Structure–function analysis of the TBP-binding protein Dr_1 reveals a mechanism for repression of class II gene transcription

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Dr_1, a repressor of class II genes, regulates transcription by a novel mechanism. Biochemical analyses reveal that Dr_1 directly interacts with the multiprotein TFIID complex. By use of the yeast two-hybrid system, we demonstrate that the association of Dr_1 with the TATA-binding protein (TBP) subunit of TFIID occurs in vivo. In addition, Dr_1 can repress transcription from TATA-containing as well as TATA-less promoters in transient transfection assays. Importantly, Dr_1-mediated repression can be reversed by overexpression of TBP in vivo. By use of diverse approaches, we mapped two distinct domains in Dr_1 required for repression. One domain is essential for the Dr_1–TBP interaction, and the second is rich in alanine residues. The TBP-binding domain of Dr_1 cannot be replaced by a heterologous DNA-binding domain in mediating repression. We demonstrate that some, but not all, transcriptional activators can reverse Dr_1-mediated repression in vivo.

[*Key Words*: Transcription; Dr_1; TBP; two-hybrid system; repression]

Received April 27, 1994; revised version accepted July 11, 1994.

Transcription initiation from class II genes is a multistep process that requires the ordered assembly of the general transcription factors (GTFs) along with RNA polymerase II (RNAPII) on class II promoters (Weis and Reinberg 1992; Drapkin et al. 1993). The first step involves the binding of general transcriptional factor TFIID to the TATA element present in many class II promoters (Buratowski et al. 1989). This is followed by the sequential assembly of TFIIA, TFIIB, TFIIF–RNAPII, TFIIE, TFIIH, and TFIIJ to form a transcriptionally competent complex (Zawel and Reinberg 1993). The binding of TFIID to the TATA element is thought to be a site for regulation (Abmayr et al. 1988; Lewin 1990; Ptashne and Gann 1990; Liberman and Berk 1991; Roeder 1991). TFIID is a multiprotein complex, composed of the TATA-box-binding protein (TBP) and a number of tightly associated polypeptides called TBP-associated factors (TAFs). TAFs are important for activation of transcription yet are apparently dispensable for basal transcription (Dynlacht et al. 1991; Tanese et al. 1991; Pugh and Tjian 1992). Most of the TAFs have been cloned and characterized (Dynlacht et al. 1993; Goodrich et al. 1993; Hisatake et al. 1993; Hoey et al. 1993; Kokubo et al. 1993, 1994; Ruppert et al. 1993; Weinzierl et al. 1993b; Yokomori et al. 1993). *Drosophila* TAF110 ($dTAF_{II}110$) and $dTAF_{II}40$ have been shown to interact with the activation domains of Sp1 and VP16, respectively (Goodrich et al. 1993; Hoey et al. 1993). The interaction of activators with TAFs is suspected to enhance one or more of the steps toward preinitiation complex formation (Choy and Green 1993; Gill and Tjian 1993).

Proteins other than TAFs are known to interact with TBP and/or TFIID to regulate promoter activity. These include the negative regulators Dr_1, NC1, NC2, and Dr_2 and positive cofactors involved in enhancing activation of transcription, such as ACF, PC1, and PC2 (Meisterernst and Roeder 1991; Meisterernst et al. 1991; Inostroza et al. 1992; Merino et al. 1993). Dr_1 and Dr_2 have been purified to homogeneity and their cDNAs isolated. Dr_2 was identified as human DNA topoisomerase I. Dr_2 interacts directly with TBP and mediates repression of basal transcription (Merino et al. 1993). Dr_2-mediated repression of basal transcription can be overcomed by activators (Merino et al. 1993). On the other hand, Dr_1 can repress both basal and activated transcription in an in vitro-reconstituted transcription assay (Inostroza et al. 1992). We demonstrated previously that Dr_1 represses transcription by precluding the association of TFIIA and/or TFIIB with the TFIID(TBP)–DNA complex (Inostroza et al. 1992). Therefore, it appears that Dr_1 is a general repressor of transcription and differs from other previously described specific repressors such as Id, IκB, Krüppel (Kr), even-skipped (eve), and engrailed (en) (Baeuerle

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and Baltimore 1988; Benezra et al. 1990; Licht et al. 1990; Han and Manley 1993).

Analysis of the primary and acid sequence of the Dr_1 clone revealed a number interesting motifs. These include glutamine- and alanine-rich regions that share some sequence homology with several DNA-binding repressors described in *Drosophila*, including Kr, eve, and en (Licht et al. 1990; Han and Manley 1993). The amino terminus of Dr_1 also possesses homology to the yeast HAP3 protein (Hahn et al. 1988). To determine the importance of the various motifs and to identify functional regions in Dr_1 required for repression of transcription, we performed an extensive mutational analysis. We mapped the TBP-binding domain of Dr_1 to a 20-amino-acid region and identified an additional region in Dr_1 that is required for repression. Overexpression of Dr_1 in yeast and mammalian cells allowed us to analyze functionally the interaction between Dr_1 and TBP/TFIID. The mechanism through which Dr_1 represses transcription in vivo was scrutinized by study of the effect of different classes of activator on Dr_1-mediated repression. The results of these studies define further the molecular mechanism of Dr_1-mediated repression.

