A common site on TBP for transcription by RNA polymerases II and III

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The TATA-binding protein (TBP) is involved in all nuclear transcription. We show that a common site on TBP is used for transcription initiation complex formation by RNA polymerases (pol) II and III. TBP, the transcription factor IIB (TFIIB)-related factor Brf1 and the pol III-specific factor Bdp1 constitute TFIIB. A photochemical cross-linking approach was used to survey a collection of human TBP surface residue mutants for their ability to form TFIIB–DNA complexes reliant on only the TFIIB-related part of Brf1. Mutations impairing complex formation and transcription were identified and mapped on the surface of TBP. The most severe effects were observed for mutations in the C-terminal stirrup of TBP, which is the principal site of interaction between TBP and TFIIB. Structural modeling of the Brf1–TBP complex and comparison with its TFIIB–TBP analog further rationalizes the close resemblance of the TBP interaction with the N-proximal part of Brf1 and TFIIB, and establishes the conserved usage of a TBP surface in pol II and pol III transcription for a conserved function in the initiation of transcription.

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Introduction

The TATA-binding protein (TBP) is an essential component of the core transcription apparatus of all three nuclear RNA polymerases. For transcription by RNA polymerase (pol) III, TBP participates as one of three subunits of the core transcription factor transcription factor IIIB (TFIIB); the other two subunits are the TFIIB-related Brf1 and the pol III-specific Bdp1. All the pol III-transcribed genes of the yeast *Saccharomyces cerevisiae* are served by its unique TFIIB; in humans, a variant TFIIB, in which Brf1 is replaced by its paralog Brf2, is required for transcription of pol III genes with upstream

promoter elements bound by the snRNA gene-specific transcription factor SNAP_C/PTF (reviewed by Geiduschek and Kassavetis, 2001; Schramm and Hernandez, 2002; White, 2002).

TFIIB is brought to its location in pol III promoters, upstream of the start site of transcription, by its assembly factor TFIIC, but can also bind independently of TFIIC, through its TBP subunit, to promoters that contain a strong TATA box (Margottin *et al.*, 1991; Joazeiro *et al.*, 1994; Dieci *et al.*, 2000). Whether assembled at the promoter by TFIIC or bound independently, TFIIB alone suffices to recruit pol III for multiple rounds of transcription and plays an essential role in formation of the open pol III initiation complex (Kassavetis *et al.*, 1998b, 2001; Hahn and Roberts, 2000).

Both Brf1 and Bdp1 have a modular organization that permits the separate analysis of individual functional domains. In particular, Brf1 consists of two major modules: an N-terminal half that is related to the pol II general transcription factor TFIIB, and a Brf1-specific C-terminal half that contains three well-conserved segments (Figure 1A). It is its C-terminal half that provides most of the affinity of Brf1 for TBP (Khoo *et al.*, 1994; Chaussivert *et al.*, 1995; Wang and Roeder, 1995; Kassavetis *et al.*, 1997, 1998a; Colbert *et al.*, 1998; Shen *et al.*, 1998). Unlike TFIIB, both yeast and human Brf1 bind stably to TBP in the absence of DNA and co-purify with TBP from cell extracts as a complex (Kassavetis *et al.*, 1991; Wang and Roeder, 1995; Mital *et al.*, 1996). The high-affinity TBP-binding surface of Brf1 lies in its C-terminal homology region 2, and interacts primarily with the convex surface and side of the first TBP pseudo-repeat (Colbert *et al.*, 1998; Kassavetis *et al.*, 1998a; Shen *et al.*, 1998). The crystal structure of a Brf1 homology region 2–TBP–DNA complex has recently been solved (Juo *et al.*, 2003).

In contrast, stable binding of the N-terminal half of Brf1 to the TBP–DNA complex has not been detected. However, this half of Brf1 retains the ability to recruit Bdp1 to a TBP–DNA complex and direct efficient transcription of supercoiled DNA. The TFIIB–DNA complex assembled with only the N-terminal half of Brf1 does not survive electrophoretic separation on native gels, but its (unperturbed) presence in solution is readily detected by photochemical cross-linking (Kassavetis *et al.*, 1998a).

While the sites of interaction of TBP with the C-terminal half of Brf1 are now well characterized by molecular genetic and structure analysis, sites conferring the lower affinity of the N-terminal half of Brf1 for TBP have not yet been mapped. Predictions based on the homology with TFIIB might suggest a conserved mode of interaction, but there are also subfamily-specific divergences of amino acid sequence between Brf1 and TFIIB

Fig. 1. A photochemical cross-linking screen for Brf1–TBP interaction. (A) Domain organization of *S.cerevisiae* Brf1. The N-proximal TFIIB-related half of Brf1 (amino acids 1–282) contains an N-terminal Zn-ribbon motif and two imperfect repeat segments (amino acids 94–164 and 189–264, respectively); the Brf-specific C-terminal half (amino acids 283–596) contains three conserved sequence segments. The ends of the protein fragments used in this study (69–365 and 408–596) are indicated. (B) DNA probes. The transcribed (template) strand sequence of the 59 bp U6 gene probe containing a TGTA mutant TATA box is shown on the bottom line. The non-transcribed strand primers used to generate three double-stranded photoprobes are shown in capital letters on the top three lines. These primers were extended by incorporation of ABdUMP (u) and the specified radioactive nucleotide (lower case letter) and subsequently extended to full length. Probes are designated according to the location of ABdUMP in the non-transcribed (N) strand. (C) Simultaneous analysis of cross-linking to bp –39/–38, –33 and –28 in the non-transcribed strand. TFIIB–DNA complexes containing hTBPm3 [wild type (wt), or mutants thereof, as indicated], Brf(2–365) and Bdp1 were assembled either in the absence (N) or presence (N/C) of Brf(408–596) and subjected to photochemical cross-linking as described in Materials and methods. Cross-linked proteins are identified at the right. Results of the quantitative analysis are summarized in Table I.

that might dictate or allow different modes of interaction. Indeed, even the strength of the extensively studied TFIIB–TBP interaction is still not known: the association with the TBP–pol II promoter complex is stable to gel-electrophoretic separation, but benefits from separate sequence-specific interactions of TFIIB with DNA directly upstream and downstream of the TATA box (Tsai and Sigler, 2000); the contribution of the direct TBP–TFIIB

interaction to the total affinity of this ternary promoter complex remains to be determined.

