Gal4-VP16 and Gal4-AH Increase the Orientational and Axial Specificity of TATA Box Recognition by TATA Box Binding Protein†

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ABSTRACT: Previous work has shown that binding of the TATA box binding protein (TBP) to the TATA box is a rate-limiting step during preinitiation complex (PIC) formation. Although the transcription of eukaryotic genes normally proceeds in one direction, studies in solution have shown that TBP lacks the information necessary to orient itself on the TATA box. Instead, yeast TBP binds TATA-containing promoters in two orientations that are related by a 180° rotation about TBP's pseudo-2-fold symmetry axis. Recruitment of PIC components by gene-specific activators is considered a primary mechanism of transcriptional enhancement. Here we ask whether activators might function, at least in part, by increasing the fraction of PICs assembled with TBP bound in the orientation necessary for transcription. We use DNA affinity cleavage and a TBP−phenanthroline−copper conjugate to monitor the orientation of TBP in the presence of the well-studied activators Gal4-VP16 and Gal4-AH. In the absence of a transcriptional activator, only 51% of the TBP·TATA box complexes were bound in the orientation necessary for the initiation of transcription. However, in the presence of saturating Gal4-VP16, 87% of the TBP bound to the TATA box was oriented correctly at equilibrium. This increase in orientational specificity corresponds to a free energy difference ($\Delta\Delta G_{obs}$) of 1.1 kcal·mol^{-1} and was accompanied by a dramatic increase in axial specificity, reminiscent of the effects of transcription factors TFIIB and TFIIA reported previously. Gal4-AH also enhanced the orientational and axial specificity of the TBP·TATA complex, although to a lesser extent. We suggest that these effects on specificity represent a variation of recruitment, since they require direct interactions between the activator and a PIC component but only increase the effective concentration of the *correctly oriented* PIC component. These findings add to increasing evidence that recruitment may encompass a broad range of mechanisms.

The rate of transcription of any single eukaryotic gene is controlled at multiple steps, including the initiation of transcription (*1*−*3*). Preinitiation complex (PIC)[1] formation, the first step in transcription initiation, is an essential control point in the transcription of protein-encoding genes by RNA polymerase II (pol II). The PIC consists of several basal transcription factors (TF) including TFIIA, TFIID, TFIIB, TFIIE, TFIIF, and TFIIH, as well as Srb and Med factors and pol II (*4*−*7*). Pol II itself does not contain the information required to identify the promoter of a gene. As a result, pol II must be placed correctly at the start site by other proteins within the PIC. Two pathways have been described by which the PIC assembles and pol II is placed correctly on the promoter. One pathway involves a stepwise assembly of basal transcription factors that begins with the binding of TFIID (*5, 8*). An alternative pathway involves the binding of a holoenzyme composed of pol II and various basal, Srb, and Med factors (*9*−*13*). In either case, TFIID is widely considered to be the basal factor responsible for initial recognition of class II gene promoters. The TFIID·DNA complex that results forms a scaffold on which the rest of the PIC can assemble in preparation for productive transcription.

TATA box binding protein (TBP), the central TFIID component (*14, 15*), is targeted by gene-specific regulators to affect transcription levels both in vivo (*16*−*19*) and in vitro (*20, 21*). The 180 C-terminal residues of TBP form a conserved core (TBP_c), consisting of two direct and highly homologous repeats (*22*−*24*). Residues responsible for contacting the TATA box are 70% (*Arabidopsis thaliana*) to 89% (*Homo sapiens*) conserved between these direct repeats and are essential for transcriptional control (*25*). Structural studies have revealed that the binding of TBP splays open the minor groove of the TATA box (consensus sequence 5′- $TATA^a/_tA^a/_t$ N-3′) (*26*), allowing multiple base-specific contacts to occur and resulting in an 80° bend in the DNA toward the major groove (*27*−*30*). Despite the high homology in the sequence and structure of the N- and C-terminal TBP_c repeats, all crystal structures of eukaryotic TBPs, including those that contain TFIIA or TFIIB, show TBP bound in one orientation to the asymmetric TATA box.

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[1] Abbreviations: TBP, TATA box binding protein; wt TBP, wild-type TATA box binding protein; yTBP, yeast TATA box binding protein; TBP_c, conserved core of TATA box binding protein; K97C, yeast TATA box binding protein variant with cysteine residue at position 97; K97C-OP, K97C yeast alkylated with 1,10-*o*-phenanthroline; OP, 1,10-*o*-phenanthroline; AdMLP, adenovirus major late promoter; PIC, preinitiation complex; pol II, RNA polymerase II; TAFs, TATA box binding protein associated factors; DTT, dithiothreitol; MPE−Fe, (methidiumpropyl)ethylenediaminetetraacetic acid (EDTA)−iron; TF, transcription factor.

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In this orientation, the N-terminal repeat of TBP_c contacts the 3′-$^{a}/_{t}A^{a}/_{t}N$ half of the TATA box while the C-terminal repeat contacts the 5′-TATA half (*27*−*34*). Significantly, genetic studies on a TBP variant with altered DNA specificity suggest the preservation of this orientation in vivo (*35*). This study reported that a TBP variant containing a single amino acid change in the C-terminal repeat preferentially recognized a TATA box with a T to C mutation in the 5′ half of the TATA box. This genetic evidence, in combination with data from crystallography, precipitated the view that TBP bound the TATA box in a single orientation and facilitated assembly of a uniquely oriented PIC capable of productive transcription from the start site (*27, 28, 36, 37*). In this paper we refer to this single orientation as the correct orientation.

