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Mutational analysis of BTAF1–TBP interaction: BTAF1 can rescue DNA-binding defective TBP mutants

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ABSTRACT

The BTAF1 transcription factor interacts with TATA-binding protein (TBP) to form the B–TFIID complex, which is involved in RNA polymerase II transcription. Here, we present an extensive mapping study of TBP residues involved in BTAF1 interaction. This shows that residues in the concave, DNA-binding surface of TBP are important for BTAF1 binding. In addition, BTAF1 interacts with residues in helix 2 on the convex side of TBP as assayed in protein–protein and in DNA-binding assays. BTAF1 drastically changes the TATA-box binding specificity of TBP, as it is able to recruit DNA-binding defective TBP mutants to both TATA-containing and TATA-less DNA. Interestingly, other helix 2 interacting factors, such as TFIIA and NC2, can also stabilize mutant TBP binding to DNA. In contrast, TFIIB which interacts with a distinct surface of TBP does not display this activity. Since many proteins contact helix 2 of TBP, this provides a molecular basis for mutually exclusive TBP interactions and stresses the importance of this structural element for eukaryotic transcription.

INTRODUCTION

TATA-binding protein (TBP) is required for transcription by all three eukaryotic RNA polymerases (1). Each type of RNA polymerase employs different TBP-containing multiprotein complexes. In the case of human RNA polymerase I, TBP is complexed with three TBP-associated factors (TAFs), TAF_I48, TAF_I63 and TAF_I110 into the SL1 complex. RNA polymerase III employs the TFIIIB complex, which consists of TBP, Brf1 and Bdp1 proteins (2). RNA polymerase II (pol II) is able to use TBP embedded within two factors: TFIID and B–TFIID (2–5). TFIID is a 700 kDa complex consisting of TBP and 13 highly conserved TAFs (6). B–TFIID is composed of TBP and one protein, BTAF1 (7).

The BTAF1 protein belongs to the family of SNF2-like ATPases (8,9). The N-terminal third of the protein (~600 amino acids) was shown to contain several discrete regions, which are able to interact independently with TBP (10–14). These regions coincide with predicted pairs of HEAT/ARM repeats (5,15). BTAF1 is highly conserved throughout evolution and its *Saccharomyces cerevisiae* ortholog, Mot1p, is also found in a stable complex with TBP (16). The ATPase domain of BTAF1/Mot1p is located within the C-terminal third. Limited mutational analysis of yeast and human TBP showed that both its concave DNA-binding and convex solvent-exposed surfaces are used in BTAF1/Mot1p interaction (10–14). BTAF1 associates stably with a significant fraction of TBP in human cell lysates (17). The resulting B–TFIID complex is able to bind promoter DNA and support basal transcription *in vitro* (17,18). The Mot1p–TBP complex displays high affinity binding to a broad range of DNA sequences (19). It is not clear what the exact role of BTAF1 in the pre-initiation complex (PIC) assembly is. BTAF1 could be accommodated in the active PIC together with basal transcription factors (BTFs) and pol II. Alternatively, BTAF1 could deliver TBP to promoter DNA and subsequently dissociate from TBP as the result of competition with BTFs or upon the action of factors such as TFIIA or NC2. Interestingly, the NC2α but not

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NC2β subunit of the NC2 complex interacts with BTAF1 and stimulates BTAF1–TBP interaction (20). *In vivo* experiments in yeast demonstrate that Mot1p is present at many active promoters and is required for TBP recruitment to at least some of them (21–23). Moreover, Mot1p was shown recently to co-occupy active promoters with TFIIB and pol II under stress conditions, which led to the proposal that stress activates Mot1p–TBP complexes (24).

BTAF1 and Mot1p are also able to dissociate TBP from DNA *in vitro*, which requires their ATPase function (9,14,18). Consequently, BTAF1 and Mot1p were proposed to act as repressors of transcription. In agreement with this, several fragments of the N-terminal part of BTAF1/Mot1p can contact the concave DNA-binding surface of TBP and inhibit its binding to DNA and consequently pol II transcription (10,11). A model was proposed in which BTAF1/Mot1p dissociates TBP from DNA by inserting its N-terminal region as a 'wedge' between TBP and DNA. Alternatively, BTAF1/Mot1p could alter the conformation of TBP–DNA complexes. Several observations indicate that BTAF1/Mot1p does not act as classical DNA helicase or a DNA tracking enzyme (25–27).

To obtain insight into the function of BTAF1 in TBP activity we have analysed BTAF1–TBP interaction both off and on the DNA. Here, we report comprehensive mapping of the TBP surfaces contacted by BTAF1. The convex surface in the region of helix 2 and the concave surface of TBP are both involved in BTAF1 binding. Strikingly, DNA binding of TBP with mutations in the concave, but not in the convex surface can be rescued by BTAF1. Moreover, BTAF1 drastically changes the TATA-box specificity of TBP, as DNA-binding mutants of TBP can be rescued for the interaction with TATA-box or TATA-less DNA by addition of BTAF1. At the same time, we show that BTAF1 does not require contacts with the DNA-binding surface of TBP to disrupt TBP–DNA complexes. We compare the ability of BTAF1 to stabilize TBP–DNA complexes with other TBP-interacting proteins, TFIIA, TFIIB and NC2. Our data indicate that BTAF1 interacts with surfaces of TBP overlapping with those of TAF1, TFIIA, NC2, Brf1 and TAF_I48 and that BTAF1 shares the ability to stabilize TBP–DNA complexes with several other regulators of TBP function.

MATERIALS AND METHODS

Proteins

TBP proteins were expressed and purified as described previously (28). Briefly, plasmids carrying GST-TBP mutant cDNAs were constructed by site-directed mutagenesis of the pET11c-GST-TBP plasmid. Protein expression was induced by addition of 0.4 mM isopropyl thiogalactopyranoside to BL21(DE3) bacteria carrying appropriate GST-TBP plasmids. After lysis, proteins were bound to glutathione-agarose beads (Sigma) and the resin was washed extensively. The last wash consisted of thrombin cleavage buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 2 mM $CaCl_2$ and 1 mM EDTA). Subsequently, thrombin was added to release TBP from the GST moiety. Supernatants containing human TBP proteins were collected and protein inhibitor cocktail (Pefabloc, Roche) and DTT were added in final concentrations of 200 μg/ml and 10 mM, respectively. Samples were dialyzed against D_{100} buffer [20% glycerol, 20 mM HEPES–KOH, pH 7.9, 100 mM KCl, 0.2 mM EDTA, 2 mM DTT, 1 mM phenylmethylsulfonyl fluoride (PMSF)] and stored at −80°C. TBP protein concentrations were normalized based on Coomassie staining and immunoblotting with TBP antibodies.