To better understand the architecture of TFIIB in the pol III initiation complex, and to assess its relationship with the pol II initiation complex, we have specifically analyzed the interaction between TBP and the N-terminal half of Brf1. For this purpose, we have exploited two resources: (i) a library of human TBP mutant proteins with

radical surface residue substitutions (Bryant *et al.*, 1996; Shen *et al.*, 1998); and (ii) variants of Brf1 split at sites that permit the assessment of interaction and function of the N-proximal half separated from the quantitatively dominant interaction of the C-terminal half. TBP mutants have been screened for protein–protein interaction defects by photochemical cross-linking, and for functional defects by transcription assay. We have identified several TBP surface residue substitutions that specifically interfere with the assembly of the N-terminal half of Brf1 into a TFIIB–DNA complex, some of them also exhibiting profound functional defects. The results reveal novel structural and functional insights into the pol III initiation complex and show, contrary to prior proposals (Colbert *et al.*, 1998; Zhao *et al.*, 2003), that the principal TBP interactions are conserved between TFIIB and the N-terminal half of Brf1.

Results

TBP surface mutations that block interaction with the N-proximal part of Brf1

The N-proximal half of Brf1 forms a relatively unstable but transcriptionally active TFIIB–DNA complex with Bdp1 and TBP (Kassavetis *et al.*, 1998a). A photochemical cross-linking assay was devised to identify mutations on the surface of TBP that generate defects in the assembly of DNA complexes lacking the C-terminal half of Brf1. For this purpose, the photoreactive thymidylate analog, ABdUMP {5-[N'-(p-azidobenzoyl)-3-aminoallyl]dUMP} (Bartholomew *et al.*, 1990) was incorporated into the non-transcribed strand of DNA fragments containing a yeast U6 (*SNR6*) promoter (Figure 1A). For unambiguous interpretation of the cross-linking assay, the promoter contained a TGTA-mutant TATA box that specifies unidirectional binding of TBPm3, and therefore of the entire TFIIB complex (Whitehall *et al.*, 1995). Three different photoprobes were generated, with the location of each photoreactive site chosen to allow maximum efficiency of cross-linking to Bdp1, the N-proximal half of Brf1 or the C-proximal half of Brf1, respectively (Kassavetis *et al.*, 1998a). All three photoprobes were combined and used in the same reaction.

To screen for mutations that weaken the interaction of TBP with the N-terminal half of Brf1, we took advantage of a human TBPm3 radical surface substitution library (Bryant *et al.*, 1996). Initially, 91 mutants spanning TBP surface residues 156–338 were screened in a photochemical cross-linking assay for defects in the assembly of TFIIB–DNA complexes dependent on the N-terminal half of Brf1 (amino acids 1–282). In this preliminary survey, assembly of this TFIIB-related half of Brf1 was measured indirectly by cross-linking of Bdp1, since the recruitment of Bdp1 and the N-terminal half of Brf1 to the TBP–DNA complex is co-dependent (Kassavetis *et al.*, 1998a). All TBP mutations significantly affecting Bdp1 cross-linking were located in its C-proximal lobe. A control assay of Bdp1 cross-linking in the presence of the C-terminal half of Brf1 (amino acids 284–596) indicated that most of these mutations were specifically affected in Brf1(1–282)-mediated assembly (data not shown).

Based on this preliminary screen, all 32 radical surface substitutions in the C-terminal lobe of hTBPm3 that retain

DNA-binding activity (Shen *et al.*, 1998) were analyzed for their effect on the assembly of the N-proximal part of Brf1, using the three-probe photochemical cross-linking assay, which separately and directly probes Brf1 and Bdp1 assembly into TFIIB–DNA complexes. The latter were formed with hTBPm3, the N-terminally truncated Bdp1(138–594), and Brf1(69–365), which contains its TFIIB-related segment, with or without Brf1(408–596), which contains the principal Brf1 binding site for Bdp1 and TBP (Figure 1A). These protein fragments were chosen for their ability to be detected by cross-linking to DNA and for optimal resolution by gel electrophoresis. The intervening amino acid residues between these two Brf1 segments (residues 366–407) are not present in other fungal Brf1s and it has been noted that intact Brf1 lacking the intervening segment is more active for specific transcription than is full-length Brf1 (Kassavetis *et al.*, 1999). Since the activities of individual TBP mutant proteins vary, the ability of TBP–DNA complexes to assemble Brf1(69–365) and Bdp1(138–594) was compared with identical binding reactions also containing Brf1(408–596) (Figure 1C). Brf1(408–596) increases the assembly of Bdp1, which, in turn, increases the assembly of the N-proximal segment of Brf1 (Kassavetis *et al.*, 1998a); cross-linking of TBP is concomitantly decreased, probably as a result of increased competition by Brf1(69–365) and Bdp1. Table I displays the four independent ratios specifying the relative cross-linking efficiencies of Brf1(69–365) in binding reactions containing only TBP and Bdp1(138–594) and reaction mixtures also containing Brf1(408–596). (Ten cross-linking efficiency ratios can be specified but the other six can be derived from this irreducible set of four ratios.) Since Brf1(408–596) can bind to the TBP–DNA complex independently of Bdp1 and Brf1(69–365), its cross-linking efficiency in DNA complexes also containing Bdp1 and Brf1(69–365) provides the most sensitive normalization for quantitatively assessing defects of Brf1(69–365) assembly due to TBP radical substitutions (Table I, column c).