In contrast to data from crystallography, studies of TBP in solution provide evidence that TBP binds the TATA box in two orientations (*38*). DNA affinity cleavage experiments showed that, in the absence of other proteins, TBP binds to the TATA box in two orientations that are related by a 180° rotation about TBP's pseudodyad axis of symmetry. TBP displayed only a slight preference for the orientation preferred in the crystal ($\Delta\Delta G_{obs} \leq 0.2$ kcal·mol^{-1}) (*39, 40*). Molecular modeling studies of the TBP·TATA complex performed by others support these solution-phase results, indicating no unfavorable interactions when TBP interacted with the TATA box in the reverse orientation (*41*). Although it has been reported that TBP does not determine the direction of transcription (*42, 43*), in vitro studies of the U6 snRNA TATA box argue that the deformability of the TATA box influences the orientation of TBP binding (*44*). Recent kinetic studies attributed the slight orientational preference at equilibrium to a more rapid assembly of the TBP·TATA correct orientational isomer (*45*). Previous affinity cleavage experiments also demonstrated that TBP lacks axial specificity as well as orientational specificity and binds in solution to several sites that overlap the TATA box (*39, 40*). The absence of orientational and axial specificity exhibited by TBP in solution suggests that TBP·TATA complexation may be a point at which other transcription factors could increase specificity and perhaps the extent of transcriptional activation.

The basal transcription factors TFIIA and TFIIB both increase TBP's orientational specificity in vitro. In the presence of saturating concentrations of TFIIA or TFIIB, 84% ($\Delta\Delta G_{obs} = 1.0$ kcal·mol^{-1}) and 80% ($\Delta\Delta G_{obs} = 0.8$ kcal·mol^{-1}), respectively, of the TBP·TATA complexes are bound in the correct orientation (*39, 40*). Moreover, TFIIA and TFIIB act additively to correctly orient 95% of the TBP bound to TATA box (*40*), an increase in specificity of 1.8 kcal·mol^{-1}. The orientational and axial restrictions placed on TBP, and thus the PIC, by TFIIA and TFIIB imply that a great majority of PICs will assemble correctly for productive transcription from the start site.

Whereas basal factors position pol II at the promoter, transcriptional activators act on both basal factors and pol II to facilitate PIC assembly (*46*−*53*). Chimeric activators Gal4-VP16 and Gal4-AH are employed widely to mimic natural transcriptional activators. These Gal4 derivatives contain a Gal4 DNA-binding domain and either the VP16 activation domain or the synthetic amphipathic helix (AH) activation domain (*54, 55*). Although both Gal4-VP16 and Gal4-AH assemble the PIC efficiently (*56*), Gal4-VP16 is considered a stronger activator (*56, 57*). Multiple studies provide evidence for direct contacts between VP16 and the N-terminal DNA binding domain of TBP (*58*−*61*), whereas direct contacts between Gal4-AH and TBP have remained elusive (*56, 62*−*64*).

In light of our findings that TBP cannot orient itself on the promoter, we wondered if a transcriptional activation domain could increase the fraction of TBP oriented productively on the TATA box at equilibrium. We assessed the effect of Gal4-VP16 and Gal4-AH on the orientational specificity of TBP using a DNA construct containing the adenovirus major late promoter (AdMLP) TATA box with upstream Gal4 binding sites. Our results indicate that Gal4-VP16 and Gal4-AH enhance the orientational and axial specificity of yTBP in a manner reminiscent of the effects of TFIIA and TFIIB reported earlier. This result suggests that activators may enhance transcription levels by stabilizing productively oriented PICs on the promoter.

EXPERIMENTAL PROCEDURES

Preparation of DNA. G5-TATA TOP is a 186mer oligonucleotide containing five Gal4 DNA binding sites (sequence 5′-CGGAGGACAGTACTCCG-3′) with two bases, CT, separating the sites. The first Gal4 site is 25 bp from the 5′ end of the oligonucleotide and the fifth Gal4 site is 35 bp upstream of the 8 bp AdMLP TATA box, which is 25 bp from the 3′ end of the oligonucleotide. The sequence of the 186mer is taken directly from pG5MLT, a gift from Danny Reinberg (Department of Biochemistry, University of Medicine and Dentistry of New Jersey) (*65*). G5-TATA BOTTOM is the complement of G5-TATA TOP. Both oligonucleotides were synthesized by HHMI Biopolymer/Keck Foundation Biotechnology Resource Laboratory, Yale University. Oligonucleotides were purified on a 12% polyacrylamide gel (1:20 bisacrylamide:acrylamide, 7 M urea) and extracted from the gel by elution into 1× TE. DNA was concentrated on a Speedvac and desalted over a Microspin G25 column (Amersham Pharmacia Biotech). To observe the TATA box, G5-TATA TOP was 3′-end-labeled with [α-^{32}P]-3′-dATP (DuPont NEN) and terminal transferase (New England Biolabs), and G5-TATA BOTTOM was 5′-end-labeled with [δ-^{32}P]ATP (DuPont NEN) and T4 polynucleotide kinase (New England Biolabs). To observe the Gal4 binding sites, G5-TATA BOTTOM was 3′-end-labeled.

Expression and Purification of K97C TBP and K97C-OP. The TBP variant K97C was expressed and purified following the procedure used previously (*40*), with the following modifications. K97C was expressed in BL21(DE3) pARG electrocompetent cells, a gift from Yichin Liu (Department of Chemistry, Yale University). The concentrated K97C-containing fractions from the SP Sepharose FPLC column were loaded onto a Heparin Hi-trap column (Pharmacia Biotech) equilibrated in buffer A [30 mM Tris-HCl (pH 7.5 at 25 °C), 10% glycerol, 50 mM KCl, 1 mM EDTA, and 1 mM dithiothreitol (DTT)] and eluted with a salt gradient of 50−600 mM KCl over 80 mL. K97C eluted at ∼380 mM KCl. Collected fractions were concentrated and buffer was exchanged to reduce KCl concentration to less than 100 mM KCl in a Centriprep-10 (Amicon). K97C was stored at −70 °C in buffer A. The concentration of purified K97C was determined spectroscopically through its absorbance at 280

and by amino acid analysis. K97C-OP was prepared by treatment of K97C with 5-iodoacetamido-1,10-phenanthroline (IAAOP) and purified by procedures described previously (*39*).

