Role of the Inhibitory DNA-Binding Surface of Human TATA-Binding Protein in Recruitment of Human TFIIB Family Members

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TATA box recognition by TATA-binding protein (TBP) is a key step in transcriptional initiation complex assembly on TATA-box-containing RNA polymerase (Pol) II and III promoters. This process is inhibited by the inhibitory DNA-binding (IDB) surface on the human TBP core domain (TBP_{CORE}) and is stimulated by promoter-specific basal transcription factors, such as two human TFIIB family members, the Pol II factor TFIIB and the Pol III factor Brf2, which is required for transcription from TATA-box-containing Pol III promoters. In contrast, the third TFIIB family member, Brf1, which is required for transcription from TATA-less Pol III promoters, does not stimulate TBP binding to the TATA box. We show here that in addition to its role in regulating TBP binding to a TATA box, the TBP IDB surface is unexpectedly involved in TBP association with all three TFIIB family members. Interestingly, the loss of IDB function has specific and diverse effects on each TFIIB family member. Indeed, the IDB and prototypical TFIIB contact surfaces of TBP, which lie on opposite sides of the TBP_{CORE}, cooperate to form the wild-type TFIIB-TBP-TATA box complex. These results reveal how, through differential usage of opposite surfaces of the TBP_{CORE}, TBP can achieve versatility in the assembly of Pol II and Pol III promoter complexes with TFIIB family proteins.

The accurate assembly of basal factors on an appropriate promoter region is critical for the correct control of the initiation of transcription (4, 10, 16, 22). Basal factors such as the TATA-binding protein (TBP), which recognizes the TATA box core promoter element, and TFIIB, which associates with TATA-box-bound TBP, play key roles in the assembly of the preinitiation complex.

TBP is unique among basal factors in that it is the only known basal factor that is involved in transcription by all three eukaryotic nuclear RNA polymerases—Pol I, Pol II, and Pol III—from promoters with and without a TATA box (10). Consistent with its central role in transcription, TBP contains a highly conserved and structured 180-amino-acid C-terminal core domain, illustrated in Fig. 1A and called TBP_{CORE}. On TATA-box-containing Pol II and Pol III promoters, TBP_{CORE} binds the TATA box and unwinds the DNA to form an unusually bent TBP_{CORE}-TATA box complex (11, 13, 14, 20). The TBP-induced bent DNA structure plays an important role in the assembly of the correct preinitiation complex. In addition to the conserved TBP_{CORE}, TBP also contains an N-terminal region that is highly divergent in both sequence and length among different species.

In humans, full-length TBP recognizes the TATA box through a regulated two-step mechanism (39). It initially binds the TATA box to form an unstable complex of low abundance containing unbent DNA and then slowly forms a stable complex containing bent DNA. During this two-step process, the N-terminal region of human TBP works together with an inhibitory DNA-binding (IDB) surface on the TBP_{CORE} to pro-

mote formation of the unbent complex. TFIIB overcomes the inhibitory effects of the IDB surface upon TBP DNA binding, greatly accelerating formation of a bent TFIIB-TBP-TATA box complex.

In contrast to the general involvement of TBP in nuclear transcription, TFIIB is restricted to Pol II transcription; two human TFIIB-related proteins, Brf1 (BRF/TFIIB90) and Brf2 (BRFU/TFIIB50), are involved in human Pol III transcription, as illustrated in Fig. 1B and C (26, 37). Brf1 promotes transcription from the gene-internal Pol III promoters in humans and yeast, whereas the human Brf2 protein, for which there is no known yeast homolog, is involved in transcription from Pol III promoters with gene-external promoter elements, such as the human U6 small nuclear RNA (snRNA) gene promoter, for which there are no known counterparts in yeast (27, 32). The basal elements of the evolutionarily restricted human U6 snRNA promoter are a proximal sequence element (PSE), which recruits a protein complex called SNAP_C/PTF (25, 26, 38), and a TATA box, which recruits TBP; SNAP_C and TBP bind cooperatively on the human U6 promoter DNA (19).

To date, TFIIB, Brf1, and Brf2 constitute the human TFIIB protein family (Fig. 1B). TFIIB contains an N-terminal region with a structured zinc ribbon (8, 41) and a structured C-terminal core domain (TFIIB_{CORE}) (1, 21, 35). TFIIB_{CORE} binds cooperatively to the second stirrup region of the saddle-shaped TBP_{CORE} on the TATA box, and the N-terminal region of TFIIB recruits a Pol II/TFIIF complex to the promoter (22). Brf1 and Brf2 contain (i) N-terminal conserved TFIIB-like zinc ribbon and core domains and (ii) unrelated C-terminal regions (26).