By this measure, TBP mutations S282E, E284R, E286R, L287E and V306E reduce the cross-linking efficiency of Brf1(69–365) 6- to 50-fold; S261E and D263R reduce cross-linking 3- to 4-fold. The defects are also evident when Brf1(69–365) cross-linking is compared with Bdp1 cross-linking in the presence of Brf1(408–596) (Table I, column d), and when Brf1(69–365) cross-linking is compared in the absence and presence of Brf1(408–596) (column b); TBP R299E also appears to be defective by these measures. Surprisingly, even the ratio of cross-linking efficiencies of Brf1(69–365) and Bdp1 in the absence of Brf1(408–596) (Table I, column a) showed an ~2- to 3-fold effect of hTBPm3 mutations S282E, E286R, L287E, R299E and V306E, despite the co-dependence of assembly of Bdp1 and Brf1(69–365). Apparently, these mutations either allow some residual assembly of Brf1(69–365) in an orientation/conformation that cross-links less efficiently, or assembly of Bdp1 in an orientation that cross-links more efficiently. It is also noteworthy that the H277E mutation appears to reduce the relative cross-linking of Bdp1 (Table I, column a). This deficit is also apparent when the cross-linking efficiency of Bdp1 assembled in the absence of Brf1(408–596) is specified relative to cross-linking of Brf1(408–596) or Brf1(69–

Table I. Quantitative analysis of cross-linking and transcription

	(a) N-Brf1 [N] Bdp1 [N]	(b) N-Brf1 [N] N-Brf1 [N + C]	(c) N-Brf1 [N] C-Brf1 [N + C]	(d) N-Brf1 [N] Bdp1 [N + C]	(e) Transcription Brf1 [N + C]	(f) Relative transcription Brf1 [N]/[N + C]
wt	(1)	(1)	(1)	(1)	(100)	0.54
K254E	1.24 ± 0.19	1.18 ± 0.31	0.78 ± 0.18	1.13 ± 0.21	ND	ND
S261E	0.84 ± 0.11	0.39 ± 0.11**	0.31 ± 0.06**	0.41 ± 0.08*	190	0.26 ± 0.03*
D263R	1.39 ± 0.32	0.28 ± 0.12**	0.25 ± 0.02**	0.31 ± 0.03**	44*	0.27 ± 0.02*
R265E	0.84 ± 0.12	0.62 ± 0.21	0.78 ± 0.19	0.97 ± 0.11	ND	ND
P267E	0.78 ± 0.11	0.97 ± 0.31	0.87 ± 0.04	1.53 ± 0.28	ND	ND
R269E	0.78 ± 0.20	0.63 ± 0.14	0.49 ± 0.12*	1.06 ± 0.32	105	0.44 ± 0.01
E271R	1.95 ± 0.35	1.51 ± 0.38	1.42 ± 0.39	2.36 ± 0.42*	82	0.57 ± 0.04
L275R	1.05 ± 0.23	1.60 ± 0.01	1.20 ± 0.30	1.38 ± 0.28	101	0.65 ± 0.08
H277E	3.48 ± 0.08**	1.09 ± 0.07	0.69 ± 0.06	1.59 ± 0.21	13**	0.49 ± 0.05
Q278E	1.43 ± 0.02	0.52 ± 0.19	0.58 ± 0.11	0.93 ± 0.16	83	0.45 ± 0.05
A279E	0.84 ± 0.20	1.20 ± 0.24	0.80 ± 0.16	0.91 ± 0.29	ND	ND
F280E	0.85 ± 0.12	1.89 ± 0.29	1.15 ± 0.22	1.34 ± 0.27	119	0.44 ± 0.01
S282E	0.44 ± 0.13*	0.36 ± 0.07**	0.17 ± 0.08**	0.19 ± 0.09**	95	0.15 ± 0.03**
E284R	0.93 ± 0.05	0.19 ± 0.07**	0.06 ± 0.00**	0.17 ± 0.02**	79	0.07 ± 0.00**
E286R	0.53 ± 0.01	0.12 ± 0.03**	0.03 ± 0.00**	0.11 ± 0.00**	80	0.05 ± 0.01**
L287E	0.36 ± 0.06**	0.08 ± 0.06**	0.02 ± 0.02**	0.03 ± 0.02**	50*	0.08 ± 0.03**
K297E	0.93 ± 0.30	1.28 ± 0.30	1.60 ± 0.35	1.20 ± 0.30	ND	ND
R299E	0.49 ± 0.00*	0.39 ± 0.19**	0.57 ± 0.22	0.37 ± 0.06**	127	0.29 ± 0.02
V306E	0.35 ± 0.12**	0.20 ± 0.00**	0.10 ± 0.01**	0.09 ± 0.02**	149	0.21 ± 0.03**
V317E	0.87 ± 0.24	1.51 ± 0.21	1.49 ± 0.17	1.19 ± 0.31	157	0.48 ± 0.02
R318E	0.72 ± 0.23	1.15 ± 0.17	2.07 ± 0.29*	1.02 ± 0.29	166	0.50 ± 0.05
A319R	0.80 ± 0.26	0.52 ± 0.09	0.42 ± 0.14*	0.48 ± 0.15*	118	0.49 ± 0.02
Y322E	0.95 ± 0.11	0.95 ± 0.02	0.41 ± 0.00*	0.40 ± 0.00**	164	0.31 ± 0.02
E323R	0.77 ± 0.11	0.72 ± 0.18	0.53 ± 0.02	0.57 ± 0.04	100	0.41 ± 0.01
E326A	0.85 ± 0.21	1.05 ± 0.12	1.28 ± 0.23	1.09 ± 0.24	ND	ND
N327E	1.46 ± 0.32	0.96 ± 0.32	1.38 ± 0.36	0.95 ± 0.26	ND	ND
P330R	1.05 ± 0.32	1.37 ± 0.13	1.99 ± 0.08	1.48 ± 0.31	ND	ND
K333E	1.25 ± 0.33	1.27 ± 0.31	1.92 ± 0.42	1.60 ± 0.27	ND	ND
G334R	1.15 ± 0.30	1.36 ± 0.39	1.32 ± 0.38	1.52 ± 0.29	ND	ND
R336E	1.37 ± 0.34	1.61 ± 0.14	1.77 ± 0.36	2.00 ± 0.25*	ND	ND
K337E	1.34 ± 0.40	1.37 ± 0.39	2.06 ± 0.40*	1.84 ± 0.37	ND	ND
T338R	1.21 ± 0.38	1.35 ± 0.12	1.58 ± 0.31	1.77 ± 0.36	ND	ND

