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GAL4 Interacts with TATA-Binding Protein and Coactivators

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A major goal in understanding eukaryotic gene regulation is to identify the target(s) of transcriptional activators. Efforts to date have pointed to various candidates. Here we show that a 34-amino-acid peptide from the carboxy terminus of GAL4 is a strong activation domain (AD) and retains at least four proteins from a crude extract: the negative regulator GAL80, the TATA-binding protein (TBP), and the putative coactivators SUG1 and ADA2. TFIIB was not retained. Concentrating on TBP, we demonstrate in in vitro binding assays that its interaction with the AD is specific, direct, and salt stable up to at least 1.6 M NaCl. The effects of mutations in the GAL4 AD on transcriptional activation in vivo correlate with their affinities to TBP. A point mutation (L114K) in yeast TBP, which has been shown to compromise the mutant protein in both binding to the VP16 AD domain and activated transcription in vitro, reduces the affinity to the GAL4 AD to the same degree as to the VP16 AD. This suggests that these two prototypic activators make similar contacts with TBP.

Transcriptional activators have been shown to stimulate in vitro the assembly of transcriptional preinitiation complexes (1, 7, 27, 51) as well as downstream events in the transcription process (92). Stimulation is thought to depend on antirepression of the inhibitory effects of chromatin (9; for reviews, see references 11 and 45) and on direct or indirect protein-protein interactions between transcriptional activators and the general transcriptional machinery. A strong argument for protein-protein interactions is the inhibitory effect ("squelching") on transcription caused by overexpression of GAL4 (2, 16) and other activators (see, e.g., references 4, 69, and 85).

Early studies with partially purified fractions of transcription factors suggested TFIID as the target for various activators in HeLa cells (1, 26, 27, 71). Subsequently, the TATA-binding proteins (TBPs) of yeast and human TFIID were shown to bind in vitro to the strong activation domains (ADs) of the viral activators VP16 (77), E1A (29, 46), Zta (49), and IE2 (20). Recently, this list has been extended by an increasing number of cellular activators, the first of which were PU.1 (19) and the tumor suppressor p53 (83). In vitro interactions have also been shown between TFIIB and VP16 (51), steroid receptors (3, 30), Rel oncogene products (37, 90), and the proline-rich activator CTF (40), as well as between TFIIH and the ADs of VP16 and p53 (87). All of these activators, with the exception of steroid receptors, have also been shown to bind TBP.

Activation in in vitro transcription systems requires more than the minimal components (RNA polymerase II [PolII], TFIIA, TFIIB, TBP, TFIIE, TFIIF, and TFIIH). The best-characterized complexes isolated from biochemical assays are the TBP-associated factors (TAFs) of the TFIID fraction in *Drosophila melanogaster* and humans (10, 62, 81, 93). Recently, TAFs have also been found in the yeast *Saccharomyces cerevisiae* (56, 59, 60). The PolII-associated "mediator" proteins of the Holo-PolII complex in yeasts (41, 42) and components of the USA fraction in mammals (14, 43, 44, 55) also condition response to activators in vitro. *Drosophila* TAF40 has been shown to have affinity for the acidic AD of VP16 (18), and *Drosophila* TAF110 has affinity for the glutamine-rich ADs of SP1 (24) and cyclic AMP-responsive element-binding protein (12). The USA component, PC4/p15, has been demonstrated to bind directly to ADs and to a TBP-TFIIA complex (14, 43).

Finally, three factors have been identified genetically as putative "coactivators" in yeasts: SUG1 (79), GAL11 (56), and ADA2 (5). Recently, SUG1 and GAL11 have been identified as components of the Holo-PolII complex (40) and SUG1 has been shown to interact directly with several ADs and TBP (80).

Clearly, true activation appears to be more complicated than originally envisioned (61) and might involve a large number of proteins that make direct or indirect contacts in different spatial and temporal arrangements with activators and the transcriptional machinery. It will be increasingly important to determine the biological relevance and mechanistic effects of individual interactions.

Here we show that the 34-amino-acid (aa) carboxy-terminal AD of GAL4 retains TBP and the putative coactivators SUG1 and ADA2. Mutations in the GAL4 AD that increase the dissociation constant of the AD-TBP complex in vitro are compromised in transcriptional activation in vivo, indicating that the observed interaction is biologically relevant.

MATERIALS AND METHODS

Strains, media, enzyme assays, and genetic techniques. Yeast strains used were 21R (*GAL4 GAL80 ura3-52 leu2-3,112 ade1*) and YJ0-Z (Δ*gal4* Δ*gal80 ura3-52 leu2-3,112 his3 ade2-101 trp1* with an integrated *GAL1-lacZ* reporter [47]). Yeast transformations were done by the lithium acetate method (32). *Escherichia coli* TG1 (70) and BL21 (78) were used for plasmid constructions and protein purifications, respectively. Standard yeast media (74) contained as carbon sources either 4% galactose (extracts for binding) or 3% glycerol plus 2% lactic acid (extracts for enzyme assays and electrophoretic mobility shift assay). α-Galactosidase activities were assayed as described previously (38) with whole-cell extracts prepared by the glass bead method (34). All assays were done at least in triplicate from independent transformants with standard deviations of less than 20%. The yeast centromeric vector pSB32 (a derivative of YCp50 [68]) was used to express physiological amounts of GAL4 and GAL4-AD fusions from the native *GAL4* promoter. For overexpression in yeast cells, the 2μm-based multicopy plasmid YEp351 (23) was used.

Plasmid constructions. Yeast TBP (*SPT15*) was isolated as a PCR fragment with primers that introduced a *Nco*I site at the start ATG, with plasmid pAB24 (a gift of A. J. Berk) as the template. PCR fragments were gel purified and ligated to plasmid pTL37N (47) for in vitro transcription. The plasmid for in vitro transcription of GAL4(Δ148–728) was described previously (47).

GAL4-NcoI-92 was constructed by ligating a *Nco*I adaptor oligonucleotide for in-frame GAL4(1–92)-AD fusions into the *Hpa*I site of GAL4 (corresponding to amino acids 92 to 94). AH, GAL4(841–875), GAL4(149–238), and the VP16,

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TABLE 1. Sequences of GAL4 AD, GAL4 mutant ADs, and ADs from other activators used

AD	Sequence[a]
GAL4 34-aa wild type	MDQTAYNAFGITTGMFNTTTMDDVYNYLFDDEDT
GAL4 mutant 7	----------P----------
GAL4 mutant 12	----------E---V------
GAL4 truncation Stop 870	----------
GAL4 truncation gal4D	----------
AH	MVPGIELQELQELQALLQQQ
GCN 4 (107–144)	MDSTPMFEYENLEDNSKEWTSLFDNDIPFTTDDVSLADKAIESTEEVSL
VP16 (420–490)	MGDELHLDGEDVAMAHADALDDFDLDMLGDGDSPGPGFTPHDSAPYGALDMADFEFEQMFTDALGIDEYGG
p53 (1–97)	FRVTAMEEPQSDPSVEPPLSQETFSDLWKLLPENNVLSPLPSQAMDDLMLSPDDIEQWFTEDPGPDEAPRMPEAAPPVAPA PAAPTPAAPAPAPSWPLSSSVPSQ
GAL4 region I (149–238)	MIDSAAHHDNSTIPLDFMPRDALHGFDWSEEDDMSDGLPFLKTDPNNNGFFGDGSLLCILRSIGFKPENYTNSNVNRLPT MITDRYTLASRS
TAT (1–13)	MEPVDPRLEPWK

