TATA-binding Protein and the Gal4 Transactivator Do Not Bind to Promoters Cooperatively*

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The yeast Gal4 protein, like many activators, binds TATA-binding protein (TBP) directly in vitro. It has been speculated that this protein-protein interaction is important for Gal4p-mediated activation of transcription, but little work has been done to test specific models involving this interaction. In this study, the effect of Gal4p on TBP-TATA binding is addressed. Specifically, it is asked if the Gal4p-TBP interaction can support cooperative binding of the two factors to promoters. It is easy to see how such an event could stimulate transcription, particularly from promoters with a non-consensus TATA box. In vitro, however, a derivative of Gal4p (Gal4-(1–93+768–881)) containing the DNA-binding, dimerization, and activation domains does not bind to promoter DNA cooperatively with either recombinant, purified TBP, or with protein from a yeast crude extract. In vivo, reporter gene experiments using promoters with differing TBP affinities reveal no major Gal4p-mediated stimulation of TBP function from weak TATA boxes, as would be predicted if the proteins bind cooperatively. Furthermore, native Gal4p and a potent Gal4p-based artificial activator lacking a TBP-binding activation domain support similar ratios of transcription from a series of promoters identical except for mutations in the TATA box. It is concluded that Gal4p and TBP do not bind cooperatively to promoters and that this mechanism does not contribute substantially to Gal4p-mediated transcriptional activation.

Gal4 is a yeast activator that regulates genes involved in the metabolism of galactose and related sugars (1). Like most activators, Gal4p contains a sequence-specific DNA-binding domain and an activation domain (AD).1 Gal4’s AD is of the “acidic” variety, a putative family that includes the well studied VP16 (2) and p53 (3) activators as well as many others. In vitro the Gal4p AD has been reported to interact with many transcription factors including TBP, TFIIIB, Gal11p, Srb4p, and others (4–6). It is generally thought that one or more of these interactions is critical for Gal4p-mediated activation. Exactly how these contacts stimulate GAL gene expression is not clear.

It is generally thought that activators recruit transcription factors to the core promoter, but details of how this occurs are lacking in most cases, and many activators have been demonstrated to mediate post-recruitment steps as well (7–9). An important goal in this area is to devise experimental tests of well defined mechanistic models both in vitro and in vivo.

TBP-promoter binding is thought to be the first committed step in the assembly of a preinitiation complex. Furthermore, many reporter gene experiments have demonstrated that transcriptional output is usually correlated with the affinity of the TATA box for TBP (10, 11), implying that TBP-promoter association can be at least partially rate-determining. Given these facts, it is appealing to postulate that this step is a target of activators, including Gal4p. Indeed, recent in vivo cross-linking studies have shown that TBP does not associate with the GAL1 promoter prior to induction of Gal4p activity (12, 13).

A specific model for how direct Gal4p-TBP contact might activate transcription is through cooperative promoter binding. In this way, a Gal4p dimer stably associated with an upstream activation sequence could stimulate TBP-core promoter binding. In this study, the Gal4p-TBP cooperative binding model is tested directly. It is shown that a Gal4 derivative containing the complete DNA-binding, dimerization, and activation domains does not bind DNA cooperatively in vitro or with either recombinant TBP purified from Escherichia coli or with TBP present in a yeast crude cell extract. Furthermore, a series of reporter gene experiments designed to detect cooperative Gal4p-TBP binding to promoters in vivo did not provide evidence of such an event. It is concluded that stabilization of TBP-TATA contacts via cooperative binding is not a major function of the Gal4 activator.

MATERIALS AND METHODS

Yeast Strains—Sc18 (GAL4, gal80, uro3–52, leu2–3, 112, his3, trp, MEL1) was used in all reporter gene assays, except for those that employed the Gal4 DNA-binding domain-Gal11 C-terminal fusion protein, for which Sc302 (gal11::HIS, gal4-190) was employed.

Proteins—Recombinant proteins His6-TBP and Gal4p(1–93+768–881) were purified as described previously (14). The concentration of each purified protein was determined by the Bradford assay and SDS-polyacrylamide gel electrophoresis followed by Coomassie Blue staining using bovine serum albumin as a standard. The sequence-specific DNA binding activity of the purified Gal4p(1–93+768–881) protein was determined by electrophoresis mobility shift assay at DNA and protein concentrations well above the Kd of the complex (~2 × 10–10 M). The TATA binding activity of the purified TBP (15) was determined similarly except that fluorescence polarization using a short, fluoresecin-labeled DNA fragment containing a consensus TATA sequence was employed to monitor binding. The protein concentrations given in this article represent the fraction of each preparation determined to be active for DNA binding.