To assess the relative cross-linking effectiveness of the N-terminal part of Brf1 and to obtain a quantitative measure for defects in complex assembly for each TBP mutant, relative cross-linking efficiencies, specified as internally standardized ratios of reactivity, were determined. Cross-linking reactivities of Brf1(69–365) ('N-Brf1'), Brf1(408–596) ('C-Brf1') and Bdp1(138–594) ('Bdp1') have been quantified after complex formation either in the absence ([N]) or presence ([N + C]) of Brf1(408–596) (Figure 1C), expressed as the ratios specified above columns a–d, and normalized to the respective wild-type ratio (first row); average deviations of two independent experiments are also specified. The four ratios in columns a–d are independent variables; six additional (dependent) ratios can be determined from this set (of four ratios). Significantly decreased or increased (** ≥ 2.5-fold; * ≥ 2-fold) relative cross-linking efficiencies, identifying TBP mutants that are aberrant for protein assembly, are highlighted. Columns e and f summarize the quantitative analysis of transcription. Column e: the absolute transcriptional activity of each TBP mutant, determined in the presence of both Brf1(69–365) and Brf1(408–596), is normalized to wild-type TBP (100%). Column f: transcriptional activity of TFIIB complexes lacking Brf1(408–596) relative to complexes containing both Brf1 fragments. The corresponding ratio for wild-type TBP is 0.54. Averages and average deviations of two determinations are shown.

365) in the presence of Brf1(408–596) (a 3- to 5-fold effect; divide the value in Table I, column c or b, respectively, by the value in column a).

Surface mutations on the C-proximal lobe of TBP that reduce transcriptional activity

TFIIB complexes containing only the N-terminal half of Brf1 are nearly fully active for TFIIC-independent transcription of supercoiled DNA (Kassavetis *et al.*, 1998a). We exploited this property of Brf1 in a transcription assay to identify TBP mutations that impair function. TFIIB–DNA complexes containing hTBPm3, Bdp1 and Brf1(2–365) were assembled with or without Brf1(408–596) for transcription of supercoiled plasmid pU₆boxB, whose nearly symmetric U6 snRNA gene TATA box generates divergent transcripts (U6_R and U6_L; Figure 2) (Whitehall *et al.*, 1995). Under the conditions of the assay, TFIIB–DNA complexes assembled with wild-type hTBPm3, Bdp1 and limiting Brf1(2–365) generated

approximately half the transcriptional activity of TFIIB–DNA complexes also containing Brf1(408–596) (Figure 2; Table I, column f, first row). This value served as the benchmark for examining the effects of a subset of 19 TBP mutations on transcriptional activity in the same assay (Table I, column f). The compensatory effect of Brf1(408–596) on transcription is shown in Table I, column e. Eight mutations generated ≥ 2-fold defects in transcription directed by TFIIB assembled with Brf1(2–365): S261E, D263R, S282E, E284R, E286R, L287E, R299E and V306E. Provision of the C-terminal Brf1(408–596) fragment compensated for the transcriptional defects of all these mutants (Table I, column e) (but less than completely for TBP D263R and L287E). The outcome of the transcription analysis strikingly mimics the results of the protein interaction assay: mutations with the most significant defects in complex formation also exhibited the most notable functional deficiencies (compare Table I, columns f, b and c).

Fig. 2. Transcription analysis. TFIIB–DNA complexes containing hTBPm3 [wild type (wt), or mutants thereof, as indicated], Brf1(2–365) and Bdp1 were assembled in the absence (N) or presence (N/C) of Brf1(408–596) for transcription of supercoiled plasmid pU₆boxB. The divergent transcripts of this template (Whitehall *et al.*, 1995), designated U₆_R (rightward) and U₆_L (leftward), are specified at the right margin. Results of the quantitative analysis are summarized in Table I. rm, the recovery marker used for normalization.

These experiments show that surface mutations on the C-proximal lobe of TBP, particularly on the TFIIB-interaction surface at the C-terminal stirrup, which interfere with the interaction between TBP and the TFIIB-related part of Brf1, also reduce transcriptional activity directed by an N-terminal fragment of Brf1. Thus, while Brf1 forms its stable complex with TBP predominantly through the C-terminal homology region II, the N-terminal (TFIIB-related) part contributes structurally and functionally in a way that is comparable to the TFIIB–TBP interaction.

A different kind of transcriptional defect is generated by the H277E mutation. In this case, it is transcription with Brf1(2–365) and Brf1(408–596) that was found to be severely defective (Table I, column e), but the residual activity was not notably affected by withholding Brf1(408–596) (column f). The high efficiency of Brf1 N-terminal fragment cross-linking relative to Bdp1 cross-linking (Table I, column a) suggests a defect in Bdp1 recruitment. This was further examined by analyzing transcription under conditions of limiting Bdp1. As shown in Figure 3, complexes formed with TBP H277E required 4- to 8-fold higher concentrations of Bdp1 to achieve their near-maximal activity compared with wild-type hTBPm3 and TBP L275R. (Additional control titrations showed that TBP E271R, F280E and R336E also behaved like the wild-type and L275R mutant proteins.) We conclude that the H277E mutation interferes with the assembly of Bdp1 into the TFIIB-promoter complex. A radical mutation at the close-by amino acid 271 (E271R) generated less extremely elevated cross-linking of N-Brf1 relative to Bdp1 (Table I, column a), also suggesting an effect on interaction with Bdp1, but this was not reflected in defective transcription (column e) or in a change of apparent affinity for Bdp1 (examined in an experiment like that in Figure 3; data not shown).