Recombinant human TFIIB was purified to near homogeneity as described previously (29). Recombinant human NC2(hisα/β) complex was purified as described previously (20). Recombinant, unprocessed TFIIA protein was a kind gift of H. Stunnenberg and H. Zhou. BTAF1 protein used for co-immunoprecipitation experiments (Figure 1A) or for electrophoretic mobility shift assay (EMSA) was supplied as a lysate of RK13 cells infected with BTAF1 and T7 RNA polymerase-encoding vaccinia viruses to overexpress full-length human recombinant BTAF1 (20).

TBP–BTAF1 solution binding assay

Equal amounts of the indicated TBP proteins (50 ng) were incubated with 5 μl of BTAF1-containing RK13 cells lysate in 500 μl of binding buffer (50 mM Tris–HCl, pH 8.0, 300 mM KCl, 1 mM EDTA, 1 mM DTT, 0.01% Triton X-100, 0.5 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin and 1 μg/ml pepstatin) for 2 h on ice. Subsequently, 100 μl of 10% protein A agarose beads equilibrated in binding buffer and 1.6 μg of purified, monoclonal 1F8 anti-TBP antibodies (18) were added to the reactions. Reactions were incubated overnight with tumbling and the beads were washed three times with binding buffer. Immunoprecipitated TBP and BTAF1 were resolved using SDS–PAGE and detected by immunoblotting with specific antibodies.

Electrophoretic mobility shift assay

EMSAs were performed as described previously with small modifications (18,20,28). Radiolabelled probe consisting of adenovirus major-late promoter fragment −53 to −12 was used. The probe contained wild-type TATA-box, except in Figure 3 where the TATAAAAG sequence was replaced by CGCAAACG as indicated. Indicated proteins were incubated without the probe in 10 μl reaction volumes for 5 min at 4°C. Reaction buffer contained 20 mM HEPES–KOH, pH 7.9, 1 mM Tris–HCl, pH 8.0, 60 mM KCl, 5 mM $MgCl_2$, 500 ng/μl BSA, 10% glycerol, 0.25 mM EDTA, 7 mM DTT and 0.1% Tween-20. Subsequently, 0.3 ng of radiolabelled probe and 2 ng of poly(dG-dC):poly(dG-dC) was added and the reactions were incubated for 30 min at 30°C. In Figure 4 ATP (40 μM) was supplied 5 min before gel loading. Electrophoresis was performed as described previously (28). Intensities of specific bands were quantified using a Storm 820 PhosphorImager (Amersham) and Image QuaNT software. The ability of BTAF1, TFIIA, NC2 and TFIIB to form ternary complexes with TBP mutant proteins and DNA (Table 2) was calculated based of the titration experiments with various amounts of transcription factors, wild-type TBP and DNA.

RESULTS

TBP mutants defective in BTAF1 interaction *in vitro*

Eighty-five TBP mutant proteins containing mutations in 57 surface exposed residues were tested for the interaction with

