MOLECULAR AND CELLULAR BIOLOGY, Sept. 1995, p. 5007–5016 Vol. 15, No. 9
0270-7306/95/$04.00+0

The Transcriptional Repressor Even-skipped Interacts Directly with TATA-Binding Protein

MOONKYOUNG UM, CHI LI, AND JAMES L. MANLEY*

Department of Biological Sciences, Columbia University, New York, New York 10027

Received 19 April 1995/Returned for modification 6 June 1995/Accepted 20 June 1995

The *Drosophila* homeodomain protein Even-skipped (Eve) has previously been shown to function as a sequence-specific transcriptional repressor, and in vitro and in vivo experiments have shown that the protein can actively block basal transcription. However, the mechanism of repression is not known. Here, we present evidence establishing a direct interaction between Eve and the TATA-binding protein (TBP). Using cotransfection assays with minimal basal promoters whose activity can be enhanced by coexpression of TBP, we found that Eve could efficiently block, or squelch, this enhancement. Squelching did not require Eve DNA-binding sites on the reporter plasmids but was dependent on the presence of the Eve repression domain. Further support for an in vivo interaction between the Eve repression domain and TBP was derived from a two-hybrid-type assay with transfected cells. Evidence that Eve and TBP interact directly was provided by in vitro binding assays, which revealed a specific protein-protein interaction that required an intact Eve repression domain and the conserved C terminus of TBP. The Eve homeodomain was also required for these associations, suggesting that it may function in protein-protein interactions. We also show that a previously characterized artificial repression region behaves in a manner similar to that of the Eve repression domain, including its ability to squelch TBP-enhanced expression in vivo and to bind TBP specifically in vitro. Our results suggest a model for transcriptional repression that involves an interaction between Eve and TBP.

It is clear by now that control of gene expression in eukaryotes involves repression as well as activation of transcription. A significant number of proteins that are capable of functioning as transcriptional repressors in various assays have been identified, and many of them are known to play key roles in a variety of important cellular and developmental processes. These include, for example, the homeodomain protein α2, which functions with other proteins to control cell type in *Saccharomyces cerevisiae* (19, 21); the homeodomain proteins Even-skipped (Eve) and Engrailed (En), which are involved in pattern formation during early *Drosophila* embryogenesis (13, 17); and in mammals, the Zn^{2+} finger-containing v-erbA oncoprotein, or thyroid hormone receptor (8), and the WT1 Wilms tumor gene product (31). These proteins all share the property that they are sequence-specific DNA binding proteins capable of recognizing binding sites in target genes and repressing transcription.

There are a number of ways in which transcriptional repressors can function, and even those whose action involves sequence-specific DNA binding can employ distinct mechanisms (for reviews, see references 26 and 39). Perhaps the simplest involves competition for DNA-binding sites, whereby the repressor interferes with binding of either an activator or a basal transcription factor, by virtue of adjacent or overlapping binding sites. A second mechanism, called quenching, involves simultaneous DNA binding by both the activator and the repressor, coupled with a protein-protein interaction that prevents the activator from functioning, for example by masking the activation domain. Thirdly, a direct repressor functions by binding DNA and then interfering, via protein-protein interactions, with the formation or activity of the basal transcription complex. This form of repression is of particular interest because the mechanism(s) involved appears to be analogous to those thought to be employed by transcriptional activators, with the exception that it leads to repression rather than activation of transcription. However, unlike the situation with activators, in which direct interactions between activation domains and basal transcription factors have been demonstrated in vitro (e.g., see references 29 and 45), a direct interaction between a defined repression domain and a basal factor has not yet been demonstrated. As has also been observed with activators, at least some repressors appear to require the function of interacting proteins; for example, α2 in *S. cerevisiae* (23) and the *Drosophila* Hairy protein (37) both interact with auxiliary proteins that are essential for full activity.

A number of repressors, like activators, have been shown to consist of a modular structure, containing separable DNA-binding and repression domains. This was shown first with the *Drosophila* Krüppel protein, which contains DNA binding Zn^{2+} fingers and a distinct repression region that is capable of blocking transcription in transfected mammalian cells when it is fused to a heterologous DNA-binding domain (27). Likewise, the Eve (14, 47) and En (15, 18) proteins contain transferable repression regions that can function in transfected *Drosophila* cells. Remarkably, all three of these repression regions are characterized by alanine richness (the Eve domain is moreover enriched in proline residues) (14, 15, 27). Transferable repression regions have been found in several mammalian proteins (1, 4, 32, 44), including those described above. Although alanine richness does not seem to be a feature of all characterized repression regions, a common theme appears to be that charged residues, particularly acidic ones, are scarce. Two artificial repression regions that function when fused to DNA-binding domains have also been described. One, in *Drosophila melanogaster*, is proline plus leucine rich (14), while the other, in *S. cerevisiae*, is highly basic (41).

Repression mediated by Eve has been studied in some detail. Initial experiments employing cotransfection assays provided evidence that the protein is a DNA-binding-site-dependent repressor but could not clearly distinguish among possible repression mechanisms (13, 17). However, subsequent work

* Corresponding author.

has provided strong support for the idea that Eve is a direct repressor. Biggin and Tjian (3) showed that Eve can repress basal transcription in vitro from binding sites situated upstream or downstream of the promoter. Subsequently, evidence was provided that Eve can interfere with transcription complex assembly in vitro, perhaps resulting in formation of nonproductive preinitiation complexes (20). Finally, Han and Manley (14) provided evidence, using transfection assays, that Eve can repress transcription from a minimal basal promoter containing Eve-binding sites upstream of the promoter. Transcription stimulated by any of several activators was also effectively repressed.

In this paper, we present data suggesting that Eve repression involves a direct interaction between Eve and the TATA-binding protein (TBP). Using cotransfection assays, we first show that Eve can squelch basal expression enhanced by TBP and that an intact repression domain is required for this activity. Additional evidence for an in vivo interaction between the two proteins is provided by a two-hybrid-type assay. We then demonstrate by an in vitro protein-protein interaction assay that Eve and TBP specifically bind to each other and that an active repression domain is essential for this interaction.

MATERIALS AND METHODS

Recombinant plasmids. The in vivo expression vector Act 5C PPA and all Eve expression plasmids, including EveABFS1 and EveABFS2, were described previously (13, 14). The TBP expression plasmid Act-TBP was described by Colgan and Manley (5). The reporter plasmid E1b TATA CAT was described by Colgan et al. (7). NP6 E1b TATA CAT, which contains six Eve binding sites located 30 bp upstream of the E1b TATA sequence, was constructed by inserting an NP6 fragment (14) into E1b TATA CAT. G5 Inr CAT and G5 TATA CAT, which contain five GAL4-binding sites upstream of the TdT Inr or E1b TATA box, respectively, were described by Colgan and Manley (5, 6). The GAL4-TBP fusion protein expression plasmids Act GAL4-TBP, Act GAL4-TBP(1-157), and Act GAL4-TBP(1-282) and the Eve-VP16 expression plasmids Actflu eveAB-VP16 and Actflu eveABCD-VP16 were constructed from Act GAL4(1-147)-TBP(1-335) and Actflu VP16 (provided by J. Colgan), respectively, by standard subcloning procedures. Sequences around the junctions of the fusion proteins were confirmed by DNA sequencing. The protein encoded by Actflu eveAB-VP16 consists of the nine-residue influenza virus (flu) epitope, Eve residues 1 to 139, and the VP16 activation domain, while the Actflu eveABCD-VP16-encoded protein is identical, except that it contains Eve residues 1 to 246 (see reference 14 for details).

pGST-dTBP and pGST-hTBP were constructed by cloning full-length dTBP cDNAs into the glutathione *S*-transferase (GST) expression vector pGEX-2TK. pGST-yTBP was kindly provided by J. Reese and M. Green. The GST-dTBP derivative Δ1-161 was constructed from the corresponding in vivo expression vector (provided by J. Colgan). PET-3a was used as in vitro transcription vector, and full-length Eve cDNA was inserted into this vector under the control of the T_7 promoter. Vectors encoding Eve mutants were made by subcloning appropriate DNA fragments from in vivo expression vectors (14) into PET-3a.