[a] Spacer amino acids are shown in small capitals. Dashes indicate amino acids identical to those of the 34-aa wild-type GAL4 sequence.

p53, GCN4, and TAT ADs were isolated as PCR fragments with primers that introduced *Nco*I sites with in-frame start ATGs at the 5′ ends. PCR fragments were either exchanged against a *Nco*I-*Hin*dIII fragment comprising GAL4(94–881) on the multicopy plasmid YEp351 and/or, for construction of GST fusion vectors, inserted into pGEX-CS (57). Construction of wild-type and mutant forms of *GAL4* in pSB32 was described previously (48). The glutathione-*S*-transferase (GST)-TBP expression vector was constructed by inserting the *Nco*I-*Bam*HI TBP fragment from pTL37N-TBP into pGEX-CS. All PCRs were performed with *Pfu* polymerase (Stratagene) or Vent polymerase (New England Biolabs), both of which contain 3′-5′ exonucleolytic proofreading activities, and with plasmid DNA as templates. The correct sequence of all junctions was confirmed by DNA sequencing with the fmole DNA sequencing system from Promega.

Expression of GST fusion proteins and affinity chromatography. GST fusion proteins were expressed and purified on glutathione Sepharose 4B as described previously (84). Protein concentrations were determined by the bicinchoninic acid method (Pierce) after incubation of beads with bound GST fusion proteins in 1% sodium dodecyl sulfate (SDS) at 72°C for 20 min. For affinity chromatography, yeast strain 21R was grown in 500 ml of yeast extract-peptone (YEP) medium with 4% galactose as the carbon source to an optical density at 600 nm of 1.2. Cells were washed with ice-cold H_2O and broken by the glass bead method in 4 ml of phosphate-buffered saline (PBS)–1.15 M NaCl in the presence of protease inhibitors. Cell debris was removed by centrifugation, and the extract was diluted with PBS–0 M NaCl to 3.7 mg of protein per ml and 290 mM NaCl. Two 13-ml aliquots of this extract were incubated with 250 μg of GST or 250 μg of a fusion between GST and the 34-aa GAL4 AD (GST-34) immobilized to 100 μl of glutathione beads each for 1 h at 4°C on a rotating wheel. The beads were washed sequentially with 12 ml each of PBS, PBS–1% Triton X-100, PBS, and 50 mM Tris · Cl (pH 8.0). GST or GST-34, plus retained proteins, were eluted with 30 mM glutathione in 50 mM Tris · Cl (pH 8.0) for 30 min at room temperature. Aliquots of eluates and extract were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and blotted onto polyvinylidene difluoride membranes. Blots were blocked with 5% nonfat dry milk and incubated with the indicated polyclonal antibodies. Secondary antibodies were horseradish peroxidase-conjugated anti-rabbit or anti-mouse antibodies for use in the ECL (chemiluminescence) protocol (Amersham).

Determination of relative binding affinities of TBP to ADs. Proteins were in vitro transcribed from the T7 RNA polymerase promoter of pTL37N and translated in rabbit reticulocyte lysate (Promega) as described previously (47). For quantitations, all binding reactions were performed in buffer B (150 mM KCl, 10 mM $MgCl_2$, 10% glycerol, 1% Tween 20, 50 mM potassium phosphate [pH 6.0]) with 10 mg of *E. coli* protein per ml as described elsewhere (80). A 1.0-μl (ca. 0.5-ng) portion of in vitro translated ^{35}S-TBP was incubated with 10 μg of each of the GST-AD fusion proteins bound to glutathione beads in a total volume of 400 μl. To minimize potential bead losses during washes, buffer B was mixed with glutathione beads to give a total bead volume of 15 μl per reaction. Mixtures were incubated on a rotating wheel at 4°C for 1 to 2 h and washed four times with 1 ml of cold buffer B each. Bead pellets were resuspended in 2× SDS sample buffer (70), boiled for 5 min, and loaded together with the ^{35}S-TBP input onto 10% Tricine gels (72). The GST-AD input was loaded next to each sample. Gels were first stained with Coomassie blue to visualize intactness of the fusion proteins and to determine potential bead losses by direct comparison with the adjacent bead input. The amount of ^{35}S-TBP bound to a given AD in relation to input TBP was determined with a PhosphorImager (Molecular Dynamics) and Imagequant software.

For determination of the dissociation constant (K_D), the GST-34 bead slurry was diluted with glutathione beads to 200 ng/15 μl, 1 μg/15 μl, and 10 μg/15 μl. Beads were either boiled in SDS sample buffer and loaded directly onto a PAGE gel (bead input control) or incubated with in vitro translated TBP as above. Gels were first stained with Coomassie blue to compare bead input controls with beads in the binding reaction and were then exposed on a PhosphorImager screen to determine the percent TBP bound relative to TBP input. The relationship between percent TBP bound and K_D is $K_D = [\text{GST-34}]_{eq}\,[\text{TBP}]_{eq}/[\text{GST-34 TBP}]_{eq}$. As $[\text{GST-34}]_{total}$ is in a large excess over $[\text{TBP}]_{total}$, $[\text{GST-34}]_{eq}$ equals approximately $[\text{GST-34}]_{total}$ and $[\text{TBP}]_{eq}/[\text{GST-34 TBP}]_{eq}$ is the determined ratio of free to bound TBP.

Gel mobility shift assays. Transformants were grown in selective media with glycerol and lactic acid as the carbon source to an optical density at 600 nm of 0.5. Cells were washed and broken by the glass bead method in 50 mM Tris · Cl (pH 7.5)–10 mM $MgCl_2$–0.25 mM EDTA–600 mM KCl–20% glycerol–1 mM dithiothreitol in the presence of protease inhibitors. Extracts were diluted to 15 mg of protein per ml (single-copy gel shift) or 5 mg of protein per ml (multicopy gel shift) with extract buffer. A 10-μl aliquot of each extract was incubated with 10 μl containing 1.5 μg of salmon sperm DNA and 5 ng (single-copy gel shift) or 1 ng (multicopy gel shift) of a GAL4 consensus-binding-site oligonucleotide in 0.1 mg of phenylmethylsulfonyl fluoride per ml for 20 min at room temperature. Oligonucleotides 5′-TCGAGCGGAGGACTGTCCTCCGG-3′ and 5′-TCGAC CGGAGGACAGTCCTCCGC-3′ were annealed to each other and end labeled with [γ-^{32}P]ATP and T4 kinase. A 0.5-μl volume of anti-GAL4 antibody (see below) was added to reaction mixtures, when indicated, after the first 5 min of incubation. Protein-DNA complexes were resolved in 4% acrylamide gels in 0.5× TBE (12.5 mM Tris, 95 mM glycine, 0.5 mM EDTA) at 10 V/cm and 4°C. Gels were exposed on either Kodak X-OMAT film or PhosphorImager screens. The gel shift bands were quantified on the PhosphorImager with Imagequant (Molecular Dynamics) software.