Gal4 Variants. Gal4-VP16 contains residues 1−147 of Gal4 attached by an amino acid linker to residues 413−490 of VP16 (*54*). Gal4-AH contains residues 1−147 of Gal4 attached to a synthetic acidic activation domain (*55*). Both were gifts from Steve Hahn (Fred Hutchinson Cancer Research Center). Gal4(1−94) was a gift of Cheng-Ming Chiang (Department of Biochemistry, Case Western Reserve University).

DNA Binding Reactions. Binding reactions were performed in a total volume of 5 μL. Gal4-VP16 (from 0.9 to 3.8 μM), Gal4-AH (1.9−3.8 μM) or Gal4(1−94) (3.8 μM) and ^{32}P-labeled DNA (200 pM) were incubated in binding buffer (5 mM $MgCl_2$, 4 mM Tris, pH 8, 60 mM KCl, 4% glycerol, and 0.1% Nonidet P-40) for 20 min at 25 °C. Final concentrations are given in parentheses. To the activator·DNA complex was added wt TBP (31 nM) or K97C-OP (140 nM) with 0.04 mg/mL BSA and 0.02 mg/mL poly dG·dC in binding buffer. The mixture was incubated for 35 min at 25 °C.

MPE−Fe Footprinting and Affinity Cleavage Reactions. MPE−Fe footprinting reactions were performed as described previously (*66*−*68*) with several modifications. Briefly, 1.5 μL of methidiumpropyl-EDTA− (MPE−) Fe solution [66.6 μM MPE (Sigma), 133.3 μM $Fe_2(NH_4)_2(SO_4)_2$ (Arcos), and 16.6 mM DTT] was added to the binding reactions prepared as described above and quenched after 5 min at 30 °C by EtOH precipitation. After washing with 70% EtOH, the DNA was dried on a Speedvac and resuspended in a 1:1 mixture of 1× TBE and formamide loading buffer (9.2 M urea, 40 mM EDTA, 0.3% xylene cyanol, and 0.3% bromophenol blue in deionized formamide). DNA affinity cleavage reactions were performed as described previously (*40*) with several modifications. Briefly, 1 μL of cleavage buffer (300 μM $CuSO_4$, 0.06% H_2O_2, and 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, washed with 70% EtOH, and dried on a Speedvac. After resuspension in a 1:1 mixture of 1× TBE and formamide loading buffer, the DNA was denatured at 95 °C and the reaction products were separated on an 8% polyacrylamide gel (1:20 acrylamide:bisacrylamide, 7 M urea) in 1× TBE at 75 W for 1.25 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 with Molecular Dynamics Storm 840 PhosphoImager and Image-Quant software. The intensity of each cleavage band was background-corrected, first by subtracting the volume at the same position in a control lane containing DNA and cleavage buffer, and second by dividing the volumes in that lane by the lowest volume or least intense band and then subtracting 1 to give a value of zero for the least intense band. Each lane was then normalized by multiplication to bring the band with the highest intensity of cleavage in each lane to a value of 10. These data were then averaged for each set of conditions to produce the final histograms.