Plasmid Constructs—The plasmids used in reporter gene assays were constructed as described previously (11). A plasmid that expresses Gal4-(1–145)-Gal11-(799–1081) in yeast was constructed as follows. pSB32Gal4 was digested with CiaI, made blunt-ended, and then cut

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1 The abbreviations used are: AD, activation domain; TBP, TATA-binding protein; HA, hemagglutinin; TF, transcription factor; PCR, polymerase chain reaction; UAS, unactivated sequence.
with BamHI. The resulting fragment was pasted into YEp351 cut with BamHI and SmalI. The resulting construct, YEpGal4, expresses Gal4-(1–145) under the control of the natural promoter of GAL4. The coding region of the Gal11 protein’s C terminus (residues 799–1081) was PCR-amplified from yeast genomic DNA using the following primers: 5′-CGA GCT CAG AAT ACC GCC TGT CGA CTC-3′ and 5′-CAC CAG TGA GAC GGG CAA CAG C-3′. The PCR products contain a consensus Gal4p binding sequence (upstream activating sequence, or UAS) (16) and a single TATA box (TATAAAA, called MLP since it corresponds to the consensus TATA box found in the adenovirus major late promoter, or TTTAAA, called T2). TATA-only is a 277-base pair PCR product derived from using UASTATA-MLP as the template and 5′-GCT CGA AGG GCC TGT CGA CTC-3′ and 5′-GCT CGA AGG CTA TGA GAG TGG G-3′ as primers. The PCR product contains a single MLP TATA box sequence.

Fluorescence Polarization Assay—A solution containing 40 nM Gal4p-(1–93+768–881) and 10 nM F-UAS21 in GD buffer (20 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM MgCl2, 1 mM EGTA, 1 mM β-mercaptoethanol, 20 μM zinc sulfate, 50 μM EDTA, 4 mM magnesium, and 10% glycerol) was incubated at room temperature for 15 min to allow Gal4-UAS complex formation. TBP was then added at the concentrations indicated in Fig. 1. The solutions were further incubated for 60 min before the polarization value was measured using a Beacon 2000 instrument (PanVera). Polarization values are reported in units of mA (17). The polarization values were converted to anisotropy value using the equation A = 2P/3 – P (A = anisotropy value, P = polarization value) and plotted against the concentration of TBP. F-UAS21 is a 21-base pair, 5′-fluorescein-labeled oligonucleotide containing a central consensus 17-base pair Gal4 binding site.

Restriction Enzyme Digestion Protection Assay—Radiolabeled UASTATA-T2 DNA (9 nM) was first incubated with Gal4p (at the concentrations indicated in Fig. 2B) on ice for 30 min in 45 μl of GTD buffer (20 mM HEPES, pH 7.5, 50 mM potassium acetate, 1 mM β-mercaptoethanol, 20 μM zinc sulfate, 50 μM EDTA, 4 mM magnesium, and 10% glycerol). Aliquots from this solution were loaded onto a 6% native polyacrylamide gel in order to verify Gal4p-DNA binding by gel retardation. Then purified TBP was added to the reactions at various concentrations. The solutions were further incubated for 30 min at room temperature. DraI (20 units, New England Biolabs) was then added, and the incubation was continued at 37 °C for 4 min before the restriction reaction was stopped by addition of 60 μl of formamide, followed by heating to 95 °C. The radiolabeled DNA was separated on a 6% denaturing polyacrylamide gel and quantitated using a PhosphorImager (PanVera). Polarization values are reported in units of mA (17). The polarization values were converted to anisotropy value using the equation A = 2P/3 – P (A = anisotropy value, P = polarization value) and plotted against the concentration of TBP. F-UAS21 is a 21-base pair, 5′-fluorescein-labeled oligonucleotide containing a central consensus 17-base pair Gal4 binding site.

RESULTS

Recombinant, Purified Gal4p-(1–93+768–881) and TBP Do Not Bind Cooperatively to Promoter DNA—Many studies of Gal4 AD-transcription factor interactions have made use of fusion proteins containing the 34-amino acid core region of the AD (residues 841–875), which is also sufficient to bind the Gal80 repressor (20, 21). In this study, a Gal4p-(1–93+768–881) fusion protein, containing a much larger fragment of the C terminus as well as the DNA-binding and dimerization domains, was employed. As shown in Fig. 1A, a fluorescence polarization experiment was done to verify that this particular Gal4p fragment binds recombinant TBP. Increasing amounts of TBP were added to a fluorescein-labeled 21-base pair oligonucleotide containing a consensus Gal4p binding site in the presence or absence of saturating amounts of Gal4p-(1–93+768–881). When the Gal4p was present, it was preincubated with the DNA to allow complex formation prior to introduction of TBP. The increase in the mA value in the presence of the Gal4 derivative (top curve) indicates association of the two proteins since little change in polarization was observed in the absence of the activator (lower curve). B, a double reciprocal plot of the data obtained in A with the Gal4 derivative present.