Antibody production and purification. A young female rabbit was given an injection of 200 μg of purified GAL4(1–140) (a generous gift of S. Vashee and T. Kadodek) and Freund's complete adjuvant (Sigma). The rabbit was boosted eight times with 100 μg of GAL(1–140) and Freund's incomplete adjuvant and finally sacrificed. For gel shifts, immunoglobulin G of the crude serum was purified on a protein A column (Pierce).

RESULTS

A 34-aa region from the C terminus of GAL4 is necessary and sufficient for transcriptional activation. Deletion analysis of the 881-aa transcriptional activator GAL4 defined two regions comprising ADs, an internal region (aa 148 to 196) and the C-terminal 113 aa (54). Interaction of the negative regulator GAL80 with aa 852 to 875 in the C terminus blocks transcription (35, 52, 53), and deletion of this region reduces transcriptional activation by 97% in vivo (79). We tested whether a 34-aa region (aa 841 to 875 [Table 1]) encompassing the GAL80-binding site independently as an AD by expressing it as a fusion to the minimal GAL4 DNA-binding domain [GAL4(1–92)-34] in a *gal4* deletion strain (Fig. 1A). Figure 1B shows that GAL4(1–92)-34 activates expression from the endogenous GAL4-regulated *MEL1* gene to a similar level to that activated by the strong AD VP16 fused to GAL4(1–92) and to a level about 14 times higher than that activated by GAL4(1–92)-AH, containing an artificial AD (17), which has been used extensively in in vitro transcription experiments. The weak activity of GAL4(1–92)-AH is not due to low expression

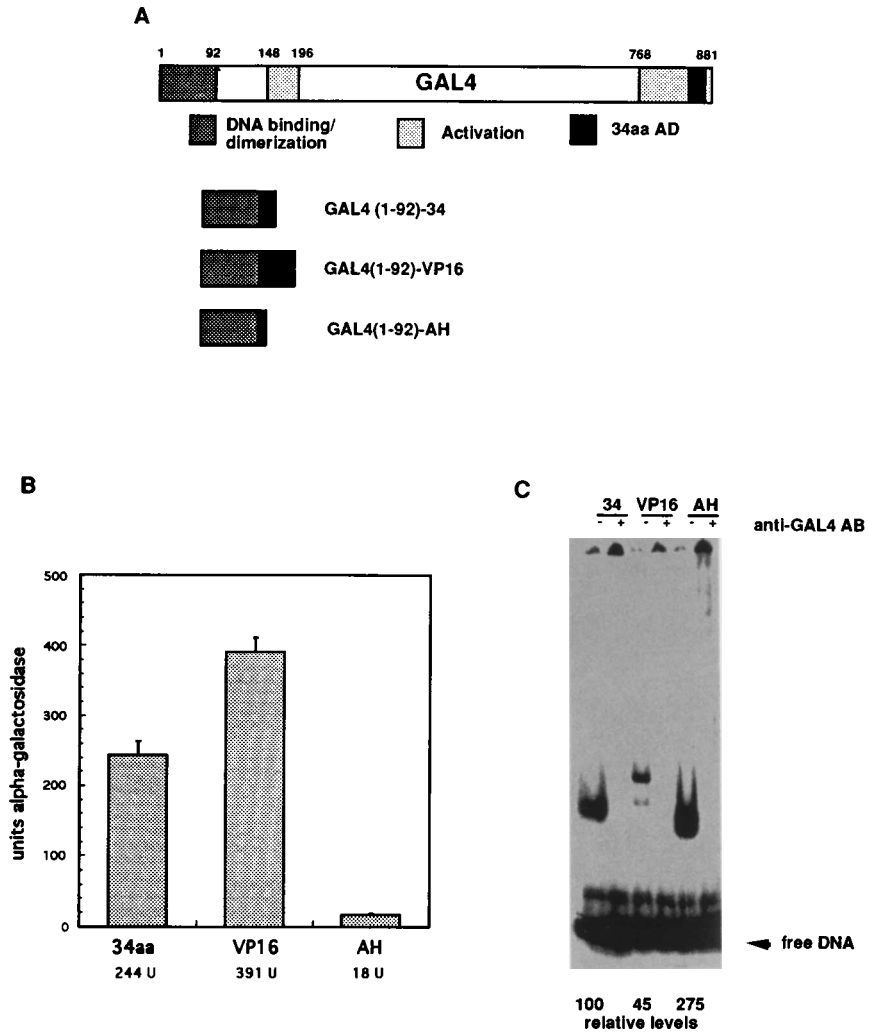

FIG. 1. A 34-aa peptide from the carboxy terminus of GAL4 is an AD. (A) Schematic presentation of the 881-aa GAL4 protein and of GAL4(1–92)-AD fusion proteins. The 34-aa AD corresponds to GAL4(841–875). (B) Activation of the endogenous *MEL1* (α-galactosidase) gene by GAL4(1–92)-AD fusions. Fusion proteins were expressed from the GAL4 promoter on a multicopy (2μm) plasmid. MEL1 activity is expressed in nanomoles of substrate conversion per minute and milligram of total protein. The reporter gene activity with GAL4(1–92) is below 5 U. (C) Electrophoretic mobility shift assay of GAL4(1–92)-AD proteins. Portions (50 μg) of whole-cell extract made from the respective GAL4(1–92)-AD transformants were incubated with 1.5 ng each of a ^{32}P-labelled GAL4 consensus-binding-site oligonucleotide and separated on a native PAGE gel. GAL4-specific complexes were identified by addition of polyclonal immunoglobulin G directed against the DNA-binding domain of GAL4. Relative amounts of retarded probe represent an average of two experiments.

levels or to interference with DNA binding. In contrast, extracts from the GAL4(1–92)-AH transformant retard 2.7- and 6.1-fold more of a radiolabelled GAL4 consensus binding site than do extracts from GAL4(1–92)-34 and GAL4(1–92)-VP16, respectively, in a gel mobility shift assay (Fig. 1C).

The activation domain of GAL4 retains TBP and putative coactivators from a whole-cell yeast extract. We overexpressed either GST or GST-34 in *E. coli* and purified the respective proteins with glutathione-Sepharose beads. Beads with immobilized fusion proteins were incubated with whole-cell yeast extract and extensively washed. Bound proteins were eluted with glutathione. Aliquots of extract and eluates were separated by SDS-PAGE and either stained with Coomassie blue or incubated with various antibodies to screen for proteins that might be specifically retained from a *GAL4 GAL80* wild-type extract.