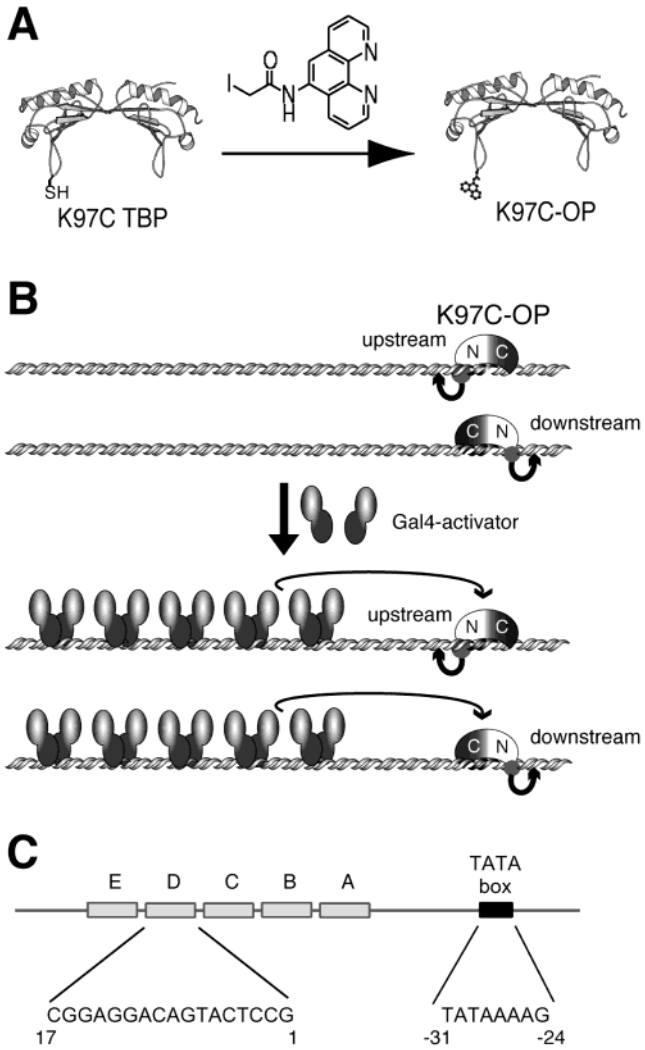
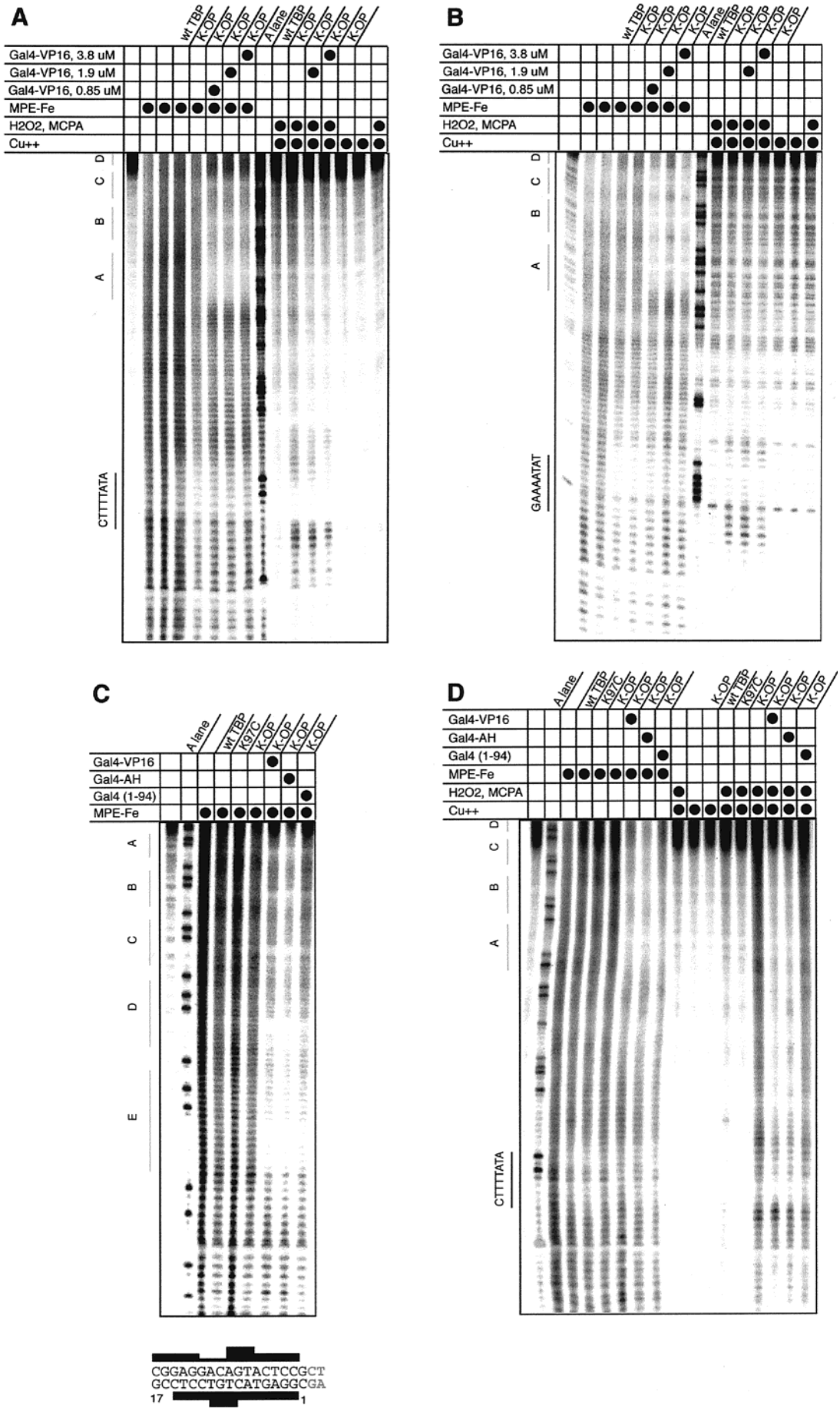


FIGURE 1: K97C-OP is a DNA affinity cleavage agent (*71*). Scheme illustrating (a) modification of K97C TBP with IAAOP (*72*) to produce K97C-OP and (b) the regions of the promoter that are expected to be cleaved, as indicated by the curved black arrows, in the presence of cupric ion when K97C-OP is bound to the TATA box in the orientations shown. N and C refer to the pseudosymmetrical halves of TBP_c formed by the N- and C-terminal direct repeats (*27*−*30*). A small dark gray ball appended to the N-terminal half of K97C identifies the location of the tethered OP−Cu complex on K97C. Gal4-derived activators are shown binding as dimers to five Gal4 binding sites located upstream of the TBP·TATA box complex. The Gal4 DNA binding domain is depicted as a dark gray oval and the activation domain as a light gray oval. The lengths of the flanking DNA are not drawn to scale in this scheme. See Materials and Methods section for experimental conditions. (c) Diagram of the DNA template indicating the locations of the five Gal4 binding sites (gray boxes labeled A−E) relative to the TATA box (black box) and the sequence and relative numbering for both as used in the text. All five Gal4 binding sites contain the same 17 bp sequence.