DNA transfection and transient-expression assays. Transient-expression assays were performed essentially as described by Han et al. (13). *Drosophila* Schneider L2 cells were maintained in M3 medium supplemented with 10% fetal bovine serum (Gibco). Cells were plated at 2×10^6 to 4×10^6 cells per 10-cm cell culture dish 1 day before DNA transfection. Transfection mixtures contained the amounts of expression plasmids indicated in the figure legends, 2 μg of a reporter plasmid and 2 μg of *copia* LTR-*lacZ* internal control plasmid. Act 5C PPA was added as necessary so that the total amount of actin 5C promoter was constant, and Gem1 was used as a carrier to adjust the total amount of plasmid DNA to 10 μg. Transfected cells were incubated for 2 days, removed from cell culture dishes by agitation, washed twice with phosphate-buffered saline, resuspended in 0.1 ml of 0.25 M Tris-HCl (pH 7.8), and frozen at −70°C. Whole-cell extracts were prepared by thawing and sonicating for 2 min, which was followed by centrifugation for 10 min in an Eppendorf Microfuge. The chloramphenicol acetyltransferase (CAT) values obtained all fell within the linear range of the reaction and were normalized for any variations in transfection efficiency by measurement of β-galactosidase activities (see reference 13 for details). To facilitate comparisons, the basal value obtained with the reporter plasmid used in each experiment was set equal to 1.0. Each experiment was done at least three times in duplicate, and the indicated values are the averages of at least three independent experiments. Western blot (immunoblot) analyses of whole-cell lysates from transfected cells were performed essentially as described by Colgan and Manley (5).

Purification of GST-TBP fusion proteins from *Escherichia coli*. *E. coli* JM101 containing plasmids encoding each of the pGST-TBP derivatives was cultured in 2× YT medium supplemented with ampicillin (200 μg/ml) at 37°C. Protein expression was induced with 0.4 mM isopropyl-β-D-thiogalactopyranoside when the optical density at 600 nm was ~0.6. After 3 h of induction, the cells were harvested by centrifugation. The pellet was resuspended in NETN (100 mM NaCl, 1 mM EDTA, 20 mM Tris-HCl [pH 8.0], 0.5% Nonidet P-40) with 0.5 mg of lysozyme per ml. The lysate was sonicated and rotated in a cold room for 1 h. Cell debris was removed by centrifugation. The supernatant was loaded on a glutathione-agarose (S) column. After extensive washing with NETN, the GST fusion proteins were eluted with elution buffer (100 mM Tris-HCl [pH 8.0], 120 mM NaCl, 20 mM reduced form of glutathione). Purified GST fusion proteins were then dialyzed in buffer containing 20 mM Tris-HCl (pH 8.0), 120 mM NaCl, 20% glycerol, 1 mM dithiothreitol, and 0.2 mM EDTA. GST fusion proteins were frozen on dry ice and stored at −70°C. Concentrations of the fusion proteins, which were determined by the Bradford method, varied from 0.2 to 0.4 mg/ml.

Protein-protein interaction assays. Wild-type and mutant Eve proteins were produced by in vitro transcription, which was followed by translation in reticulocyte lysate (Promega) in the presence of [^{35}S]methionine. Two micrograms of each purified GST-TBP fusion protein was incubated with 20 μl of glutathione-agarose beads in 100 μl of NETN for 1 h at 4°C, with rocking. The glutathione-agarose-GST-TBP complexes were recovered by centrifugation, and the supernatants were discarded. Simultaneously, equal amounts of [^{35}S]methionine-labeled Eve proteins (1 μl) were also incubated with 20 μl of glutathione-agarose in 40 μl of NETN for 1 h at 4°C. After centrifugation, the supernatants were transferred to tubes containing the agarose-GST-TBP complexes. Radiolabeled Eve proteins were incubated with agarose-GST-TBP for 1 h at room temperature with shaking. After extensive washing with NETN, the bound proteins were eluted twice by incubation with 30 μl of elution buffer at room temperature for 30 min. The combined elutions were diluted with 2× sample buffer, boiled, and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and fluorography.

RESULTS

Eve can squelch TBP-enhanced expression. We previously used cotransfection assays to investigate the possible roles of the general transcription factors TBP and TFIIB in activated transcription. For example, we showed that overexpression of TBP could significantly enhance expression from minimal TATA-containing promoters, indicating that, under the conditions employed, TBP is limiting for RNA polymerase II transcription in vivo (5). Consistent with this, overexpression of TFIIB did not significantly affect promoter activity, although a mutant TFIIB was shown to act as a strong, dominant negative inhibitor of a specific transcriptional activator (7). Therefore, we decided to examine whether similar approaches might indicate a functional interaction between Eve and a basal factor. A series of cotransfection experiments with Eve and TFIIB failed to suggest a relationship between these factors (results not shown). However, similar assays involving TBP and Eve have provided evidence for a functional interaction between these two proteins.

In a first set of experiments, we took advantage of the fact that overexpression of *Drosophila* TBP in transfected Schneider cells enhanced very significantly, and in a concentration-dependent manner, expression from minimal TATA-containing promoters (5). This phenomenon provided an opportunity to determine whether Eve might be able to squelch (12) TBP-enhanced transcription. Previous studies showed that repression by Eve was, in all cases studied, completely dependent on the presence of Eve-binding sites in the reporter plasmid (e.g., see references 13 and 14). However, we reasoned that if Eve and TBP could in fact interact in vivo, then perhaps TBP-enhanced transcription would be sensitive to Eve expression in the absence of DNA-binding sites. Figure 1 provides evidence that this is the case. In this experiment, an amount of TBP expression vector (0.2 μg of Act-TBP) sufficient to enhance transcription from a minimal TATA-containing reporter plasmid (E1b TATA CAT) ~40 fold was included in transfection mixtures, together with increasing amounts of Eve expression vector (Act eve). Strikingly, strong repression, nearly to the

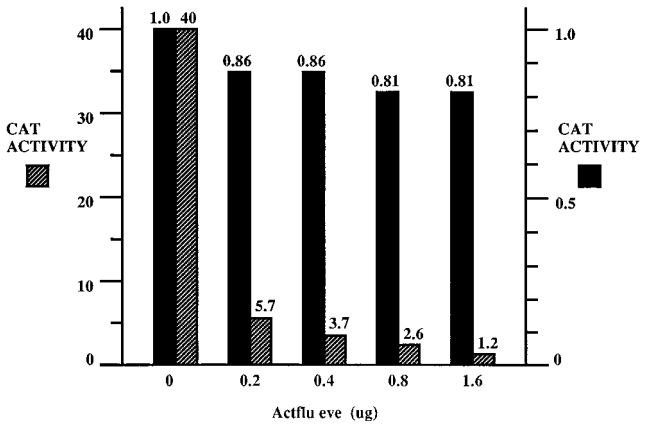

FIG. 1. Eve can repress TBP-enhanced transcription in the absence of Eve-binding sites. A 2-μg amount of the reporter plasmid E1b TATA CAT and the indicated amounts of Eve expression plasmid Actflu eve were cotransfected into *Drosophila* Schneider L2 cells with and without the TBP expression plasmid Act-TBP. In this and all subsequent experiments, the total amount of expression plasmid in each sample was adjusted to 1.8 μg by the addition of Act 5C PPA, and 2 μg of an internal control plasmid, *copia* LTR-*lacZ*, which was not affected by TBP overexpression was also included. The graph displays CAT activities normalized via assays of β-galactosidase activity (see Materials and Methods). The scale on the left presents values obtained in the presence of 0.2 μg of Act-TBP (hatched bars), while that on the right gives values in the absence of Act-TBP (black bars).

basal level, was detected despite the absence of Eve-binding sites in the reporter plasmid. In contrast, and consistent with previous experiments, Eve was found to have essentially no effect on CAT expression in the absence of exogenous TBP (Fig. 1). To ensure that this phenomenon was not unique to E1b TATA CAT, we tested another promoter previously shown to be responsive to elevated TBP concentrations, the *Drosophila* metallothionein minimal promoter (met TATA CAT [5]). TBP-enhanced expression from this promoter was also found to be inhibited by Eve (~8 fold; results not shown), while basal expression was not significantly affected (see below).

The fact that Eve squelched TBP-enhanced but not basal expression is consistent with the existence of an interaction between Eve and the exogenous TBP. However, it was possible that the effect was indirect and that Eve squelched by interacting with some other general transcription factor. By this model, basal transcription from E1b TATA CAT would have escaped inhibition because it was so weak that there were sufficient levels of the hypothetical target factor even after squelching. To test this idea, we examined the effect of Eve expression on the basal activities of several additional minimal promoters lacking Eve-binding sites (Table 1). The strengths of these promoters (as measured by CAT activities) varied by 100-fold, from activities that were approximately 10-fold lower (met TATA CAT) to 10-fold higher (hsp TATA CAT) than the activity of E1b TATA CAT in the presence of coexpressed TBP. One of the promoters (*copia* CAT) was TATA lacking, and the other two (met TATA CAT and hsp TATA CAT) contained TATA boxes (all have been described previously [5]). In each case, coexpression of Eve had at most a very minor effect (maximum inhibition of 1.6-fold), which was less by a factor of nearly 10 than the efficient inhibition of TBP-enhanced expression (Table 1). These results indicate that Eve does not squelch an essential endogenous basal factor and support the idea that Eve interacts with transfected TBP.