Results

The association of Dr_1 with TBP is mediated by a 20-amino-acid amphipathic helical region contained within Dr_1

We have shown previously that Dr_1 interacts with TBP in gel mobility-shift assays and in Far-Western analysis (Inostroza et al. 1992). To scrutinize further the specificity of this interaction, we analyzed whether Dr_1 could interact with TFIIB and RAP30 (the small subunit of TFIIF), as well as the activation domain of the viral transactivator VP16. Interactions were analyzed by use of glutathione *S*-transferase (GST) fusion proteins (GST–TFIIB, GST–RAP30, GST–VP16, and GST–TBP as a control). The proteins were purified to apparent homogeneity (Fig. 1A), and equal amounts of each fusion protein were immobilized on glutathione–agarose columns. The ability of different columns to retain recombinant Dr_1 (rDr_1) was monitored by Western blot analysis with anti-Dr_1 antibodies. As shown in Figure 1A, the interaction of Dr_1 with TBP is highly specific as only the GST–TBP column retained Dr_1.

To delineate region(s) of Dr_1 that interact with TBP, amino- and carboxy-terminal truncation deletions of Dr_1 were constructed (Fig. 1B). Mutant proteins were expressed as GST fusions in bacteria, purified to apparent homogeneity (Fig. 1B, middle), and immobilized on glutathione–agarose columns. The ability of Dr_1 columns to retain human TBP (hTBP) was monitored by Western blot with anti-hTBP antibodies (Fig. 1B). Analyses with amino-terminal truncations indicated that the first 78 amino acids of Dr_1 were dispensable for the interaction with hTBP (Fig. 1B, lane 7). However, further removal of 22 amino acids from the amino terminus (mutant Δ1–100) completely abolished its ability to interact with hTBP (Fig. 1B, lane 8). Amino acids were also deleted from the carboxyl end of Dr_1. GST–Dr_1 mutants Δ166–176 and Δ151–176 retained a considerable amount of hTBP (Fig. 1B). In contrast, GST–Dr_1 mutants Δ130–176 and Δ101–176 did not bind detectable amounts of hTBP (Fig. 1B). In summary, analyses with terminally truncated Dr_1 mutants indicated that amino acids between residues 78 and 151 were required to interact with hTBP (see summary in Fig 1B). To delineate further the residues in this region necessary for the interaction with hTBP, small in-frame internal deletions were constructed (Fig. 1C). Consistent with the results derived from the truncated Dr_1 mutants, in-frame internal deletions between residues 1 and 69 had no effect on Dr_1 binding to hTBP (data not shown). However, analysis of in-frame internal deletions between residues 69 and 157 revealed that two regions appear important for the hTBP–Dr_1 interaction (see summary in Fig. 1C). The first region is located between amino acid 85 and 99; the other is from amino acid 113 to 140. To determine whether both regions of Dr_1 are required for the interaction with hTBP, small peptides from both regions were constructed as GST fusion proteins (Fig. 2A). A Coomassie blue stain of the purified GST–Dr_1 peptides is shown in Figure 2B. The ability of the GST–Dr_1 peptides to interact with hTBP was assayed as in Figure 1B. As expected, hTBP bound to the GST–Dr_1(79–150) column (Fig. 2B, lane 3). Interestingly, GST–Dr_1(79–111), but not the GST–Dr_1(101–150) column, retained hTBP (Fig. 2B lanes 4,5). This finding was surprising as in-frame internal deletions between amino acids 113 and 140 of GST–Dr_1 abrogated the Dr_1-hTBP interaction (Fig. 1C, lanes 6,7). Thus, the results obtained with the GST–Dr_1 peptides seemed inconsistent with results obtained with the deletion mutants. We shall address this apparent discrepancy below. Nevertheless, the analyses with the GST–Dr_1 peptides indicated that amino acids 79–111 of Dr_1 are sufficient to mediate its association with TBP. It is of interest that this 30-amino-acid stretch has a putative α-helical structure with hydrophobic and hydrophilic residues displayed on different sides of the helix (Fig. 2C).

In vivo interaction of Dr_1 and hTBP in yeast

To corroborate the in vitro biochemical studies on the hTBP–Dr_1 interaction, we expressed Dr_1 and hTBP in *Saccharomyces cerevisiae* and studied their interaction by use of the two-hybrid system (Fields and Song 1989; Ruppert et al. 1993; Staudinger et al. 1993). Dr_1 was expressed as a fusion protein with the DNA-binding domain of the bacterial repressor LexA. This domain enables Dr_1 to enter the nucleus and bind to DNA containing LexA-binding sites. hTBP, the target of Dr_1, was expressed as a fusion protein with the acidic activation domain (AAD) of Gal4. The interaction between the two fusion proteins brings the AAD of Gal4 into proximity of the promoter and activates transcription of the reporter gene, in this case *lacZ*, bearing LexA-binding sites. Yeast expressing both LexA–Dr_1 and hTBP–AAD, but not ei-