RESULTS

DNA Affinity Cleavage Provides a Direct Readout of the Effects of Transcriptional Activators on the Orientational and Axial Specificity of TBP. DNA affinity cleavage was used to examine the orientational and axial specificity of yTBP bound to the AdMLP TATA box in the presence and absence of transcriptional activators Gal4-VP16 and Gal4-AH (*69*−*71*) (Figure 1). The DNA affinity cleavage reagent K97C-OP (Figure 1A) was constructed by alkylating the yTBP variant K97C, which contains a reactive cysteine at


A
wt TBP
K-OP
A lane
Gal4-VP16, 3.8 uM
Gal4-VP16, 1.9 uM
Gal4-VP16, 0.85 uM
MPE-Fe
H2O2, MCPA
Cu++
CTTTTATA
B
GAAAATAT
C
Gal4-VP16
Gal4-AH
Gal4 (1-94)
K97C
CGGAGGACAGTACTCCGCT
GCCTCCTGTCATGAGGCGA
17
1
D

position 97 within the N-terminal stirrup, with 5-iodoacetamido-1,10-phenanthroline (IAAOP) (*72*). Addition of cupric ion, hydrogen peroxide, and a reducing agent to K97C-OP generated a reactive yet nondiffusible OP−Cu complex (*73, 74*) able to abstract hydrogen atoms from the deoxyribose backbone and effect DNA cleavage. Previous control experiments demonstrated that K97C itself did not induce DNA cleavage, even in the presence of cupric ion, hydrogen peroxide, and a reducing agent (*39, 40*). K97C that had been subjected to a mock alkylation reaction with 1,10-phenanthroline itself also failed to cleave DNA (*39, 40*). An additional control experiment detected no cleavage when K97C-OP was replaced with wild-type (wt) yTBP alkylated elsewhere with IAAOP (*39, 40*). Taken together, these results indicate that the DNA cleavage observed in the presence of K97C-OP is due to the copper−phenanthroline complex located at position 97.

Previous DNA affinity cleavage studies revealed that yTBP itself binds the TATA box as a mixture of two orientational isomers and several axial isomers (*39, 40*). In the absence of other transcription factors, K97C-OP shows little if any preference (51:49 ratio) for the orientation in which the N-terminal repeat (containing residue 97) binds the downstream end of the TATA box, the same orientation seen in the crystal structures of eukaryotic TBP (*27*−*34*). Were Gal4-VP16 or Gal4-AH to have no influence on the orientational specificity of K97C-OP, we would expect K97C-OP to produce a cleavage pattern that closely resembled that observed in the absence of activator, with relatively equal cleavage on the upstream and downstream sides of the TATA box. However, were Gal4-VP16 or Gal4-AH to confer orientational specificity on K97C-OP, then we would expect a cleavage pattern shifted to one side of the TATA box (Figure 1B). Increased cleavage on the downstream side of the TATA box would indicate an increased population of correctly oriented TBP•TATA box complexes; increased cleavage on the upstream side of the TATA box would indicate an increased population of incorrectly oriented complexes.

Assembly of Gal4-VP16, Gal4-AH, and TBP on TATA-Containing DNA. To characterize the effects of Gal4-VP16 and Gal4-AH on the orientational and axial specificity of TBP, we first determined the concentration of K97C-OP required to minimally protect our target DNA, which contained a central TATA box and five upstream Gal4 sites. MPE−Fe footprinting experiments (*45, 68*) were performed with a ^{32}P-end-labeled, 186 base pair (bp) DNA fragment containing five 17 bp Gal4 binding sites located 35 bp upstream of the AdMLP TATA box (Figure 1C) (*65*). Titration experiments performed in the absence of Gal4-VP16 and Gal4-AH indicated modest protection of the AdMLP TATA box (TATAAAAG) from MPE−Fe cleavage in the presence of 140 nM K97C-OP. Maximal protection was observed at positions −22 and −33 on the coding strand and positions −21 to −34 on the noncoding strand (Figure 2A,B). (The TATA box spans positions −24 to −31.) The location of the MPE−Fe footprint of K97C-OP is consistent with previous experiments that made use of various radical footprinting reagents and TBP (*45, 75, 76*). The MPE−Fe footprints of the K97C-OP•TATA complexes mirrored, in terms of size and concentration dependence, analogous footprints of complexes containing K97C or wt yTBP (Figure 2C,D and data not shown).

We next determined the concentration of Gal4-VP16 necessary to saturate the five Gal4 binding sites upstream of the TATA box in the presence of K97C-OP. Addition of between 0.85 and 3.8 μM Gal4-VP16 protected both strands of each of the upstream Gal4 binding sites from MPE−Fe (Figure 2A−C). Footprints were observed equally between positions 9−11 on the noncoding strand of each 17 bp Gal4 binding site; faint protection was observed between positions 2−8 and 12−15 (Figure 1C). Clear protection of the coding strand was observed between positions 7−9 and faint protection between positions 2−6 and 13−17. This level of protection by a Gal4 derivative at all available binding sites is consistent with previously reported DNase I footprints of analogous Gal4-VP16•DNA complexes (*77*−*79*). Analogous experiments were performed with Gal4-AH, a weaker activator, and Gal4(1−94), which lacks an activation domain. The five Gal4 binding sites are equally saturated at 3.8 μM concentration of each Gal4 protein (Figure 2C,D). The protection of Gal4 binding sites by Gal4-AH and Gal4(1−94) mirrored, in both pattern and saturation, the protection observed with Gal4-VP16.

It should be noted that the extent of protection of the TATA box decreased as the concentration of Gal4-VP16 increased. This observation has been reported previously (*80*) and results presumably from competition between the VP16 activation domain and the TATA box for the DNA binding surface of TBP (*25, 61, 81, 82*). Previous work has shown that a Gal4 activation domain lacking a DNA binding domain sequesters TBP and inhibits binding of the TATA box by virtue of complex formation in solution (*83*). Since previous control experiments demonstrated that DNA affinity cleavage requires bound TBP, the existence of free K97C-OP (or free K97C-OP•Gal4-VP16 complexes) in these experiments should not influence the extent or location of DNA cleavage.