TABLE 1. Eve squelches only TBP-activated transcription[a]

Reporter plasmid	CAT activity		Fold inhibition
	Act 5C PPA	Actflu eve	
copia CAT	30	33	0.91
met TATA CAT	3.5	2.8	1.3
hsp TATA CAT	350	220	1.6
E1b TATA CAT + Act-TBP	42	3.0	14

[a] A 2-μg amount of each reporter plasmid was cotransfected with 0.8 μg of Actflu eve or 0.8 μg of Act 5C PPA, except for E1b TATA CAT, which was cotransfected with 0.2 μg of Act-TBP plus 0.8 μg of Actflu eve or 0.8 μg of Act 5C PPA. The CAT activities shown were determined as described in Materials and Methods. Fold inhibition is the ratio between the CAT activity observed in the presence of Actflu eve and the CAT activity observed in its absence.

Squelching requires a functional repression domain. If the squelching that we observed was related to the DNA-binding-site-dependent repression activity of Eve, then it should require the alanine- and proline-rich repression region that we described previously (14). To test this, we analyzed the abilities of a series of Eve derivatives to squelch TBP-enhanced expression. As shown previously (14), differences in protein accumulation (measured by Western blotting) were minimal and could not be responsible for differences in activity. The results, some of which are illustrated in Fig. 2, reveal a pattern extremely similar to that observed in our previous experiments. (To facilitate comparisons, relative repression values [from reference 14] are listed in parentheses at the bottom of the graph and relative squelching values are given at the top.) Sequences within the alanine- and proline-rich region encompassed by Eve regions C and D (residues 140 to 247) were essential for efficient squelching, and neither the alanine-rich C domain nor the proline-rich D domain was sufficient (Fig. 2A). Regions dispensable for repression, such as A, E, and F, were also unnecessary for squelching. Further, we previously dissected the C-D repression region and identified shorter derivatives that retained full or partial repression activity, including a minimal 57-residue sequence (C2D2) that contains the majority of the alanine and proline residues, and retained nearly full repression activity for both activated and basal expression (14, 15a). Figure 2B shows that these derivatives all had similar activities in the squelching assay. For example, the Eve derivative containing the C2D2 region (Eve ABC2D2) was an extremely effective squelcher, while the protein containing the C and D1 regions, which had weaker repression activity, likewise showed somewhat weaker squelching activity.

We previously described a 29-residue artificial repression region (FS1) that when fused to the Eve homeodomain imparted a high level of sequence-specific repression activity, which was very similar to that observed with Eve itself (14). However, a related derivative, FS2 (see Fig. 8 below for amino acid sequences), was nearly inactive in repression. Figure 2B shows that the two fusion proteins displayed very similar properties in the squelching assay; EveABFS1 was extremely effective in blocking TBP-mediated activation, while the FS2-containing derivative was much less effective. Taken together, these results provide evidence for a functional interaction between TBP and each of two repression regions shown previously to have the ability to act as direct repressors in vivo.

All but one of the proteins described above contained an intact homeodomain. The derivative that we tested with a lesion in this region (AbCDEF, which contains an in-frame deletion that removes 25 residues) was inactive in squelching (Fig. 2A), despite being stable and localized to the nucleus (14). We believe that this reflects a role for the homeodomain in the protein-protein interaction between TBP and the Eve repression domain, a view supported by data presented below.

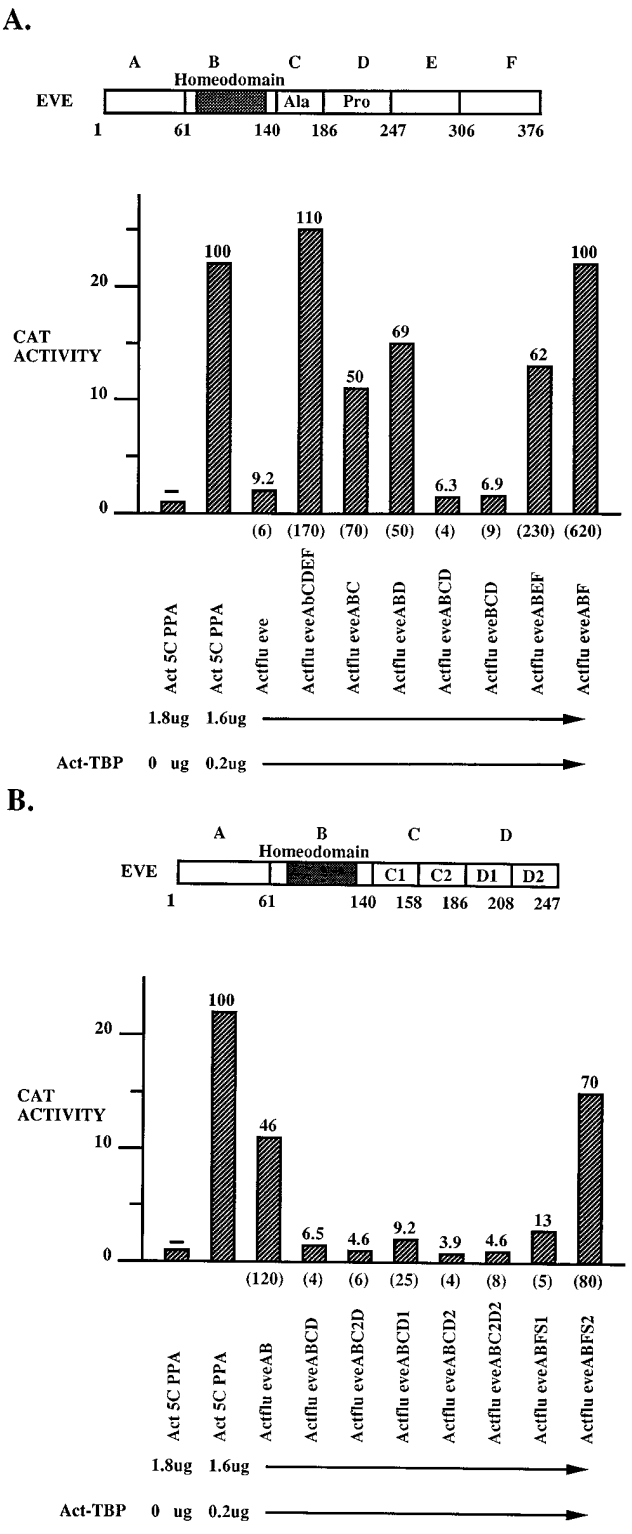

FIG. 2. Eve alanine- and proline-rich regions are required for binding-site-independent repression. The reporter plasmid E1b TATA CAT, Act-TBP, and the indicated Eve expression plasmids were cotransfected into Schneider cells. The scale on the left of each graph displays the actual, β-galactosidase-normalized CAT activities. The numbers above each bar display relative CAT activities, in which the value resulting from cotransfection of Act-TBP in the absence of any Eve expression plasmids was taken as 100%. Corresponding binding-site-dependent repression values, which are taken from reference 14, are shown in parentheses below each bar. (A) The repression domains C and D are necessary for binding-site-independent repression of TBP-activated transcription. The diagram at the top depicts the domains of full-length Eve, which were originally defined by Han and Manley (14). Domain C is rich in alanine (46%), and domain D is rich in proline (25%). (B) Minimal Eve repression domains also squelch TBP-activated transcription in the absence of Eve-binding sites. The diagram at the top of the panel depicts the subdivided repression region of Eve, also as defined by Han and Manley (14).

However, an alternative explanation was suggested by the results described by Ohkuma et al. (36), which showed that the homeodomain protein Engrailed could repress transcription in vitro by competition with TFIID for binding to the TATA box (the homeodomain-binding site is A-T rich). If this were the mechanism of squelching, then a prediction would be that Eve should inhibit TATA-containing promoters in the absence of TBP overexpression, and elevated levels of TBP would in fact lessen the inhibition by altering the competition. However, analysis of the effect of Eve expression on several TATA-containing promoters lacking Eve-binding sites indicated that Eve cannot compete with TFIID for the TATA box in vivo. The activity of the very weak E1b TATA promoter was not significantly affected by Eve in the absence of exogenously expressed TBP (Fig. 1), while the stronger met TATA and hsp TATA promoters were likewise only very slightly inhibited (Table 1). These findings are not consistent with the idea that squelching was due to competition for the TATA box.