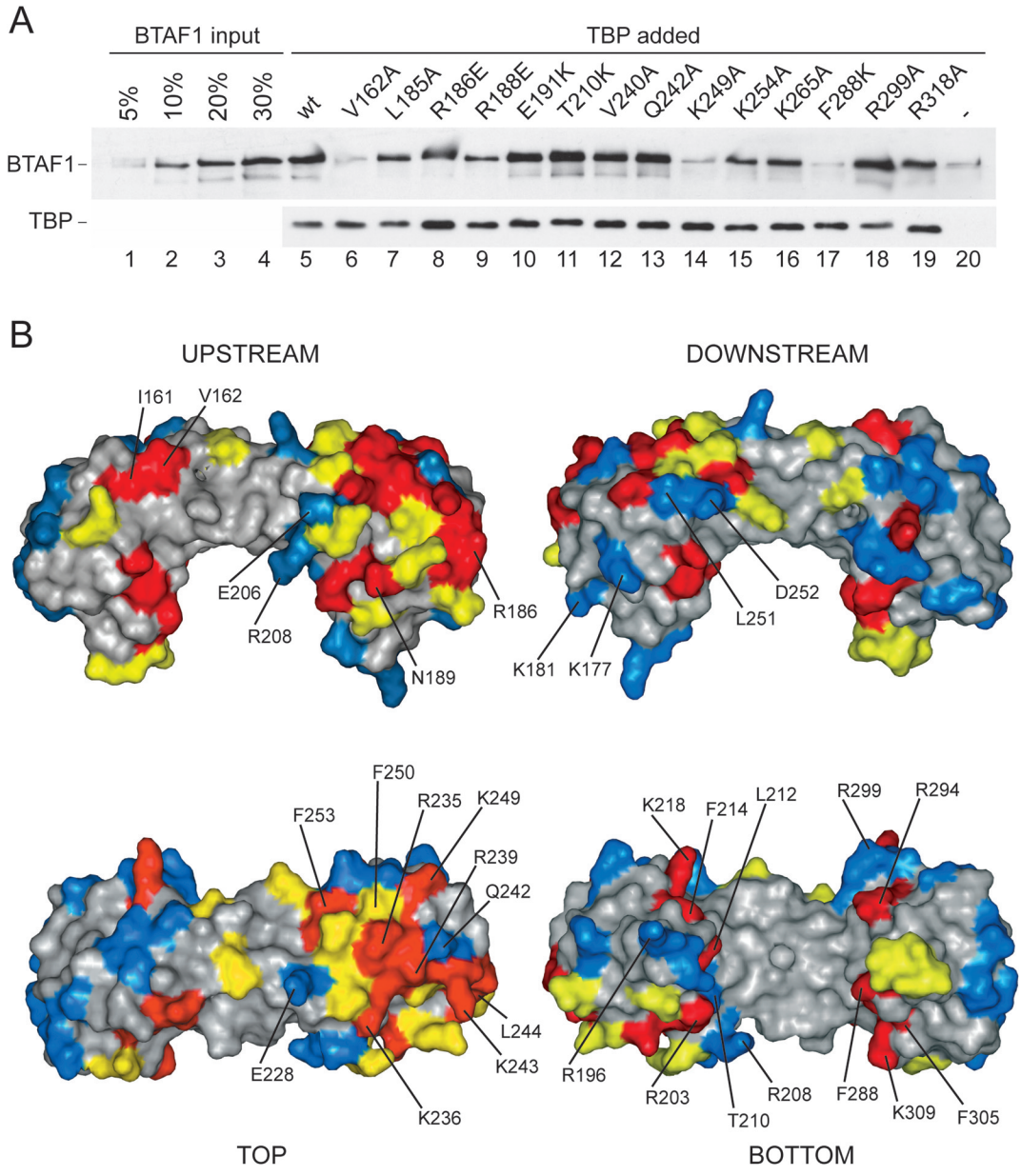

Figure 1. Interaction of BTAF1 with mutant TBP proteins. (**A**) Representative results of BTAF1–TBP interaction assay in solution. Binding reactions were performed as described in Materials and Methods. Lane 5 contained wild-type TBP (50 ng), lanes 6–19 contained indicated mutant TBP proteins (50 ng) and no TBP was added to lane 20. Lanes 5–20 contained 5 µl of BTAF1-enriched lysate. BTAF1 and TBP proteins were co-immunoprecipitated using TBP antibody. Indicated amounts of BTAF1-containing lysate were loaded as the input control (lanes 1–4). Proteins were visualized using specific antibodies. (**B**) Human TBP model (30) showing results of BTAF1–TBP interaction. Top left—view from upstream DNA; top right—view from downstream DNA (start site); bottom left—view on the top (convex) TBP surface; and bottom right—view on the DNA-binding (concave) TBP surface. Residues affected in BTAF1–TBP interaction by mutations in TBP are indicated (Table 1, column 2): red, <25% of the wild-type TBP interaction; yellow, 25–50% of the wild-type interaction; and blue, 50–100% of the wild-type interaction. Double mutations were marked in blue only if they retained 50–100% of the wild-type interaction.

the BTAF1 transcription factor. Mutants were designed to encompass various surfaces of TBP, including convex and concave sides (Figure 1B and Table 1). TBP mutants were mixed with saturating amounts of BTAF1 from cell lysates highly enriched in full-length BTAF1. BTAF1–TBP complexes were recovered with 1F8 TBP monoclonal antibody directed against residues 56–97 within the N-terminus of human TBP. Several mutants showed a complete loss of interaction with BTAF1 (Figure 1A and Table 1, column 2). Analysis of the mutants revealed that two major regions of TBP are important for interaction with BTAF1. The first region mapped to the upper side of the first TBP repeat in the area of helix 2 (Figure 1B, top view). Exposed charged residues R235, K236, R239 and K243 in helix 2 (H2) were particularly sensitive to mutagenesis, both to alanine and to opposite charge (Table 1). Additionally, some mutants (in residues L244, K249, F250 and F253) within the loop between H2 and strand 1′ (S1′) and in S1′ were also affected (Figure 1B, top view). The surface seemed to extend towards the side of TBP, since mutation of residues within the helix 1 and the loop linking H1

Table 1. Formation of the BTAF1–TBP, TBP–DNA and BTAF1–TBP–DNA complexes

TBP mutant[a]		Complex formation BTAF1–TBP[b]	TBP–DNA[c]	BTAF1–TBP–DNA[d]	BTAF1–TBP–DNA[e]/ TBP–DNA
	wild-type	+	+	+	1.0
	I161A	–	–	±	/
	V162A	–	–	+	/
	K177A	+	+	+	1.7
H1	K181E	+	±	+	4.1
H1	L185A	±	–	+	6.0
H1	L185K	+	±	+	3.4
H1	**R186A**	–	–	+	4.7
H1	**R186E**	–	+	±	0.3
	R188A	±	+	±	0.2
	R188E	±	+	–	0.0
	R188E/ T210K	–	–	–	/
	R188E/ L287A	–	+	–	0.1
	N189A	–	±	±	1.1
	N189E	–	±	–	0.4
	N189K	–	–	+	/
S2	E191K	±	–*	+	/
stirrup	K195A	+	+	+	1.3
stirrup	R196A	+	–*	+	/
S3	**R203A**	–	–*	–	/
S3	**R203E**	–	–*	+	/
	R205A	±	+	+	0.8
	R205E	±	–	–	0.9
	E206K	+	+	+	0.6
	R208E	+	–*	+	/
S4	T210A	+	–*	+	/
S4	T210K	+	–*	+	/
S4	**L212A**	–	–*	+	/
	F214A	–	–*	±	/
S5	**K218A**	–	–*	+	14.7
S5	**K218E**	–	–*	±	/
H2	E228A	+	+	+	1.7
H2	E228K	+	+	+	0.8
H2	R231A	±	n.d.	n.d.	n.d.
H2	R231E	+	+	+	0.3
H2	L232A	±	+	+	1.6
H2	L232K	+	+	±	0.5
H2	**R235A**	–	+	±	0.3
H2	**R235E**	–	n.d.	n.d.	n.d.
H2	**K236A**	–	+	±	0.3
H2	**K236E**	–	+	+	0.2
H2	**R239A**	–	+	±	0.2
H2	**R239E**	–	+	+	0.1
H2	V240A	+	±	+	3.1
H2	V240L	±	±	+	2.6
H2	**V240D**	–	±	±	0.8
H2	V240K	±	–	+	/
H2	Q242A	+	+	+	1.3
H2	Q242K	+	+	+	1.4
H2	**K243A**	–	+	–	0.1
H2	**K243E**	–	+	±	0.1
	L244K	–	–	–	/
	K249A	–	–	±	/
	F250A	+	+	+	0.7
	F250K	–	–	–	/
S1′	L251A	+	+	+	0.8
S1′	D252A	+	±	+	2.4
S1′	**F253A**	–	–	–	/
S1′	**F253K**	–	–	–	/
S1′	K254A	±	–	+	/
	K265A	±	–	+	11.0
H1′	R269A/ E271A	+	+	+	0.5
H1′	L275A	+	±	+	2.5
H1′	L275K	+	+	+	0.7
	Q279A	+	+	+	1.5
stirrup’	E284R	±	+	+	0.3
stirrup’	E284N/ E286A	±	+	+	1.5
stirrup’	E286R	±	+	+	0.4
stirrup’	L287A	±	–*	+	/
stirrup’	**F288A**	–	n.d.	n.d.	n.d.
stirrup’	**F288K**	–	–*	±	/
S3′	**R294A**	–	–*	+	/
	I296A	+	+	+	0.7
	K297A	–	–	±	/
	K297A/R299A	–	–	±	/
	R299A	+	–*	+	/
	F305A	–	–*	±	/
	F305K	–	–*	±	/
S5′	**K309A**	–	–*	+	/
S5′	**K309E**	–	–*	±	/
H2′	R318A	±	–	+	/
H2′	E320A	±	–	±	/
H2′	E323A/E326A	+	–	+	4.6
H2′	N327A	+	±	+	3.0
H2′	K333A	+	±	+	3.2
	R336A	+	±	+	3.1

