Virtually unidirectional binding of TBP to the AdMLP TATA box within the quaternary complex with TFIIA and TFIIB

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Background: The TATA box binding protein (TBP) is required by all three RNA polymerases for the promoter-specific initiation of transcription. All eukaryotic TBP–DNA complexes observed in crystal structures show the conserved C-terminal domain of TBP (TBP_c) bound to the TATA box in a single orientation that is consistent with assembly of a preinitiation complex (PIC) possessing a unique polarity. The binding of TBP to the TATA box is believed to orient the PIC correctly on the promoter and can function as the rate-limiting step in PIC assembly. Previous work performed with TBP from *Saccharomyces cerevisiae* (yTBP) showed that, despite the oriented binding of eukaryotic TBP observed in crystal structures, yTBP in solution does not orient itself uniquely on the adenovirus major late promoter (AdMLP) TATA box. Instead, yTBP binds the AdMLP as a mixture of two orientational isomers that are related by a 180 degree rotation about the pseudo-dyad axis of the complex. In addition, these orientational isomers are not restricted to the 8 bp TATA box, but rather bind a distribution of sites that partially overlap the TATA box. Two members of the PIC, general transcription factor (TF) IIB and TFIIA individually enhance the orientational and axial specificity of yTBP binding to the TATA box, but fail to fix yTBP in a single orientation or a unique position on the promoter.

Results: We used an affinity cleavage assay to explore the combined effects of TFIIA and TFIIB on the axial and orientational specificity of yTBP. Our results show that the combination of TFIIA and TFIIB affixes yTBP in virtually a single orientation as well as a unique location on the AdMLP TATA box. Ninety-five percent of the quaternary TBP–TFIIA–TFIIB–TATA complex contained yTBP bound in the orientation expected on the basis of crystallographic and genetic experiments, and more than 70% is restricted axially to the 8 bp sequence TATAAAAG.

Conclusions: Although yTBP itself binds to the TATA box without a high level of orientational or axial specificity, our data show that a small subset of general TFs are capable of uniquely orienting the PIC on the AdMLP. Our results, in combination with recent data concerning the pathway of PIC formation in yeast, suggest that transcription could be regulated during both early and late stages of PIC assembly by general factors (and the proteins to which they bind) that influence the position and orientation of TBP on the promoter.

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Introduction

Gene expression in eukaryotes is regulated in large part by control of the rate of transcription initiation (for recent reviews see [1–3]). For genes that encode proteins, transcription initiation requires assembly of a class II preinitiation complex (PIC) on a core promoter. The PIC in eukaryotes consists of RNA polymerase II (pol II), Srb and Med factors, and general transcription factors (TF) TFIIA, TFIIB, TFIID, TFIIE, TFIIF, and TFIIH (reviewed in [4–6]). In higher eukaryotes, the general factor TFIID is comprised of the TATA binding protein (TBP) and at least eight additional TBP-associated factors (TAFs) (reviewed in [7,8]). Although the full scope of their function is continually being evaluated [8–11], TAFs enhance transcription of certain genes in response to transcriptional activators and coactivators (reviewed in [12–14]). Nevertheless, the TBP subunit of TFIID binds the TATA box in the absence of TAFs and assembles a PIC capable of basal levels of transcription, and TAF-independent transcription has been observed in the presence of the cofactor PC4 [15]. The rate of PIC formation at a particular promoter depends on many factors that include the accessibility of the chromatin-embedded promoter [16], the presence of protein activators and repressors [17], and the presence of several DNA sequence elements including the TATA box [18,19].

Central role of TBP during formation of the PIC

Previous work identified the essential role of the TFIID subunit TBP in controlling the efficiency of PIC formation. Early experiments performed in vitro revealed that PIC formation can begin when TBP binds its DNA target site, the TATA box (consensus TATA a/t A a/t N [20]), and bends the DNA toward the major groove by approximately 80 degrees. Once assembled, the uniquely structured TBP–TATA complex functions as a scaffold for subsequent binding of TFIIB, followed by TFIIF in conjunction with pol II and finally TFIIE and TFIIH (reviewed in [4,14]). TFIIA can assemble into the PIC at any point after TBP [21]. PIC assembly can also follow a pathway in which a pol II holoenzyme [22,23] containing RNA polymerase II and various general factors and coactivators (but not TBP [24]) (reviewed in [25,26]) binds the TBP–TATA complex in a single step. No matter which pathway is followed, formation of a stable, functional, class II PIC demands direct interactions between TBP and the core promoter (in most cases; for an interesting exception, see reference [27]) emphasizing the importance of TBP in transcription initiation.

The central role of TBP in PIC formation has stimulated considerable research on the structure of eukaryotic TBP, alone and in complex with DNA and general TFs TFIIA and TFIIB [28–39]. Early studies of the free, conserved C-terminal domain of yeast and plant TBP (TBP_c) showed a structure containing two subdomains related by a pseudo-two-fold symmetry axis [28,29,35]. Later studies on binary TBP_c–TATA box complexes demonstrated that this symmetry extends to the interface with the TATA box [30,31,36,37,39,40]. The amino acids within TBP_c that contact the asymmetric 8 bp AdMLP or CYC1 TATA boxes are 70% (*Arabidopsis thaliana*) and 89% (*Homo sapiens*) conserved between the two subdomains. In spite of this high degree of symmetry, all eukaryotic TBP_c–TATA crystal structures published to date show the protein bound in a single orientation to the asymmetric TATA box [30–34,36,37,39]. In this orientation, the C-terminal TBP subdomain interacts with the more conserved 5′ TATA half-site and the N-terminal subdomain interacts with the less conserved 3′ a/t A a/t N half site. Ternary complexes containing TBP, DNA and either TFIIA or TFIIB show conservation of the contacts between TBP_c and DNA; TFIIA contacts the N-terminal subdomain of TBP_c and TFIIB contacts the C-terminal subdomain [32–34,38]. The orientation of TBP_c in crystal structures, in addition to genetic evidence that this orientation is preserved in vivo [41], has led to the prevailing view that TBP binds to the TATA box in the single orientation observed in the crystal structures, nucleates assembly of an orientationally defined PIC, and initiates transcription in one direction [30,31, 40,42–44].