As shown by the Coomassie blue-stained gel in Fig. 2, very little protein from the extract was retained on the GST or GST-34 resin. Binding of GAL80 to the immobilized peptide was used as a positive control. When incubated with the extract, the 48-kDa band that was recognized by the anti-GAL80 antibody in the extract was clearly detected in the GST-34 eluate but not in the GST eluate (Fig. 2). No protein was detected when the extract was made from a *gal80* deletion strain, and a greatly enhanced signal was observed in the GST-34 eluate when the extract was made from a GAL80-overproducing strain (data not shown).

TBP was also retained by the GAL4 AD, as is evident in Fig. 2. Antibody to yeast TBP (yTBP) cross-reacts with the 27-kDa TBP in the GST-34 eluant but not in the GST eluant. Two additional proteins, the putative coactivators SUG1 and ADA2, were also detected in the GST-34 eluate. Two observations argue that the retention of TBP, SUG1, and ADA2 is specific. Antibodies directed against yeast TFIIB (Fig. 2) or the GAL6 protein (88) (data not shown) recognized the respective proteins in the extract but failed to detect them in either eluate. Second, proteins that cross-hybridized with antibodies in the extract were not retained in either eluate. We conser-

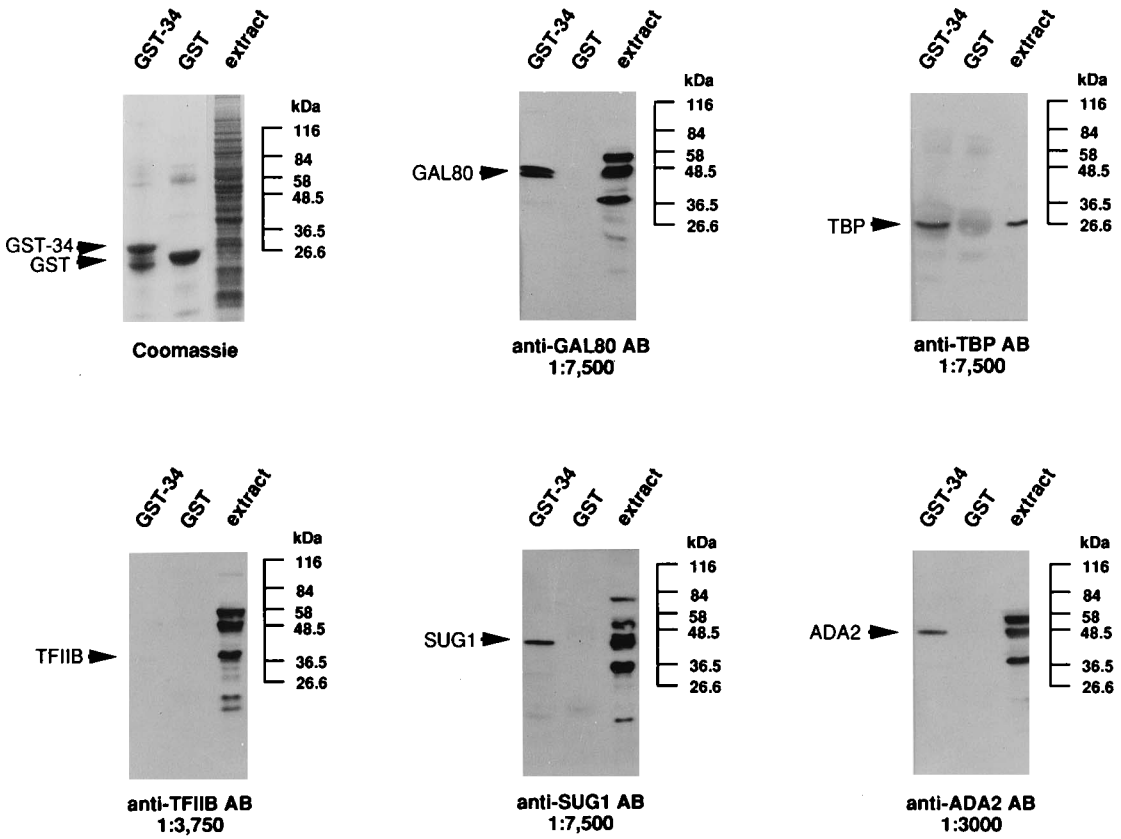

FIG. 2. The 34-aa AD of GAL4 retains TBP and coactivators from a crude extract. Yeast whole-cell extract was incubated with either GST or GST-34 immobilized on glutathione-Sepharose beads. After extensive washing, GST or GST-34, plus the proteins bound to them, were eluted with glutathione. Equal aliquots of each eluate were separated by SDS-PAGE and either stained with Coomassie blue or transferred to polyvinylidene fluoride membranes and incubated with the indicated antibodies. The major 25-kDa band in the GST-34 lane of the Coomassie blue-stained gel is a GST degradation product of GST-34; the proteins in the 55- to 60-kDa range were retained from *E. coli* during the initial purification on glutathione beads. The anti-TBP antibody was raised against recombinant TBP proteolytically cleaved from a GST fusion and shows weak cross-reactivity with GST.

vatively estimate that about 30 to 50% of total GAL80 and TBP and about 10 to 25% of total SUG1 and ADA2 were retained by GST-34. For GAL80 and TBP, the amount bound may represent most of that available to interact with the GAL4 AD: GAL80, which is present in a molar excess over GAL4 (22), is stably engaged with endogenous GAL4 (6, 47, 58), and TBP in PolI and PolIII transcription complexes (for reviews, see references 66, 73, and 86) may not interact with the AD. Yeast TFIIB was weakly (about 0.5 to 1% of total TFIIB) and apparently unspecifically retained by GST-34, since it was also weakly retained by GST (Fig. 2). The SUG1-AD interaction has been analyzed in detail elsewhere (80). In the following, we investigate the TBP-GAL4 AD interaction.

yTBP can bind to the activation domain of GAL4 independently of additional proteins. Since initially we used a crude extract, binding of TBP to GST-34 could occur by direct protein-protein interactions or indirectly through coactivators or TAFs. We wished to test whether GAL4 AD and TBP could directly interact. For this and the following experiments, we used the same conditions that we developed to reproduce in vivo interactions between GAL80 and mutant forms of the 34-aa AD (55b) (see Materials and Methods). As shown in Fig. 3A and B, both in vitro translated yTBP and *E. coli* produced recombinant yTBP (a gift from M. Van Hoy, J. Simmons, and T. Kodadek) bound tightly to GST-34 but not to GST, demonstrating that the interaction is direct. Next, we tested whether binding also occurs with immobilized TBP and with the AD in a context different from GST-34. Therefore we purified and immobilized a GST-yTBP fusion and in vitro translated a GAL4 protein with an intact carboxy terminus but a large internal deletion [GAL4(Δ148–728) (47)]. We used GAL4(Δ148–728) because transcription and translation of the complete *GAL4* gene yields only a small percentage of full-length protein (47). GAL4(Δ148–728) binds efficiently to GST-TBP but does not bind to GST (Fig. 3C).