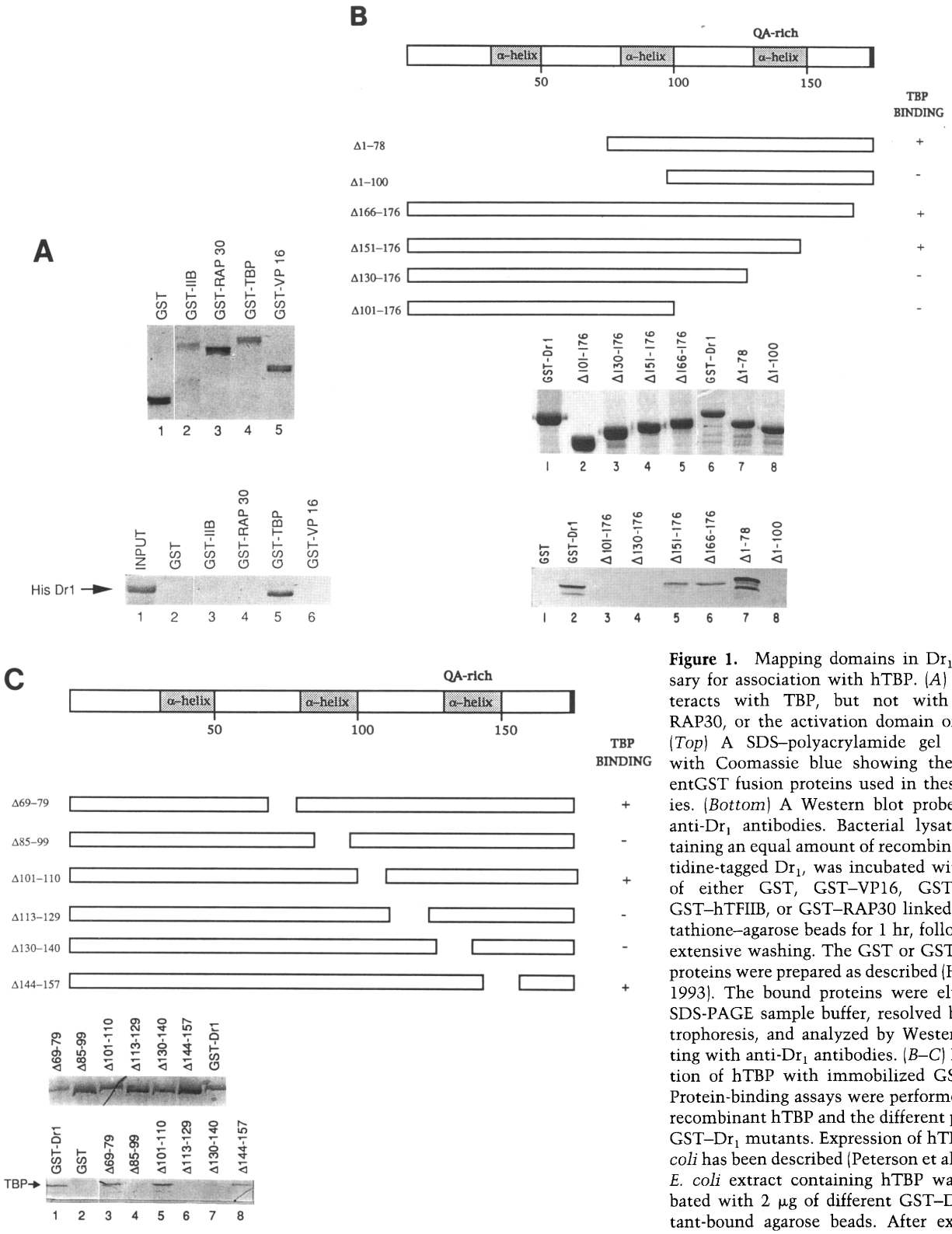

Figure 1. Mapping domains in Dr_1 necessary for association with hTBP. (*A*) Dr_1 interacts with TBP, but not with TFIIB, RAP30, or the activation domain of VP16. (*Top*) A SDS–polyacrylamide gel stained with Coomassie blue showing the differentGST fusion proteins used in these studies. (*Bottom*) A Western blot probed with anti-Dr_1 antibodies. Bacterial lysate, containing an equal amount of recombinant histidine-tagged Dr_1, was incubated with 2 μg of either GST, GST–VP16, GST–hTBP, GST–hTFIIB, or GST–RAP30 linked to glutathione–agarose beads for 1 hr, followed by extensive washing. The GST or GST fusion proteins were prepared as described (Ha et al. 1993). The bound proteins were eluted in SDS-PAGE sample buffer, resolved by electrophoresis, and analyzed by Western blotting with anti-Dr_1 antibodies. (*B–C*) Interaction of hTBP with immobilized GST–Dr_1. Protein-binding assays were performed with recombinant hTBP and the different purified GST–Dr_1 mutants. Expression of hTBP in *E. coli* has been described (Peterson et al. 1990). *E. coli* extract containing hTBP was incubated with 2 μg of different GST–Dr_1 mutant-bound agarose beads. After extensive washing, the bound proteins were loaded on SDS-PAGE and transferred to nitrocellulose. The blots were analyzed with anti-hTBP antibody. (*B*) Amino and carboxy terminal deletions of Dr_1. (*Top*) A schematic representation of the different Dr_1 truncated proteins; (*middle*) the different GST–Dr_1 proteins stained with Coomassie blue; (*bottom*) a Western blot displaying the amount of hTBP bound to the different GST–Dr_1 columns. (*C*) Internal deletions of Dr_1. (*Top*) Schematic representation of the different Dr_1 internal deletions; (*middle*) the different GST–Dr_1 proteins stained with Coomassie blue; (*bottom*) Western blot showing amounts of hTBP bound to the different GST–Dr_1 columns.