Bidirectional binding of TBP to asymmetric TATA boxes in solution

Recently, we reported affinity cleavage experiments whose results forced re-examination of how PIC orientation and the direction of transcription are determined [45,46]. These experiments showed that yeast TBP (yTBP) binds the asymmetric TATA boxes in the AdML and CYC1 promoter as mixtures of two orientational isomers that differ by a 180 degree rotation about the pseudo-dyad axis of the complex. Although the orientational isomer observed by crystallography is preferred, it is only marginally (0.3 kcal mol^{-1}) more stable than the isomer in which yTBP is rotated 180 degrees [46]. This difference in binding free energy corresponds to a 60:40 ratio of the two isomers at equilibrium. Indeed, molecular dynamics simulations detected no unfavorable interactions when TBP was forced to bind in this rotated orientation [47], suggesting that the two isomeric complexes possess similar free energies. Further affinity cleavage experiments indicated that in addition to two orientational isomers, the yTBP–TATA complex assembles as a mixture of several axial isomers in which yTBP binds a distribution of sites displaced by up to 3 bp toward either end of the 8 bp TATA box [46]. These experiments demonstrated that, in the absence of other proteins, the TBP–TATA complex is more accurately described as a mixture of two orientational and several axial isomers.

The observation that TBP binds DNA as a mixture of orientational and axial isomers led us to question whether the orientational and/or axial specificity of TBP might be provided by other components of the PIC. In the stepwise assembly pathway, the TBP–TATA complex acts as a scaffold for the binding of general factors TFIIA and TFIIB [4,14,48]. TFIIB and TFIIA both interact directly with TBP as well as with the DNA within and surrounding the TATA box [32–34,38,49,50]. By contacting both the DNA and protein members of the TBP–TATA complex, TFIIB and TFIIA could help orient TBP in one direction and/or restrict TBP's axial position on the TATA box. If so, then TFIIA and TFIIB would function in a manner analogous to NFATp, which uses a similar strategy to orient the heterodimeric bZIP transcription factor AP-1 on its binding site [51–55].

Earlier results showed that TFIIA and TFIIB individually increase the orientational specificity of yTBP for the TATA box by 0.5–0.7 kcal mol^{-1}, but neither is sufficient to specify a unique orientation to the complex [46]. Here we explore the combined effects of TFIIA and TFIIB on the axial and orientational specificity of yTBP. Our results show that TFIIA and TFIIB act together to affix yTBP in virtually a single position and orientation on the AdMLP TATA box. Ninety-five percent of the complexes contain TBP bound in the single orientation expected on the basis of crystallographic and genetic experiments, and more than