Since GAL4 has also been reported to have a weak internal AD (54) (see above), we tested binding of TBP to this region [GAL4(149–238)] as well as to the acidic ADs of VP16 (82), p53 (13), GCN4 (25), AH (17), and TAT (63, 64) (Table 1). As shown in Fig. 4, TBP binds efficiently to the GAL4 34-aa AD (aa 841 to 875) and to the strong ADs of VP16 and p53. In addition, TBP binds weakly but reproducibly to the GCN4 AD and to AH. TBP binds very weakly if at all to GAL4(149–238), TAT(1–13), and GST itself. Note that another region of TAT (aa 36 to 50) has been implicated in transcriptional activation and does bind TBP (36). After adjustment for protein levels, we conclude from Fig. 4 that TBP binds the GAL4 C-terminal AD as strongly as or more strongly than it binds the other acidic ADs tested but that it does not bind the GAL4 proposed internal AD.

Binding of TBP to GST-34 is independent of charge and highly salt stable. TBP is a strikingly basic protein (21, 28). It is therefore tempting to assume that the acidic ADs would bind the basic TBP protein predominantly by charge interaction.

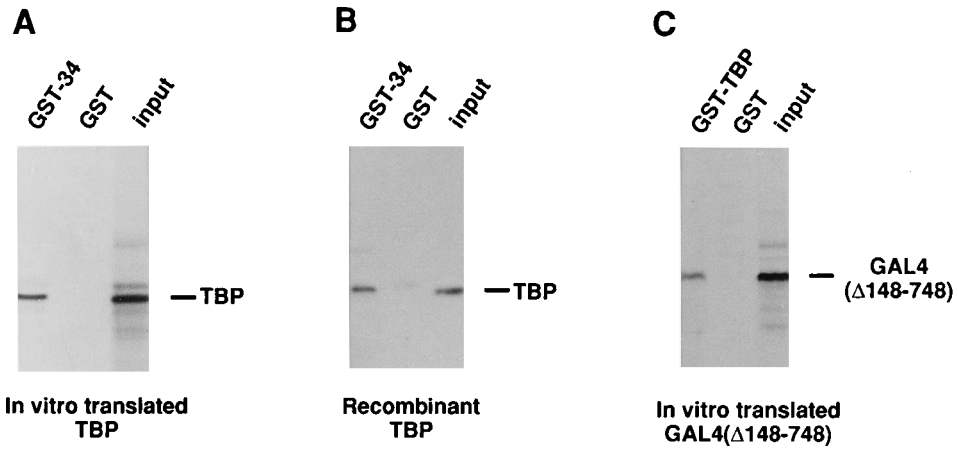

FIG. 3. The interaction between the AD of GAL4 and yTBP is direct. Indicated GST fusion proteins bound to glutathione beads were incubated with in vitro translated or purified proteins. (A) Autoradiogram after incubation of beads with in vitro translated yTBP. For the input control, the same amount of in vitro translated yTBP (ca. 0.5 ng) was loaded directly onto the gel. (B) Western immunoblot after incubation of beads with purified yTBP. (C) The AD of GAL4 binds to immobilized TBP. In vitro translated GAL4(Δ148–728) was incubated with GST and GST-yTBP, respectively.

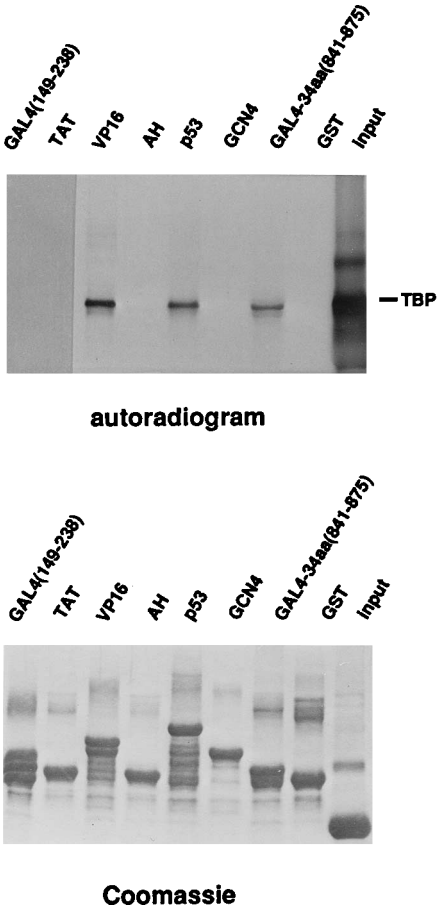

FIG. 4. TBP interacts with different acidic ADs but not with the internal AD (aa 149 to 238) of GAL4. In vitro translated TBP was incubated with ADs (Table 1) fused to GST in the presence of *E. coli* protein competitor. After extensive washing, bound TBP was eluted with SDS sample buffer (see Materials and Methods).

Acidic ADs have indeed been proposed to function mainly by virtue of their negative charges (reviewed in reference 75). More recent observations, however, suggest that negative charge is not the most important determinant for the potency of ADs and instead stress the importance of bulky hydrophobic amino acids in "acidic" ADs (8, 48, 50, 65).

In a direct approach to determining the importance of electrostatic and hydrophilic interactions in the binding between GST-34 and TBP, we determined the effect of increasing salt concentrations. In vitro translated yTBP was incubated with GST-34 bound to glutathione beads in the presence of 10 mg of soluble *E. coli* protein per ml as a nonspecific competitor. Beads with bound proteins were extensively washed and aliquoted into four samples. Half of each sample was used as input control, and the other half (5 μl of bead slurry) was incubated with buffer B plus NaCl (20 μl) with final NaCl concentrations as indicated. Proteins in the supernatant or bound to settled beads were denatured in SDS sample buffer and loaded onto an SDS-PAGE gel in parallel with the input controls. Even in the presence of 1.6 M NaCl, TBP remained completely in the bead pellet (Fig. 5). The presence of TBP in the bead pellet was due to stable binding to GST-34 and not to nonspecific precipitation of TBP under high-salt conditions, because TBP is found in the supernatant when mixed with GST beads instead of being bound to GST-34 beads (data not shown). Binding of GAL80 to GST-34 under the same conditions was also insensitive to salt (data not shown).

Binding of TBP to mutants of the GAL4 AD correlates with activation. To establish the relationship between binding in vitro and activation of transcription in vivo, we compared the characteristics of binding and activation for AD and TBP mutants. First we expressed a set of GST-34 mutant proteins (Table 1). These fusion proteins contain GAL4 carboxy-terminal ADs that resulted from an oligonucleotide saturation mutagenesis. Chosen mutants showed altered activation potential when the mutated region was exchanged for the wild-type 34-aa AD of GAL4 expressed from its own promoter on a centromere-containing plasmid (48).