Eve-binding sites increase repression of TBP-enhanced transcription. During the course of the above experiments, we observed that transcription squelched by Eve did not fall below the basal level of expression observed in the absence of cotransfected TBP (e.g., Fig. 1). However, our previous studies indicated that in the presence of binding sites, Eve efficiently repressed basal transcription (14). Therefore, we wished to determine whether Eve-binding sites would allow more efficient repression of TBP-enhanced transcription. To test this directly, we compared the abilities of Eve to repress TBP-enhanced expression from reporter plasmids that differed only by the presence or absence of Eve-binding sites, and the results are shown in Fig. 3. The results show that increasing concentrations of Eve effectively blocked TBP-enhanced expression from the plasmid lacking Eve-binding sites but were unable to reduce transcription below basal levels (which are displayed in the leftmost columns). In contrast, in the presence of Eve-binding sites, Eve not only inhibited TBP-enhanced transcription more efficiently but also reduced expression to well below basal levels. These results suggest that the interactions between Eve and its target (i.e., TBP or TFIID) are more effective when Eve is bound to upstream DNA than when it is not. A possible mechanistic basis for this is discussed below.

Eve and TBP interact in a two-hybrid-type assay. As an independent test that Eve and TBP interact in vivo, we employed a modification of the yeast two-hybrid assay (10; see also reference 30). This involved cotransfection of Schneider cells with appropriate expression plasmids plus a CAT reporter plasmid. In a first experiment, we used two types of expression plasmids (see Materials and Methods for details). The first type encoded the yeast GAL4 DNA-binding domain, either alone or fused to full-length TBP. The second type expressed the VP16 activation domain, either alone or fused to the Eve AB or ABCD regions. The latter plasmids also encoded an influenza virus hemagglutinin epitope tag at the N termini of the proteins. The reporter plasmid contained five GAL4-binding sites located upstream of a 23-bp fragment encompassing the TdT initiator (Inr) element. An Inr was used to prevent possible interactions between GAL4-TBP and a TATA box. The rationale for the experiments was that an Eve-TBP inter-

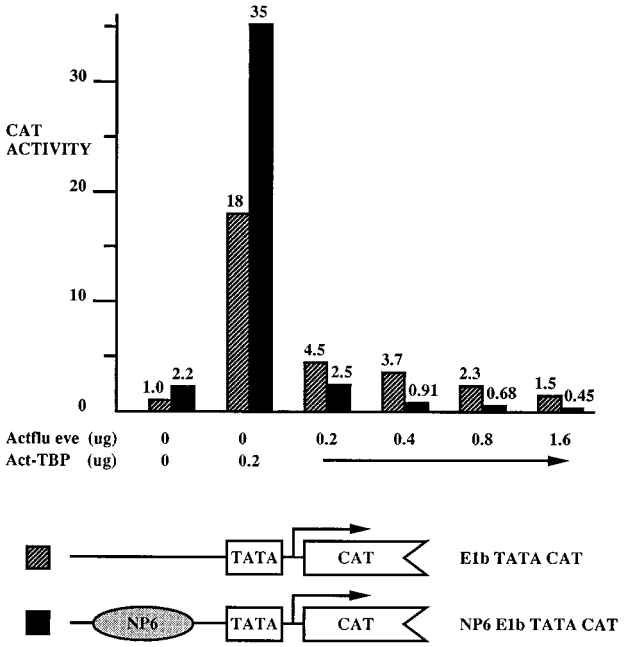

FIG. 3. Repression of TBP-enhanced transcription is more efficient from a template containing Eve-binding sites. Act-TBP and increasing amounts of Act-flu eve were cotransfected with E1b TATA CAT or NP6 E1b TATA CAT as indicated. The reporter plasmids are diagrammed at the bottom of the figure. The graph displays normalized CAT activities. The hatched bars denote results from experiments with E1b TATA CAT, and the black bars indicate the results with NP6 E1b TATA CAT.

action would bring the VP16 activation domain to the promoter via the GAL4 DNA-binding domain, resulting in increased CAT expression. A possible complication stemmed from the fact that VP16 and TBP have been shown to interact in vitro (e.g., see reference 45). However, controls with several different VP16 derivatives failed to provide evidence for a TBP-VP16 interaction in this assay (results not shown; see also below).

The results of CAT assays from transfections with different combinations of the plasmids described above are shown in Fig. 4A. When transfected alone, all of the expression plasmids but one had no significant effect on CAT activity. Somewhat surprisingly, however, GAL4-TBP resulted in significant, concentration-dependent (not shown) increases in CAT expression. This was unexpected, because we had shown previously that overexpression of TBP with an Inr plasmid similar to the one used here repressed, rather than activated, transcription (5). Activation by GAL4-TBP required the conserved C terminus of TBP and not the N-terminal glutamine-rich region (results not shown; see also below). When the GAL4-alone expression plasmid was cotransfected with any one of the three VP16 plasmids, no activation was detected. Likewise, cotransfection of VP16 and GAL4-TBP plasmids did not enhance expression above the value seen with GAL4-TBP alone. Coexpression of Eve AB-VP16 with GAL4-TBP enhanced CAT activity slightly, somewhat less than 2-fold. However, when Eve ABCD-VP16 and GAL4-TBP were coexpressed, a significant enhancement of ~5-fold was observed. Although modest, this level of activation has been detected reproducibly and supports the existence of an interaction between TBP and the Eve repression domain. The higher activity observed with Eve ABCD-VP16 relative to Eve AB-VP16 was not due to higher protein accumulation. As determined by Western blotting (Fig. 4C), the levels of ABCD-VP16 were in fact somewhat lower than those of AB-VP16.

The above results encouraged us to investigate the behavior of several GAL4-TBP mutant derivatives. One reason for doing this was to identify possible TBP mutants that were unable to bring about activation by themselves but which retained the ability to interact with Eve and thereby to activate transcription in the two-hybrid assay. This could conceivably lead to a higher fold activation due to the reduced background from the GAL4-TBP derivative alone and at the same time could allow us to begin to define the region of TBP responsible for the interaction with Eve. Figure 4B displays the results obtained with two GAL4-TBP derivatives, GAL4-TBP(1-157) and GAL4-TBP(1-282). The former consists of essentially only the species-specific N terminus of TBP, while the latter lacks ~70 C-terminal residues. (Because both proteins were inactive by themselves, a TATA-containing reporter plasmid and higher amounts of expression vectors were used in the experiment illustrated in Fig. 4B.) GAL4-TBP(1-157) failed to activate transcription when cotransfected with expression vectors encoding any of the three VP16 derivatives. GAL4-TBP(1-282) was also inactive when expressed with either VP16 or Eve AB-VP16. In contrast, coexpression of GAL4-TBP(1-282) and Eve ABCD-VP16 resulted in an ~16-fold activation of CAT expression. These results taken together provide strong evidence for an interaction between residues in the conserved core of TBP and the Eve repression domain.

TBP and Eve physically interact. The results described above support the existence of a functionally relevant interaction between Eve and TBP in vivo. To provide evidence that this involves a direct, physical interaction between the two proteins, we employed the GST fusion protein interaction assay. For this, we first constructed an in-frame fusion between GST and a cDNA encoding full-length *Drosophila* TBP (dTBP [Fig. 5]). GST-dTBP and GST were expressed in *E. coli*, purified (Fig. 5A), and immobilized on glutathione agarose beads. [^{35}S]methionine-labeled Eve was produced by in vitro transcription-translation, and aliquots were mixed with the appropriate beads (see Materials and Methods). After extensive washing, proteins were eluted with buffer containing 20 mM glutathione, resolved by SDS-PAGE, and subjected to fluorography. The results (Fig. 5B) show that Eve did not bind detectably either to the glutathione beads (mock) or to the beads containing GST. Eve also did not bind to a GST-*Drosophila* TFIIB (GST-dTFIIB) fusion protein (results not shown). However, significant binding of Eve to GST-dTBP was detected, supporting the existence of a specific interaction between the two proteins. Because both Eve and TBP are DNA-binding proteins, it was important to show that the interaction did not depend on contaminating DNA, which could conceivably tether the two proteins (24). This was tested by determining the effect of including ethidium bromide (EtBr) in the binding buffer to disrupt possible DNA-protein interactions (24). The results (Fig. 5B) indicate that EtBr not only did not reduce the interaction but in fact increased binding to nearly quantitative levels (~3-fold, as judged by phosphorImager analysis). While we can currently only speculate on the significance of this enhancement (see Discussion), the results provide strong evidence that DNA is not necessary for the interaction between Eve and TBP.