Discussion

Brf1 and TFIIB interact with the C-terminal lobe of TBP in a functionally and structurally equivalent manner

The high degree of sequence similarity of the general pol II transcription factor TFIIB and the N-proximal domain of

Fig. 3. A Bdp1 recruitment defect: The Bdp1 concentration dependence of transcription directed by TFIIB assembled with Brf1(2–365), Brf1(408–596) and wild-type or mutant hTBPm3. Transcription directed by wild-type hTBPm3 (squares), TBPm3 L275R (circles) and TBPm3 H277E (X) is shown, separately normalized to its maximum value (with 200 fmol Bdp1 for the wild-type and L275R proteins, and with 400 fmol Bdp1 for TBPm3 H277E).

Brf1 suggests that they might bind to the TBP–DNA complex in a similar way. However, experiments to determine the orientation of this N-proximal Brf1 segment relative to the TBP–DNA complex are complicated by the tethering that is imposed by the stable interaction of Brf1's C-terminal homology segment 2 with the N-terminal convex surface and side of TBP: the existence of this dominant TBP-binding site in Brf1 makes it difficult to infer the properties of weaker interactions. Failure to take this dominance into account may have been at the root of prior suggestions, based on experiments with intact Brf1, that the C-terminal stirrup of TBP either does not interact with Brf1 or that this interaction does not play a significant role in transcription (Colbert *et al.*, 1998; Zhao *et al.*, 2003).

We have confronted this issue by taking advantage of the fact that: (i) Brf1 can be split to separate the high affinity TBP-binding C-terminal half from the N-terminal half; (ii) the N-terminal half of Brf1 directs accurately initiating transcription in the absence of the C-terminal half; and (iii) both halves of Brf1 together assemble into a

Fig. 4. A model of the complex of yeast Brf1 amino acids 76–273 with TBP, based on the structures of the human TFIIB–TBP–DNA and *P.woesei* TFB–TBP–DNA ternary complexes (PDB codes 1C9B and 1D3U). **(A)** The alignment of two Brf1, TFIIB and TFB segments that interact with the C-terminal stirrup of TBP. The TBP amino acids known to interact with human TFIIB are shown above the alignment and TBP amino acids known to interact with the archaeal TFB are shown below the alignment. Archaeal TBP residues are numbered according to their human counterparts. H-bonds to specific TBP residues, indicated by vertical dotted lines, are color-coded: blue, unique to the structure of the hTFIIB complex; green, unique to the structure of the archaeal TFB complex; red, unique to the model of the Brf1 complex; orange with black frame, common to the model of the Brf1 complex and one or both of the structures of the TFB and TFIIB complexes. The alignment of the entire Brf1 segment is presented in Supplementary figure 1. **(B)** The model. Left: threading of Brf1 (green) into TFIIB (backbone axis in red). The locations of 9 amino acids in the C-terminal stirrup of TBP (blue) whose mutations generate notable effects on protein–DNA cross-linking and transcription are specified. H277 (magenta) is proposed to interact with Bdp1. Center and right: the key H-bond interactions of the TBP–TFIIB structure and the TBP–Brf1 model are highly similar. Backbone axes are traced for TBP (blue) and the relevant TFIIB (red) and Brf1 (green) segments. Hydrogen bond donor (light blue balls)–acceptor (red balls) pairs are emphasized, and the corresponding pairs of amino acids are identified. The side chains of these amino acids and of other TBP residues implicated in Brf1 and Bdp1 interaction are also shown.

highly stable TFIIB–DNA complex that is indistinguishable from the TFIIB complex formed with intact Brf1 (Kassavetis *et al.*, 1998a). We have also taken advantage of a human TBP library with radical amino acid substitutions distributed over 91 surface residues (Bryant *et al.*, 1996), and have designed a three-probe site-specific photochemical cross-linking assay that allows detection and quantification of protein–DNA interactions in complexes that are not stable to electrophoretic separation in gels.

Our analysis shows that TBP radical substitutions S282E, E284R, E286R, L287E and V306E, all in or adjacent to the C-terminal stirrup of TBP, substantially reduce the Bdp1-dependent association of Brf1(69–365) with the TBP–DNA complex; S261E, D263R and R299E reduce the binding of Brf1(69–365) more modestly (Figure 1; Table I); the locations of these residues are shown in Figure 4, and are further discussed below. The same TBP substitutions substantially reduce the transcriptional activity of TFIIB–DNA complexes in the absence of the stabilizing effect of Brf1 homology region 2

(Figure 2; Table I). Three of these TBP residues, E284, E286 and L287, also play a critical role in binding TFIIB, with individual amino acid substitutions resulting in 10- to 50-fold lower binding affinities (Bryant *et al.*, 1996; Tang *et al.*, 1996).

We conclude from these results that TFIIB and the N-proximal domain of Brf1 share a common binding site on TBP, and that the resulting orientation of the N-proximal half of Brf1 is functionally relevant for the transcription factor activity of TFIIB. Our conclusions are supported by the following lines of evidence.

The C-terminal stirrup of TBP is a shared binding site. The three TBP radical mutations in the C-terminal stirrup that decrease the affinity of TBP for TFIIB, namely E284R, E286R and L287E (Bryant *et al.*, 1996), are also the most defective in assembling the TFIIB-related domain of Brf1 (Table I, column c). Two of the other five radical substitutions that diminish Brf1(69–365) assembly, S282E and V306E, involve amino acid side chains in, or adjacent to, the C-terminal stirrup of TBP. This is direct evidence for a common binding site.

