention of yeast viability (the N-terminal 240 amino acids, the C-terminal 108 amino acids, 68 amino acids between residues 312 and 372, and 17 amino acids between 253 and 269), leaving just 161 Bdp1 residues that are essential (26, 28). Nevertheless, there are multiple sites of interaction between Bdp1 and the Brf1-TBP-DNA complex that independently suffice for TFIIB-DNA complex formation and transcription, and this has been an impediment to identifying the individual sites within Brf1 that are involved in Bdp1 interaction. This work presents the first analysis and identification of Brf1 residues that are involved in assembling Bdp1 into the TFIIB-DNA complex.

Structure-informed mutagenesis of Brf1 (441 – 506) identifies two separated segments that are involved in Bdp1 assembly. Brf1 residues Asn441, His443, and Leu444 lie above the convex surface of the C-terminal lobe of TBP (Fig. 4B). This region of Brf1 is anchored to TBP by weak van der Waals interaction of Brf1 residues Arg440 and Leu445 with TBP residues Glu228 and Tyr229, respectively. Since this weakly anchored region lies adjacent to the (GS)₆ linker, we considered the possibility that the observed defect of Bdp1 assembly by Brf1c N441A/H443A/L444A might be due to an indirect effect, altering the path of Brf1 on the convex surface in a way that would eliminate three polar contacts mediated by the deep intrusion of the Brf1 helix H21 residue Thr448 between TBP helices H2 and H2’ (Fig. 1A) (7). The evidence indicates that this is not the case: 1) the single mutations within this cluster were also defective in Bdp1 assembly; 2) with the exception of Pro446 and Ala472, all Brf1 side chains not involved in TBP interaction on the convex surface of TBP were mutated, and none displayed as severe a defect in Bdp1 assembly (Table 1). The amino acid sequence context of this Bdp1-binding epitope, PRNLHLLP, is only conserved within the Ascomycota subphylum Saccharomycotina, and divergence is observed even within this subphylum among the species Candida, Debaryomyces, Pichia, and Clavispora (consensus: PRNLVKNLP). One possible implication of the sequence divergence in this region is that the selective significance that is provided by this site of Bdp1-Brf1 interaction and therefore its overall importance is not dominant in the context of the full-length proteins.

The second Bdp1-binding segment, defined by the deleterious effect of substitutions at Brf1 residues Leu182, Arg485, Ile486, Ile488, and Leu490 lies at the outward facing, exposed surface of Brf1 helix H24 at the N-terminal side of TBP (Fig. 4B). The helix H24 region of Brf1 is a major interface with TBP, contributing six intersubunit hydrogen bonds in yeast (four in mammals) (7), and is conserved from yeast to mammals (29 – 31). However, the residues identified here as important for Bdp1 assembly are also conserved between the anciently diverged (1.0 – 1.2 Myr (32, 33)) fungal phyla Ascomycota (U/L/J/R(V/L)/U/E) and Basidiomycota (U/J/R(L/V)/U/X) and partly retained in chordate Brf1 (U/V/L/ELM(Q/E)) (supplemental Fig. S2). If this Bdp1-Brf1 interface is retained in vertebrate Brf1, it is noteworthy that this part of the Brf1 helix H24 is clearly absent from the Brf1 paralogue Brf2 (34). A prior in vivo analysis (35) of the effect of sequence substitutions in 19 residues of Brf1 between amino acid residues 453 and 508 did not reveal any growth defect that could be ascribed to a defect in interaction with Bdp1. Given that 18 of those substitutions replaced charged amino acids, this negative result is consistent with our analysis: only one charge reversal, R485E, substantially impaired Bdp1 assembly (Table 1). In fact, most of the Brf1 residues identified here as important for Bdp1 assembly contain large hydrophobic side chains (Leu444, Leu482, Ile486, Ile488, and Leu490). TFIIB-DNA complexes are distinctive in that they are salt- and heparin-resistant (12). The interaction of Bdp1 with Brf1 helix H24 may be predominantly hydrophobic, and this may contribute to the stability of TFIIB-DNA complexes at high salt concentrations.