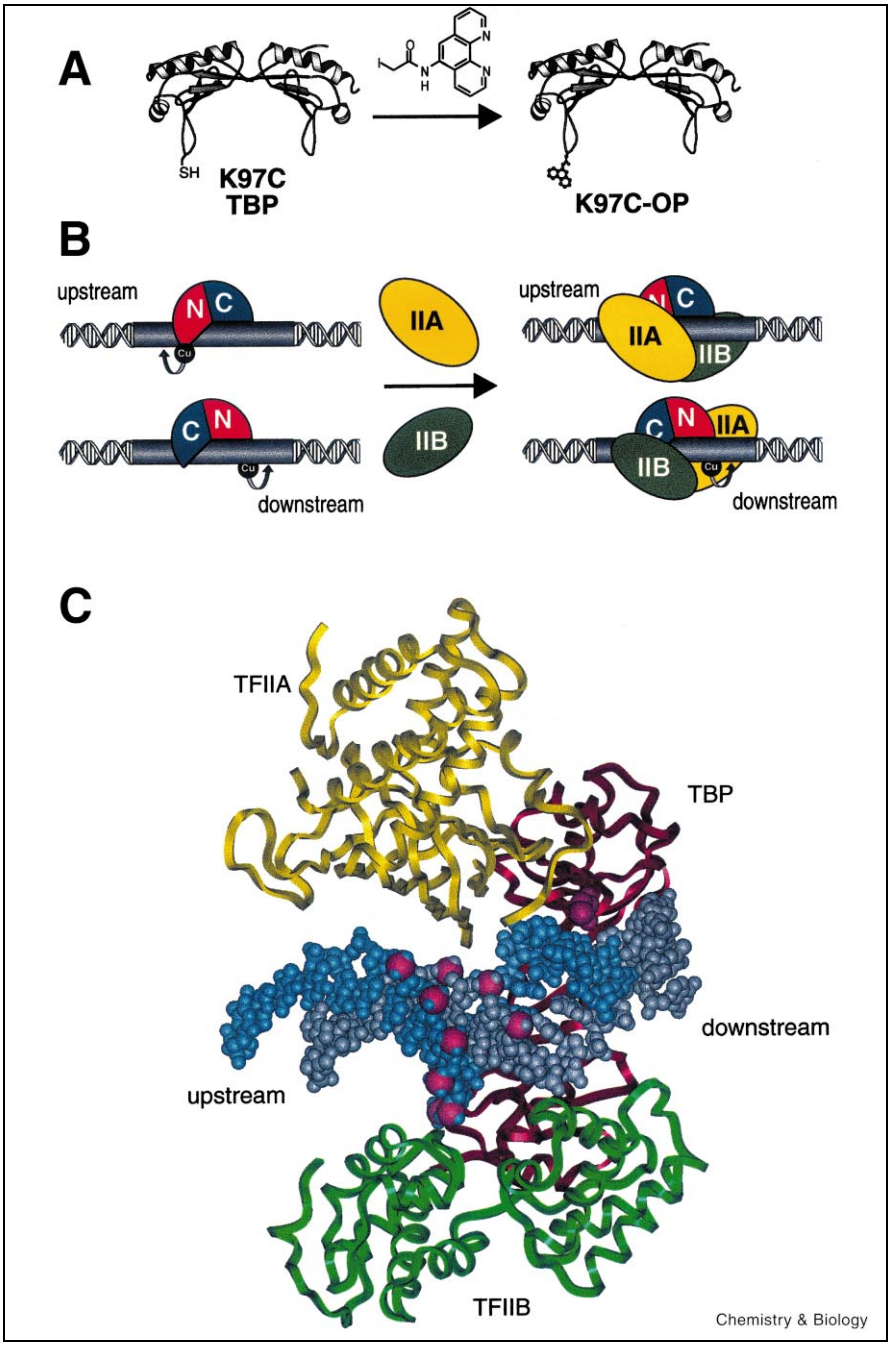


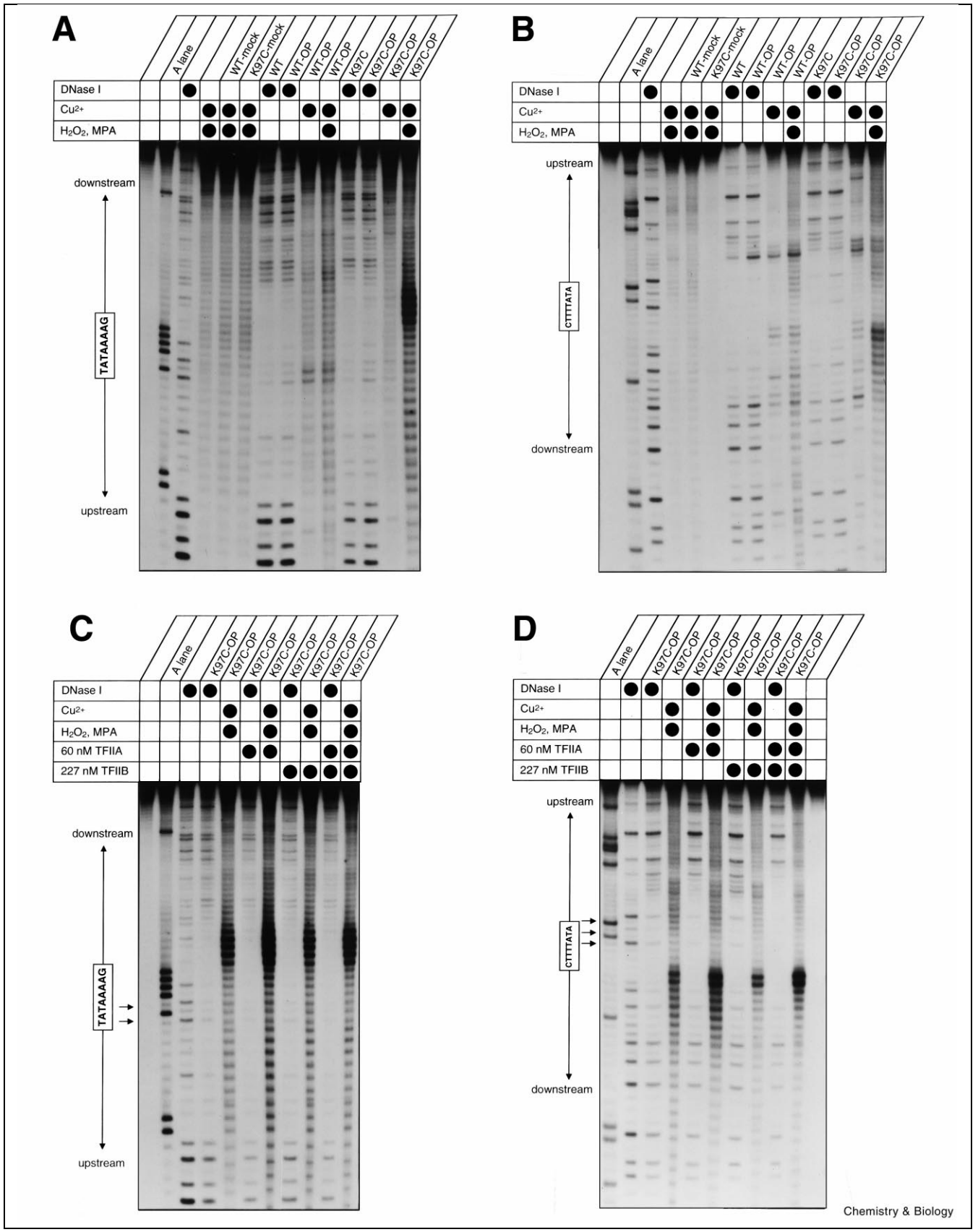
Figure 1. K97C-OP is a DNA affinity cleavage agent [58,59]. Scheme illustrating **(A)** modification of K97C TBP with the affinity cleavage reagent IAAOP to produce K97C-OP and **(B)** the regions of the promoter that are expected to be cleaved, as indicated by the gray arrows, in the presence of copper ion when K97C-OP is bound to the TATA box in the orientations shown. N and C refer to the pseudo-symmetrical halves of TBP_c formed by the N- and C-terminal direct repeats, respectively. Cu identifies the location of the tethered OP–Cu complex on K97C. IIA and IIB refer to TFIIA and TFIIB, respectively. The lengths of the flanking DNA are not drawn to scale in this scheme. Note that the tethered OP–Cu complex is obscured from view by TFIIA bound upstream of the TATA box. See Materials and methods for experimental conditions. **(C)** Molecular model of the quaternary K97C-TFIIB–TFIIA–TATA complex [32,33] generated on the basis of ternary complex crystal structures. TFIIA is in yellow, TBP in red, TFIIB in green, the coding strand of the TATA box is gray, and the non-coding strand is blue. The cysteine residue at position 97 of TBP is shown in red (and is enlarged for clarity) near the interface with TFIIA. The complex is viewed from the major groove of the TATA box, rotated 90° relative to the orientation in **(B)**. This view best illustrates the position of TBP residue 97 in the minor groove of the DNA and the relative locations of TFIIA and TFIIB. In addition, by highlighting the locations of minimal DNA cleavage (red spheres), this view also illustrates that the combined effects of TFIIA and TFIIB on TBP orientation represent a true shift in the binding equilibrium and not simply steric protection of the DNA by protein.