Although the assembly of the TFIIB-related domain of Brf1 and Bdp1 into the TBP–DNA complex is co-dependent, cross-linking of Brf1(69–365) in our three-probe assay is more affected by radical substitutions at S282, E286, L287 and V306 than is cross-linking of Bdp1 (Table I, column a). It is known that the N-terminal half of Brf1 (and not Bdp1) cross-links efficiently to base pair –28 of the non-transcribed strand (Kassavetis *et al.*, 1998a); base pair –28 is oriented to cross-link to any protein that interacts with the C-terminal stirrup of TBP, and yeast TFIIB likewise cross-links efficiently to the same DNA site (G.A.Kassavetis, unpublished observations).

Effects on transcription. The evidence indicates that the orientation of the N-terminal half of Brf1 in the TFIIB–DNA complex remains the same whether or not it is co-assembled with the C-terminal half or present as intact Brf1. The N-terminal half of Brf1, Bdp1 and TBP form a TFIIB–DNA complex that is fully functional for transcription of *SNR6* (the U6 snRNA gene) as supercoiled DNA (Figure 2; Kassavetis *et al.*, 1998a). TFIIB–DNA complexes lacking the N-terminal half of Brf1 are transcriptionally nearly inert. Thus, the N-terminal half of Brf1 maintains the interactions that are necessary for binding pol III and initiating transcription in TFIIB–DNA complexes lacking the C-terminal half of Brf1. This conformation is destabilized by single mutations in the C-terminal stirrup of TBP, resulting in a 7- to 10-fold decrease in transcription. The C-terminal half of Brf1 stabilizes this conformation, since it increases the cross-linking efficiency of the N-terminal half of Brf1 to the base pair –28 site (Kassavetis *et al.*, 1998a). The C-terminal Brf1 segment also substantially or completely repairs the transcriptional defect generated by the S282E, E284R, E286R and L287E mutations (Table I, compare columns e and f). The starkly contrasting ability of a E284R/E286R/L287E triple TBP mutant to function for transcription in the presence of intact Brf1 (Shen *et al.*, 1998) reflects the very high local concentration of the TFIIB-related N-terminal half of Brf1 when tethered by the more stably TBP-bound C-terminal half. Interactions with Bdp1 also hold the N-terminal half of Brf1 in place: TBP residue H277, which lies just above the C-terminal stirrup (Figure 4B), appears to be involved in the assembly of Bdp1 (Table I; Figure 3). This is consistent with a role for Bdp1 in stabilizing and constraining the interaction of the TFIIB-related domain of Brf1 with the C-terminal TBP stirrup. Bdp1 also stabilizes intact Brf1 in the TFIIB–DNA complex: TFIIB binds to a Brf1–TBP–DNA complex (presumably displacing the N-terminal domain of Brf1), but does not bind to a TFIIB–DNA complex (Colbert *et al.*, 1998).

Architecture of the initiation complex. The orientations of TFIIB, the TFIIB-related half of Brf1, and TFB (their archaeal homolog) in their respective promoter complexes must be similar, because their Zn ribbon domains execute similar functions through interactions with structurally related domains of their cognate RNA polymerases. Deletions and point mutations in the N-terminal domains containing the zinc ribbon motif and adjacent sequence of Brf1, TFIIB and archaeal TFB display two similar properties: a defect in binding the cognate polymerase and an alteration in transcriptional start site selection. Mutant Brf1 and TFIIB proteins display a third property: a

transcriptional defect that occurs at a step subsequent to polymerase recruitment (Pinto *et al.*, 1994; Bushnell *et al.*, 1996; Pardee *et al.*, 1998; Cho and Buratowski, 1999; Hawkes and Roberts, 1999; Ranish *et al.*, 1999; Bell and Jackson, 2000; Hahn and Roberts, 2000; Kassavetis *et al.*, 2001).

Modeling Brf

The effects of mutations in the TBP C-terminal stirrup on assembly of Brf1(2–365) into the TFIIB–DNA complex can be rationalized by modeling the TFIIB-related domain of Brf1 onto the structure of the TFIIB–TBP–DNA complex (Nikolov *et al.*, 1995) (Figure 4). Residues 76–273 of Brf1 have been modeled into human TFIIB and its archaeal homolog, *Pyrococcus* TFB, in their respective ternary complexes with TBP and DNA [Schwede *et al.*, 2000; Brookhaven Protein Data Bank (PDB) entries 1C9B and 1D3U, respectively; Littlefield *et al.*, 1999; Tsai and Sigler, 2000]. The amino acid sequence alignment used for threading also aligns the secondary structure of Brf1 with the 10 TFIIB helices in the 1C9B structure (Supplementary figure 1, available at *The EMBO Journal* Online). The amino acids forming H-bonds with the C-terminal stirrup of TBP in the TFIIB and TFB complex structures and in the Brf1 model are identified in Figure 4A. The left panel of Figure 4B is a ribbon-style representation of Brf1(76–273) (green) modeled into the structure of the human TFIIB (red backbone trace)–TBP (blue)–DNA (not shown) ternary complex. TBP residues implicated in interaction with Brf1(69–365) are space-filled in dark blue; H277, which is implicated in Bdp1 interaction, is in pink. The alignment between Brf1 and human TFIIB generates a 2-amino acid insertion between TFIIB helices BH5 and B1', and a 3-amino acid insertion between helices BH1' and BH2' (Supplementary figure 1), which does not distort the comparable conformations of Brf1 and TFIIB. The middle panel of Figure 4B highlights polar and ionic hydrogen bonds between TFIIB and TBP; the right-hand panel highlights TBP residues implicated in Brf1 and Bdp1 interaction in this work and potential hydrogen bonding between Brf1 and TBP. The relevant hydrogen bonds of the TFIIB and TFB complexes and the Brf1–TBP complex model are specified in Supplementary table I.