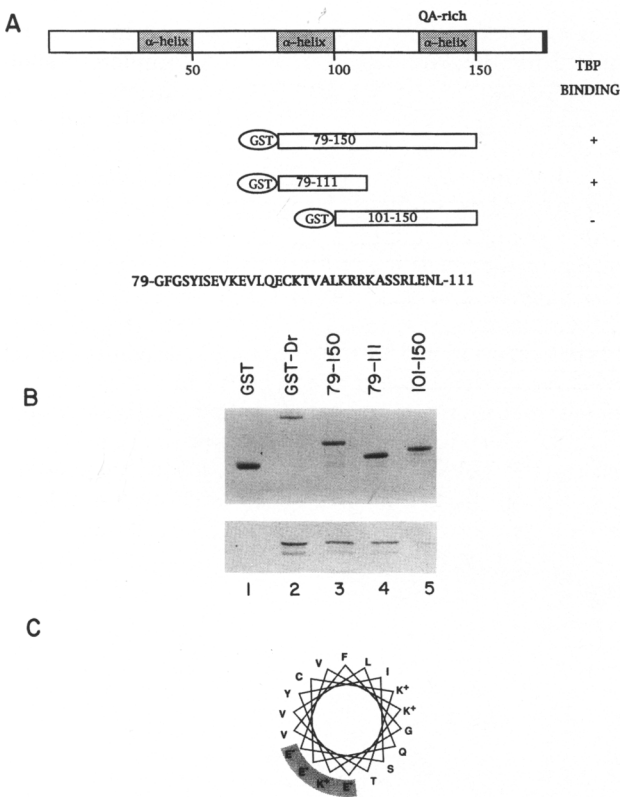

Figure 2. Interaction of hTBP with immobilized GST–Dr_1 peptides. (*A*) Schematic representation of the different GST–Dr_1 peptides. The amino acid sequence (in single-letter code) of residues 79–111 of Dr_1 is shown (*bottom*). (*B*) (*Top*) The different GST–Dr_1 peptides stained with Coomassie blue. The ability of the different GST–Dr_1 peptides to associate with hTBP was analyzed by use of the same binding assay described in Fig. 1. hTBP bound to the different GST columns was eluted in SDS-PAGE sample buffer, resolved by electrophoresis, and analyzed by Western blotting with anti-hTBP antibodies (*bottom*). (*C*) Helical wheel depiction of Dr_1 residues required for TBP binding.

ther fusion alone, produced high levels of β-galactosidase activity (see legend to Fig. 3). The interaction is specific, as deletion of the last 180 amino acids of hTBP abrogated the β-galactosidase activity (data not shown). To analyze regions of Dr_1 required for interacting with hTBP in vivo in yeast, deletions were introduced into the LexA–Dr_1 yeast expression vector. Consistent with the in vitro biochemical studies, deletion of amino acids 85–99 completely abolished the interaction with hTBP (Fig. 3A), whereas there was only a 15% reduction in β-galactosidase activity when amino acids 15–33 were deleted (Fig. 3A). The inability of LexA–Dr_1 Δ85–99 to interact with hTBP was not attributable to the instability of the protein, as Western blots of yeast whole-cell extract expressing LexA–Dr_1, or its mutated derivative, demonstrated that these proteins were expressed at comparable levels (Fig. 3B). In agreement with the in vitro biochemical studies, deletion of amino acids 113–140 of LexA–Dr_1 also diminished the Dr_1–TBP interaction in vivo. The β-galactosidase activities of mutants Δ113–129 and Δ130–140 were reduced by ~60% and ~90%, respectively (Fig. 3A). We have inferred that the effect of these two deletions was indirect and resulted from perturbations of the overall structure of Dr_1 (see below). The yeast in vivo analysis was in good agreement with our in vitro studies demonstrating that Dr_1 can associate with