FIG. 1. DNA-bound Gal4p-(1–93+768–881) binds to TBP. A, fluorescence polarization assay. Increasing amounts of TBP were added to a fluorescein-labeled 21-base pair oligonucleotide containing a consensus Gal4p binding site in the presence or absence of saturating amounts of Gal4p-(1–93+768–881). When the Gal4p was present, it was preincubated with the DNA to allow complex formation prior to introduction of TBP. The increase in the mA value in the presence of the Gal4 derivative (top curve) indicates association of the two proteins since little change in polarization was observed in the absence of the activator (lower curve). B, a double reciprocal plot of the data obtained in A with the Gal4 derivative present.

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TATA sequence (11). The DNA fragment also contained a single high affinity Gal4p binding site about 100 base pairs upstream of TATA. The DNA was first incubated in the presence or absence of Gal4-(1–93768–881). Then various amounts of TBP were added, and the reactions were allowed to reach equilibrium. Dra1 was added to digest the DNA lacking a bound TBP (Fig. 2A), allowing the determination of whether the presence of bound Gal4-(1–93768–881) increased the degree of TBP-dependent protection of the DNA from Dra1 cleavage. The parameters of the experiment were designed so that even a modest effect would be detected. The TATA-T2 DNA was present at a concentration (9 nM) comparable to the $K_d$ of the complex ($\sim 10^{-8}$ M). Thus, even when the TBP level reaches an equimolar concentration, the DNA will not be saturated with TBP at this dilution unless the presence of the Gal4 derivative increases the affinity of TBP for the DNA. However, even in the absence of a Gal4p effect, some binding will be observed, making it easy to verify that the experiment is working. In this concentration range, if the presence of a Gal4p derivative on the DNA increased the affinity of TBP even by a modest 5–10-fold, it would have a substantial and easily measured effect on the fraction of DNA protected from Dra1 cleavage. Independent measurements determined that the dissociation constant of the Gal4p-(1–93768–881) complex with this DNA is $\sim 2 \times 10^{-10}$ M (data not shown). Since the DNA concentration is well above this level, almost all of the active Gal4p derivative added is expected to bind DNA. For reasons discussed in detail previously (22), this experiment was repeated at several different Gal4p derivative/DNA stoichiometries.

As shown in Fig. 2B, gel shift analysis of a fraction of the aliquots used for Dra1 cleavage confirmed that the Gal4p derivative, when present, was indeed loaded stoichiometrically onto the DNA substrate. Therefore, under these conditions, the presence of a stably bound Gal4 dimer on the DNA should facilitate binding of TBP if the two proteins bind cooperatively. However, as shown in Fig. 2C, the apparent affinity of TBP for the DNA, as measured by Dra1 protection, was unaffected by the presence of Gal4-(1–93768–881) within experimental er-
We conclude that the two purified polypeptides do not bind to DNA cooperatively in vitro.

**TBP (TFIID) Does Not Bind Preferentially to Gal4-bound Promoter DNA**—An obvious concern about the Fig. 2 experiment, conducted with two purified proteins, might not reflect the in vivo situation. Specifically, TBP is thought to function as part of larger transcription complexes (23, 24) that could exhibit different promoter- and/or Gal4p-binding properties than isolated TBP. To address the question of whether or not Gal4p can stabilize binding of a more biologically relevant form of TBP to promoters, a binding assay was carried out with a crude whole cell extract. Equivalent amounts of two DNAs containing identical core promoter sequences were mixed with Gal4-(1–768–881) dimer was present in a slight excess over the UAS-containing DNA (1.5:1) and the absolute concentration of the active protein was more than 10 times above the $K_d$, ensuring saturation of the UAS-containing DNA. This was confirmed by gel shift analysis (data not shown). The reaction mixture was then incubated with extract prepared from yeast that express a triply epitope (HA)-tagged TBP (18). Subsequently, immobilized anti-HA antibodies were added into the reaction to immunoprecipitate the HA-tagged TBP and the labeled DNA associated with TBP. The amount of TBP was limiting with respect to the DNA, so if the activator derivative enhances the affinity of TFIIID or any other TBP-containing complex for DNA, then the longer, activator-bound DNA should be enriched in the immunoprecipitate relative to the DNA lacking an activator binding site. In other words, in this competition experiment, the activator-bound DNA should out-compete the shorter DNA if there is cooperative binding.