70% is restricted axially to the 8 bp TATA box. Recent data on the pathway of PIC formation in yeast have been interpreted as showing TFIIA to be involved in the first step and TFIIB to join the complex with the holoenzyme [24]. Our results, in combination with this data, suggest that transcription could be regulated during both early and late stages of PIC assembly by general factors (and the proteins to which they bind) that influence the position of TBP on the promoter.

Results and discussion

DNA affinity cleavage provides a direct read-out of the orientation of TBP on the TATA box

We examined the orientational and axial specificity of yTBP bound to the AdMLP TATA box within the quaternary complex containing TFIIA and TFIIB using DNA affinity cleavage [56–59]. Our general strategy is illustrated in Figure 1. The yTBP variant K97C, containing a reactive cysteine at position 97 within the N-terminal stirrup, was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A lane
WT-mock
K97C-mock
WT
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H2O2, MPA
downstream
TATAAAAG
upstream
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WT-mock
K97C-mock
WT
WT-OP
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CTTTTATA
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modified with IAAOP [59], a 1,10-phenanthroline (OP) reagent that specifically alkylates cysteine residues (Figure 1A). Addition of cuprous ion, a reducing agent, and hydrogen peroxide to K97C-OP generated a reactive yet non-diffusible OP–Cu complex [60,61] able to abstract hydrogen atoms from the deoxyribose backbone and affect DNA cleavage. According to the orientation of TBP observed in binary and ternary crystal structures [30–34,36–38], the sulfur of cysteine 97 is located in the DNA minor groove, within 13 Å of 6 bp downstream of the TATA box and more than 17 Å from the nearest bp upstream of the TATA box. Thus, if the quaternary complex containing K97C-OP, TFIIA, TFIIB and DNA (Figure 1C) contains yTBP bound in the single orientation seen in the crystal structure, addition of cuprous ion, a reducing agent, and hydrogen peroxide should generate cleavage on only the downstream side of the TATA box. If the quaternary complex contains yTBP bound in two orientations, however, then cleavage on both sides of the TATA box should be observed (Figure 1B). Although TFIIA contacts the N-terminal TBP subdomain near position 97, the primary contacts are to residues along the S2 and S3 strands of yTBP which lead to and away from the loop containing position 97 [33,34]. Control experiments indicate that K97C-OP assembles effectively into a quaternary complex with TFIIA, TFIIB, and DNA under conditions very similar to those used for ternary complex formation with TFIIA or TFIIB alone (see Materials and methods for details).

Orientational and axial specificity of TBP within a TFIIA–TFIIB–TBP–TATA quaternary complex

Our first step towards determining the effects of bound TFIIA and TFIIB on the orientational and axial specificity of TBP–TATA interactions made use of DNase I footprinting methods [62,63] to identify conditions under which a limiting amount of K97C-OP TBP assembled into a quaternary TFIIA–TFIIB–TBP–TATA complex. Experiments were performed with a 5′-^{32}P-labeled 80 bp DNA fragment containing bp −64 through +16 of the AdMLP [64]. Titration experiments indicated that the AdMLP TATA box (TATAAAAG) was only modestly protected from DNase I cleavage in the presence of 30 nM K97C-OP (Figure 2A,B). Addition of either 60 nM TFIIA′ or 227 nM TFIIB$_c$, truncated versions of TFIIA and TFIIB containing their conserved regions (see see Materials and methods), slightly expanded the DNase I footprint of the binary complex by 4 bp upstream on both strands of the DNA (Figure 2C,D). These changes are consistent with observed DNase I and hydroxy-radical footprints of analogous ternary complexes reported elsewhere [65–67]. Addition of both 60 nM TFIIA′ and 227 nM TFIIB$_c$ to form the yTBP–TATA–TFIIA′–TFIIB$_c$ quaternary complex did not expand the footprint of either ternary complex, but rather increased the level of protection within the footprint (Figure 2C,D). Both observations are consistent with previous reports [20,68]. In particular, faint bands at positions −32 and −30 on the coding strand, and positions −32, −30, and −28 on the non-coding strand (the TATA box contains positions −31 to −24) were present within the region protected by yTBP alone or a combination of yTBP and either TFIIA′ or TFIIB$_c$. In contrast, the bands were fully protected in the presence of yTBP, TFIIA′ and TFIIB$_c$ (Figure 2C,D, see arrows). The DNase I footprint produced by the K97C-OP–TATA–TFIIA′–TFIIB$_c$ complex mirrored, in terms of size and concentration dependence, the analogous footprints produced by quaternary complexes containing K97C and wild type (WT) yTBP in place of K97C-OP (Figure 2A,B and data not shown). These footprinting experiments, taken with the affinity cleavage results described below, indicate that most, if not all, of the available K97C-OP that is bound to DNA is assembled within a specific quaternary complex with TFIIA′ and TFIIB$_c$.