A functional repression domain is required for TBP binding. We then wished to determine whether the Eve repression domain is required for the interaction with TBP. For this, we prepared a number of Eve derivatives labeled with [^{35}S]methionine by in vitro transcription-translation and determined their ability to bind GST-dTBP (Fig. 6). Eve ABCD, which as de-

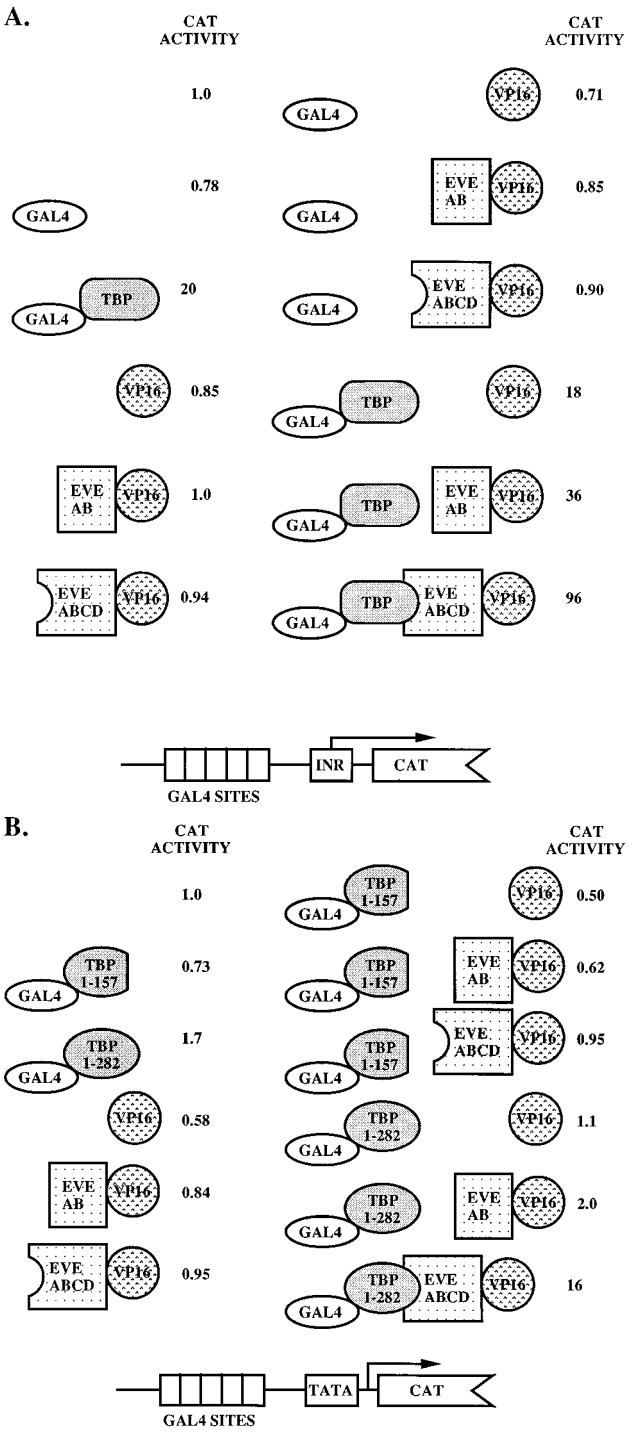

FIG. 4. Eve and TBP interact in a two-hybrid assay. (A) GAL4-TBP and Eve ABCD-VP16 interact to activate transcription in vivo. Act GAL4 or Act GAL4-TBP plasmid (0.05 μg) and/or Actflu-VP16, Actflu eveAB-VP16, or Actflu eveABCD-VP16 plasmid (2 μg) was cotransfected with the reporter plasmid G5 Inr CAT, which is diagrammed at the bottom. The values shown are the normalized CAT activities. (B) TBP C-terminal residues 158 to 282 are sufficient for interaction with the Eve repression domain. Act GAL4-TBP(1-157) or Act GAL4-TBP(1-282) plasmid (0.5 μg) and/or Actflu-VP16, Actflu eveAB-VP16, or Actflu eveABCD-VP16 (5 μg) was cotransfected with the reporter plasmid G5 E1b TATA CAT, which is diagrammed at the bottom. (C) Accumulation of flu Eve-VP16 fusion proteins. Whole-cell lysates were prepared from cells transfected with Actflu eveAB-VP16 (lanes 1 and 3) or Actflu eveABCD-VP16 (lanes 2 and 4), plus (lanes 3 and 4) or minus (lanes 1 and 2) Act GAL4-TBP(1-282). flu-Eve fusion proteins were detected by Western blotting. The sizes of protein markers (in kilodaltons) are indicated on the left.

scribed above retains strong repression and squelching activity, also retained the ability to interact with TBP (although binding was slightly reduced relative to that of wild-type Eve [Fig. 6A]). In contrast, binding was undetectable with variants lacking the CD region. Figure 6A shows that Eve ABEF and ABF were not retained on the GST-dTBP resin. Likewise, Eve AB, which contains the homeodomain and Eve N terminus, was unable to interact with GST-dTBP (see Fig. 8 below). These results indicate that sequences contained within the CD region, which includes residues essential for repression and squelching, are also essential for binding TBP.

As described above for squelching and previously for repression (14), the CD region can be further subdivided to produce fragments that have various degrees of activity. Two of these Eve derivatives, which both have very high repression and squelching activities, were produced by in vitro transcription-translation, and the ability of each to bind GST-dTBP was determined. The results (Fig. 6B) were again consistent with those from the activity assays. Eve ABCD2 and ABC2D2 (recall that C2D2 is the minimally defined repression region) both bound GST-dTBP with efficiencies similar to that of wild-type Eve, which correlates with their ability to function very effectively as repressors. Taken together, these results indicate that the region of Eve required for repression is also required for binding TBP, and conversely that regions dispensable for repression are also dispensable for TBP binding.

We also tested whether the CD region could by itself bind GST-dTBP but were unable to detect an interaction (results not shown). This suggests that the B domain (homeodomain) is required along with the repression domain for TBP binding. (The N-terminal A domain is not required; results not shown.) Whether the homeodomain makes direct contacts with TBP or functions indirectly, for example by stabilizing a specific conformation of the repression domain, is unclear. However, the fact that the Eve-TBP interaction was resistant to EtBr indicates that the role of the homeodomain is distinct from that of DNA binding.

The conserved C-terminal core of TBP is sufficient for Eve binding. The two-hybrid interaction assay described above suggested that sequences within the evolutionarily conserved C-terminal domain of TBP mediate the in vivo interaction between TBP and Eve. To determine whether this region of TBP is also responsible for the in vitro interaction, we tested the ability of several GST-TBP fusion proteins, which are diagrammed in Fig. 7A, to bind [^{35}S]methionine-labeled Eve. The proteins, purified from *E. coli*, are shown in Fig. 7B, and the results of binding assays, performed as above, are shown in Fig. 7C. Deletion of the species-specific N-terminal region of TBP (mutant Δ1-162) had no effect on binding, indicating that sequences within the C-terminal core were sufficient. We also purified GST human and yeast TBP (hTBP and yTBP, respectively) fusion proteins and found that Eve could bind to these derivatives with efficiencies comparable to that of GST-dTBP (Fig. 7C). Taken together, these results indicate that Eve rec-

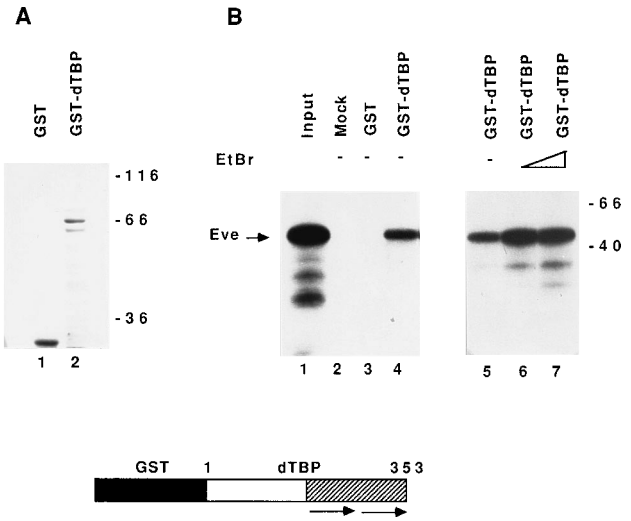

FIG. 5. Eve interacts with immobilized dTBP. (A) Coomassie blue stain of an SDS-polyacrylamide gel containing GST and GST-dTBP (2 μg) used in the protein-protein association assay shown in panel B. The sizes of molecular mass markers (in kilodaltons) are indicated on the right. (B) In vitro-translated Eve binds to immobilized dTBP. In vitro-transcribed and translated ^{35}S-labeled Eve was incubated with either buffer plus glutathione-agarose beads (lane 2), or 2 μg of GST (lane 3) or 2 μg of GST-dTBP (lanes 4 to 7) bound to glutathione-agarose beads. The input lane (lane 1) was loaded with the amount of Eve used in each of the binding reaction mixtures. The Eve-GST-dTBP complexes were eluted with glutathione and analyzed by SDS-PAGE as described in Materials and Methods. The reactions were done in the absence of EtBr (lanes 2 to 5) or in the presence of EtBr at a concentration of 100 μg/ml (lane 6) or 400 μg/ml (lane 7). The position of Eve in the gel is indicated. A schematic diagram of GST-dTBP is also shown. The two imperfect direct repeats present in the C-terminally conserved region of dTBP are represented by arrows.