Three of the four TBP residues in the C-terminal stirrup whose mutants substantially decrease binding of Brf1(69–365), S282, E284 and E286, are engaged in side chain-specific polar/ionic bonds with Brf1 in the modeled structure; the fourth TBP residue, L287, makes van der Waals contacts with two Brf1 residues. The S282 side-chain can form a polar bond with the ϵ -amino group of Brf1 K166; the corresponding TFIIB residue, K200 (Figure 4A), makes an alternative main chain polar bond. TBP E284 can form an ionic bond with Brf1 R135, the counterpart of TFIIB R169. Brf1 cannot form the equivalent hydrogen bond between TBP E286 and TFIIB K188, but can form an alternative side chain-specific ionic bond between TBP E286 and Brf1 R219; a polar bond, TBP E286–TFB S246, is formed at the equivalent location of the archaeal complex (Figure 4A). Two additional hydrogen bonds in the TFIIB–TBP–DNA complex can also be maintained by Brf1: the side-chain-specific TBP Q278–TFIIB S205 bond is retained in TBP

Q278–Brf1 T171; TBP K337, which forms an ionic bond with TFIIB D243, can re-orient to form an alternative ionic bond with Brf1 E216. The detrimental effect of TBP V306E can also be readily explained in the modeled Brf1–TBP–DNA complex, which predicts a like-charge clash with Brf1 E216.

Three other Brf1 binding-defective mutations on the lateral surface of TBP (S261E, D263E and R299E) are not validated in the modeled structure and can be presumed to impair other interactions. These residues lie in the vicinity of Brf1 residue 439 in the crystal structure of the Brf1 homology region 2–TBP–DNA complex (Juo *et al.*, 2003) and may interact with the bridging amino acid 274–365 segment of Brf1(69–365). Cross-linking by the Y322E mutant TBP is also somewhat aberrant: low relative cross-linking of Brf1(69–365) (Table I, column c) and of Bdp1 in the presence of both Brf1 segments (column d). Amino acid Y322 interacts with Brf1 homology region II (Juo *et al.*, 2003). The Y322E mutation has the potential to stabilize binding by the C-terminal Brf1 fragment (Table I, column c) and co-stabilize Bdp1 (column d). The A→R mutation at the nearby residue 319, which also contacts the C-terminal Brf1 fragment, generates similar effects on cross-linking.

It is also worth noting that the transcription factor NC2 (Dr1/Drap1) has been shown to repress certain pol II genes by specifically blocking the TFIIB interactions site of TBP (Goppelt *et al.*, 1996; Cang *et al.*, 1999; Kamada *et al.*, 2001) and to inhibit pol III transcription by interfering with the assembly of Brf1 (White *et al.*, 1994). NC2β helix H25 contacts TBP R299 and H277 and should interfere with the assembly of both Bdp1 and Brf1 on the C-terminal lobe of TBP. Both NC2α and NC2β would interfere with the binding of Brf1 C-terminal homology region 2 to the N-terminal lateral surface of TBP.

We have also modeled human Brf1 and human Brf2 into the structure of the human TFIIB–TBP–DNA complex (analysis not shown). Human Brf1 forms all the hydrogen bonds proposed for yeast Brf1, except for TBP K337–yBrf1 E216 (hBrf1 S221); however, a mere 1.5 Å displacement of the unstructured backbone between TFIIB helices BH2' and BH3' would allow this bond to form. Human Brf2, which clearly interacts with the C-terminal stirrup of TBP (Zhao *et al.*, 2003), proved difficult to model because the amino acid sequence of its second TFIIB-related repeat diverges so significantly. A reasonable alignment based on sequence and the predicted secondary structure of Brf2 is provided by looping out 11 amino acids between helices BH5 and BH1' (between the two globular repeat domains of TFIIB; Supplementary figure 1) and 20 amino acids between helices BH3' and BH4' (at the surface of the second globular repeat domain of TFIIB). This structure also maintains the potential hydrogen bonds of yeast Brf1. The loop introduced between BH5 and BH1', which protrudes near TBP helix H1', would clearly be a candidate for additional interactions with TBP and/or Bdp1; the large loop introduced between helices BH3' and BH4' would clearly be a candidate for interaction with SNAPc.

In examining the modeled structures, it becomes clear that TFIIB has the potential for forming alternative hydrogen bonds to the C-terminal stirrup of TBP. For example, S249 of human TFIIB can maintain the same

hydrogen bond with TBP E286 that is made by S246 of archaeal TFB. The potential for alternative interactions may be a prerequisite for transcriptional activation at certain promoters. In this regard, a view of the TFIIB–TBP–DNA complex as a static and unique structure makes it difficult to envision how the modest E186D mutation in yeast TBP (human E284D) would debilitate transcription at some pol II promoters in an upstream activation sequence-dependent process (Virbasius *et al.*, 2001), unless some rearrangement of interaction between TFIIB and the C-terminal stirrup of TBP were required.

Brf1 and DNA binding

If Brf1 can maintain most or all of the hydrogen bonding of the TFIIB–TBP complex, why does stable association of its N-terminal half with TBP and DNA require Bdp1? Clearly, base-specific and non-specific TFIIB–DNA interactions, both upstream and downstream of the TATA box (Lagrange *et al.*, 1996, 1998; Tsai and Sigler, 2000), contribute significantly to the stability of the TFIIB–TBP–DNA complex. The binding affinity of the specific TFIIB–TBP (binary) complex has not been measured, and it would not be surprising to find that it is relatively low. [Exploratory experiments (L.S.Martel and A.J.Berk, unpublished) have indicated a weak interaction. An early study—the only published work that we know of—in which the TFIIB–TBP interaction was examined in the absence of DNA (Ha *et al.*, 1993), pointed to the formation of an aberrant complex since it did not involve the C-terminal stirrup of TBP.] It is possible that the N-terminal half of Brf1 does not interact significantly with DNA either alone or in the presence of Bdp1. This would be consistent with finding that two-dimensional hydroxyl radical DNA footprinting does not (readily) distinguish between TFIIB–DNA complexes containing full-length Brf1 and the C-terminal half of Brf1 (Colbert *et al.*, 1998). We suggest that the protein–protein interactions of the N-terminal half of Brf1 and TFIIB with TBP are of very similar strengths. Additional DNA contacts of TFIIB stabilize its TBP–DNA complex, and the strong protein interaction site in the C-terminal half of Brf1 (Juo *et al.*, 2003) stabilizes the Brf1–TBP–DNA complex.