Unlike TFIIB, Brf1 largely utilizes its unique C-terminal region to recognize and form a stable complex with TBP (6, 36), although a weak association between the N-terminal re-

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FIG. 1. Structure of human TBP_{CORE} and the human TFIIB family proteins and requirement of the TFIIB family members for corresponding promoters. (A) Molecular surface representation of the promoter-bound human TBP_{CORE} viewed from upstream of the TATA box (20). Residues in red and yellow are in the center and on the border, respectively, of the IDB surface of TBP, and residues in dark blue are not a part of the IDB surface. Residues in cyan are on the TFIIB interaction surface in the second stirrup region of TBP (21, 35). Bent DNA is shown in green. (B) Schematic structure of the human TFIIB family members TFIIB, Brf1, and Brf2 (26). The locations of the structured zinc (Zn) ribbon (green) and core domain (blue) of TFIIB and those of the corresponding regions in the other proteins are indicated. The purple boxes in the Brf1 structure indicate conserved regions II and III between human and yeast Brf1. (C) Differential requirement of TFIIB, Brf2, and Brf1 for four model Pol II (AdML and U1 snRNA) and Pol III (U6 snRNA and adenovirus VAI) promoters along with the characteristics of these promoters (18, 27, 32, 36).

gion of Brf1 and TBP exists (12, 23). Consistent with these findings, a Brf1 contact surface of TBP differs from the TFIIB contact surface and overlaps the IDB surface; this surface is involved in transcription from Brf1-dependent Pol III promoters (6, 28, 40). Interestingly, Brf2 shares features with both TFIIB and Brf1 in its interaction with TBP. On the one hand, Brf2 behaves like TFIIB because they both utilize the conserved TFIIB core domain for association with TBP (5) and

recognize the second stirrup region of TBP similarly to initiate transcription from Pol III and Pol II promoters, respectively (40). On the other hand, Brf2 and Brf1 recognize a shared residue on the IDB surface of TBP (5, 6, 28). Nevertheless, although full-length human TBP TATA box recognition is inhibited by the TBP IDB surface (39) and stimulated by TFIIB and Brf2 (5, 17, 22, 40), the specific role, if any, of the IDB surface and its function in TBP interaction with the TFIIB family proteins are unknown.

Here we have analyzed the effect of an extensive set of human TBP IDB surface mutations on recruitment of the human TFIIB family proteins to the TATA box. We show that the complete IDB surface is involved in recruitment of all three TFIIB family proteins, although surprisingly, the role is different for each TFIIB family member. Indeed, remarkably, even though the IDB surface and TFIIB contact surface of TBP are located on opposite sides of the TBP_{CORE} (Fig. 1A), they both promote TBP association with TFIIB and cooperate to form the wild-type TFIIB-TBP-TATA box complex. These results reveal the versatility of the human TBP and TFIIB family proteins in the assembly of Pol II and Pol III promoter preinitiation complexes.

MATERIALS AND METHODS

Protein synthesis and preparation. Glutathione *S*-transferase (GST) fusions to full-length wild-type and mutant human TBP, TFIIB, and Brf1 molecules as well as a 6× His-tag fusion to human Brf2 were synthesized in *Escherichia coli* (27, 39). All full-length single-amino-acid-substitution TBP mutants, wild-type TBP and R188E mutant TBP with the N-terminal 157 amino acids deleted (TBP_{CORE} and TBP_{CORE/R188E}) have been described previously (39). All combined TBP amino acid substitutions were generated de novo by oligonucleotide site-directed mutagenesis of a wild-type TBP coding sequence. The nucleotide sequence of the entire coding sequence of all TBP molecules was determined. The GST fusion to the human TFIIB_{CORE} (residues 106 to 316) expression construct will be described elsewhere (T. Tubon and W. Herr, unpublished results).

Protein preparations were done as described previously, with the GST moiety of the GST fusion protein removed by proteolysis (27, 39). Importantly, the purified TBP proteins were not normalized by any activity assay, such as for DNA-binding activity or transcriptional activity, but rather by determination of molecular quantity by Coomassie staining after sodium dodecyl sulfate-polyacrylamide gel electrophoresis and by immunoblot analysis with the N-terminal-specific monoclonal antibody SL30 (24) for full-length wild-type and mutant TBP molecules and with a C-terminal polyclonal TBP antibody (Geneka Biotechnology) for full-length and N-terminally truncated TBP molecules (39). To ensure the accuracy of protein purification and normalization procedures, we examined each normalized mutant TBP molecule by transcription from a set of four promoters in vitro, and each exhibited the wild-type level of activity for transcription from at least one of the four promoters (40), excluding the possibility that inactive samples are due to denatured TBP molecules. Preparation of SNAP_C synthesized in baculovirus was done as described previously (9).