FIGURE 2: MPE−Fe footprinting (*66*−*68*) and OP−Cu affinity cleavage analysis (*69*−*71*) of the interactions between K97C-OP TBP and the AdMLP TATA box in the absence or presence of Gal4-VP16. (A, B) Representative footprint and cleavage reactions in the presence and absence of Gal4-VP16 on the noncoding and coding strands, respectively. The DNA cleaving agents present in each reaction are indicated above each lane. K-OP refers to K97C-OP; MPE−Fe indicates hydroxy radical footprinting reactions; and Cu^{2+}, H_2O_2, and MCPA indicate affinity cleavage of the DNA by the OP−Cu complex. Wt TBP refers to wild-type yeast TBP. A lane in which the DNA has been cleaved at all adenosine bases for sequencing purposes is labeled as A lane. The TATA box is labeled by the sequence on the respective strand and a black bar to the left of each gel. Gal4 sites are indicated by gray bars to the left of each gel, labeled A−D, and correspond to those shown in Figure 1C. (C, D) Footprint and cleavage controls on 5′ and 3′ end-labeled noncoding strands, respectively. Abbreviations and labeling of Gal4 sites and the TATA box are as described for panels A and B. Graphic depiction of a representative MPE−Fe footprint on a Gal4 site is shown below panel C. The Gal4 binding sequence is shown in black with the 2 bp spacer in gray. The black bars represent a quantitative measure of the protection from hydroxy radical cleavage by Gal4-VP16. All Gal4 sites A−E followed the same pattern of protection in the presence of Gal4-VP16, Gal4-AH, or Gal4(1−94). The numbering specified in Figure 1 and used in the text is shown below the Gal4 sequence for reference.

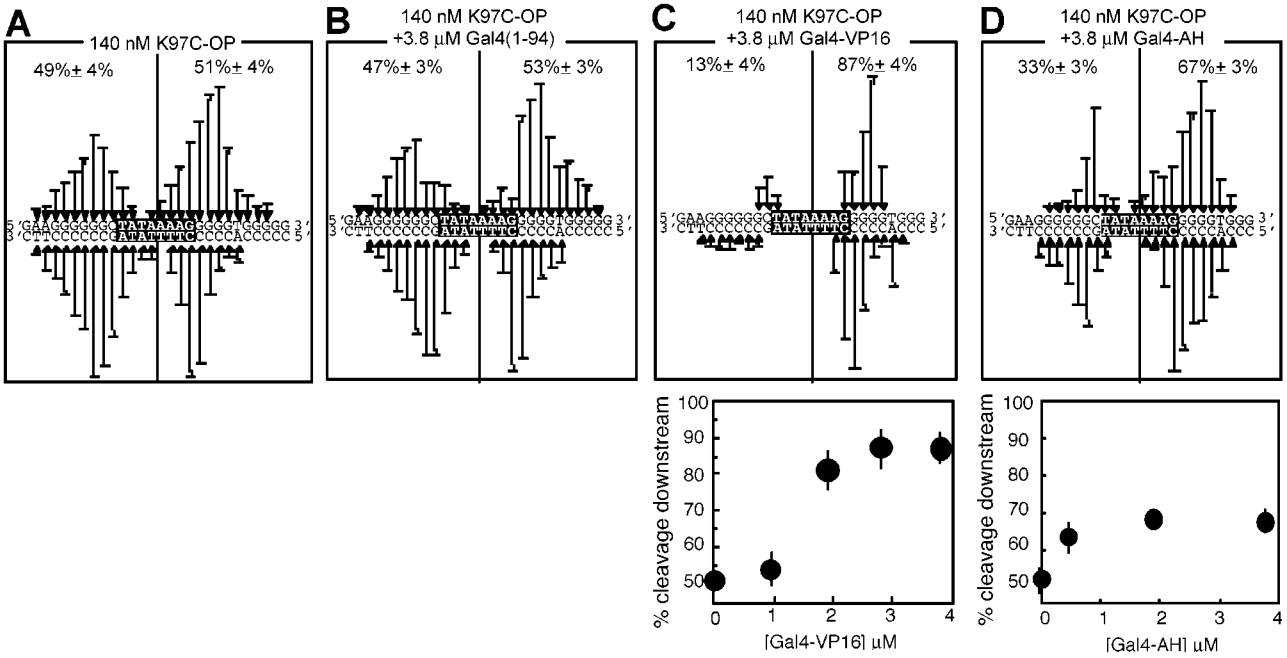


FIGURE 3: Cleavage patterns reveal that Gal4-VP16 and Gal4-AH orient TBP. Histograms illustrating cleavage at each base pair of the AdMLP TATA box and flanking DNA by K97C-OP (A) alone, (B) in the presence of the Gal4 DNA binding domain, (C) in the presence of Gal4-VP16, and (D) in the presence of Gal4-AH. The extent of cleavage at each position above a cupric ion, hydrogen peroxide, mercaptopropionic acid control is proportional to the length of the arrow. Error bars indicate the standard deviation of 3−8 trials. The TATA box is shaded gray. Graphs below panels C and D show the concentration dependence of the ability of activators Gal4-VP16 and Gal4-AH, respectively, to orient TBP. The percentage of total cleavage by K97C-OP that occurred on the downstream half of the TATA box is plotted as a function of activator concentration.