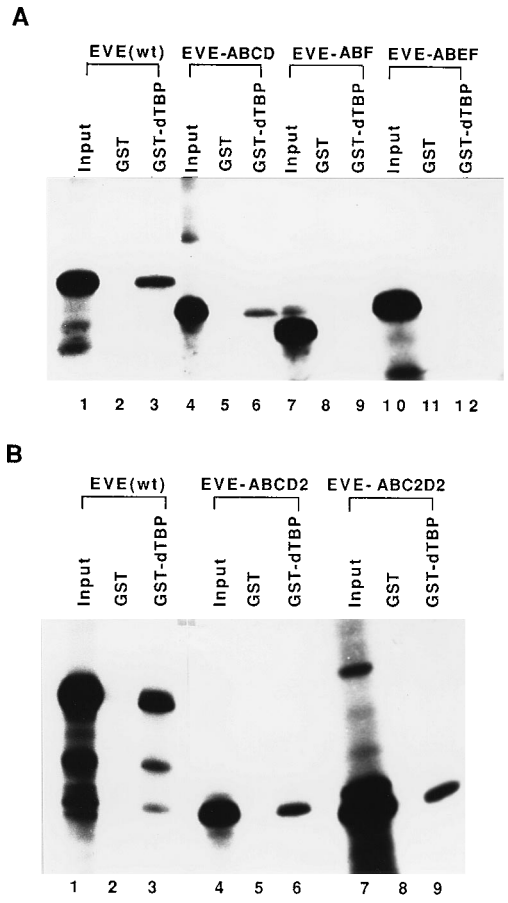

FIG. 6. The Eve repression domain is required for interaction with immobilized dTBP. [^{35}S]methionine-labeled Eve and the indicated derivatives were produced by in vitro transcription-translation, and equal amounts (Input) of Eve proteins were incubated with either 2 μg of GST or 2 μg of GST-dTBP linked to glutathione-agarose beads. After extensive washing, the bound proteins were eluted, resolved by electrophoresis, and visualized by fluorography as described in the legend to Fig. 6. wt, wild type.

ognizes a highly conserved sequence or structure in the C-terminal region of TBP.

An artificial repression region also binds TBP. As described above, the artificial repression region FS1 displays properties very similar to those of the Eve repression domain. Therefore, we wished to determine whether the 29-residue FS1 peptide could mediate TBP binding in the context of the Eve ABFS1 protein. For this, [^{35}S]methionine-labeled Eve ABFS1 was produced by in vitro transcription-translation as described above, and its ability to bind immobilized GST or GST-dTBP was determined. As shown in Fig. 8, Eve ABFS1 bound GST-dTBP at least as well as wild-type Eve but did not bind at all to GST alone. The FS1 region was essential for binding, since the Eve AB region by itself was completely inactive.

As mentioned above, FS2 is a second artificial sequence that is related to FS1 but is greatly reduced in both repression and squelching activity. (The sequences of both FS1 and FS2 are shown at the bottom of Fig. 8.) The existence of this related pair of sequences allowed us to test further the correlation between direct repression (or squelching) activity in vivo and the ability to bind TBP in vitro. Therefore, Eve ABFS2 was produced by in vitro transcription-translation, and its ability to bind GST-dTBP was compared with that of Eve ABFS1. Extending the pattern observed above with different Eve derivatives, ABFS2 bound GST-dTBP much less efficiently than did ABFS1 (Fig. 8). In agreement with the ability of Eve ABFS2 to mediate very weak repression (14), a very low level of binding was observed. Taken together, our results show that two different repression regions can each mediate binding to TBP in vitro and bring about squelching of TBP-enhanced transcription in vivo.

DISCUSSION

The data described above indicate that the *Drosophila* transcriptional repressor Eve interacts directly with TBP. The squelching and two-hybrid assays are indicative of an in vivo interaction between Eve and TBP, with the relevance of this to repression supported by the perfect correspondence between the protein domains required for repression on the one hand and for squelching and the two-hybrid interaction on the other. Evidence that these in vivo interactions are reflective of a direct association between the two proteins came from the in vitro protein-binding assays, with the relevance to repression again established by the requirement for a functional repression domain. Our data thus demonstrate that a sequence-specific DNA-binding transcriptional repressor can interact with a component of the basal transcription machinery. Our findings also support the idea, which was previously suggested by others (20), that the mechanisms employed by certain activators and repressors may be related.

Squelching by Eve was found to be specific for TBP-enhanced transcription. That is, basal expression from the same minimal reporter plasmids in the absence of exogenously expressed TBP was essentially unaffected by Eve. Since TBP, or TFIID, is required in both cases, why was only the former

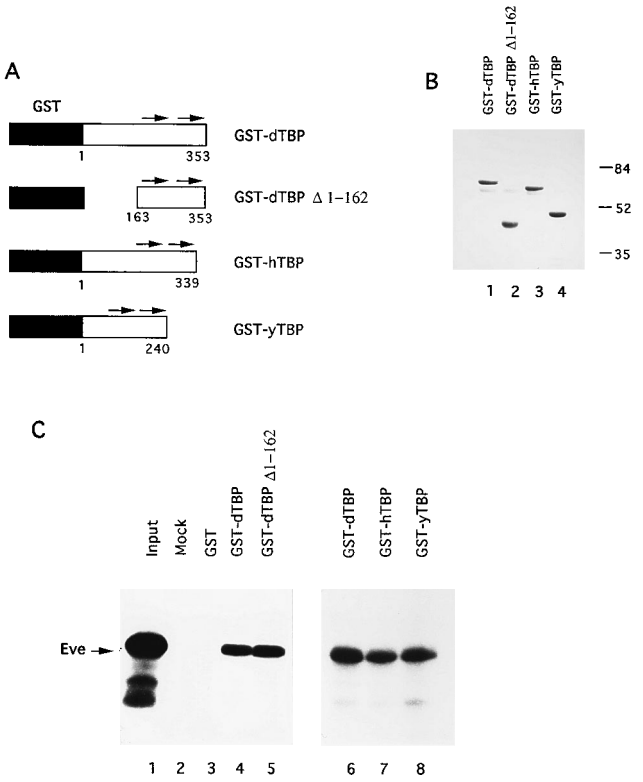

FIG. 7. Eve interacts with the conserved C-terminal core of TBP. Protein binding assays were performed by using [^{35}S]methionine-labeled Eve and the indicated GST-TBP fusion proteins. (A) Structures of GST-TBP fusion proteins. *Drosophila*, human, and yeast GST-TBP constructs are shown. The two direct repeats in the conserved C terminus of TBP are represented by arrows. Numbers indicate amino acid residues. (B) Coomassie blue staining of a protein gel containing 2 μg of GST-TBP fusion proteins used in protein binding assays shown in panel C. (C) Binding of in vitro-translated ^{35}S-labeled Eve to GST-TBP fusion proteins. In vitro-translated Eve was incubated with buffer (lane 2), GST (lane 3), or the indicated GST-TBP fusion protein (lanes 4 to 8). The bound proteins were eluted and analyzed by PAGE as described in the legend to Fig. 6.