As shown in Fig. 3B, the ratios of the two DNAs in the immunoprecipitated fraction was identical to that in the input in the absence of the Gal4p derivative (compare lanes 3 and 5). This confirms that TBP binds to both molecules with equal affinity, as expected. More importantly, when Gal4-(1–93+768–881) was also included in the solution at a saturating level, the result was the same (compare lanes 4 and 5). This demonstrates that TBP, even in the context of TFIIID and/or other larger transcription complexes present in the extract, has no preference for Gal4p-bound DNA. It is concluded that the two proteins do not bind cooperatively to DNA even in the context of a crude extract.

**Probes for Cooperative Gal4p-TBP Binding to Promoters in Vivo**—Although the data shown above reveal no evidence of cooperative binding between Gal4-(1–768–881) and TBP, even in the context of a crude extract, it is conceivable that the result would be different in vivo. This could be due to the fact that chromatin, rather than naked DNA, is the template, or that full-length Gal4p behaves differently than the derivative employed for the in vitro assays, or other differences between the in vivo and in vitro milieu.

To probe for cooperative binding of native Gal4p and TBP in vivo, a series of reporter gene experiments were employed. A fundamental tenet of the cooperativity model is that when the activator is established on the promoter, it should facilitate binding of TBP to the TATA box significantly relative to the DNA lacking an activator binding site. In other words, in this competition experiment, the activator-bound DNA should out-compete the shorter DNA if there is cooperative binding.

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of plasmids contained a single Gal4p binding site from the native MEL1 promoter located about 100 base pairs upstream of TATA, while the other did not. When basal transcription was measured from plasmids lacking the Gal4p binding site, the expected reduction in lacZ expression was observed as the affinity of the TATA region for TBP was reduced (Table I). As is also shown in Fig. 4A, the relative levels of Gal4p-activated lacZ expression from the series of plasmids containing the activator binding site tracked the basal data very closely, when each data set was normalized to the output observed for the plasmid containing the consensus TATA box region, e.g. T2 = TTTAAAA. Each set of data was normalized to the expression level observed when the reporter plasmid contained a consensus TATA box. B, same as A, except that all of the plasmids contained a Gal4p binding site. In this case, the two sets of data were obtained in otherwise congenic strains that expressed native Gal4p or Gal4-(1–143)-Gal11-(799–1081).

### TABLE I

| In vitro affinity | Basal (no UAS) | Gal4p-activated (MEL1) | Gal4-Gal11-activated |
|------------------|----------------|------------------------|----------------------|
|                  | β-Gal | MLP | β-Gal | MLP | β-Gal | MLP |
| % of MLP          |       |     |       |     |       |     |
| MLP              | 100   | 100 | 3522  | 100 | 546   | 100 |
| T5               | 80    | 16  | 20    | 7   | ND    | ND  |
| T2               | 60    | ND  | 16.8  | 5.9 | 53    | 8.0 |
| A1               | 30    | 11  | 10.5  | 3.7 | 33    | 5.0 |
| G2               | 2     | ND  | 1.6   | 0.6 | ND    | ND  |

of plasmids that differed only in the sequence of the TATA box and the presence or absence of a Gal4p binding site. Basal represents the data obtained using the plasmids lacking an activator site. Gal4p-activated represents the data obtained using the plasmids containing the UAS. MLP represents the consensus TATA sequence of the adenovirus major late promoter (5'-TATAAAA). T5, T2, and A1 represent different single nucleotide substitution in the TATA box region, e.g. T2 = TTTAAAA. Each set of data was normalized to the expression level observed when the reporter plasmid contained a consensus TATA box. B, same as A, except that all of the plasmids contained a Gal4p binding site. In this case, the two sets of data were obtained in otherwise congenic strains that expressed native Gal4p or Gal4-(1–143)-Gal11-(799–1081).