DNA probe preparation and electrophoretic mobility retardation assay. DNA probe preparation and electrophoretic mobility retardation assays were performed as described previously (39). DNA probes for electrophoretic mobility retardation assays were generated by PCR amplification of the human U6 (positions -70 to -14) and AdML (positions -38 to -17) promoter regions by use of 5'-³²P-labeled universal (USP) and reverse (RSP) sequencing primers as described previously (39). The sequences of the human U6 promoter region (positions -70 to -14) and the AdML promoter region (positions -38 to -17) are tatgcttaccgttaactgaaagtatttcgttctgctTATATAtctgtggaa and gggggcTAT AAAAGgggggtggg, respectively, with the TATA boxes indicated by uppercase letters. All the binding reactions were performed at 30°C for 30 min. For the TBP-TFIIB family protein binding reactions, the amount of TBP protein used for each probe preparation was that determined to provide optimal formation of the unbent TBP-TATA box complex (TBP_{FL} complex). In general, 4 to 20 ng (4.4 to 22 nM) of TBP and 50,000 cpm (0.2 to 1 nM) of ³²P-labeled DNA probes were used. The formation of the TBP_{FL} complex in these studies is consistent

with the maximum levels of TBP-TATA box complex formation reported previously (3, 30). The amount of the TFIIB family proteins used was that determined to provide maximum cooperative binding with TBP. By use of Coomassie staining after polyacrylamide gel electrophoresis and comparison to bovine serum albumin titration, we estimate that the molar ratios of TFIIB, Brf2, and Brf1 over TBP are roughly 60:1, 12:1, and 2.5:1, respectively. Compared to the TBP_{FL} complex, the significantly increased formation of the TFIIB-TBP-TATA box complex indicates that a large fraction of active TBP molecules do not bind to DNA in the binding reaction of TBP alone with DNA, which is consistent with the view of the intrinsically weak DNA-binding activity for full-length human TBP (3, 30). For the TBP-SNAP_C binding reactions, the amounts of the wild-type TBP and SNAP_C proteins used were those determined to provide maximum cooperative binding.

RESULTS

Figure 1A shows a molecular surface representation of the promoter-bound human TBP_{CORE} viewed from upstream of the TATA box (11, 20). Residues in red (e.g., R188) are in the center of the IDB surface and play the most important role in IDB function; residues in yellow are less important and are on the border of the IDB surface; and residues in dark blue lie outside of the IDB surface (39). In addition, residues in cyan (e.g., E286) on the opposite side of TBP represent the known human TFIIB contact surface in the second stirrup region of TBP, as previously determined structurally and functionally (3, 21, 30, 31, 35). Figure 1B shows the described structures of the human TFIIB family proteins, and Fig. 1C illustrates the differential use of the TFIIB, Brf2, and Brf1 proteins on four model Pol II and Pol III promoters analyzed previously (40) along with the known characteristics of these promoters.

Selective effects of IDB surface mutations of human TBP on interaction with the human TFIIB family proteins. We examined the effects of amino acid substitution of residues (i) in the center, (ii) on the border, and (iii) outside of the IDB surface (39) in full-length human TBP on TBP interaction with TFIIB, Brf2, and Brf1. (Note that, in this report, we use the word "interaction" to denote "action on each other," which may be direct or indirect, and use the word "contact" to denote "physical touching.") As shown in Fig. 2, we assayed TBP interaction with TFIIB (panel A), Brf2 (panel B), and Brf1 (panel C) on TATA-box-containing DNA by electrophoretic mobility retardation assays. For each assay, the even-numbered lanes contain the added TFIIB family member. Simple examination of the three panels in Fig. 2 reveals the strikingly selective effects of IDB surface mutations on TFIIB family member interaction with the TBP-TATA box complex, because the odd-numbered lanes lacking TFIIB family protein generate the same pattern in each panel, as they are identical samples, and the even-numbered lanes generate very different patterns, thus producing the very different overall look for each panel in the figure.