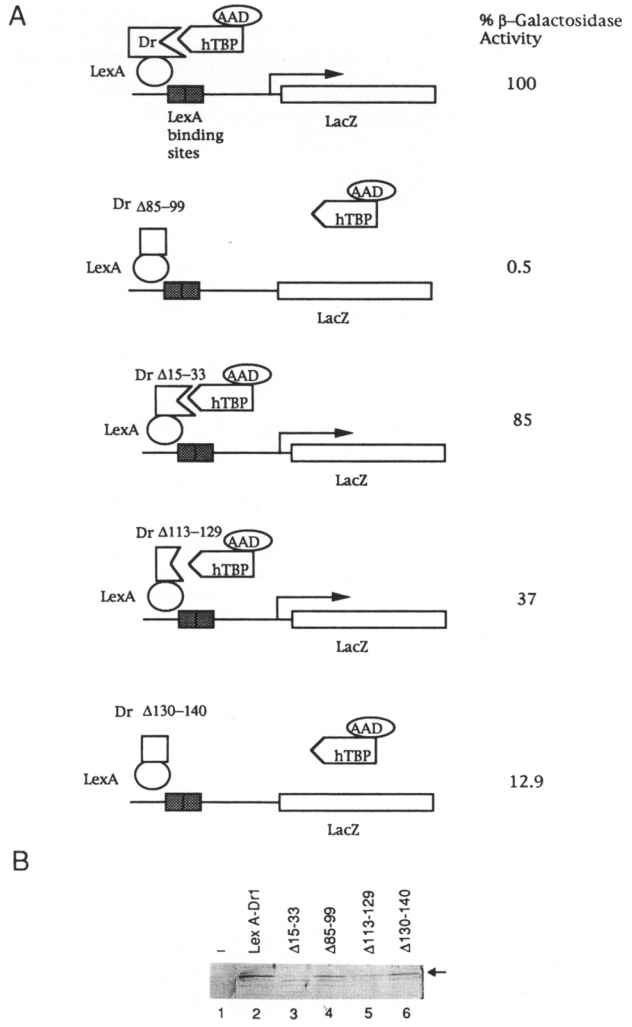

Figure 3. In vivo interaction of Dr_1 and hTBP in yeast. (*A*) Dr_1 domain required for the interaction with hTBP. Full-length Dr_1 or its derivatives were expressed as fusion proteins with the DNA-binding domain of LexA. hTBP was expressed as a fusion protein with the AAD of Gal4. Expression vectors were transformed either alone, or in combinations, into a yeast strain bearing a *lacZ* reporter with two copies of LexA-binding sites. The β-galactosidase activity obtained with either hTBP–ADD (10 U/mg) or LexA–Dr_1 (23 U/mg) alone is negligible when compared with activity from yeast transformed with both (1651 U/mg). β-Galactosidase activity was expressed as percent activity. (*B*) Protein extracts from yeast cells containing LexA–Dr_1 or its mutants were analyzed by Western blot with anti-Dr_1 antibodies.

TBP and that amino acids 85–99 of Dr_1 are critical to this interaction.

Two regions of Dr_1 are required for the inhibition of transcription of the adenovirus major late promoter in vitro

To define further the mechanism of Dr_1-mediated repression, the effect of different in-frame internal deletion mutants was assayed in transcription in a system reconstituted with purified GTFs, RNAPII, and the adenovirus major late (AdML) promoter. Mutant proteins (Fig. 4A), were expressed in *Escherichia coli* as histidine fusions and purified by nickel-affinity chromatography (Fig. 4B). The result of one of the representative transcription assays is shown in Figure 4C. Deletion of amino acids from residue 2 to 79 of Dr_1 had no effect on the ability of Dr_1 to repress transcription (lanes 5–10 and data not shown). However, removal of amino acids 85–99, which are required for the interaction with TBP, completely abolished the activity of Dr_1 (lanes 11–13). Interestingly, a second domain in Dr_1, between residues 144 and 157, was also found to be required for repression of transcription in vitro (lanes 17–19). This domain is rich in alanine residues. Although deletion of residues 113–140 of Dr_1 diminished the binding to TBP in vitro and in vivo, deletion of these residues in yeast was without effect on the repressing activity of Dr_1 (Fig. 4C,D; see below). Taken together, it is possible that deletion of amino acids between 113 and 140 have changed the conformation of Dr_1 such that the region needed for TBP interaction is not accessible. However, in the context of a transcription complex, Dr_1 undergoes conformational change(s), exposing the TBP-binding domain. We favor this possibility because amino acids 113–140 are also dispensable for repression of transcription in vivo in transfected HeLa