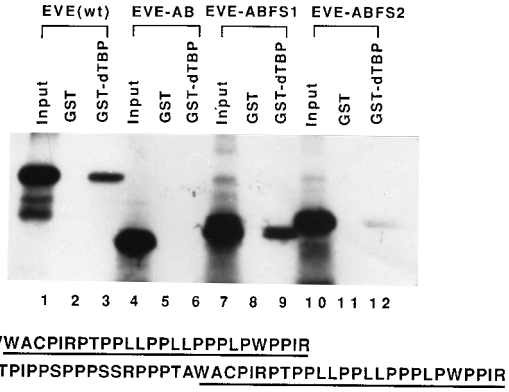

FIG. 8. Interaction of Eve derivatives FS1 and FS2 with immobilized dTBP. The indicated Eve derivatives were produced by in vitro transcription-translation as described above, and equal amounts were incubated with 2 μg of GST or 2 μg of GST-dTBP bound to glutathione-agarose. Protein complexes were eluted and analyzed as described in the legend to Fig. 6. The primary sequences of FS1 and FS2 are shown at the bottom of the figure. The region that is identical in both peptides is underlined. wt, wild type.

subject to squelching? One possibility is that exogenously expressed TBP was not efficiently assembled into TFIID, and free, or partially assembled, TBP is a better target for Eve than is fully assembled TFIID. However, recent results indicate that at least a fraction of TBP exogenously expressed in Schneider cells is bound to TBP-associated factors (TAFs) (9), which is consistent with data indicating that cotransfected TBP can effectively cooperate with a number of different activators (e.g., see references 2, 5, 9, and 22). Presumably for such cooperation, TBP must be incorporated into TFIID, since to date only TFIID has been found capable of mediating activation in vitro (e.g., see references 38 and 46). It is also possible that other differences between endogenous and exogenous (i.e., newly synthesized) TBP, for example subcellular localization and/or association with other basal transcription factors, influence their ability to interact with Eve. Perhaps there are changes in the conformation of TFIID that occur during assembly of the initiation complex, and only when Eve and endogenous TFIID are both bound to DNA, which occurs in natural, DNA-binding-site-dependent repression, is the Eve-TBP interaction sufficiently strong to block transcription. This idea is supported by our finding that repression of TBP-enhanced transcription by Eve from a reporter plasmid containing Eve-binding sites was significantly more efficient than was squelching from the same plasmid lacking binding sites.

We previously showed that the Eve repression domain (CD) was functional when fused to the heterologous GAL4 DNA-binding domain (14), suggesting that the homeodomain is not essential for repression activity. The experiments described here, however, provide evidence that the homeodomain is necessary for the interaction of Eve with TBP. One possible explanation for this apparent discrepancy is that the role of the homeodomain in the TBP interaction is relatively nonspecific (e.g., to stabilize or solubilize the hydrophobic repression domain) and can be fulfilled by other sequences such as the GAL4 DNA-binding domain. Another explanation derives from the fact that GAL4-CD-mediated repression is relatively weak (14), and more recent experiments have shown that some promoters containing GAL4-binding sites cannot be repressed by GAL4-CD, although they can be repressed by the homeodomain-containing GAL4-BCD derivative (unpublished data). This suggests that the homeodomain can play a role in Eve repression apart from DNA binding, which could, as our data suggest, be to participate in the interaction with TBP.

Several other proteins that are able to influence transcription negatively and to bind TBP have been identified previously. Two, DR1 (16) and DR2 (or topoisomerase I [35]), were characterized as activities in nuclear extracts that can repress basal transcription in vitro. (Two activities with similar properties, NC1 and NC2, had been described previously [33, 34].) These proteins appear likely to function as more general repressors of transcription, since neither has specific DNA-binding activity. Nonetheless, certain properties of DR1 are noteworthy in light of our studies of Eve. Like Eve, DR1 can inhibit both basal and activated transcription. DR1 appears to do this by binding TBP and preventing association with TFIIA and/or TFIIB, which is certainly a plausible mechanism by which Eve might function (see below). It is notable that DR1 contains an alanine-rich region that shares some similarity with the repression region of Eve (16). Recent studies have shown that this region is in fact required for repression, although TBP binding appears to be mediated by a separate region (49). Finally, the adenovirus E1A 12S and 13S proteins have been shown to have the ability to repress the activity of a variety of enhancer elements, perhaps through interactions with a common, basal factor (reference 40 and references therein). Consistent with the idea that this factor could be TBP, both E1A proteins have

been found to interact in vitro with TBP (25), although the relevance of this binding to enhancer repression is unknown.

As mentioned in the introduction, a number of sequence-specific DNA-binding proteins that function as transcriptional repressors have been identified, and several of these may have features in common with Eve. Like Eve, the *Drosophila* Krüppel protein contains an alanine-rich region that can function as a repression domain (27). However, unlike Eve, Krüppel appears unable to repress transcription in an activator-independent fashion, and it has been suggested that Krüppel may function by a quenching mechanism involving interactions with certain specific activators (28, 50). In addition, also unlike Eve, Krüppel can, depending on its concentration in transfected cells, be an activator as well as a repressor (42, 43). In mammalian systems, the v-*erbA* oncogene product, which is the unliganded thyroid hormone receptor (TR), is a strong repressor that has been well characterized. Initial experiments (8) suggested that TR can function as a direct repressor (i.e., through interactions with basal factors), and this has more recently been confirmed by in vitro transcription assays showing that TR can prevent formation of productive preinitiation complexes (11). Protein interaction assays revealed that TR can bind TFIIB, and no interaction with TBP was detected. One explanation for this difference between Eve and TR is that, like activators, repressors can function in diverse ways involving interactions with distinct general factors. However, it is well established that TR can also function as a transcriptional activator, and it is presently not known whether the TR-TFIIB interaction is indeed relevant to repression. Recently, the murine homeodomain protein Msx-1 was shown to mediate repression via alanine-plus-proline-rich regions and to interact with DNA-basal factor complexes in gel shift assays (4). In both transfection and in vitro transcription assays, repression was independent of Msx-1-binding sites on the DNA template, suggesting a potentially important role for interactions between Msx-1 and one of the basal factors, which our data suggest could be TBP.

How might the Eve-TBP (or Eve FS1-TBP) interaction lead to transcriptional repression? Although additional work is certainly required to answer this question, we suggest two possibilities. First, Eve may function in a manner similar to that suggested for DR1 (16). That is, Eve would interact with TBP (TFIID) subsequent to DNA binding by both factors and by so doing would interfere with binding of other general factors such as TFIIA and/or TFIIB. A second model envisions that Eve functions even earlier in preinitiation complex assembly, by either blocking or destabilizing the interaction of TBP (TFIID) with DNA. Our observation that addition of EtBr to binding reaction mixtures enhanced the Eve-TBP interaction can be taken as evidence in support of this; EtBr likely removes contaminating *E. coli* DNA bound to the GST-dTBP protein, and because this increases Eve binding, it could be that Eve and DNA in essence compete for binding to TBP. If this is the case, then an interesting question is whether Eve functions solely by preventing TBP (TFIID) from binding DNA or whether it might be able to displace TBP (TFIID) that is already bound. Finally, although our data support the idea that a TBP-Eve interaction is likely important for repression, they do not address the question of whether it could be sufficient. If current models for transcriptional activation provide a precedent (for a review, see reference 48), then it could be that multiple protein contacts with distinct factors will be required for efficient repression. Some of these interactions may be mediated by corepressors, such as the Groucho protein in *D. melanogaster* (37). However, whatever the detailed mechanism, our studies indicate that the function of a direct repressor such as Eve may involve an interaction with TBP.

ACKNOWLEDGMENTS

We are grateful to J. Colgan and K. Han for providing plasmids and much valuable advice, and to M. Hosford for preparing the manuscript.

This work was supported by NIH grant GM 37971.

M. Um and C. Li contributed equally to this work.

REFERENCES

1. **Baniahmad, A., A. C. Kohne, and R. Renkawitz.** 1992. A transferable silencing domain is present in the thyroid hormone receptor, in the v-erbA oncogene product and in the retinoic acid receptor. EMBO J. **11:**1015–1023.
2. **Berkenstam, A., M. D. M. Vivanco Ruiz, D. Barettino, M. Horikoshi, and H. G. Stunneberg.** 1992. Cooperativity in transactivation between retinoic acid receptor and TFIID requires an activity analogous to E1A. Cell **69:**401–412.
3. **Biggin, M. D., and R. Tjian.** 1989. A purified *Drosophila* homeodomain protein represses transcription *in vitro*. Cell **58:**433–440.
4. **Catron, K. M., H. Zhang, S. C. Marshall, J. A. Inostroza, J. M. Wilson, and C. Abate.** 1995. Transcriptional repression by Msx-1 does not require homeodomain DNA-binding sites. Mol. Cell. Biol. **15:**861–871.
5. **Colgan, J., and J. L. Manley.** 1992. TFIID can be rate limiting *in vivo* for TATA-containing, but not TATA-lacking, RNA polymerase II promoters. Genes Dev. **6:**304–315.
6. **Colgan, J., and J. L. Manley.** 1995. Cooperative interactions between basal promoter elements enhance transcription *in vivo*. Proc. Natl. Acad. Sci. USA **92:**1955–1959.
7. **Colgan, J., S. Wampler, and J. L. Manley.** 1993. Interaction between a transcriptional activator and transcription factor IIB *in vivo*. Nature (London) **362:**549–553.
8. **Damm, K., C. C. Thomson, and R. M. Evans.** 1989. Protein encoded by v-erbA functions as a thyroid-hormone receptor antagonist. Nature (London) **339:**593–597.
9. **Farmer, G., and J. Colgan.** Unpublished data.
10. **Fields, S., and O. Song.** 1989. A novel genetic system to detect protein-protein interactions. Nature (London) **340:**245–246.
11. **Fondell, J. D., A. L. Roy, and R. G. Roeder.** 1993. Unliganded thyroid hormone receptor inhibits formation of a functional preinitiation complex: implications for active repression. Genes Dev. **7:**1400–1410.
12. **Gill, G., and M. Ptashne.** 1988. Negative effect of transcriptional activator GAL4. Nature (London) **334:**721–724.
13. **Han, K., M. S. Levine, and J. L. Manley.** 1989. Synergistic activation and repression of transcription by *Drosophila* homeobox proteins. Cell **56:**573–583.
14. **Han, K., and J. L. Manley.** 1993. Transcriptional repression by the *Drosophila* Even-skipped protein: definition of a minimal repression domain. Genes Dev. **7:**491–503.
15. **Han, K., and J. L. Manley.** 1993. Functional domains of the *Drosophila* Engrailed protein. EMBO J. **12:**2723–2733.