Gal4-VP16 and Gal4-AH Increase the Orientational Specificity of TBP, Although to Different Degrees. To test the effect of Gal4-VP16 on TBP's orientational specificity, we incubated 140 nM K97C-OP (the concentration that only modestly protected the TATA box) with between 0.85 and 3.8 μM Gal4-VP16 and assessed the ratio of TBP·TATA orientational isomers by DNA affinity cleavage (Figures 2A,B and 3C). Only a negligible increase in the orientational specificity of K97C-OP was observed at the lowest concentration of Gal4-VP16 (0.85 μM). The ratio of TBP·TATA orientational isomers observed (54:46) was close to that observed in the absence of activator (51:49). However, higher concentrations ($\geq$1.9 μM) of Gal4-VP16 had a clear and measurable effect on yTBP orientation. At 1.9 μM Gal4-VP16, the ratio of orientational isomers was approximately 80:20 in favor of the orientation observed by crystallography. The maximum increase in TBP orientational specificity was observed at a Gal4-VP16 concentration of 2.8 or 3.8 μM. Under these conditions, 87% of the TBP·TATA complex isomers were oriented correctly in the presence of 3.8 μM Gal4-VP16; only 13% of the complexes formed in the unproductive orientation (Figure 3C). This 87:13 ratio corresponds to a free energy difference ($\Delta\Delta G_{obs}$) of 1.1 kcal·mol^{-1} between the two TBP·TATA orientational isomers in the presence of 3.8 μM Gal4-VP16, clearly favoring the correct orientation. This ratio is comparable to those seen in the presence of saturating concentration of TFIIA (84:16) or TFIIB (80:20) (*39, 40*).

In comparison with Gal4-VP16, Gal4-AH enhanced the orientational specificity of K97C-OP only modestly, and Gal4(1−94) had no statistically significant effect (Figures 2C,D and 3). Again, we incubated 140 nM K97C-OP with between 0.48 and 3.8 μM Gal4-AH to determine the maximum ratio of TBP·TATA orientational isomers by DNA affinity cleavage. At the lowest concentration of 0.48 μM Gal4-AH, 63% of TBP orientational isomers were bound correctly. Increasing the concentration of Gal4-AH from 1.9 to 3.8 μM only minimally changed the orientational isomer ratio, with 68% and 67% of TBP bound in the transcriptionally productive orientation, respectively. The 67:33 ratio of TBP·TATA orientational isomers represents a $\Delta\Delta G_{obs}$ of 0.4 kcal·mol^{-1} in favor of the correct orientation (Figure 3D). In contrast, a free energy difference of less than 0.1 kcal·mol^{-1}, equivalent to a 54:46 ratio, separated the orientational isomers of TBP in the presence of 3.8 μM Gal4-(1−94) (Figure 3B). Together the DNA affinity cleavage data indicate that the orientational specificity of TBP can be enhanced significantly by interactions between transcriptional activation domains and TBP and that orientational specificity, at least in this case, correlates with activator strength.

Gal4-VP16 and Gal4-AH Increase the Axial Specificity of TBP. In addition to their effects on TBP's orientational specificity, both Gal4-VP16 and Gal4-AH increased TBP's axial specificity as judged by a decrease in the width of the cleavage pattern produced in their presence (Figure 3). In the absence of any activator, the cleavage pattern produced by K97C-OP spanned 30 bp centered on the TATA box. However, in the presence of 3.8 μM Gal4-VP16 and Gal4-AH, the cleavage pattern was localized to 8 and 11 bp, respectively, on the downstream side of the TATA box and 9 bp in both cases upstream of the TATA box. Similarly, quantitative analysis of the MPE−Fe footprints of TBP bound to the TATA box indicate that axial specificity is conferred on TBP by the activators (Figure 4). In the absence of activator, the MPE−Fe footprint of K97C-OP spanned 12 and 14 bp centered around the TATA box on the coding and noncoding strands, respectively. In the presence of 3.8 μM Gal4-VP16, the footprint narrowed to 7 bp on the coding

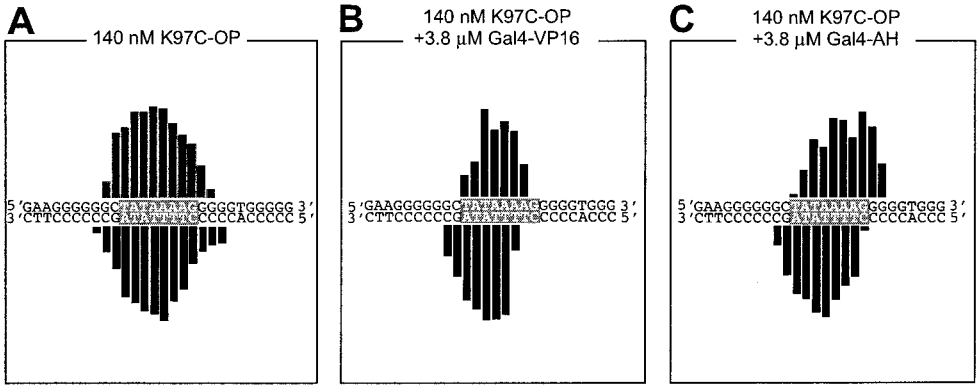


FIGURE 4: Quantitative analysis of MPE−Fe footprints of K97C-OP bound to the TATA box demonstrate that axial specificity is conferred on TBP by Gal4-VP16 and Gal4-AH. Histograms illustrating the degree of protection by K97C-OP at each base pair of the AdMLP TATA box and flanking DNA (A) alone, (B) in the presence of Gal4-VP16, and (C) in the presence of Gal4-AH. The extent of protection at each nucleotide from cleavage by MPE−Fe above background is proportional to the length of the bar. The TATA box is shaded gray.

strand and 8 bp on the noncoding strand. Even the weaker activator Gal4-AH narrowed the MPE−Fe footprint of K97C-OP to 10 bp on both the coding and noncoding strands. By contrast, the DNA binding domain Gal4(1−94) had no effect on the width of the cleavage pattern, indicating that the mere presence of protein bound upstream does not enhance axial specificity. This result emphasizes the role of the VP16 and AH activation domains (and not the Gal4 DNA binding domain itself) in the control of TBP specificity. Although the asymmetry present in yTBP itself cannot specify precise binding directly over the TATA box (*84*), this specificity can be achieved through interactions with Gal4-VP16 and Gal4-AH.