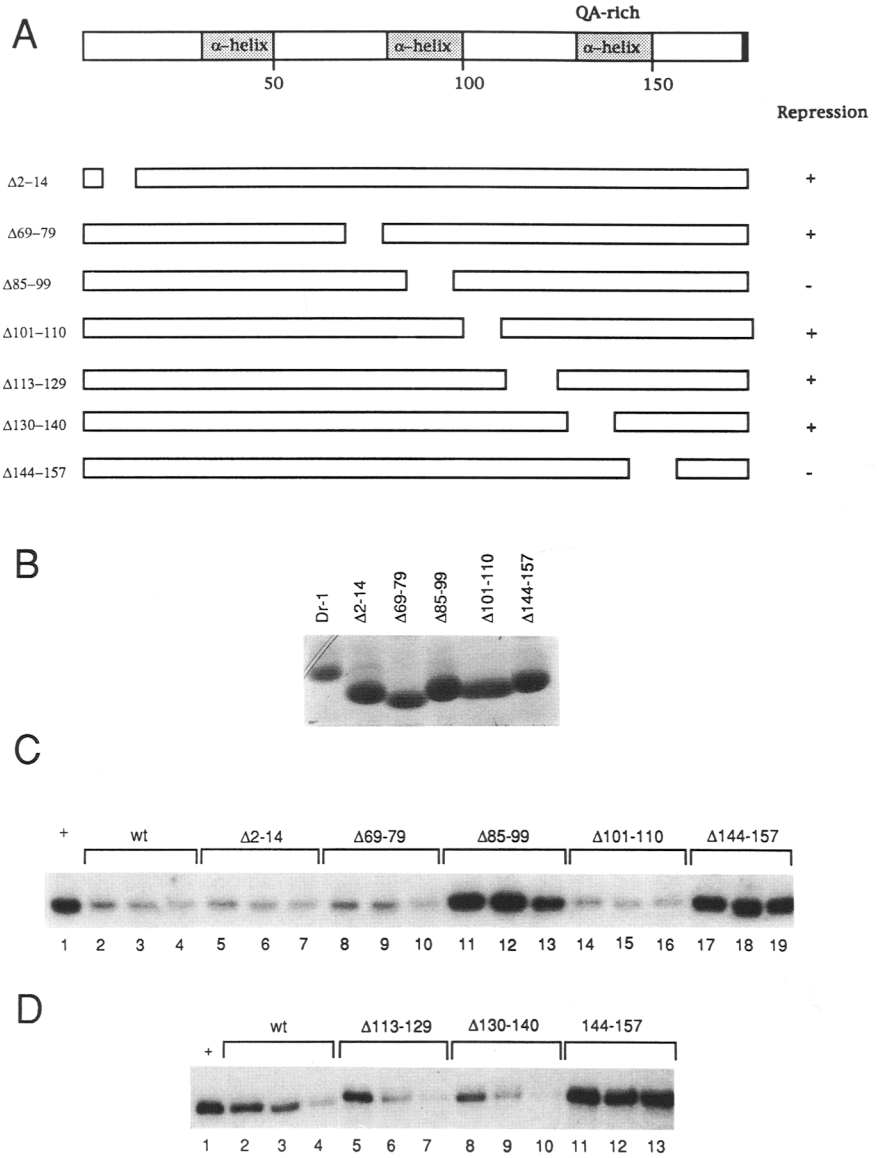

Figure 4. Dr_1 domains required for repression of transcription of the AdML promoter in vitro. (*A*) Schematic representation of the different Dr_1 mutant proteins. (*Right*) Symbols denote the relative activity of the different Dr_1 proteins. (*B*) Coomassie blue staining of an SDS–polyacrylamide gel containing the different Dr_1 mutant proteins. The proteins were expressed in *E. coli* with histidine tags at the amino-terminal end and purified by nickel-affinity chromatography (Hoffmann and Roeder 1991). (*C,D*) Autoradiographs of transcription assay. Transcription reaction mixtures containing the AdML promoter were reconstituted with purified general transcription factors, RNAPII and 10, 20, and 40 ng of Dr_1 or its mutants. Factors added were as follows: yTBP [5 ng, S-Sepharose (Maldonado et al. 1990)], rTFIIB [15 ng, phosphocellulose fraction (Ha et al. 1991)], RNAPII [22 ng, DEAE-5PW (Lu et al. 1991)], rTFIIE [30 ng, Sephacryl 200 (Peterson et al. 1991)], TFIIF [35 ng, TSK–phenyl superose (Flores et al. 1992)], TFIIH [42 ng, hydroxylapatite (Flores et al. 1992)], TFIIA [120 ng, hydroxylapatite (Flores et al. 1992)], TFIIJ [0.27 μg, phenyl superose (Flores et al. 1992)].

cells (see below). These observations thus reconciled the earlier conflicting results with the GST–Dr_1 mutants and the GST–Dr_1 peptide (cf. Fig. 1C, lanes 6 and 7 with Fig. 2C, lane 5). In summary, our in vitro findings indicate that two separable domains of Dr_1 are required for repression of the AdML promoter (see summary in Fig. 4A). One of the domains (amino acids 79–100) is required for interaction with TBP and the other region (amino acids 144–157), which is rich in alanine residues, most likely represents a repressing domain.

Association of Dr_1 with hTBP is required for the repression of class II gene expression in HeLa cells

In our previous studies we demonstrated that Dr_1 interacted with TBP and that this interaction results in repression of class II transcription in vitro. To substantiate the biological relevance of these studies, we studied the effect of Dr_1 on class II gene expression in vivo. Dr_1 was expressed transiently under the cytomegalovirus (CMV) immediate early promoter in HeLa cells in the presence of either TATA-containing or TATA-less promoters driving a luciferase reporter. The effect of Dr_1 on expression of the AdML, SV40 early, human heat shock (Hsp70), and the TATA-less human β-DNA polymerase promoters is shown in Figure 5A. The luciferase activity declined precipitously from all four promoters with increasing concentrations of Dr_1 expression vector. To analyze the specificity of the Dr_1 inhibition observed in vivo, Dr_1 expression vectors carrying different deletions were analyzed. Of particular interest were mutations in the residues mediating interaction with TBP (residues 85–99) and the alanine-rich motif (residues 144–157) found to be necessary for repression in vitro. Removal of the TBP-binding domain eliminated the ability of Dr_1 to repress transcription from the SV40 promoter (Fig. 5B) and other promoters (data not shown). The failure of Δ85–99 to repress transcription was not attributable to instability or sequestration of the mutated protein in the cytosol because mutated protein is detected in the nuclei in an amount comparable to that of the wild type (Fig. 5B, inset). No effect was observed when several other regions of Dr_1 were deleted (Fig. 5B, mutants Δ130–140, Δ51–68). When the requirement for the alanine-rich motif was analyzed, we found, consistent with the in vitro observations, that this motif was required to sustain repression of the AdML (Fig. 5C) and the human Hsp70 promoters (data not shown). However, this domain was dispensable for repression of transcription of the SV40 early promoter (Fig. 5C). This result is perhaps related to studies demonstrating that the TATA motif of the SV40 early promoter is unique in that it is not able to mediate activation by the $E1A_{12S}$ protein (Simon et al. 1988) and by some other enhancers (Wefald et al. 1990).