To determine relative TBP affinities, mutant ADs were expressed and purified as GST fusion proteins from *E. coli* and incubated with yTBP (Fig. 6A). In our assay, about 0.5 ng of in vitro translated TBP was mixed with 4 mg of *E. coli* competitor protein and then incubated with 10 μg of immobilized

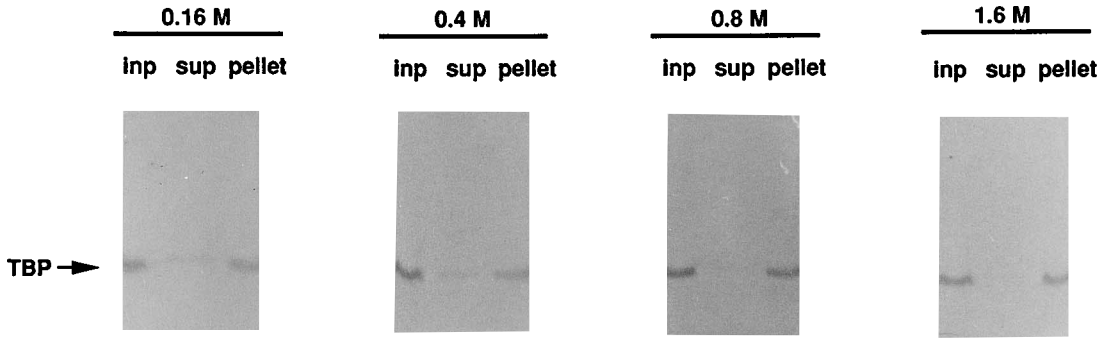

FIG. 5. The interaction between TBP and GST-34 is not disrupted by high salt concentrations. TBP bound to GST-34 was incubated in the presence of different NaCl concentrations. After a 5-min incubation at room temperature, beads and supernatants were separately mixed with SDS sample buffer and loaded together with input controls onto 10% Tricine gels. Abbreviations: inp, input; sup, supernatant.

GST-AD proteins corresponding to about 1 μg of the isolated AD. Under conditions where 35% of input TBP bound to the wild-type AD, less than 0.1% of the complex mixture of *E. coli* proteins was retained (compare the autoradiogram in Fig. 6A with the Coomassie blue-stained gel in Fig. 6B). We have repeated these binding assays several times with different batches of fusion proteins and in vitro translated TBP. We have also tested different buffer compositions, different pHs, and different competitor proteins. Relative binding affinities were very reproducible and under all conditions resulted in the same relative order for TBP binding. It is important to note that our in vitro binding studies were not designed to represent binding conditions (e.g., activator levels) in the cell but to determine relative binding affinities or binding constants. They were performed under nonsaturating (equilibrium) conditions, as we determined the same minimal binding constant, 3×10^{-7} M, for the GST-34-TBP interaction when we decreased the GST-34 concentration 10- or 50-fold (Fig. 6C; see also Materials and Methods).

For measuring in vivo potencies (Fig. 7A), we tried to approach physiological conditions as closely as possible. As described previously (48), mutant ADs were expressed in the context of the full-length GAL4 protein and at the very low level that is physiological for GAL4 in a strain lacking chromosomal *GAL4* and *GAL80*. Our reasoning was as follows: (i) expression of ADs in the context of GAL4(1–92) requires overexpression to give moderate levels of reporter gene activation (55c), even though strong GAL4-binding sites are nearly saturated by endogenous GAL4 in vivo (89); and (ii) internal regions of GAL4 appear to contribute to activation in a way that is mechanistically different from the effect of the C-terminal AD (55c), prompting us to keep potential other functions of GAL4 constant. To account for any differences in protein levels, we determined relative amounts of GAL4 by gel mobility shift assay (Fig. 7B) and used expression of the endogenous *MEL1* gene as the reporter. We chose the *MEL1* gene rather than the widely used *GAL1-GAL10* promoter-based reporters because *MEL1* is regulated by a single GAL4-binding site, thus eliminating any cooperative effects of multiple GAL4 dimers on DNA binding or activation. In vivo titration experiments have indicated a linear relationship between DNA binding and transcription for *MEL1* (89). As shown in Fig. 7C, transcriptional output under physiological conditions in vivo and TBP binding under equilibrium conditions correlate well for the tested set of GAL4 mutant ADs.

TBP L114K is compromised in binding to GST-34. We translated 29 TBP mutants (91) (generously provided by R. G. Roeder and coworkers) in vitro and tested their binding to GST-34. Each mutant contained a single-amino-acid change in the conserved 180-aa core domain. Only one of the mutants, L114K, showed significantly reduced affinity to GST-34 (data not shown). TBP L114K has been identified by Kim et al. as one of three mutants that are defective in activated but not basal transcription in vitro. L114K was shown to be compromised in binding to the VP16 AD; the other two mutants were defective in TFIIB (L189K) and in TATA (K211L) interactions (39). We compared the extent of reduction in TBP L114K affinity for GST-34 and a fusion between GST and the AD of VP16 (GST-VP16). Both ADs had the same six- to eightfold decrease in affinity to the L114K mutant relative to wild-type TBP (Fig. 8). These results support the importance of specific interactions of the GAL4 and VP16 ADs with TBP and suggest that both ADs make similar contacts with TBP.

DISCUSSION

The 34-aa GAL4 AD retains TBP and coactivators. GAL4 has served as a paradigm for studies of eukaryotic transcriptional activators, yet no direct interaction between GAL4 or any other yeast activator and their transcriptional targets has been reported. In an attempt to identify proteins that physically interact with its AD, we initially applied whole-cell yeast extract to an immobilized peptide that corresponds to the primary AD of GAL4 (GST-34) and to a control (GST). We found that both TBP and the putative coactivators SUG1 and ADA2 bound to GST-34. The direct or indirect binding of ADA2 was a surprise, since previous work suggested that ADA2 interacted with VP16 and GCN4 but not GAL4 (59). However, we found that in whole-cell extract, ADA2 was retained by the ADs of both GAL4 and VP16 and that the interaction with GAL4 was actually stronger than the one with VP16 (55a).