In detail, as expected (40), none of the three TFIIB family proteins alone bound to the human U6 promoter DNA probe (Fig. 2, lanes 2). Full-length wild-type TBP bound to the TATA box to form an unbent complex of low abundance that we refer to as the TBP_{FL} complex (lanes 3 and 21). Individually, the addition of TFIIB (Fig. 2A), Brf2 (Fig. 2B), and Brf1 (Fig. 2C) retarded the mobility of the TBP_{FL} complex, forming TFIIB-, Brf2-, and Brf1-specific TBP-TATA box complexes (compare lanes 4 to lanes 3 and lanes 22 to lanes 21).

As described previously, mutations on the IDB surface of TBP led to the formation of an abundant and bent TBP-TATA

box complex that we refer to as the TBP_{FL}* complex (Fig. 2, compare lanes 9, 11, 13, 15, 17, and 19 to lanes 3 and 21) (39). The TBP_{FL}* complex has been demonstrated previously to be TATA box specific (39). Surprisingly, each of the four central IDB mutations exhibits the same effect upon association with a TFIIB family member, but the effect differs for each TFIIB family member. With TFIIB, all the mutations on the IDB surface led to formation of the same robust complex of non-wild-type mobility (Fig. 2A, compare lanes 10, 12, 14, 16, 18, and 20 to lanes 4 and 22; see arrowhead). These TBP mutant TFIIB-TBP-TATA box complexes possess a faster electrophoretic mobility than the wild-type TFIIB-TBP-TATA box complex. In addition, both the radical and alanine mutations on the IDB surface led to formation of the mutant TFIIB-TBP-TATA box complex, with the same altered electrophoretic mobility (data not shown).

With Brf2 and Brf1, the central set of four IDB mutations displayed deleterious effects, albeit different effects for each of these two proteins. They prevented Brf2-TBP-TATA box complex formation without affecting TBP_{FL}* complex formation (Fig. 2B, compare lanes 14, 16, 18, and 20 with lanes 13, 15, 17, and 19, respectively). In stark contrast, however, Brf1 had a large effect on activated DNA binding caused by the IDB mutations: it greatly inhibited TBP_{FL}* complex formation by these IDB-defective mutants (Fig. 2C, compare lanes 14, 16, 18, and 20 with lanes 13, 15, 17, and 19, respectively).

Mutations on the border of the IDB surface showed selective effects on TBP association with Brf2 and Brf1. The R205E mutation had little, if any, effect on Brf2 and Brf1 complex formation (Fig. 2B and C, compare lanes 10 to lanes 4 and 22), whereas the V240D mutation prevented Brf2 and Brf1 association with the TBP-TATA box complex (compare lanes 11 and 12). In conclusion, the IDB surface is differentially involved in the recruitment of the TFIIB family of proteins to TATA-box-containing DNA in the assembly of the preinitiation complex.

The effects of mutations on the IDB surface of human TBP on interaction with the human TFIIB family of proteins were observed for both the Pol III human U6 promoter TATA box (Fig. 2) and the Pol II adenovirus major late (AdML) promoter TATA box (data not shown), indicating that the human TBP IDB surface interacts with the TFIIB family of proteins similarly for these two TATA-box-containing Pol II and Pol III promoters. These two TATA boxes possess very different flanking sequences (see Materials and Methods). Thus, the role of the human TBP IDB surface in recruitment of the TFIIB family of proteins is probably independent of specific TATA-box-flanking sequences.

Mutations on the IDB surface of human TBP do not affect its interaction with the human snRNA promoter-specific factor SNAP_C. In contrast to human TBP interaction with TFIIB family members, the IDB surface mutations have little, if any, effect on cooperative binding with another basal transcription factor, SNAP_C, as shown in Fig. 3. As described previously, SNAP_C alone binds to the human U6 promoter PSE to form a SNAP_C-DNA complex (lane 2) of low abundance (19). TBP binds to the U6 TATA box to form both the unbent TBP_{FL} and the bent TBP_{FL}* complexes (lane 3) (39). As reported previously, with the higher TBP concentrations used in this assay, for unknown reasons TBP forms two TBP_{FL}*-like complexes (40). As expected, TBP bound to U6 promoter DNA cooper-

FIG. 2. Differential effects of mutations of the IDB surface of TBP on its interaction with TFIIB, Brf2, and Brf1. Electrophoretic mobility retardation analyses of full-length wild-type and mutant TBP-molecule interaction in the presence (even-numbered lanes) and absence (odd-numbered lanes) of TFIIB (A), Brf2 (B), and Brf1 (C) on the human U6 promoter. Three classes of TBP mutations that were located (i) outside the IDB surface (K181E and E191K; lanes 5 to 8), (ii) on the border of the IDB surface (R205E and V240D; lanes 9 to 12), and (iii) in the center of the IDB surface (R188E, K236E, R239E, and K243E; lanes 13 to 20) were analyzed. The TBP-TATA box complexes with unbent and bent DNA are labeled TBP_{FL} and TBP_{FL}*, respectively. The arrowhead indicates the IDB surface-specific mutant TFIIB-TBP-TATA box complexes with a characteristic altered electrophoretic mobility. *, nonspecific complex.