DISCUSSION

Activators Increase Transcription from Specific Promoters. Gene-specific activators that enhance transcription can be divided functionally into those that (1) remodel nucleosomes, (2) favor PIC assembly, and/or (3) facilitate postinitiation events (*47, 85*). Evidence for activator involvement in PIC assembly derives from documented direct interactions between activators and several PIC components (*52*), including TBP (*58, 86*). These contacts are believed to facilitate recruitment (*47, 49*−*53*), in which PIC formation is enhanced by bound activators that bind basal factors and increase their local concentration near the promoter (*52*). Recruitment is supported by a variety of studies. For example, fusion of TBP to a heterologous DNA binding domain increases the effective concentration of TBP near the promoter and enhances transcription both in vivo and in vitro. This "activator bypass experiment" is consistently cited as strong evidence that recruitment is an important component of transcriptional activation (*49, 87*−*91*). In a similar way, Gal11P, a mutant of the pol II holoenzyme component Gal11, interacts with the Gal4 dimerization domain and triggers gene activation in the absence of an activation domain (*92, 93*). Additional support for recruitment derives from the observation of enhanced transcription when PIC components are overexpressed in the absence of an activator (*94, 95*). Finally, recruitment in its simplest form is supported by the observation that transcriptional activation does not occur when an activation domain is disconnected from a DNA binding domain (*96*).

However, other studies suggest that recruitment in its simplest form cannot fully and always account for transcriptional activation. For example, recent findings suggest that Gal4 and TBP do not bind cooperatively to either the AdMLP or a promoter containing a nonconsensus TATA box (*97*). Additionally, Gal4-VP16 fails to stimulate the levels of TBP bound to the TATA box in multiple reports (*56, 62, 98*). Further, the activation domains of both Gal4 and VP16 compete with the TATA box for the same binding surface of TBP (*61, 83*). As a result, a "hand-off" model of activation has been proposed in which the activator releases TBP before TBP binds to the TATA box (*58*). These findings that Gal4 and VP16 do not enhance binding of TBP to the TATA box imply that these activators use an alternative form of recruitment to enhance transcription.

Activators May Increase the Formation of Correctly Oriented PICs on a Promoter. Although increasing the effective concentration of a basal factor at the promoter is one way to facilitate PIC assembly, another way is to prevent the formation of nonproductive PICs (*52, 99, 100*). Footprinting experiments (*76, 101*) suggest that only a small fraction of assembled PICs produce basal levels of transcription (*102*−*104*), while cross-linking studies have shown that active PICs are oriented (*105*). By disfavoring the formation of inappropriately assembled PICs, activators facilitate productive PIC assembly (*100, 102, 106*). Studies have shown that activators are necessary only in early steps of PIC assembly and are dispensable later (*99, 107*). Once the assembling PIC is committed to a productive conformation, activators are rendered obsolete and are no longer needed to stabilize the complex. Significantly, reports suggest that this activation mechanism is not necessarily mutually exclusive with simple recruitment (*96, 100*). Indeed, our results support a mechanism of orientational recruitment in which activators alter the equilibrium between active and inactive PIC isomers by stabilizing correctly oriented PICs more than incorrectly oriented PICs.

Gal4-VP16 and Gal4-AH Orient TBP on the TATA Box. Our data show that the proportion of correctly oriented TBP•TATA complexes is increased significantly by Gal4-VP16. In addition, Gal4-VP16 axially restricts TBP to the TATA box, limiting the number of overlapping sites that TBP samples when binding promoter DNA. Saturating concentrations of Gal4-AH elicit a far milder effect on TBP•TATA box orientation; only 67% of the complexes were oriented correctly when Gal4-AH was bound upstream. Still, Gal4-AH axially limited the binding of TBP over the TATA box.

In contrast, Gal4(1−94), which lacks an activation domain, had no effect on orientational or axial specificity. These results support the idea that activators can enhance PIC assembly by reducing the fraction of nonproductive PICs assembled on the promoter. Since our cleavage experiments are performed at equilibrium, the significant effect of Gal4-VP16 on TBP orientation demands that Gal4-VP16 interacts with TBP in an activator·TBP·TATA ternary complex, a variation of the "hand-off" model (*58*).

The relative enhancements by Gal4-VP16 and Gal4-AH on the orientational specificity of the TBP·TATA complex correlate well with multiple reports that Gal4-AH is a weaker activator than Gal4-VP16 (*56*, *57*). Still, the effect on the orientational and axial positioning of TBP on the TATA box by Gal4-AH supports the existence of Gal4-AH·TBP interactions. The absence of any orientational enhancement by Gal4-(1−94) further strengthens the role of the VP16 and AH activation domains in mediating the orientational and axial specificity of the TBP·TATA complex. The mere presence of proteins bound 35 bp upstream of the TBP·TATA complex was insufficient to confer orientational specificity.

It is interesting that previously reported "activator bypass" experiments (*49*, *87*−*91*) could be interpreted to support orientational recruitment of TBP by activators. This alternative interpretation recognizes that the TBP-DNA binding domain chimeras reported are—by virtue of their structure—orientationally restricted in binding to the promoter. Thus, the time spent sampling nonproductive TBP·TATA box orientations decreases, thereby increasing the formation of productively oriented PICs. Similarly, an alternative interpretation of the results describing recruitment through overexpression of PIC factors (*94*, *95*) could be drawn from the increase in local concentration of basal factors at the promoter. Rather than compensating for the absence of cooperativity with activators, a larger population of more stable, correctly oriented PICs assembled could overcome the need for orientational positioning by activators. We have now shown that two basal factors, TFIIA and TFIIB, and two activators, Gal4-VP16 and Gal4-AH, enhance the orientational and axial specificity of TBP for the TATA box. It is likely that the orientation of TBP, and thus the PIC, is determined by the influence of several components of the transcriptional machinery, including basal and gene-specific factors.

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