Affinity cleavage of the AdMLP by K97C-OP in the presence of both TFIIA′ and TFIIB$_c$ was compared to analogous reactions containing either TFIIA′ or TFIIB$_c$ to assess the individual and combined effects of these factors on the orientational and axial positioning of TBP on the TATA box (Figures 2C,D and 3). In all cases, the cleavage patterns produced by K97C-OP favored the 3′ half of the binding site on both DNA strands, consistent with a cleavage agent located in the DNA minor groove [69]. Ninety-five percent of the total cleavage elicited from the quaternary K97C-OP–TFIIA′–TFIIB$_c$–TATA complex occurred downstream of the TATA box; only 5% occurred upstream (Figure 3D). This ratio indicated that a free energy difference ($\Delta\Delta G_{obs}$) of 1.8 kcal mol^{-1} separated the two orienta-

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Figure 2. DNase I footprinting and OP–Cu affinity cleavage analysis of the interactions between K97C-OP TBP and the AdMLP TATA box in the absence or presence of TFIIA′ and/or TFIIBc. **(A)** and **(B)** Footprint and cleavage controls on the coding and non-coding strands, respectively. The DNA cleaving agents present in each reaction are indicated above each lane. DNase I indicates footprinting reactions while Cu^{2+}, MPA, and H_2O_2 indicate affinity cleavage of the DNA by the OP–Cu complex. WT refers to wild type yeast TBP, TFIIA refers to TFIIA′, TFIIB refers to TFIIBc. WT-mock and K97C-mock refer to control affinity cleavage experiments in which non-alkylated WT yTBP and K97C were used in place of K97C-OP. WT-OP refers to WT yTBP that was alkylated as a negative control. **(C)** and **(D)** Footprint and cleavage reactions on coding and non-coding strands, respectively, with and without TFIIA and TFIIB as indicated above each lane. Abbreviations are the same as for **(A)** and **(B)**. Arrows indicate bands partially protected by K97C-OP with either TFIIA or TFIIB that are fully protected in by the quaternary complex.

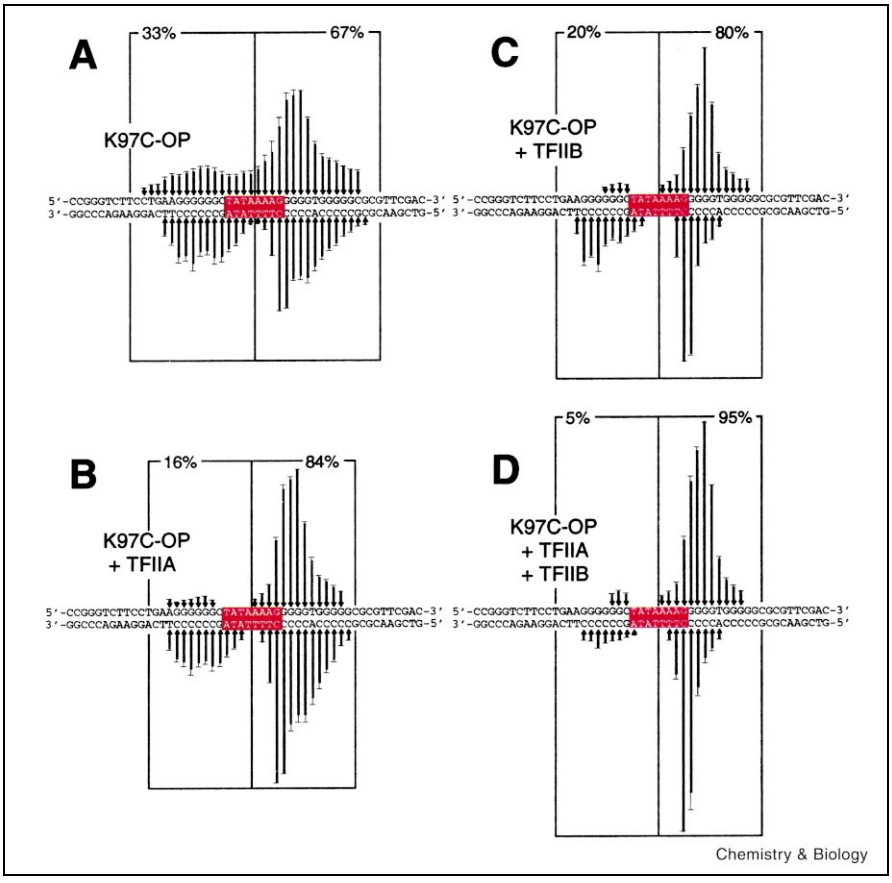

Figure 3. Cleavage patterns reveal that TFIIA and TFIIB orient TBP. Histograms illustrating cleavage at each bp of the AdMLP TATA box and flanking DNA by K97C-OP in **(A)** binary complex; **(B)** ternary complex with TFIIA; **(C)** ternary complex with TFIIB; and **(D)** quaternary complex with TFIIA and TFIIB. The extent of cleavage at each base above a cupric ion, hydrogen peroxide, mercaptopropionic acid control is proportional to the length of the arrow. Error bars indicate the standard deviation of at least five trials. The TATA box is shaded red.

tional isomers of TBP within the quaternary complex. By comparison, free energy differences of 1.0 and 0.8 kcal mol^{-1} separated the orientational isomers of TBP within the TFIIA′–K97C-OP–TATA and $TFIIB_c$–K97C-OP–TATA ternary complexes, respectively (Figures 3B,C and 4). The cleavage data revealed that TBP exhibited significantly greater orientational specificity when assembled in the quaternary K97C-OP–TFIIA′–$TFIIB_c$–TATA complex than in binary complex with DNA alone or ternary complex with either TFIIA or TFIIB. Thus, TFIIB and TFIIA, when acting together, effectively orient TBP on the asymmetric AdMLP TATA box. The cleavage data also showed that TBP exhibited greater axial specificity when assembled within the quaternary yTBP–TFIIA′–$TFIIB_c$–TATA complex than in ternary complex with either TFIIA or TFIIB (Figure 3). Although the asymmetry present in yTBP itself cannot specify unidirectional binding to the TATA box, these data show that a unidirectional PIC is specified on the AdMLP by a small subset of the general TFs.