FIG. 3. Mutations on the IDB surface of TBP do not affect its interaction with the snRNA promoter-specific factor SNAP_C . Electrophoretic mobility retardation analysis of full-length wild-type (lanes 3 and 4) and mutant (lanes 5 to 8) TBP-molecule interaction in the presence (even-numbered lanes) and absence (odd-numbered lanes) of SNAP_C on the human U6 promoter. Complexes described in the text are labeled on the left.

actively with SNAP_C and led to formation of the SNAP_C -TBP-TATA box complex, with increased abundance and slightly retarded electrophoretic mobility compared to those of the SNAP_C -DNA complex (compare lane 4 to lanes 2 and 3) (19). In contrast to its effects on interactions with the TFIIB family proteins, the R188E mutation in the center of the IDB surface did not show any evident effect on TBP interaction with SNAP_C , as did the non-IDB surface mutation K181E (compare lanes 4, 6, and 8). Thus, of the two human U6 promoter TBP-interacting proteins Brf2 and SNAP_C , only Brf2 interacts with the IDB surface of TBP, indicating that the IDB-defective effects on TFIIB family member interaction with TBP on DNA are not universal among basal transcription factors.

The nonconserved N-terminal region of human TBP is not essential for the role of the IDB surface on TBP_{CORE} interaction with TFIIB family proteins. The wild-type IDB surface inhibits TBP DNA binding and DNA bending (39). These two properties can be separated because the N-terminal region of human TBP is necessary for formation of the unbent TBP_{FL} complex but is not necessary for the inhibition of DNA binding by the IDB surface (39). We therefore asked whether the N-terminal region of human TBP is required for the effect of the IDB surface on interaction with the TFIIB family proteins. Figure 4 illustrates TFIIB (panel A), Brf2 (panel B), and Brf1 (panel C) interaction with the full length and the core of wild-type and IDB-defective R188E mutant TBP molecules on the human U6 promoter DNA probe. Both the wild-type TBP_{CORE} and $\text{TBP}_{\text{CORE/R188E}}$ exhibited properties similar to those of the corresponding full-length TBP molecules for interaction with TFIIB and Brf2 (Fig. 4A and B, compare lanes 8 and 7 to lanes 4 and 3 and lanes 10 and 9 to lanes 6 and 5),

FIG. 4. The N-terminal region of human TBP is not essential for the role of the IDB surface on TBP_{CORE} in interaction with TFIIB, Brf2, and Brf1. Electrophoretic mobility retardation analyses of full-length (lanes 3 and 4) and N-terminally deleted (lanes 7 and 8) wild-type TBP- and R188E mutant TBP (lanes 5, 6, 9, and 10)-molecule interaction in the presence (even-numbered lanes) and absence (odd-numbered lanes) of TFIIB (A), Brf2 (B), and Brf1 (C) on the human U6 promoter DNA. *, nonspecific complex. Symbols: open circles, unbent TBP_{FL} complex; filled circles, bent TBP_{FL} * complex; open triangles, wild-type-mobility TFIIB family protein-TBP-TATA box complexes; filled triangle, altered-mobility mutant TFIIB-TBP-TATA box complex; filled diamonds, wild-type-mobility bent TBP_{CORE} -TATA box complexes; open squares, wild-type-mobility TFIIB family protein-TBP_{CORE}-TATA box complexes; filled square, altered-mobility mutant TFIIB-TBP_{CORE}-TATA box complex.

FIG. 5. N-terminal region of human TFIIB is not essential for the role of the IDB surface on TBP_{CORE} interaction with TFIIB. Electrophoretic mobility retardation analysis of full-length wild-type (lanes 4 to 6) and R188E mutant (lanes 7 to 9) TBP-molecule interaction in the presence (even-numbered lanes) and absence (odd-numbered lanes) of full-length (lanes 5 and 8) and N-terminally deleted (lanes 6 and 9) TFIIB molecules on the AdML promoter.

indicating the formation of altered-mobility full-length and core TBP_{R188E} complexes with TFIIB (compare lanes 4 and 6, and compare lanes 8 and 10). As for interaction with Brf1, Brf1 inhibited the DNA-binding activity of both full-length TBP_{R188E} and TBP_{CORE/R188E} (Fig. 4C, compare lanes 9 and 10 to lanes 5 and 6), although, as also evident in Fig. 2C, the TBP_{R188E} mutant can still bind weakly to Brf1 to form a Brf1-TBP_{R188E}-TATA box complex of low abundance (compare lanes 4, 6, 8, and 10). Therefore, in contrast to its role in unbent TBP_{FL} complex formation, the N-terminal region of human TBP is not essential for the role of the IDB surface on the TBP_{CORE} in its interaction with the three TFIIB family proteins, indicating that the role of the IDB surface in TFIIB family member interaction is an autonomous activity of the conserved TBP_{CORE}.