Materials and methods

Proteins

hTBPm3 and its mutants were purified as described previously (Bryant *et al.*, 1996). Pol III, Brf1(2–365), Brf1(69–365), Brf1 (408–596), Bdp1 and Bdp1(138–594) were purified and assayed as cited previously (Kassavetis *et al.*, 2001). Brf1 fragment purifications followed their intact Brf1 counterpart.

Photochemical cross-linking

The U6 gene-derived 59 bp photochemical cross-linking probes containing 5-[N-(p-azidobenzoyl)-3-aminoallyl]-deoxyuridine monophosphate at three specific sites were generated as described previously (Kassavetis *et al.*, 1998a) with the oligonucleotides shown in Figure 1A. Protein–DNA complexes were formed for 60 min at 21°C in 20 µl volume containing 40 mM Tris–Cl (pH 8.0), 7 mM MgCl₂, 3 mM β-mercaptoethanol, 5% (v/v) glycerol, 100 µg/ml bovine serum albumin and 45 mM NaCl. Standard reaction mixtures contained 2 fmol of each photoprobe (–39/–38^N, –33^N, –28^N; 6 fmol total), 200 ng poly(dG–dC), 100 fmol hTBPm3 (or mutant thereof), 225 fmol Brf1(69–365), 100 fmol Brf1(408–596) when present, and 300 fmol Bdp1(138–594). UV irradiation and nuclease treatment prior to electrophoresis were carried out as described previously (Bartholomew *et al.*, 1995), with the

following modifications: cross-linked complexes were digested with DNase I (8 U DNase I; 10 min at 37°C), denatured for 3 min at 95°C and quickly chilled on ice. Samples were then digested with micrococcal nuclease (10 U; 10 min at 37°C), mixed with SDS sample buffer, boiled and loaded onto 13% SDS polyacrylamide gels. Dried gels were analyzed by phosphorimage plate analysis. The internal ratios of Brf1(69–365), Bdp1 and Brf1(408–596) cross-linking in Table I were used to characterize the interactions of individual TBPm3 mutants. Note that cross-linking efficiencies of Brf1(69–365) were not normalized to cross-linking of TBPm3, since the concentration of the Brf1(69–365) was limiting for formation of TFIIB–DNA complexes lacking the Brf1(408–596) fragment. TBPm3 binds independently of Brf1(69–365) but not vice versa; in consequence, the relative efficiency of Brf1(69–365) and of Bdp1 is expected to increase for TBPm3 mutants that fail to bind stoichiometrically to the DNA probe.

Transcription

TFIIB–DNA complexes were formed for 60 min at 20°C in 20 µl of reaction buffer containing 40 mM Tris–HCl, pH 8.0, 7 mM MgCl₂, 3 mM dithiothreitol, 100 µg/ml bovine serum albumin, 8% (v/v) glycerol, 50 mM NaCl with 100 ng supercoiled pU₆boxB DNA (Whitehall *et al.*, 1995), 100 fmol hTBPm3 (or mutant thereof), 300 fmol Brf1(2–365), 100 fmol Brf1(408–596) (as indicated), 300 fmol Bdp1 and 10 fmol pol III. [Note that Brf1(2–365) was used in place of Brf1(69–365) because the N-terminal Zn ribbon region is essential for transcription in the absence of the C-terminal half of Brf1 (data not shown)]. Multiple rounds of transcription at 20°C were started by adding 5 µl of reaction buffer containing 1 mM ATP, 1 mM CTP, 1 mM GTP and 125 µM [α -³²P]UTP, and stopped after 30 min by adding 155 µl stop solution (10 mM Tris–HCl, 3 mM EDTA, 0.2% SDS). Samples were precipitated, processed for denaturing gel electrophoresis and quantified by phosphorimage plate analysis.

Modeling of Brf1

Saccharomyces cerevisiae Brf1 was modeled into the structures of the human TFIIB–TBP–DNA complex (PDB entry 1C9B) and the *Pyrococcus woesei* TFB–TBP–DNA complex (PDB entry 1D3U) at the Swiss Model Server (<http://www.expasy.org/swissmod/SWISS-MODEL.html>), and the orientation of side chains was analyzed and/or optimized for hydrogen bonding with Swiss-Pdb Viewer (Guex and Peitsch, 1997); <http://us.expasy.org/spdbv/>). The automated alignment of Brf1 to TFB and TFIIB PDB entries by Swiss Model closely matched independent alignments (Clustal_W 1.81) from diverse Brf1, TFIIB and TFB sequences (16 each) and was used for threading Brf1 into individual TFB and TFIIB structures. Human Brf1 was similarly modeled into the TFIIB structure. Modeling of human Brf2 into the TFIIB structure was based on an alignment of 10 vertebrate and urochordate Brf2 sequences (both complete and partial) relative to Brf1 and TFIIB (16 each) and the predicted secondary structure of Brf2. Both analyses suggested that Brf2 contains additional sequence, conserved among Brf2s, that significantly expand the separation between TFIIB helices BH5 and BH1', and also between helices BH3' and BH4'. Secondary structure predictions were performed at the META PredictProtein Server (<http://cubic.bioc.columbia.edu/pp/>). RasMol 2.7.1 (<http://www.bernstein-plus-sons.com/>) was used to generate Figure 4B.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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