It is useful to examine the cleavage data in the context of the modeled structure for the TBP–TFIIA–TFIIB–TATA complex generated on the basis of the TBP–TIIA–TATA and TBP–TFIIB–TATA ternary complexes (Figure 1C) [30–34,36–38]. The positions on the DNA cleaved maximally by K97C-OP (four on the coding strand and two on the non-coding strand) are those which are closest (within 13 Å) to the OP–Cu reagent tethered to position 97 of TBP, as measured from the sulfur of residue 97. Notably, the positions on the DNA cleaved minimally by K97C-OP (shown as deep red spheres in Figure 1C) are not shielded from cleavage by TFIIA or TFIIB. This observation confirms that the effects of these general factors on TBP specificity represent a true shift in the binding equilibrium and not steric protection of the DNA by protein.

Relative roles of protein–DNA and protein–protein interactions

TFIIA and TFIIB could increase TBP's orientational and axial specificity through protein–protein interactions, protein–DNA interactions, or through a combination of both effects. For example, TFIIA and TFIIB could interact preferentially with the sequence or structure of the DNA immediately upstream or downstream of the TATA box. Alternatively, increased orientational and/or axial specificity could result if TFIIA and TFIIB binding caused small changes in TBP conformation that exaggerated the small, albeit finite, differences in the DNA-contact surfaces of the C- and N-terminal subdomains [45].

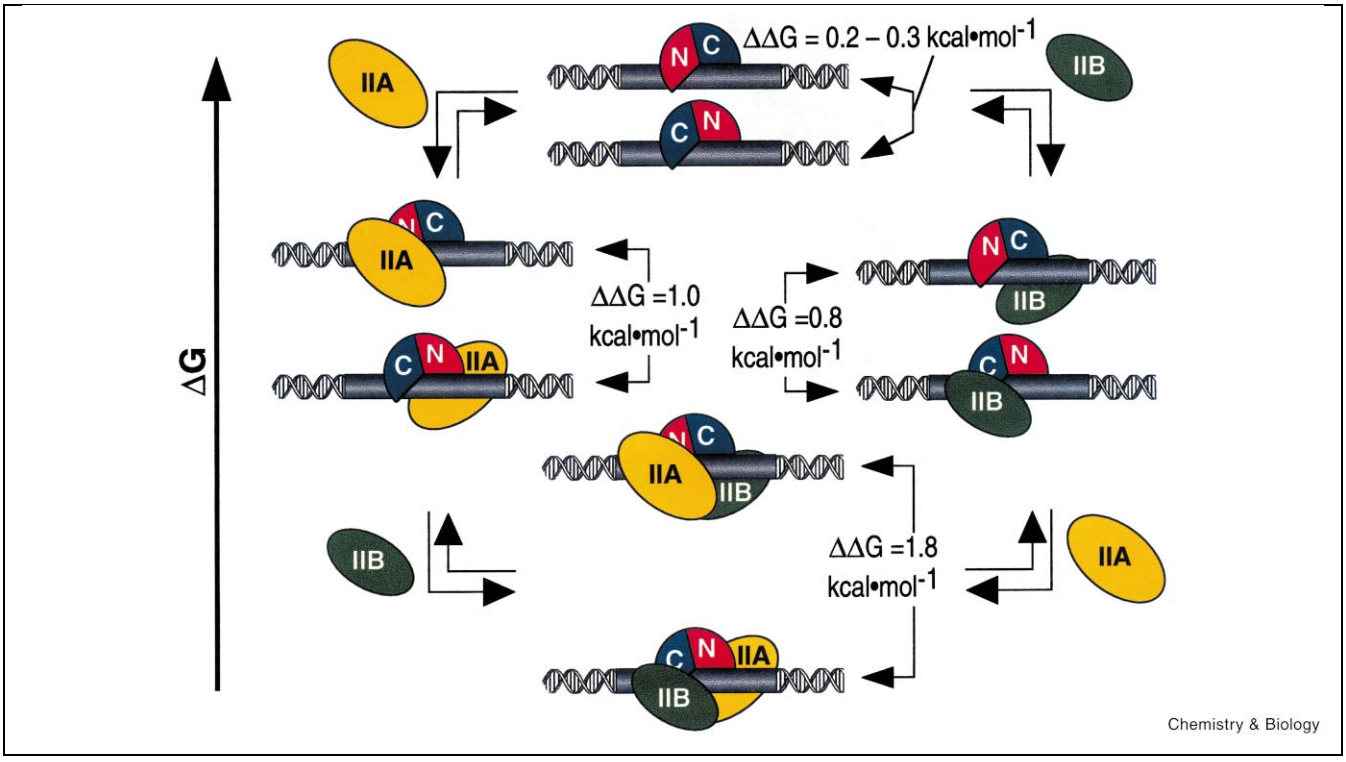

Figure 4. The effects of TFIIA and TFIIB on TBP's orientation are additive. Scheme illustrating the relative Gibbs free energies (ΔG) of the orientational isomers of TBP–TATA binary complexes, TFIIA–TBP–TATA and TFIIB–TBP–TATA ternary complexes and the quaternary TFIIA–TFIIB–TBP–TATA complex. Note that $\Delta\Delta G$'s of the orientational isomers of the quaternary complex are equal to the sum of the $\Delta\Delta G$'s of the orientational isomers of the two ternary complexes.