In addition to the human U6 promoter TATA box (Fig. 4), the same results were obtained on the AdML promoter TATA box (data not shown), indicating that the evolutionarily diverged N-terminal region has no evident influence on the IDB surface of human TBP in recruitment of the TFIIB family proteins to TATA-box-containing Pol II and Pol III promoters.

The N-terminal region of human TFIIB is not essential for interaction with the IDB surface of human TBP. The locations of the known human TFIIB_{CORE} contact and IDB surfaces of TBP—on opposite sides of TBP (Fig. 1A)—suggest that the IDB surface cannot directly contact the TFIIB_{CORE}. Full-length TFIIB, however, was used in the experiments shown in Fig. 2A and 4A, and thus the TFIIB N terminus (e.g., the zinc ribbon [Fig. 1B]) could be involved. Therefore, we asked

whether the N-terminal region of TFIIB influences TFIIB interaction with the IDB surface of TBP.

Figure 5 shows full-length TFIIB and TFIIB_{CORE} interaction with the IDB surface of TBP on the AdML promoter. Interestingly, the TFIIB_{CORE} displayed the same altered mobility effect on the TFIIB-TBP-TATA box complex with the full-length IDB-defective R188E mutant TBP molecule as the full-length TFIIB molecule did (compare lanes 5 and 8 with lanes 6 and 9). This result indicates that the N-terminal region of TFIIB has no evident influence on the interaction of TFIIB with the IDB surface of TBP (compare lanes 5 and 6, and compare lanes 8 and 9). Combined with the TBP N-terminal deletion analysis shown in Fig. 4, these results suggest that the effect of the IDB surface on TBP interaction with TFIIB is an intrinsic property of the TBP_{CORE} and TFIIB_{CORE} and probably does not result from direct contact between the IDB surface of TBP and TFIIB. Thus, the human TBP IDB surface is involved in interaction with, but does not directly contact, human TFIIB.

The IDB surface and the prototypical TFIIB contact surface of human TBP cooperate to interact with TFIIB. For interaction with TFIIB, we noticed that the altered mobility of the TFIIB-TBP-TATA box complex induced by mutations on the IDB surface of TBP (Fig. 2A) is similar to that induced by mutations in the prototypical TFIIB contact region in the second stirrup of TBP (e.g., E284R and E286R [Fig. 1A]) (40). Consistent with this similarity, both of these mutant TFIIB-TBP-TATA box complexes contain bent DNA (39; data not shown). These observations led us to test in a side-by-side comparison whether the altered-mobility complexes are related to each other and whether the IDB surface and the second stirrup region of TBP cooperate to bind TFIIB.

As shown in Fig. 6, wild-type TBP bound to DNA to form an unbent TBP_{FL} complex of low affinity (lane 3), and addition of TFIIB retarded this complex to an abundant and bent wild-type TFIIB-TBP-TATA box complex (lane 4; the nature of the unbent or bent DNA conformation in all of the complexes described for Fig. 6 was determined with DNA-bending probes [39; data not shown]). The IDB-defective R188E TBP mutation led to formation of an abundant and bent TBP_{FL}* complex (lane 5), and addition of TFIIB retarded this complex to an abundant and bent mutant TFIIB-TBP_{R188E}-TATA box complex with an altered electrophoretic mobility (lane 6; see arrowhead). The second-stirrup-region E284R TBP mutation, which has been shown to contact TFIIB in vitro (3, 21, 29, 35) and in vivo (31), resulted in formation of both unbent TBP_{FL} and bent TBP_{FL}* complexes of low affinity (lane 7) compared to the wild-type unbent TBP_{FL} complex and the R188E bent TBP_{FL}* complex, respectively (compare lane 7 to lanes 3 and 5). The addition of TFIIB retarded both the TBP_{FL} and TBP_{FL}* complexes to an abundant and bent mutant TFIIB-TBP_{E284R}-TATA box complex (lane 8) with the same altered mobility as the mutant TFIIB-TBP_{R188E}-TATA box complex (compare lane 8 to lanes 6 and 4; see arrowhead). Remarkably, the combined TBP_{R188E+E284R} mutant retained the bent TBP_{FL}* complex formation property of the TBP_{R188E} mutant (compare lanes 5 and 9), but, unlike either of the single mutants, was unaffected by TFIIB in our assay (compare lanes 9 and 10, and compare lane 10 to lanes 6 and 8). Thus, unexpectedly, TFIIB completely failed to associate with the TBP-