[a]TBP mutants deficient in TBP–BTAF1 complex formation in co-immunoprecipitation experiment are highlighted in bold. Structural elements of TBP are indicated.
[b]Analysed in co-immunoprecipitation experiments; plus, >50% of the wild-type TBP–BTAF1 complex; plus-minus, 25–50% of the wild-type TBP–BTAF1 complex; minus, <25% of the wild-type TBP–BTAF1 complex. Results were averaged from two to four independent measurements.
[c]Analysed in EMSA; plus, >50% of the wild-type TBP–DNA complex; plus-minus, 25–50% of the wild-type TBP–DNA complex; minus, <25% of the wild-type TBP–DNA complex. Asterisk, TBP residues interacting with DNA in the crystal structure (30).
[d]Analysed in EMSA; plus, >50% of the wild-type BTAF1–TBP–DNA complex; plus-minus, 25–50% of the wild-type BTAF1–TBP–DNA complex; minus, <25% of the wild-type BTAF1–TBP–DNA complex.
[e]Ratio calculated from intensities of TBP–DNA and BTAF1–TBP–DNA complexes measured in the same experiment. Ratio was set to 1.0 for wild-type TBP. Slash, low abundance of the TBP–DNA complex prevents calculation of the ratio.
n.d., not determined.

with the stirrup (R186, N189) also affected the interaction (Figure 1B, upstream view). At the same time, mutants in the vicinity of helix 1 (K177 and K181) facing the transcription start site were not affected (Figure 1B, downstream view). This suggested that BTAF1 does not engage the whole side of the first repeat of TBP. Interestingly, the surface centred at helix 2 was encircled by residues, where mutations had limited or no effect on the interaction with BTAF1. This included residue E228 towards the centre of the upper surface of TBP (Figure 1B, top view). From the downstream-facing side the interacting surface was enclosed by non-affected residues L251 and D252 (Figure 1B, downstream view). From the upstream-facing side residues E206 and R208 were not affected by mutations (Figure 1B, upstream view). Thus, the mutagenesis delineated the specific BTAF1-binding surface on top and side of the first TBP repeat.

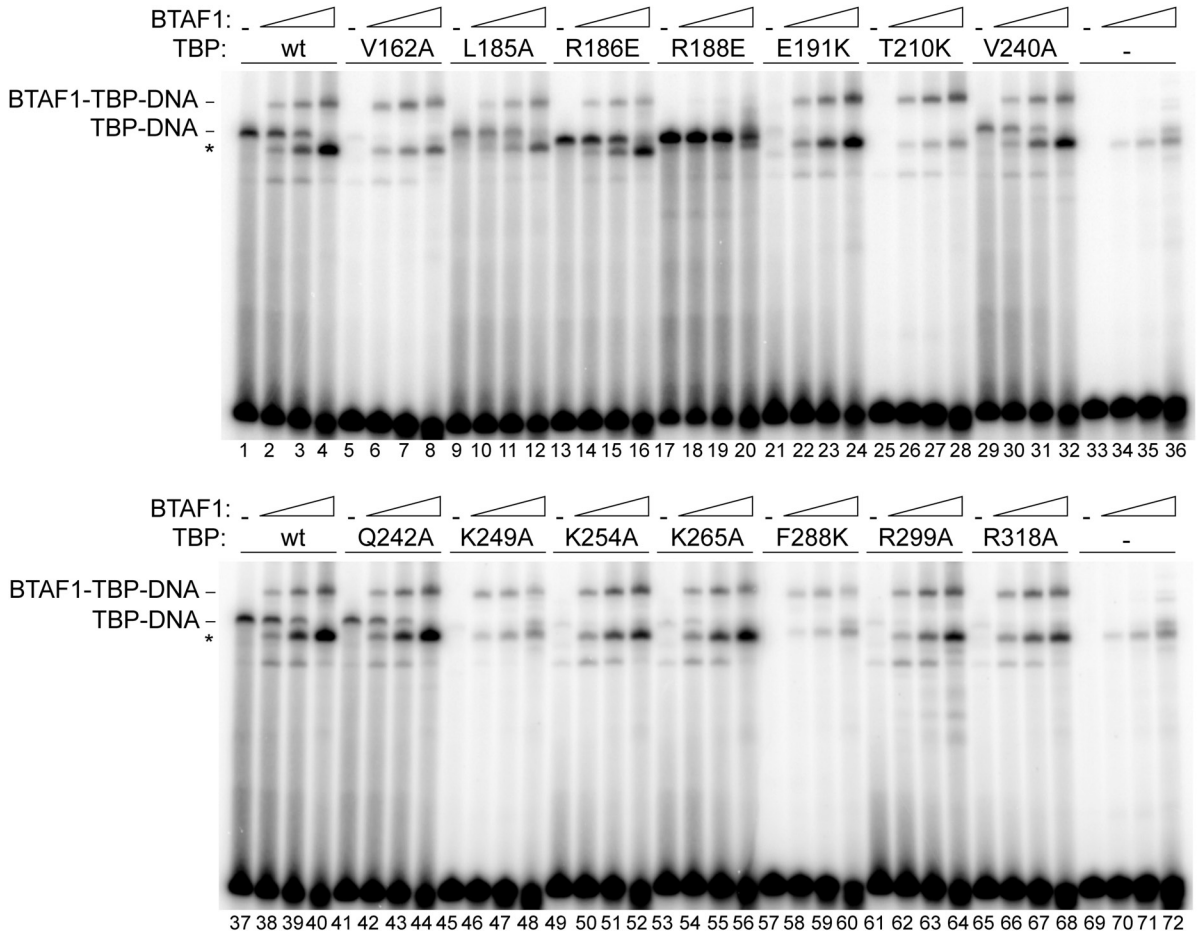

Figure 2. Analysis of the BTAF1–TBP–DNA complex formation by selected TBP mutants. EMSA reactions were performed as described in Materials and Methods with equal amounts of indicated TBP proteins (50 ng) and with the increasing amounts of BTAF1-containing lysate (0.022, 0.067 and 0.2 µl) as indicated. Lanes 33–36 and 69–72 did not contain TBP. Positions of TBP–DNA and BTAF1–TBP–DNA complexes are shown. Asterisk denotes a complex different from BTAF1–TBP–DNA originating from the BTAF1-enriched lysate.