Several lines of evidence suggest that the increased orientational and axial specificity provided to TBP in the quaternary complex with TFIIA and TFIIB results from direct interactions of TFIIA and TFIIB with DNA and not changes in the inherent specificity of TBP itself. First, the TFIIA–TBP–TATA and TFIIB–TBP–TATA ternary complex structures [32–34,38] reveal direct interactions between TFIIA and TFIIB with the DNA immediately upstream (TFIIA) or upstream, downstream and within the major groove (TFIIB) of the TATA box. Second, the structure of TBP in ternary complex with TFIIA or with TFIIB is virtually superimposable (rmsd for TBP is 0.4 Å in both cases) with the structure of TBP in binary complex with DNA and the protein structures are characterized by equivalent temperature factors [32–34,38]. Third, in vitro selection experiments performed with human TFIIB identified a preference of this protein for a distinct sequence of 7 bp located immediately upstream of the TATA box [50]. In particular, TFIIB preferred a G:C pair located three nucleotides upstream of the TATA box. Five of the 7 bp identified in the selection, including the crucial G:C pair, are found upstream of the AdMLP TATA box. Only 4 of the 7 bp are found downstream of the AdMLP TATA box, and this set does not include the critical G:C bp. Selection and bidirectional transcription experiments in archeal systems found that TFB, the archeal homolog of TFIIB, has an even stronger DNA recognition element to which it binds, orienting the PIC [70,71]. Ebright and coworkers have also suggested that TFIIA exhibits sequence selectivity [50]. These data emphasize the important contribution of TFIIA and TFIIB to the orientational and axial specificity of TBP.

Finally, support for the conclusion that the orientational and axial specificity of the quaternary complex results from interactions of TFIIA and TFIIB with DNA derives from the observation that the effects of TFIIA and TFIIB are additive, not cooperative (Figure 4). If both TFs acted on TBP directly to increase its ability to recognize the 8 bp TATA box, their individual effects on TBP structure or dynamics could magnify each other resulting in an overall effect that was greater than the sum of their respective individual contributions. As described above, previous work revealed that the two orientational isomers of the binary TBP–TATA complex are separated by a free energy difference of 0.3 kcal mol^{-1} with the more stable isomer the one observed in the crystal structure. TFIIA and TFIIB individually increased the orientational specificity of TBP; the two orientational isomers of the ternary TFIIA–TBP–TATA and TFIIB–TBP–TATA complexes

were separated by 1.0 kcal mol^{-1} and 0.8 kcal mol^{-1}, respectively. In the quaternary complex, 95% of the DNA cleavage occurred on the downstream side of the TATA box, indicating that 95% of the K97C-OP bound to the TATA box was oriented with its N-terminal subdomain positioned over the downstream half of the TATA box. This increase in orientational specificity in the presence of both TFIIA and TFIIB corresponds to a $\Delta\Delta G$ of 1.8 kcal mol^{-1}, a value that is precisely the sum of the two $\Delta\Delta G$'s of the ternary complexes. This observation supports the conclusion that TFIIA and TFIIB function additively orient TBP on the TATA box. Were TFIIA and TFIIB to cooperate in a positive or negative manner to influence TBP orientation, then the difference in the free energies should be greater than or less than, respectively, the sum of the differences of the free energies of the two ternary complexes [72].

Potential roles for upstream activators and the holoenzyme in determining TBP orientation and the direction of transcription

Analysis of our findings in light of recent results on PIC formation in yeast cell extracts [24] suggests that TBP's orientational and axial specificity can be fixed at several stages along the assembly pathway. Ranish et al. recently used an immobilized promoter assay to provide indirect evidence for a two-step PIC assembly pathway in yeast. In this model, TFIID and TFIIA bound the HIS4 TATA box (TATATAATA) to form a stable PIC intermediate in the absence of holoenzyme. This intermediate then bound holoenzyme (which contains TFIIB) in a second step to complete the PIC. The upstream activator Gal4-AH stimulated transcription by increasing the extent of PIC formation through interactions with TFIID and TFIIA; the upstream activator Gal4-VP16 accomplished the same goal through interactions with TFIID, TFIIA, and the holoenzyme itself [24]. The importance of TFIIA in PIC assembly is emphasized by the observation that extracts deficient in TFIIA activity are defective in the assembly of all PIC subcomplexes [19,66,73]. Therefore, by bringing TFIIA to the promoter, activators increase the effective concentration of TFIIA near the TATA box, and increase the fraction of TBP molecules bound in the correct orientation and axial location to the DNA. This in turn, would cause a greater fraction of the PICs assembled to be correctly oriented on the promoter. In a similar way, since TFIIB is part of two of the three isolated holoenzyme complexes [74–76], TBP's orientational specificity could also be controlled during the second assembly step. The combined effects of TFIIA and TFIIB on the orientational and axial specificity of TBP imply that the directionality of PIC formation – and hence, the direction of transcription – can be fixed at several different stages. We suspect that the assembly of a PIC of defined orientation likely involves the combined effects of a number of TFs.