The 34-aa AD used here contains the GAL80-interacting domain. In earlier studies, Ma and Ptashne (54) reported that deletion of this domain had a relatively minor effect on its ability to activate transcription. However, these studies were done with highly overexpressed GAL4 (ADH1-GAL4, on a 2μm plasmid). Highly overexpressed full-length GAL4, but not GAL4 deleted for the 34-aa AD, is a strong squelcher in vivo and causes severe retardation of growth rates (our unpublished results). These authors also reported that a fragment consisting of the 30 C-terminal amino acids (aa 851 to 881) was a weak activator when overexpressed as a fusion with the DNA-binding domain of GAL4. In contrast, we found that the C-terminal 34 aa (aa 841 to 875) served nearly as well as VP16 as an activator. In this regard, we note that the interaction of the

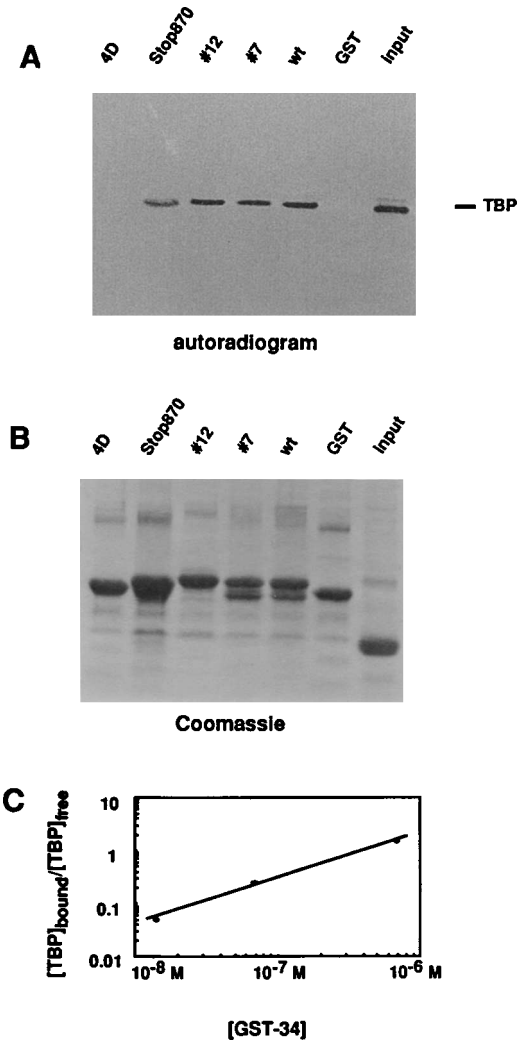

FIG. 6. Binding of yTBP to wild-type (wt) and mutant GAL4 ADs. ADs (Table 1) were fused to GST. In vitro translated TBP (ca. 0.5 ng) was incubated with 10 μg of each of the GST-AD fusion proteins (corresponding to about 1 μg of AD peptide) bound to glutathione beads in the presence of 4 mg of total *E. coli* protein as competitor. Beads were eluted with 2× SDS sample buffer and separated by SDS-PAGE. Gels were stained with Coomassie blue and then subjected to autoradiography. (A) Autoradiogram. (B) Coomassie blue-stained gel before autoradiography. GAL4 ADs are indicated above each lane of the autoradiogram. In the lane marked input, 0.5-fold the amount of in vitro-translated protein used in the binding experiment was loaded directly onto the gels. (C) The relationship between [GST-34] and $[TBP]_{bound}/[TBP]_{free}$ is linear, demonstrating that in vitro binding studies were done under equilibrium conditions. We used 6.9×10^{-7} M (10 μg/400 μl) GST-34 in our standard binding reaction. Both axes are logarithmic.

SUG1 coactivator with GAL4 requires aa 841 to 852 (79, 80). The other identified AD in GAL4 (aa 148 to 196), failed to bind TBP under our conditions (Fig. 4) and does not activate transcription at low expression levels (data not shown).

We demonstrate that binding of TBP to the AD of GAL4 is direct and specific by several criteria. Other yeast proteins tested had no detectable affinity to GST-34, whereas TBP clearly bound GST-34 but not GST. When in vitro translated TBP was mixed with a several million-fold excess of a complex mixture of *E. coli* proteins, 35% of the input TBP but no more than about 0.1% of the *E. coli* proteins were retained by GST-34. The GST control showed that the affinity is restricted to the 34-aa AD from GAL4. This interaction is not dependent on the context since it can be in the context of both GST-34 and GAL4(Δ148–729), with either the AD immobilized or the TBP immobilized.

The TBP-AD complex was not disrupted by high salt concentrations, implying that it is not dependent on ionic interactions. The formation of the TBP-AD, however, may have different salt requirements. Binding did not correlate with charge densities. For instance, GAL4 AD mutant 12 has a higher negative net charge but a lower TBP affinity than did mutant 7 and TBP L114K is strongly reduced in binding to the acidic ADs of GAL4 and VP16 in spite of gaining a positive charge. In contrast to the salt resistance of the interaction between GST-34 and TBP shown here, Stringer et al. (77) demonstrated that human TBP can be eluted from a VP16 AD column by 0.5 M NaCl, implying that the acidic amino acids were essential for interaction with TBP. The in vivo importance of ionic interactions was supported by experiments in which sequential removal of acidic amino acids from VP16 carrying half of its AD resulted in a gradual decrease in activation potential (8). However, other mutational analysis pointed to the importance of hydrophobic amino acids (8). Mutational analysis of the GAL4 AD did not show this gradual loss of activation potential with increased removal of acidic amino acids. We proposed a model for the structure of this AD as a two-sided β-sheet, with one side, which is rich in bulky hydrophobic amino acids and devoid of charge, making contacts with the transcription apparatus (48). Preliminary experiments in our laboratory confirm that VP16-TBP complexes are less salt stable than GAL4-TBP complexes. Charge may therefore make a direct contribution to the TBP interaction in the case of VP16 and an indirect one in the case of GAL4, in which negative charges may be important for the exposure of a hydrophobic contact surface in an aqueous environment. Alternatively, interactions of both ADs with TBP may be hydrophobic, but the correct exposure of critical hydrophobic amino acids in the VP16 AD might be sensitive to high ionic strength.

Biological significance of the observed affinity between TBP and acidic ADs. We determined the dissociation constant for the interaction between immobilized GST-34 and in vitro translated TBP as minimally 3×10^{-7} M. This is the minimum binding constant, since we assumed for its determination that both immobilized GST-34 and in vitro translated TBP are completely active in their ability to interact with each other. TBP binding to the GAL4 AD was as strong as or stronger than binding to the other ADs tested in Fig. 4. This moderate affinity is consistent with the idea that (i) interactions between TBP and activators are transient and (ii) interactions with the proposed minute amounts of activators in vivo may require the close proximity of activators and TBP achieved by their high-affinity template binding. Squelching experiments showed that high levels of GAL4-VP16 exclusively inhibited activated transcription when GAL4-VP16 was not bound to template but inhibited both activated and basal transcription in vivo once it was bound to template (4, 5). These observations suggest that upon template binding, the activator can directly or indirectly titrate out a general transcription factor.