The second TBP region, which was affected in BTAF1 binding by mutations was the DNA-binding, concave surface. The majority of residues shown to contact DNA in the crystal structure of human TBP were also sensitive to mutagenesis (Table 1) (30). These included amino acids R203, L212, F214 and K218 from the first TBP repeat and residues F288, R294, F305 and K309 from the second TBP repeat (Figure 1B, bottom view). Our mutagenesis identified only four DNA-interacting residues, which had no effect on BTAF1 interaction upon mutation. These included R196 in the first stirrup, R208 and T210 in S4 and R299 in S4′ (Figure 1B, bottom view).

The second interacting surface was not restricted to the DNA-binding region of TBP. Instead, it seemed to span upwards on the upstream-facing side of the second TBP repeat, as residues I161 and V162 were also sensitive to mutagenesis (Figure 1B, upstream view). Collectively, the second TBP region involved in BTAF1 interaction includes its concave surface and small parts on one side of the second repeat.

BTAF1 affects TBP–DNA interaction

To corroborate the results presented above, we tested the TBP mutant proteins for their ability to form ternary complexes with BTAF1 and TATA-box DNA. Results of these experiments are summarized in Table 1. As described previously, wild-type TBP was able to form a specific complex with the TATA-box (Figure 2, lanes 1 and 37) (18,20). Several mutants, such as V240A and Q242A, also formed DNA complexes with similar mobility (Figure 2, lanes 29 and 41; data not shown). A specific set of mutants (e.g. R186E and R188E) migrated slightly faster when complexed with DNA (Figure 2, compare lanes 1, 13 and 17; data not shown). Those mutants were described previously to bend DNA under similar conditions without formation of an intermediate, non-bend complex (28). The difference in mobility of TBP–DNA complexes with the previous study is probably owing to the length of DNA used. Several TBP mutant proteins were unable to form a TBP–DNA complex (Figure 2 and Table 1). These fell within two groups. The first consisted of mutants in residues involved directly in DNA binding as determined by crystallography (marked by an asterisk in Table 1) (30). The second group consisted of residues, which do not contact DNA in the crystallographic structure (30). The behaviour of these mutants may indicate changes in conformation or plasticity of TBP.

Addition of BTAF1 to the wild-type TBP resulted in the formation of the slower migrating BTAF1–TBP–DNA

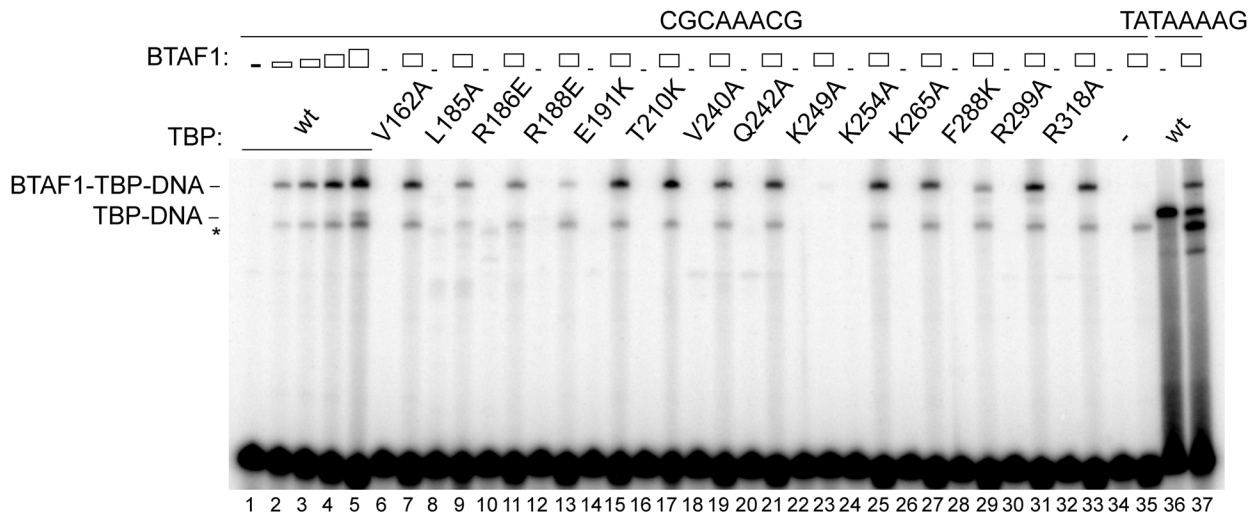

Figure 3. BTAF1–TBP–DNA complex formation on TATA-less DNA. EMSA reactions were performed as described in Materials and Methods. Lanes 1–35 contained TATA-less DNA probe (CGCAAACG), whereas lanes 36 and 37 contained TATA-box DNA probe (TATAAAAG). Various TBP mutant proteins (50 ng) were added as indicated. Lanes 2–5 received 0.017, 0.033, 0.067 and 0.2 µl of BTAF1 lysate. Odd lanes 7–37 received 0.067 µl of BTAF1 lysate. Asterisk denotes a complex different from BTAF1–TBP–DNA originating from the BTAF1-enriched lysate.

complex, consistent with the results published previously (Figure 2, lanes 1 to 4 and 37 to 40) (9,18,20). The specificity of this complex was confirmed by supershift with BTAF1 antibodies (data not shown) (20) and by sensitivity to ATP (Figure 4). Most strikingly, the pattern of BTAF1–TBP–DNA ternary complex formation did not follow the results obtained in direct BTAF1–TBP binding assay (Figure 2 and Table 1, compare columns 2 and 4). BTAF1 was able to induce DNA binding for the majority of the DNA-binding surface TBP mutants (Table 1, compare columns 3 and 4). This included also mutants defective for BTAF1 binding in solution. Only few mutants in the DNA contacting residues failed to form the ternary complex. These included a radical mutation in F288 (F288K), which has been shown to intercalate between DNA bases in the crystal structure of TBP and K309E and R203A, which contact the phosphate backbone of DNA. This indicates that BTAF1 drastically alters the DNA binding properties of TBP. In fact, BTAF1 interaction circumvents the requirement of TBP to tightly interact with DNA. Additionally, this finding is consistent with the expectation that BTAF1 does not engage the concave surface of TBP when forming the complex with DNA.

The opposite results were obtained using TBP mutants in the convex surface. In this case the efficiency of BTAF1–TBP–DNA complex formation in comparison with TBP–DNA could be determined since most of the TBP mutants in this region retained DNA-binding activity. All helix 2 mutants affected in BTAF1–TBP complex formation in solution were also severely compromised in BTAF1–TBP–DNA complex assembly when compared with wild-type TBP (Table 1, column 5). In contrast, residues in helix 2, which were able to form the BTAF1–TBP complex, such as E228 and Q242, were also competent in the formation of the BTAF1–TBP–DNA complex with a similar efficiency as wild-type TBP. These results suggest that BTAF1 engages exclusively the upper surface of TBP when it is bound to DNA.