Significance

Steps in the initiation of transcription involve recruitment of the transcriptional machinery – the six general TFs, TFIID, TFIIA, TFIIB, TFIIE, TFIIF, TFIIH, as well as RNA polymerase II and the Srb and mediator factors – to the promoter to form a PIC [77]. Numerous experiments performed in vitro [42,78,79] and in vivo [2,3,17,43] indicate that, at many (but not all [80]) promoters, the interaction of the TBP-containing factor TFIID with DNA represents a rate-limiting step in PIC formation and thus transcription initiation, and a direct relationship exists between the extent of TBP binding to a promoter and the extent of transcriptional activation [2,3]. Previous work has shown that, in the absence of other factors, TBP alone cannot specify the formation of a PIC of unique polarity. Here we show that specificity can be achieved by the combined action of TFIIA and TFIIB. This result, coupled with recent evidence that PIC assembly may proceed along a pathway in which the TFIIB-containing holoenzyme binds subsequent to TFIID and TFIIA [24], suggests that activators that function through TFIIA or TFIIB, or both, could activate transcription by indirectly orienting TBP, and thus the PIC, on the promoter. It may be that activated transcription is not only a result of recruitment of basal factors to a promoter, but also the result of orienting the PIC efficiently in the correct direction for productive transcription.

Materials and methods

Oligonucleotides were synthesized by HHMI Biopolymer–Keck Foundation Biotechnology Resource Laboratory at Yale University (New Haven, CT, USA). DNase I was purchased from Worthington Biochemistry Corporation (Lakewood, NJ, USA). [γ-^{32}P]ATP was purchased from DuPont NEN (Wilmington, DE, USA). DTT was purchased from Boehringer Mannheim (Indianapolis, IN, USA). Image Quant™ software and the Storm 840 phosphorimager were purchased from Molecular Dynamics (Sunnyvale, CA, USA). BSA was purchased from New England Biolabs (Beverly, MA, USA). poly dG–dC was purchased from Pharmacia Biotech (Piscataway, NJ, USA). Storage phosphor screens were from Molecular Dynamics.

Expression and purification of K97C TBP and K97C-OP

The TBP variant K97C was expressed and purified following the procedure used by Kim et al. [30] to prepare WT TBP, and was stored at −70°C in a buffer containing 30 mM Tris–HCl (pH 7.5 at 25°C), 10% glycerol, 50 mM KCl, 1 mM EDTA, and 1 mM dithiothreitol (DTT). The concentration of purified K97C was determined spectroscopically through its OD_{280} and by amino acid analysis. K97C-OP was prepared by treatment of K97C with 5-iodoacetamido-1,10-phenanthroline (IAAOP) and purified using procedures described previously [46].

TFIIA′ and TFIIB$_c$

TFIIA′, a fully functional deletion variant of yeast TFIIA [33], was stored at −70°C in 30 mM Tris pH 7.5, 10% glycerol, 5 mM DTT, 600 mM KCl, and 2 mM EDTA. TFIIB$_c$, the protease-resistant C-terminal core of yeast TFIIB, was stored at −70°C in 20 mM HEPES pH 7.9, 1 mM EDTA, 10% glycerol, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 150 mM potassium acetate, 50 mg ml^{-1} BSA and 0.1% Nonidet P-40.

Quaternary complex formation

With WT yTBP, complete quaternary complex formation was observed

with 114 nM $TFIIB_c$ and 30 nM TFIIA′. However, with K97C, quaternary complex formation was observed only after a two-fold increase in the concentration of $TFIIB_c$ and TFIIA′ to 227 nM and 60 nM, respectively. Binding reactions were performed in a total volume of 5 μl. To TBP (30 nM), $TFIIB_c$ (227 nM) and TFIIA′ (60 nM) were added followed by 5′ ^{32}P-labeled DNA (200 pM). Final concentrations are given in parentheses. The mixture was incubated for 30 min at 25°C with 0.04 mg ml^{-1} BSA and 0.02 mg ml^{-1} poly dG–dC in binding buffer (5 mM $MgCl_2$, 4 mM Tris pH 8, 60 mM KCl, 4% glycerol, 0.1% Nonidet P-40). TFIIA′ and $TFIIB_c$ were separately diluted to the desired concentrations in binding buffer and then added to the binding reactions. The DNA used for footprinting and affinity cleavage contained residues −64 to +16 of AdMLP with a centrally located TATAAAAG TATA box.

DNase I footprinting and affinity cleavage reactions

DNase I footprinting reactions were performed as described previously [46] with several modifications. Briefly, 1.5 μl (0.006 units) of DNase I diluted 1:500 in 5 mM $MgCl_2$ and 5 mM $CaCl_2$ was added to the binding reactions prepared as described above and quenched after 30 s by addition of 4 μl of formamide loading buffer (9.2 M urea, 40 mM EDTA, 0.3% xylene cyanol, 0.3% bromophenol blue in deionized formamide). DNA affinity cleavage reactions were performed as described previously [46] with several modifications. Briefly, 1 μl of cleavage buffer (300 μM $CuSO_4$, 0.06% H_2O_2, 30 mM mercaptopropionic acid in binding buffer) was added to the binding reaction prepared as described above and incubated for 3 h at 25°C. DNA was EtOH precipitated, denatured at 95°C and the reaction products were separated on a 12% polyacrylamide gel (1:20 acrylamide:bis-acrylamide, 7 M urea) in 1×TBE at 70 W for 1.5 h. Gels were dried prior to exposure to a storage phosphor screen.

Analysis of affinity cleavage reactions

Relative cleavage intensities were determined by volume integration of individual cleavage bands using a Molecular Dynamics Storm 840 phosphorimager and Image-Quant™ software. The intensity of each cleavage band was background-corrected by dividing the volumes in that lane by the lowest volume or least intense band, and subtracting 1 to give a value of zero for the least intense band. Each lane was then normalized by multiplication to bring the highest intensity of cleavage in each lane to a value of 10. These data were then averaged for each set of conditions (quaternary versus ternary versus binary complexes) to produce the final histograms.

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