FIG. 6. Combined mutation of the TBP IDB surface and second stirrup region blocks TBP interaction with TFIIB. Electrophoretic mobility retardation analysis of full-length wild-type and mutant TBP-molecule interaction in the presence (even-numbered lanes) and absence (odd-numbered lanes) of TFIIB on the AdML promoter. TBP mutations on the IDB surface (R188E; lanes 5 and 6) and in the second stirrup region (E284R; lanes 7 and 8) and combined mutation (R188E+E284R; lanes 9 and 10) were analyzed. The arrowhead indicates the mutant TFIIB-TBP-TATA box complexes with an altered electrophoretic mobility. Symbols: open circles, unbent TBP_{FL} complexes; filled circles, bent TBP_{FL}^* complexes; blue triangle, wild-type TFIIB-TBP-TATA box complex; red triangles, altered-mobility mutant TFIIB-TBP-TATA box complexes.

TATA box complex only when there were mutations on both the prototypical second-stirrup TFIIB contact surface and the IDB surface of TBP.

These results emphasize the importance of the novel role of the IDB surface on TFIIB interaction. These experiments reveal that each of these two regions of TBP, which lie on opposite sides of the TATA-box-bound TBP molecule, leads to formation of a specific type of mutant TFIIB-TBP-TATA box complex (Fig. 6, arrowhead). In wild-type TBP, these two surfaces function together to promote association of the TBP-TATA box complex with TFIIB. These two regions probably function differently because the TFIIB interaction with the second stirrup region is probably a direct contact, whereas its interaction with the IDB surface is probably indirect, which may be achieved through a conformational change of TBP, TFIIB, or the DNA.

DISCUSSION

For transcription from a TATA-box-containing promoter, recognition of the TATA box by TBP and subsequent recruitment of the TFIIB family proteins are critical for building up correct Pol II and Pol III preinitiation complexes (4, 10, 16, 22). We investigated the roles of the IDB surface of human

TBP in recruitment of the human TFIIB family proteins to TATA-box-containing Pol II and Pol III promoters by the use of electrophoretic mobility retardation assays. We used this method because it permits the detection of complexes of low abundance and the separation of complexes containing different conformations and subunit compositions. By this semi-quantitative assay, we detected and analyzed qualitative effects induced by mutations in TBP.

We found that mutations on the IDB surface of human TBP display selective effects on TBP interaction with each human TFIIB family protein. Importantly, our results reflect the activities of human TBP. It is currently unknown whether the DNA binding and TFIIB family interaction properties of the human TBP IDB surface have been conserved in yeast, in which TBP has been more extensively analyzed.

Diverse roles of the IDB surface of human TBP in recruitment of the human TFIIB family proteins. The results of the mutations of the IDB surface of human TBP on interaction with the human TFIIB family proteins are striking. Essentially, the four central IDB mutations demonstrate the same specific, yet different, defect pattern for each interaction with TFIIB, Brf2, and Brf1 (Fig. 2). With TFIIB, they induce formation of a non-wild-type altered-mobility TFIIB-TBP-TATA box complex; with Brf2, they inhibit its association with the TBP-TATA box complex; and with Brf1, they permit inhibition of the activated TBP binding to the TATA box. These activities, especially the responses of TFIIB and Brf1 to the IDB mutations, are surprising. The IDB mutations could influence the DNA-binding affinity or specificity of TBP or could affect TFIIB family member interaction with DNA. Discrimination among these and other explanations will require further analysis.

Whatever the case, these results suggest that for the assembly of preinitiation complexes, as key polymerase-specific bridging factors between TBP and Pol II or Pol III, TFIIB, Brf2, and Brf1 differentially interact with a specific surface of a shared target in the transcriptional apparatus, namely the TBP IDB surface. Thus, during evolution, the TFIIB family members maintained their association with the highly conserved TBP molecule but altered the ways in which they interact with it, perhaps, in this way, enhancing the variety of regulatory strategies in different promoter contexts.