BTAF1 alleviates TATA-box requirement of TBP

Our findings indicated that BTAF1 can stabilize TATA-binding of TBP mutants compromised in their DNA binding surface. It has been shown that Mot1p binding to TBP alters its specificity of DNA recognition (19). To extend this observation to BTAF1 we tested whether TATA-box sequences are required for BTAF1-TBP-DNA complex formation. We used a DNA probe carrying AdML promoter with TATAAAAG sequences mutated to CGCAAACG and analysed DNA binding of a set of TBP mutants. As expected, wild-type TBP was completely deficient in binding to the non-TATA DNA (Figure 3, compare lanes 1 and 36). However, addition of BTAF1 fully rescued its interaction with DNA (Figure 3, compare lanes 4 and 37). Similar to results with the TATA probe (Figure 2), binding of many TBP mutants to the non-TATA DNA could be rescued by BTAF1. The relative efficiencies of rescue did not differ significantly between the two probes. For example, R186E and F288K are less efficiently rescued when compared with wild-type TBP both on TATA and non-TATA probe. These results strengthen the observation that BTAF1 dramatically changes DNA interaction properties of TBP.

BTAF1 does not require efficient interaction with the concave surface of TBP for its dissociation from DNA

Based on observations that BTAF1 can directly contact the concave surface of TBP (Table 1) (10,11), we proposed that BTAF1 may dissociate TBP from DNA by inserting its N-terminal regions between TBP and DNA. This ATP-dependent step could require BTAF1 contacting the concave surface of TBP to disrupt its interaction with DNA. We have tested this hypothesis by selecting TBP mutants, which were defective in the formation of TBP–DNA and BTAF1–TBP, but were able to form the BTAF1–TBP–DNA complex on TATA-box containing DNA (Figure 4, lanes 1–18)

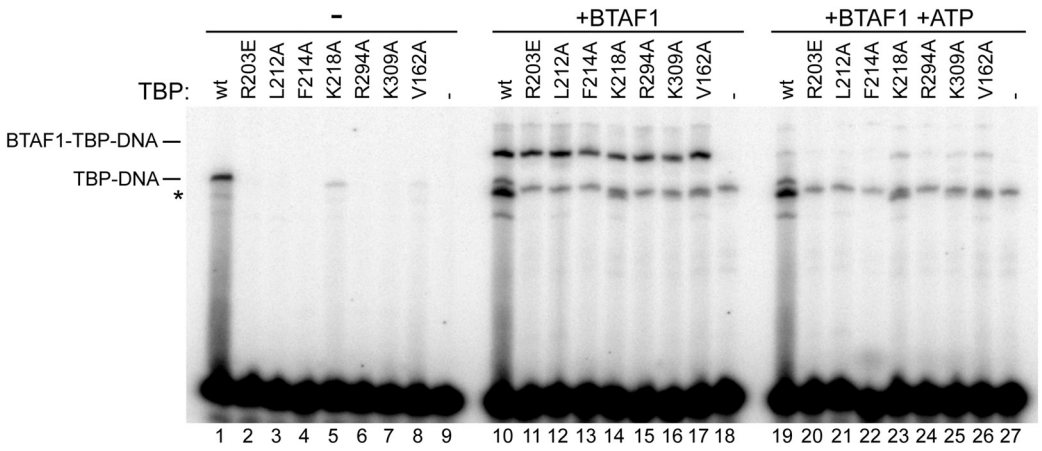

Figure 4. BTAF1 disrupts mutant TBP–DNA complexes. EMSA was performed as described in Materials and Methods using a TATA-box DNA probe. Equal amount (50 ng) of wild-type TBP or indicated mutant TBP proteins were assayed alone (lanes 1–9), with 0.067 µl of BTAF1 lysate (lanes 10–27) and in the presence of 40 µM ATP (lanes 19–27).

(Table 1). Upon addition of ATP these BTAF1–TBP–DNA complexes were disrupted with similar efficiency as the wild-type complex (Figure 4, lanes 19–27). Similar results were obtained using TATA-less DNA probe (data not shown). Thus, BTAF1 does not require binding to the concave surface residues in order to dissociate TBP from DNA.

TBP mutants specifically defective in BTAF1, TFIIA and NC2 interaction

Several TBP-interacting factors, including TFIIA and NC2, were implicated in binding to the first repeat of TBP (31–35). Therefore, we used DNA-binding assay to identify TBP mutants, which are specifically defective in the interaction with BTAF1, TFIIA, NC2 or TFIIB. Wild-type TBP was able to form complexes with BTAF1, TFIIA, NC2 and TFIIB as expected (Figure 5, lanes 1–5) (20,28). Several TBP proteins with mutations in the first repeat exhibited lack of binding to one of the factors tested (Table 2 and data not shown). For example, R188E was specifically affected in the formation of complexes with BTAF1 or NC2, but not with TFIIA and TFIIB (Figure 2, compare lanes 1–5 and 6–10). K243E mutant was affected in the formation of complexes with BTAF1 and TFIIA, but not with NC2 or TFIIB (Figure 5, compare lanes 1–5 and 11–15). The TBP(K243E)–TFIIA–TATA complex (lane 13) seems to be migrating slightly slower, but we do not consider this significant as it was not observed reproducibly (data not shown). Thus, we identified TBP mutants, which are selectively defective for BTAF1/TFIIA or BTAF1/NC2 interaction.

Next, we analysed whether similarly to BTAF1 other factors could rescue the activity of DNA-binding defective TBP mutants. This was also motivated by findings of others that binding of some yeast TBP mutants to DNA can be rescued by TFIIA or NC2 (32,35,36). We analysed our set of TBP mutants and Table 2 indicates that both TFIIA and NC2 could rescue a specific set of mutants. In contrast, TFIIB was markedly less effective in stabilizing DNA binding of TBP mutants (Table 2, column 7). Together, these data suggest that BTAF1, TFIIA and NC2 may similarly influence TBP binding to DNA, whereas TFIIB differs from the other factors tested. Only limited set of TBP mutants could be differentially