The evaluation of the relevance of the observed binding of TBP to the GAL4 AD in vitro was a primary objective of this study. So far, different strategies have been used to support in vitro binding data. One approach has been to test whether a disruption of an observed in vitro interaction abolishes activated but not basal transcription in vitro. TAFs fit this requirement since in reconstituted systems they are exclusively needed for activated transcription. The most convincing example of this type may be the interaction between VP16 and human TFIIB (hTFIIB). Two hTFIIB mutants with double point mu-

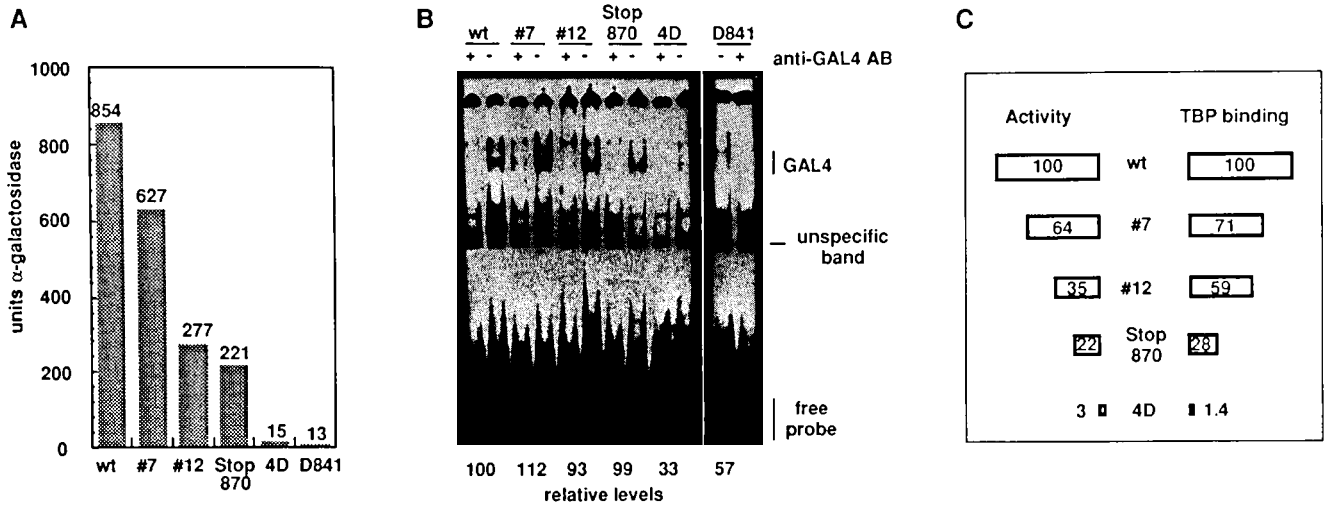

FIG. 7. TBP binding in vitro and activation in vivo correlate for point mutations and truncations in the GAL4 AD. Mutant ADs were exchanged against the wild-type (wt) AD in GAL4. (A) Activation of the endogenous *MEL1* gene by wild-type and mutant GAL4s. GAL4 proteins were expressed at physiological levels, i.e., in single copy and from the GAL4 promoter. (B) Determination of relative GAL4 levels by electrophoretic mobility shift assay. The relative amount of probe shifted in the GAL4 band is indicated below the autoradiogram and represents the average of three independent experiments. GAL4-containing complexes were identified by incubation with an antibody directed against the DNA-binding domain of GAL4. (C) Graphic presentation of relative TBP affinities and transcriptional activation for GAL4 wild-type and mutant ADs. *MEL1* reporter activities are corrected for differences in relative protein levels and for the residual activity of GAL4(1–841) (lanes D841). Relative TBP affinities represent the average of several experiments with at least four different preparations of fusion protein per construct and different batches of in vitro translated TBP.

tations have strongly decreased affinity to VP16 and are also defective in activated but not basal in vitro transcription (67). Similarly, a single point mutation in TBP, L114K, has been identified that effects binding to VP16 and activated but not basal transcription (39). We find that the same mutation is also defective in interaction with the GAL4 34-aa AD, supporting the importance of this interaction for transcriptional activation. It also provides the first indication that these two ADs contact the same surface of TBP, which is consistent with results of recent experiments demonstrating that the GAL4, VP16, and LEU3 ADs directly compete for binding to TBP (44a). A different argument to support the relevance of the VP16-hTFIIB interaction has been the observation that in an in vitro system the first slow step in preinitiation complex formation is the binding of TFIIB and that this step is accelerated by GAL4-AH or GAL4-VP16 (7, 51). The interpretation of this result has been that TFIIB is recruited to the TBP-TATA complex by direct binding to acidic activators.

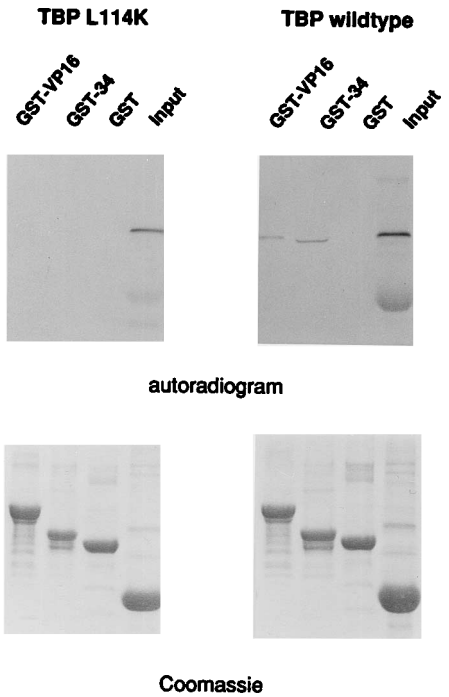

FIG. 8. TBP L114K is defective in interaction with the GAL4 and VP16 ADs. In vitro translated wild-type and mutant TBPs were loaded directly onto an SDS-PAGE gel (input) or were incubated with the indicated GST fusion proteins in the presence of 4 mg of total *E. coli* proteins as competitor. Gels were stained with Coomassie blue (lower panel) and then subjected to autoradiography (upper panel).

Considering the potential for artifacts in in vitro systems, the correlation of observed in vitro affinities with transcriptional activation in vivo appears to be a straightforward and simple alternative. A mutant form of an amino-terminal VP16 AD half-site (F442P) that has been known to drastically compromise activation in vivo (8) also shows strongly decreased affinity to TBP (31), TFIIB (51), TFIIH (87), and PC4 (14). The effect on TFIIB binding, however, could not be reproduced by Goodrich et al. (18). Other VP16 mutations that decreased reporter gene activation upon transient transfections (8) also decreased binding to yTBP, even though the strength of the effects of these mutations on binding and on activation correlated only within subgroups (31). Similarly, effects of mutations of the E1A AD on in vivo activation and in vitro human TBP binding correlated in most but not all instances (45). A more detailed study showed that all seven point mutations in the E1A AD that caused significantly reduced binding to TBP were also severely compromised in activation but that not all mutations that affected activation in vivo were compromised in TBP binding (15). These results suggested that interactions of TBP with two viral acidic activators, VP16 and E1A, are necessary but may not be sufficient for activation.

Here we expressed at physiological levels point mutations and truncations in the AD of a cellular activator, GAL4. Activation of an endogenous gene, regulated solely by a single GAL4-binding site, directly correlates with the affinities of the tested ADs for yTBP. This result, together with TBP L114K being compromised both in the interaction with the GAL4 AD and in activated transcription, suggests that the TBP-AD interaction is a rate-limiting step in transcriptional activation by GAL4.

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ADDENDUM

While this paper was in review, Silverman et al. demonstrated that ADA2 is retained by GAL4-VP16 from crude extracts (76).

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