In contrast to its diverse effects on interaction with the human TFIIB family members, the central IDB mutation R188E had no evident effect on cooperative interaction with the human Pol II and Pol III snRNA promoter-specific factor $SNAP_C$ (Fig. 3). Therefore, not all basal transcription factors use the IDB surface in cooperative binding with TBP on TATA-box-containing DNA. Interestingly, the IDB surface overlaps with the Pol II-specific TFIIA contact surface of TBP (3, 7, 29); it will be interesting to determine the role, if any, of the TBP IDB surface in its interaction with Pol II-specific basal factors such as TFIIA.

In addition to its independent function on the human Pol II U1 and Pol III U6 snRNA promoters and its interaction with basal factors such as $SNAP_C$ and TFIIA, in human cells TBP also exists in many different types of stable multisubunit complexes, including the Pol II-specific TFIID and B-TFIID complexes and the Pol III-specific TFIIB complex (16, 22, 26, 33). The human TFIIB complex includes TBP, Brf1, and the gen-

eral human Pol III factor B" (Bdp1) and is required for transcription from human Pol III TATA-less promoters (26). Thus, the TBP-Brf1 interaction and the role of the IDB surface in this interaction likely reflect a natural mechanism of TBP function within the Pol III TFIIB complex. In contrast, TBP, Brf2, and Bdp1, which are required for U6 snRNA gene transcription, can be viewed either as a loosely associated TFIIB complex or as individual free basal transcription factors for transcription from human Pol III TATA-box-containing promoters (26, 27). In either case, the role of the IDB surface of TBP in interaction with Brf2 probably reflects a role in the assembly of the human Pol III TATA-box-containing preinitiation complex. For Pol II transcription from promoters directing the synthesis of mRNA transcripts, the IDB surface may also play a role within the TFIID complex, because the N-terminal region (2, 15, 34) and the HMG-like region (17a) of the largest TBP-associated factor, TAF1, interacts with a surface of TBP overlapping the IDB surface. It will be interesting to determine whether the IDB surface plays a specific role in TFIID function in addition to a role in TFIIB recruitment.

Two surfaces of human TBP are involved in TFIIB recruitment. Although crystallographic studies have demonstrated that TFIIB_{CORE} contacts the second stirrup region of TBP_{CORE} (21, 35), we found that the entire IDB surface of TBP affects full-length TBP interaction with full-length TFIIB (Fig. 2). The fact that the N-terminal regions of TBP and TFIIB are not required for this effect (Fig. 4 and 5) surprisingly suggests that the interaction between the IDB surface of TBP and TFIIB is indirect. Interestingly, mutations of both the IDB surface and the second stirrup region of TBP individually led to formation of the same aberrant-mobility TFIIB-TBP-TATA box complex. The formation of mutant TFIIB-TBP-TATA box complexes with the same altered electrophoretic mobility is unlikely, owing to dissociation of the wild-type TFIIB-TBP-TATA box complex during electrophoresis, because dissociation of a complex during electrophoresis will result in smeared or absent bands. In our case, the unique electrophoretic mobility of the altered-mobility complex is specifically induced by mutations of the IDB and the second stirrup surfaces of human TBP (Fig. 6). We conclude that these mutant complexes may possess a distinct conformation. Our previous studies (39) demonstrated that the DNA-bending angle of the IDB-defective R188E mutant TFIIB-TBP_{R188E}-TATA box complex is greater than that of the wild-type TFIIB-TBP-TATA box complex. This difference, however, would make the TFIIB-TBP_{R188E}-TATA box complex migrate more slowly, not faster, as we observed. Thus, there is probably a nonbending angle effect that alters the mobility. Importantly, the combined mutations in the IDB and second stirrup TFIIB contact surfaces prevented TBP interaction with TFIIB (Fig. 6), indicating that these two surfaces of TBP cooperate for TBP interaction with TFIIB in ways that are not revealed by crystallographic studies (11, 20, 21, 35).

In conclusion, we have revealed a role for the IDB surface of free human TBP in promoting its interaction with the three human TFIIB family members. Thus, the IDB surface plays both negative (for DNA binding) (39) and positive (for TFIIB family protein interaction) (this study) roles in initiation complex formation, illustrating how a single surface of a regulatory molecule can play opposing regulatory roles. The interaction

with the TFIIB family members illustrates how a family of *trans* factors can target the same autoinhibitory surface of a key regulator to assemble different preinitiation complexes. The many modes of intermolecular interaction regulated by the IDB surface reveal a diverse range of regulatory mechanisms provided by a small surface of a key regulator of transcription.

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