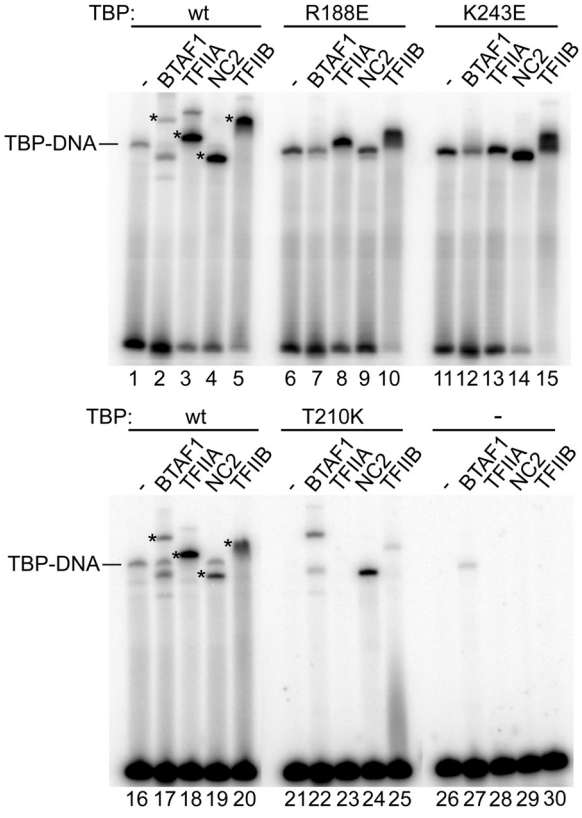

Figure 5. Analysis of the ability of selected TBP mutants to form TBP–DNA complex with BTAF1, TFIIA, TFIIB and NC2. EMSA reactions were performed as described in Materials and Methods with 50 ng of indicated TBP proteins. Reactions contained 0.1 µl of BTAF1 lysate, 0.01 µl of recombinant, unprocessed TFIIA (identical result was obtained using endogenous, HeLa cells-derived TFIIA), 3 ng of recombinant NC2 (hisα/β) (20) and 120 ng of recombinant TFIIB (10) as indicated. Asterisks on the left side of the lanes show specific complexes formed with indicated factors. Differences in mobility of specific quaternary complexes are probably owing to the faster migration of the corresponding mutant TBP–DNA complexes.

recruited to DNA by various factors. For example, T210K TBP mutant could only be rescued by BTAF1 and NC2, but not by TFIIA and TFIIB (Figure 5, compare lanes 16–20 and 21–25).

Table 2. Analysis of TBP–DNA complexes with various transcription factors

TBP mutant	Complex formation[a]					
	TBP–DNA	BTAF1–TBP–DNA	BTAF1–TBP–mDNA[b]	TFIIA–TBP–DNA	NC2–TBP–DNA	TFIIB–TBP–DNA
wild-type	+	+	+	+	+	+
V162A	–	+	+	+	±	–
L185A	–	+	–	±	–	±
R186E	+	±	–	+	±	–
R188E	+	–	–	+	–	+
N189E	±	–	n.d.	–	±	+
E191K	–	+	+	+	+	–
K195A	+	+	n.d.	+	+	+
R208E	–	+	n.d.	±	+	–
T210K	–	+	+	–	+	–
V240A	±	+	+	+	±	±
Q242A	+	+	+	+	±	±
K243E	+	±	n.d.	–	+	+
K249A	–	±	–	–	–	–
K254A	–	+	+	+	+	–
K265A	–	+	+	+	+	±
F288K	–	±	–	–	–	–
R299A	–	+	+	+	+	±
R318A	–	+	+	+	+	–

[a]Analysed in EMSA as described in Materials and Methods; plus, >50% of the wild-type TBP–DNA complex with indicated factor; plus-minus, 25–50% of the wild-type TBP–DNA complex with indicated factor; minus, <25% of the wild-type TBP–DNA complex with indicated factor.
[b]mDNA—EMSA probe bearing the mutated TATA-box (CGCAAACG).
n.d., not determined.

DISCUSSION

BTAF1 interacts with an extended surface of TBP

We have presented an extensive analysis of residues of TBP involved in binding to the BTAF1 transcription factor. Two surfaces of TBP are involved in BTAF1 interaction: upper surface around helix 2 and parts of helix 1 and the concave, DNA-binding surface. Several residues within those TBP regions have been implicated previously in BTAF1 or Mot1p binding. Residue K243 of human TBP was shown to be essential for BTAF1 binding *in vivo* (10). Similarly, single mutations K127L, K138L and K145L (K225, K236 and K243 in human TBP numbering) within the region of helix 2 of yeast TBP were shown to abolish Mot1p binding either in solution or in EMSA (11,13). Also, double mutations K133L/K138L, K133L/K145L and K138T/Y139A displayed similar lack of Mot1p binding (12,14). Likewise, the triple mutation in the concave surface of human TBP (I292F/V301T/L303V) resulted in lack of BTAF1 binding *in vivo* and in the yeast two-hybrid (10). Mutations V71E and V161E (human V169 and V259) in the concave surface of yeast TBP also prevented Mot1p interaction (11). Our analysis extends these previous results and constitutes the first extensive mapping study of the BTAF1–TBP interaction surface. Given the similarity between human BTAF1 and yeast Mot1p we expect that similar residues of yeast TBP are involved in Mot1p interaction.

Common TBP interaction surface for all TAFs

The upper TBP surface engaged by BTAF1 shows a striking overlap with the surfaces proposed to be contacted by a pol II TAF (TAF1), a pol III TAF (Brf1), a pol I TAF (TAF_I48), TFIIA and NC2 (31–35,37–40). This result bears important implications for the structural basis of the assembly of various TBP-containing complexes. It was proposed that TBP is able to form functionally unique complexes based on its mutually exclusive interactions with TAFs. The excluding interactions, which were experimentally confirmed include TAF1, TAF2 and TAF_I48 (40,41), TFIIA and NC2 (42,43), BTAF1 and NC2 (20), Mot1p and TFIIA (14). Our results provide the structural basis for the two latter results. Also, we speculate that the overlap in TBP binding surfaces of various TAFs is responsible for the formation of the B–TFIID complex, which besides BTAF1 lacks other TAFs (7).

The comparison of TBP-interacting fragments of BTAF1 with TAF1, Brf1 and TAF_I48 indicates that BTAF1 may bind TBP in a unique way. BTAF1 contacts both convex and concave TBP surfaces with ~600 residues region (10,18). Similarly, two regions within first 800 residues of yeast Mot1p were implicated in TBP interaction (11,44). These domains are probable to be structured into several HEAT/ARM repeats, which consist of two-three helices tightly bound by hydrophobic interactions (15). In contrast, TAF1, Brf1 and TAF_I48 interact with TBP by means of much shorter domains, 70–230 amino acids in length (37,38,40,45) and these bear no resemblance to HEAT/ARM repeats. Interestingly, TBP-interacting domains of TAF1 and Brf1 seem to undergo a significant reorganization of their secondary structure upon TBP binding (38,45). BTAF1 must also change its conformation in order to free the concave TBP surface for the DNA binding. At present, it is not known whether HEAT/ARM repeats of BTAF1 also undergo extensive secondary structure change or whether any changes are restricted to adjustments in the relative HEAT/ARM repeats position.