15a. **Han, K., and J. L. Manley.** Unpublished data.

16. **Inostroza, J. A., F. H. Mermelstein, I. Ha, W. S. Lane, and D. Reinberg.** 1992. Dr1, TATA-binding protein-associated phosphoprotein and inhibitor of class II gene transcription. Cell **70:**477–489.
17. **Jaynes, J. B., and P. H. O'Farrell.** 1988. Activation and repression of transcription by homeodomain-containing proteins that bind a common site. Nature (London) **336:**744–749.
18. **Jaynes, J. B., and P. H. O'Farrell.** 1991. Active repression of transcription by the engrailed homeodomain protein. EMBO J. **10:**1427–1433.
19. **Johnson, A. D., and I. Herskowitz.** 1985. A repressor (MATα2 product) and its operator control expression of a set of cell type specific genes in yeast. Cell **42:**237–247.
20. **Johnson, F. B., and M. A. Krasnow.** 1992. Differential regulation of transcription preinitiation complex assembly by activator and repressor homeodomain proteins. Genes Dev. **6:**2177–2189.
21. **Keleher, C. A., C. Goutte, and A. D. Johnson.** 1988. The yeast cell-type specific repressor α2 acts cooperatively with a non-cell-type-specific protein. Cell **53:**927–936.
22. **Kerr, L. D., L. J. Ransone, P. Wamsley, M. J. Schmitt, T. G. Boyer, Q. Zhou, A. J. Berk, and I. M. Verma.** 1993. Association between proto-oncoprotein Rel and TATA-binding protein mediates transcriptional activation by NF-κB. Nature (London) **365:**412–419.
23. **Komachi, K., M. J. Redd, and A. D. Johnson.** 1994. The WD repeats of Tup1 interact with the homeodomain protein α2. Genes Dev. **8:**2857–2867.
24. **Lai, J.-S., and W. Herr.** 1992. Ethidium bromide provides a simple tool for identifying genuine DNA-independent protein associations. Proc. Natl. Acad. Sci. USA **89:**6958–6962.
25. **Lee, W. S., C. C. Kao, G. O. Bryant, X. Liu, and A. J. Berk.** 1991. Adenovirus

E1A activation domain binds the basic repeat in the TATA box transcription factor. Cell **67:**365–376.
26. **Levine, M., and J. L. Manley.** 1989. Transcriptional repression of eukaryotic promoters. Cell **59:**405–408.
27. **Licht, J. D., M. J. Grossel, J. Figge, and U. M. Hansen.** 1990. Drosophila Krüppel protein is a transcriptional repressor. Nature (London) **346:**76–79.
28. **Licht, J. D., M. Ro, M. A. English, M. Grossel, and U. Hansen.** 1993. Selective repression of transcription activators at a distance by the *Drosophila* Krüppel protein. Proc. Natl. Acad. Sci. USA **90:**11361–11365.
29. **Lin, Y.-S., and M. R. Green.** 1991. Mechanism of action of an acidic transcriptional activator *in vitro*. Cell **64:**971–981.
30. **Liu, F., and M. R. Green.** 1990. A specific member of the ATF transcription factor family can mediate transcription activation by the adenovirus E1A protein. Cell **61:**1217–1224.
31. **Madden, S. L., D. M. Cook, J. F. Morris, A. Gashler, V. P. Sukhatme, and F. J. Rauscher III.** 1991. Transcriptional repression mediated by WT1, the Wilms tumor gene product. Science **253:**1550–1553.
32. **Madden, S. L., and F. J. Rauscher III.** 1993. A structure-function analysis of transcriptional repression mediated by the WT1 Wilms tumor suppressor protein. Oncogene **8:**1713–1720.
33. **Meisterernst, M., and R. G. Roeder.** 1991. Family of proteins that interact with TFIID and regulate promoter activity. Cell **67:**557–567.
34. **Meisterernst, M., A. L. Roy, H. M. Lieu, and R. G. Roeder.** 1991. Activation of class II gene transcription by regulatory factors is potentiated by a novel activity. Cell **66:**981–993.
35. **Merino, A., K. R. Madden, W. S. Lane, J. J. Champoux, and D. Reinberg.** 1993. DNA topoisomerase I is involved in both repression and activation of transcription. Nature (London) **365:**227–232.
36. **Ohkuma, Y., M. Horikoshi, R. G. Roeder, and C. Desplan.** 1990. Engrailed, a homeodomain protein, can repress *in vitro* transcription by competition with the TATA box-binding protein transcription factor IID. Proc. Natl. Acad. Sci. USA **87:**2289–2293.
37. **Paroush, Z., R. Finley, T. Kidd, S. Wainright, P. Ingham, R. Brent, and D. Ish-Horowitz.** 1994. Groucho is required for *Drosophila* neurogenesis, segmentation and sex determination and interacts with Hairy-related bHLH proteins. Cell **79:**805–815.
38. **Pugh, B. F., and R. Tjian.** 1990. Mechanism of transcriptional activation by Sp1: evidence for coactivators. Cell **61:**1187–1197.
39. **Renkawitz, R.** 1990. Transcriptional repression in eukaryotes. Trends Genetics **6:**192–197.
40. **Rochette-Egly, C., C. Fromental, and P. Chambon.** 1990. General repression of enhancer activity by the adenovirus-2 E1A proteins. Genes Dev. **4:**137–150.
41. **Saha, D., J. M. Brickman, N. Lehming, and M. Ptashne.** 1993. New eukaryotic transcription repressors. Nature (London) **363:**648–652.
42. **Sauer, F., and H. Jäckle.** 1991. Concentration-dependent transcriptional activation or repression by Krüppel from a single binding site. Nature (London) **353:**563–566.
43. **Sauer, F., and H. Jäckle.** 1993. Dimerization and the control of transcription by Krüppel. Nature (London) **364:**454–457.
44. **Shi, Y., E. Seto, L. S. Chang, and T. Shenk.** 1991. Transcriptional repression by YY1, a human GL1-Krüppel-related protein, and relief of repression by adenovirus E1A protein. Cell **67:**377–388.
45. **Stringer, K. F., C. J. Ingles, and J. Greenblatt.** 1990. Direct and selective binding of an acidic transcriptional activation domain to the TATA-box factor TFIID. Nature (London) **345:**783–786.
46. **Tanese, N., B. F. Pugh, and R. Tjian.** 1991. Coactivators for a proline-rich activator purified from the multisubunit human TFIID complex. Genes Dev. **5:**2212–2224.
47. **TenHarmsel, A., R. J. Austin, N. Savenelli, and M. D. Biggin.** 1993. Cooperative binding at a distance by *even-skipped* protein correlates with repression and suggests a mechanism of silencing. Mol. Cell. Biol. **13:**2742–2752.
48. **Tjian, R., and T. Maniatis.** 1994. Transcriptional activation: a complex puzzle with few easy pieces. Cell **77:**5–10.
49. **Yeung, K. C., J. A. Inostroza, F. H. Mermelstein, C. Kannabiran, and D. Reinberg.** 1994. Structure-function analysis of the TBP binding protein, Dr1, reveals a mechanism for repression of class II gene transcription. Genes Dev. **8:**2097–2107.
50. **Zuo, P., D. Stanojevic, J. Colgan, M. Levine, and J. L. Manley.** 1991. Activation and repression of transcription by the gap proteins *hunchback* and *Krüppel* in cultured *Drosophila* cells. Genes Dev. **5:**254–264.