TBPc-Brf1c variants D449K, E463K, E468K, L495A, K504E, and E506K displayed a more modest (2-fold) defect in Bdp1 assembly. In fact, only three TBPc-Brf1c variants (D458K, E484K, and E497K) assembled Bdp1 with mean cross-linking efficiencies that consistently closely matched or exceeded the reference TBPc-Brf1c. We propose that the Bdp1-binding interface of Brf1c is extensive and that additional sites along the entire segment of Bdp1c that was resolved in the structure of Brf1c-TBPc-DNA ternary complex (7) contribute to the affinity of Brf1c for Bdp1c in the TFIIB-DNA complex. The N-terminal end of Brf1c and Brf1 helix H24 provide the high affinity binding sites for Bdp1, with numerous weaker sites of Bdp1 interaction in between, as well as C-terminal of helix H24.

The segment of Bdp1 that interacts with Brf1c (439 – 596) has been localized by deletion analysis primarily to amino acids 410 – 476, with Bdp1 residues 477 – 492 also significantly contributing to Brf1 binding (Fig. 6). The SANT domain (residues 415 – 472), which is the most highly conserved Bdp1 motif (43% identity between S. cerevisiae and H. sapiens (36)), constitutes a large part of this Bdp1 segment. Although there is a strong similarity between SANT domains and the DNA-binding domain of Myb-related proteins, the involvement of the SANT domain in protein-protein interaction has also been documented (37 – 39). It is an intriguing possibility that the co-conservation of helix H24 of Brf1s and the SANT domain of Bdp1s results from a conserved interaction between these domains. In this regard, Brf1 residues Leu182, Arg485, Ile486, and Leu490 (and TBP residue Glu525), which are involved in Bdp1 binding face upstream (relative to the direction of transcription). The region of Bdp1 that cross-links to bp – 33 on the nontranscribed strand just upstream of the SNR6 TATA box also largely coincides with the SANT domain (between Bdp1 residues 425 and 485) (40). The amino acid 419 – 455 segment of the Bdp1 SANT domain can be reasonably compared with the structure of the human KIAA 1915 protein’s SANT domain (37% identity, 59% similarity; Protein Data Bank entry 2CU7), which forms a compact helix-loop-helix-loop-helix structure; two additional α helices are predicted within the residue 456 – 491 segment of Bdp1. This latter segment would need to be extended in order to span the ~60-Å distance between Brf1c helix H24 and the N terminus of Brf1c. It is likely that the loss of Brf1 binding by any deletion that encroaches into the Bdp1 410 – 492 region results at least in part from a gross alteration of Bdp1 tertiary structure. A more precise specification of residues in the SANT domain region of Bdp1 that are involved in Brf1 interaction will require site-directed mutagenesis and determination of structure.

An additional outcome of our analysis is the finding that most of the
binding affinity of Brf1n (Brf1(1–382)) for Bdp1n is contributed by Bdp1n
(1–382) (Fig. 6A). Since the TFIIB-related Brf1n(1–282) segment forms a transcriptionally active but unstable TFIIB-DNA complex with Bdp1n and TBP (R502D/K504E and E506K/D508K) also displayed a severe defect in tran-
scription, and this should aid in fine mapping the Brf1n-Bdp1n interface.

C-terminal truncations of TBPC-Brf1c to amino acids 510 and 497
essentially abolished transcription of a linear DNA, yet had little effect
on Bdp1 assembly (Fig. 2). As already noted, this observation is largely in
agreement with Ref. 22. However, TBPC-Brf1c cluster mutants K501E/
K504E and E506A/D508A also displayed a cold-sensitive growth pheno-
type (mutant II.16 (35)) similar to K594A/K595A (mutant III.2), for which genetic evi-
dence of an interaction with downstream DNA sequence
was presented in a recent study (35).

It has been proposed that E506A/D508A alters the conformational flexi-
bility of Brf1, since this mutation effectively shields a nearby site from
proteolysis during purification (35).

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