Regulation of TBP–DNA interactions through helix 2 region

We show that the DNA binding properties of TBP are influenced significantly by BTAF1, since the BTAF1–TBP–DNA complex can be formed on TATA-less DNA (Figure 3). The change in TATA-box specificity of B–TFIID is consistent with

properties previously ascribed to the yeast Mot1p–TBP complex (19). Interestingly, the affinity of Mot1p–TBP for a TATA sequences is not higher as compared with free TBP. This suggests that the binding to mutated TATA is not owing to the general increase in affinity of Mot1p–TBP or B–TFIID for DNA, but is rather a result of the changed mode of TBP–DNA interaction. Moreover, human TBP proteins with mutations in the DNA-interacting residues can be rescued for ternary complex formation with BTAF1 (Figure 3). Interestingly, our analysis indicates that TFIIA and NC2 are also able to stabilize TBP–DNA complexes of TBP mutants otherwise defective in DNA binding (Figure 5 and Table 2). This property of TFIIA and NC2 has been demonstrated before for certain DNA-binding defective yeast TBP mutants (K110L, K120L, K127L, K201L and K218L in the case of TFIIA, and K110L, L114K, K120L, L189K and K201L in the case of NC2) (32,35,36). In contrast, TFIIB does not possess such activity towards most of the mutants tested (Table 2) (36). In this respect our study represents the first comprehensive comparison of the DNA complexes formed by a uniform set of TBP mutants with four different TBP-interacting proteins.

Intriguingly, BTAF1, TFIIA and NC2 utilize the TBP region, which overlaps with the inhibitory DNA-binding (IDB) surface of TBP (28). Mutations in the IDB residues (e.g. R188, K236, R239 and K243) lead to the facilitated isomerization from a TBP–DNA complex containing unbent to bent DNA and to the stabilization of TBP binding to DNA. Moreover, the non-conserved N-terminal part of yeast TBP was proposed to interact with helix 2 of the DNA-bound TBP (46). Deletions of the N-terminus of human TBP result in enhanced DNA binding and bending (28,47). Moreover, DNA binding of some yeast TBP mutants can be rescued by removal of the N-terminus (32). In addition, the combination of deletion of the non-conserved N-terminal TBP part and mutations in the IDB region of TBP results in yeast lethality, suggesting that these two regions of TBP cooperate (48). Our results suggest that factors binding to the region of helix 2 could influence TBP-DNA interaction by a common mechanism involving actions on the IDB surface or displacement of the N-terminal part of TBP from the TBP core. Notably, TFIIB was previously shown to cooperate with the IDB surface and enhance DNA binding and bending by TBP (28,35,49). In the experimental conditions described above we do not generally detect TFIIB-mediated enhancement of TBP mutants binding to DNA (Figure 5). Therefore, ability of BTAF1, TFIIA and NC2 to recruit DNA-defective TBP mutants to DNA is an activity distinct from the one described for TFIIB. This may reflect the fact that TFIIB uses a distinct TBP surface for interaction. Since stable TBP–DNA contacts are not required for TBP recruitment to DNA in our EMSA (Figures 2 and 3), we favour the hypothesis that BTAF1, TFIIA and NC2 influence an early step in the TBP–DNA complex formation.

Mechanism of BTAF1-mediated TBP removal from DNA

Several mechanisms for ATP-dependent TBP dissociation from DNA by BTAF1 have been postulated (5,10,11,19,26). The observation that BTAF1 interacts with the DNA-binding surface of TBP in solution is central to one of them (10,11). BTAF1 has been proposed to insert its N-terminal domain between TBP and DNA, thereby dislodging TBP from DNA. Since the DNA upstream of the TBP-binding site does not seem to be absolutely required for this action (19,26), we have focused on the role of the concave TBP surface. Using a panel of mutants, we show that BTAF1 does not require stable interaction with the DNA-binding surface of TBP in order to dissociate TBP from DNA (Figure 4). Previous observations in yeast utilized Mot1p and two different DNA-binding deficient mutants of yeast TBP (K127L and spm3). However, mutant K127L is situated on the edge of helix 2, contacts DNA backbone via a water-molecule and is solvent-exposed (11,30). Another study used the relaxed specificity yTBP spm3 mutant (I194F/V203T/L205V) within the concave surface (25). A complication in the analysis of this mutant is that it is still able to interact with TATA-box DNA. Thus, our data represent the first extensive study of multiple TBP molecules with mutations buried within its DNA-binding surface. Our data suggest that a mere competition between BTAF1 and DNA is not sufficient to explain BTAF1-mediated TBP–DNA dissociation and that a more complicated mechanism is at work. It is possible that BTAF1/Mot1p interaction with DNA is involved in TBP removal (26). BTAF1 could compete with TBP for the minor groove of TBP-bound DNA. Alternatively, it could contact the major groove of DNA opposite of TBP-bound DNA and change its structure. However, the fact that Mot1p is able to disrupt a TBP–DNA complex on constrained minicircle DNA seems to disfavour the latter hypothesis (26). BTAF1 could also dissociate TBP from DNA by changing the conformation of TBP (19), without the need for direct interactions with the concave TBP surface. Interestingly, biophysical data suggest that Mot1p and TBP do not dissociate from DNA as a complex, but rather as separate proteins (19). These observations should motivate development of (biophysical) methods to investigate conformational alterations in the TBP molecule itself.

TBP mutant proteins as tools to study BTAF1 function

Our results show that the surface of TBP involved in BTAF1 binding overlaps with surfaces contacted by other factors. However, we were able to select several evolutionarily conserved residues differently affected for the binding of BTAF1, TFIIA and NC2 (Figure 5). Such mutations can be used to dissect separate functions of distinct TBP complexes both *in vitro* and *in vivo*. Most importantly, both TFIIA and NC2 were suggested to antagonize BTAF1 or Mot1p binding to TBP (14,20). By using specific TBP mutants the role of BTAF1 in TBP function could be studied without the interference from other factors.

Taken together, we present a comprehensive study of the TBP surfaces involved in BTAF1 transcription factor binding. This allowed the investigation of various aspects of BTAF1 function, such as stabilization of TBP binding to DNA and dissociation of TBP–DNA complexes. We also suggest a central role of helix 2 interacting factors, BTAF1, TFIIA and NC2, in facilitation of TBP binding to DNA.

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