The inhibition of transcription by Dr_1 in vitro can be overcome by increasing the concentration of TBP in the reconstituted transcription system (Inostroza et al. 1992). This previous finding is in agreement with the studies described above demonstrating that the interaction between Dr_1 and TBP is a requirement for inhibition. These observations prompted us to analyze whether overexpression of hTBP in vivo could overcome Dr_1-mediated repression. As anticipated, transfection of a Dr_1 expression vector resulted in repression of transcription from the SV40 early promoter as demonstrated by a 75% reduction of luciferase activity (Fig. 5D, cf. lanes 1 and 2). Repression was partially alleviated when an hTBP expression vector was cotransfected (Fig. 5D, lanes 3–5). At the highest concentration of transiently expressed hTBP, the inhibition of transcription was overcome by >60% (Fig. 5D, cf. lanes 1 and 5) and approached levels similar to those observed when hTBP was overexpressed in the absence of Dr_1 (Fig. 5D, cf. lane 5 with 6–9). The effect of TBP on Dr_1 repression appears to be specific because the same amount of transiently expressed hTFIIB (lanes 10–14) or hTFIIA (lanes 15–19) had no effect. The coexpression of both hTBP and hTFIIB slightly stimulated antirepression (lanes 20–23).

It is known that TBP exists in vivo in a large multisubunit protein complex known as TFIID (Pugh and Tjian 1991; Zhou et al. 1992). Our in vivo transfection experiments, therefore, prompted us to investigate whether Dr_1 could associate with TFIID. This was analyzed by use of immunoprecipitations with anti-Dr_1 antibodies and a partially purified TFIID fraction. As shown in Figure 5E, TBP was coimmunoprecipitated by anti-Dr_1 antibodies as detected by Western blot with anti-hTBP antibodies (Fig. 5E, lane 5). Because the above result does not rule out the possibility that the observed interaction was with free TBP present in the TFIID fraction, we reinvestigated the interaction with antibodies raised against $hTAF_{II}150$, a subunit of TFIID (Weinzierl et al. 1993). Affinity-purified TFIID [eTFIID, (Zhou et al. 1992)] was mixed with Dr_1 and proteins immunoprecipitated with anti-TAF or anti-hTBP antibodies. These antibodies immunoprecipitated Dr_1 as detected in a Western blot with anti-Dr_1 antibodies (Fig. 5F, lanes 1,2). The immunoprecipitation of Dr_1 by anti-$hTAF_{II}150$ and anti-hTBP (data not shown) antibodies was dependent on TFIID (Fig. 5F, lane 3). These results, together with the transfection experiments (Fig. 5D), demonstrate that Dr_1 can associate with the TFIID complex.

Dr_1-mediated repression is position dependent

To investgate further the role of TBP in Dr_1-mediated repression, we analyzed whether recruitment of Dr_1 to the promoter must occur via an interaction with TBP or whether the TBP-binding domain of Dr_1 could be replaced by a heterologous DNA-binding domain. Full-length Dr_1 or amino acids 124–168 (Dr_1–QA) of Dr_1, which contained the alanine-rich domain apparently important for repression, were fused in-frame to the DNA-binding domain of Gal4. GAL4–Dr_1 and GAL4–Dr_1–QA were expressed transiently under the SV40 early promoter in HeLa cells in the presence of a luciferase reporter directed by the AdML promoter with five copies of the Gal4 DNA-binding site upstream of the TATA element (5× Gal4–AdMLP). Surprisingly, neither GAL4–