### DISCUSSION

Several experiments have been conducted to probe for cooperative binding of Gal4p, or derivatives thereof, and TBP to promoter DNA. In vitro, several biochemical approaches were taken, including experiments conducted with purified recombinant TBP (Fig. 2) and with TBP in the context of a crude yeast
extract (Fig. 3). In the latter case, most or all of the TBP should be associated with other transcription factors such as TAFs in multi-protein complexes such as TFIID. In all cases, the results of these experiments were consistent with independent binding of the Gal4p derivative and TBP. No evidence was found for cooperativity. Likewise, a series of reporter gene experiments designed to probe for Gal4p-TBP binding cooperativity on the promoter of reporter plasmids in vivo failed to provide evidence for such an event (Fig. 4). In particular, the similar results obtained in these experiments (Fig. 4B) using native Gal4p and Gal4-Gal11 argues persuasively against Gal4p-TBP binding cooperativity that relies on a direct protein-protein interaction, since the Gal4-Gal11 fusion protein presumably cannot bind directly to TBP because it lacks an activation domain.

This result is consistent with biochemical studies of the Gal4p-TBP interaction. We have shown that the Gal4 AD and TBP cannot bind to TBP simultaneously (47), presumably because they recognize overlapping sites on the underside of the TBP saddle. It also agrees with mutagenesis studies, which showed that mutations in TBP that interfere with AD binding are located on the DNA-binding surface of TBP (4, 27). We note that the VP16 AD also apparently contacts the DNA-binding surface of TBP (28–30), perhaps suggesting that the results of this study will prove applicable to other TBP-binding activators as well. On the other hand, p53 and heat shock factor, both of which are considered to be acidic activators and which bind to TBP in vitro (31–34), have been reported to bind to DNA cooperatively with TBP in vitro (34, 35), although there is no evidence that this mechanism contributes substantially to activated transcription in vivo. Therefore, it will be important to assess possible activator-TBP cooperativity as a contributor to activated transcription on a case-by-case basis.

If Gal4p-TBP contacts do not support cooperative binding to promoters, what is the mechanistic function of this protein-protein interaction? One possibility is that Gal4p-TBP interaction may play a role in the initial association of TBP with the promoter during initiation. In vivo cross-linking experiments have shown clearly that TBP does not associate with the TATA box of the GAL1 promoter until Gal4p activity has been induced (12, 13), so Gal4p either directly or indirectly plays an important role in this event. Since many inhibitors of TBP-TATA interactions are known (28, 36–40), one model is that the activator could help to compete these negative regulators from TBP and then "hand-off" the protein to the TATA box (41). This type of model would be consistent with the inability of TBP to bind Gal4p and DNA simultaneously as well as the in vivo cross-linking result. In this view, association of TBP with the promoter during reinitiation (i.e. rounds 2–n of transcription) is not an activator-regulated event (42), consistent with the reporter gene studies reported here (Fig. 4).

Another possibility that must be considered is that the Gal4 AD-TBP association is not biologically relevant. The Gal4 AD, like that of many acidic activators, has been reported to bind to a wide variety of different proteins, and it seems unlikely that all of these contacts are functionally relevant. One of the stronger arguments for special consideration of the Gal4 AD-TBP complex was the observation, first reported by Melcher et al. (4) and later by Wu et al. (5), that the binding affinities of various Gal4 AD mutants (and of other ADs) for TBP correlate well with the ability of these domains to support activated transcription in vivo when substituted for the native AD of Gal4p. However, Melcher and Johnston have found that the same correlation exists when one measures the affinity of mutant forms of the Gal4 AD for bacterial lysozyme, which is clearly not a biologically relevant contact.2 This calls into question the value of these in vitro binding/in vivo potency correlations for assessing Gal4 AD-binding partners. Another argument in favor of activator-TBP contacts being important in vivo is the observation that the direct fusion of TBP to sequence-specific DNA-binding domains can support activated transcription in vivo in the absence of an activation domain (43–45). However, it is not clear that these artificial experiments reflect the real mechanism of activation mediated by native transcription factors. Furthermore, more detailed studies of these DNA-binding domain-TBP fusions revealed that they are much weaker and context-sensitive activators than fusions of DNA-binding domains to polIII holoenzyme components such as Gal11p (26). It may be the case that, although low levels of transcription can be achieved by artificial TBP recruitment, the bulk of activator-mediated stimulation is due to recruitment of the polymerase holoenzyme and chromatin remodeling/modification complexes.

In summary, this study was designed to test the discrete mechanistic hypothesis that protein-protein interactions between Gal4p and TBP are important for activated transcription because they support cooperative binding of the two proteins to promoters. The data reported here argue against such a model. Whereas there is evidence that Gal4p does bind cooperatively to promoters with the general transcription machinery in vivo (46), it seems likely that this is through contacts with components of the RNA polymerase II holoenzyme rather than with TBP.

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