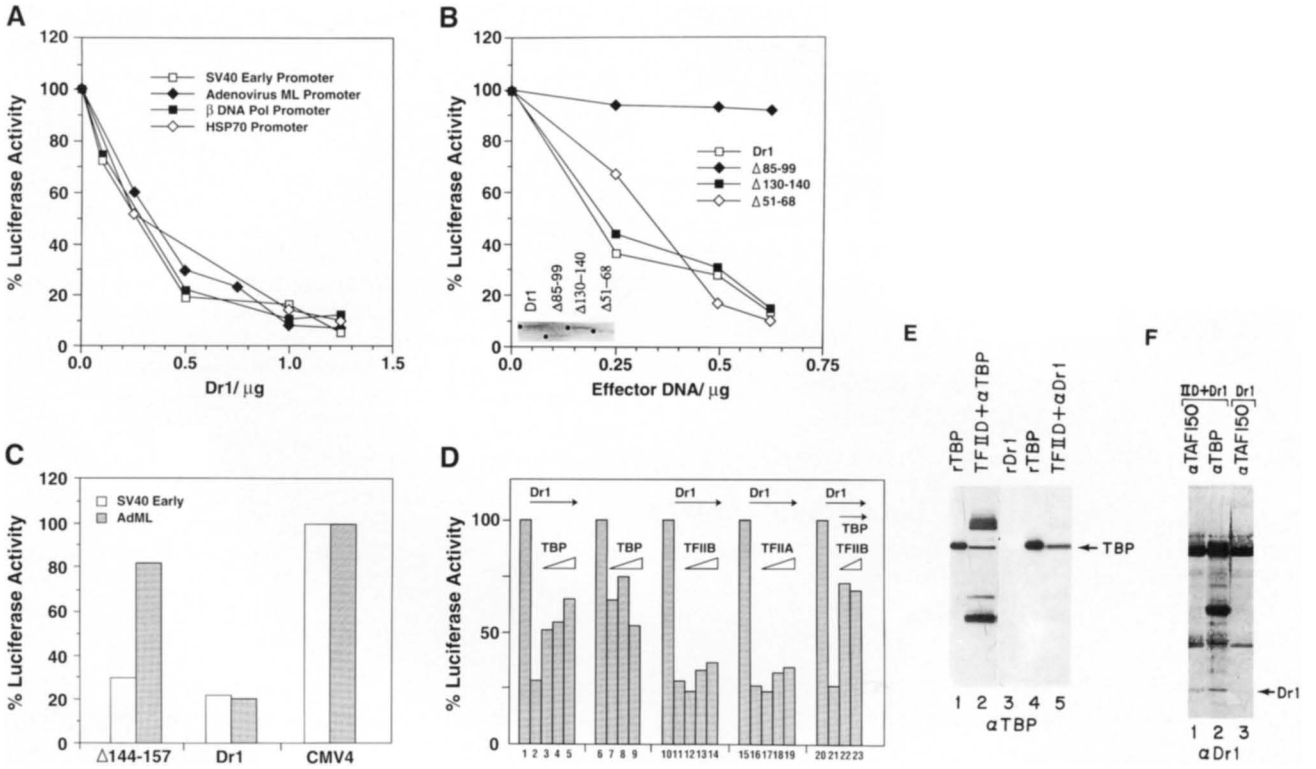

Figure 5. Repression of class II genes expression in vivo by Dr_1. (*A*) Dr_1 represses transcription from both TATA-containing and TATA-less promoters. Full-length Dr_1 was expressed under the CMV major immediate early promoter. Different amounts of the Dr_1 expression plasmid were cotransfected into HeLa cells with either 1 μg of SV40 early, 2 μg of each of AdML, or heat shock (Hsp-70), or β-DNA polymerase promoters driving luciferase reporter genes. Forty-eight hours after transfections, cells were harvested, and cell extracts were assayed for luciferase activity. The CMV expression vector alone had no repressive effect on the promoters used in the study. (*B*) The TBP-binding domain of Dr_1 is required for repression of the SV40 early promoter. Full-length Dr_1 or its mutated derivatives were expressed under the CMV major immediate early promoter. Different amounts of Dr_1 expression plasmids, together with SV40 promoter-driven luciferase reporter, were transfected into HeLa cells by lipofectamine. The effects of different deletions on repression are presented as percent luciferase activity. (*Inset*) A Western analysis of nuclear extracts prepared from HeLa cells transfected with HA epitope-tagged Dr_1 mutants with mAb 12CA5 (Field et al. 1988). (*C*) The requirement of alanine-rich region (residues 144–157) of Dr_1 for repression of SV40 and AdML promoters. HeLa cells were transfected with 650 ng of different effector DNAs with either 1 μg of SV40 or 2 μg of AdML promoters as described above. Forty-eight hours after transfection, cells were harvested and the cell extracts were assayed for luciferase activities. The effects of deletion of residues 144–157 on repression are presented as percent luciferase activity. (*D*) Dr_1-mediated repression of SV40 early promoter is reversed by hTBP. HeLa cells were cotransfected with 2 μg of SV40 promoter and 0.3 μg of Dr_1 DNA in the absence (lanes *2,11,16,21*) or presence of increasing amounts (10, 20 and 40 ng) of either CMV–hTBP (lanes *3–5*), CMV–TFIIB (lanes *12–14*), or CMV–TFIIA (lanes *17–19*) expression vectors. In lanes *22* and *23*, cells were transfected with 0.3 μg of Dr_1, and 40 ng of TBP together with increasing amounts of TFIIB (10 and 20 ng) expression vectors, as indicated. As a control, cells were also transfected with the SV40 promoter with increasing amounts (10, 20, and 40 ng) of a TBP expression vector (lanes *7–9*). Forty-eight hours after transfections, cells were harvested and the cell extracts were assayed for luciferase activities. Luciferase activity of extract from cells transfected with the SV40 promoter alone was arbitrarily set as 100% (lanes *1,6,11,16,20*). (*E*) Immunoprecipitation reactions of TFIID with hTBP monoclonal antibodies, or anti-Dr_1 antibodies. TBP was visualized by use of anti-hTBP antibodies in a Western analysis from immunoprecipitation reactions of a TFIID fraction [S-Sepharose (Maldonado et al. 1990)] with TBP-monoclonal antibodies (lane *2*), or anti-Dr_1 antibodies (lane *5*). (Lanes *1,4,3*) Bacterially expressed hTBP and Dr_1, respectively. The arrow denotes TBP. (*F*) Western blot analysis of the Dr_1 protein coimmunoprecipitated from either a purified TFIID fraction mixed with purified native Dr_1 by use of TAF150 (lane *1*) or TBP (lane *2*) monoclonal antibodies or purified native Dr_1 with TAF150 monoclonal antibody. Dr_1 was visualized with anti-Dr_1 antibodies.

Dr_1 nor GAL4–Dr_1–QA were capable of repressing the expression of the reporter promoter (Fig. 6). As expected, when not fused to the DNA domain of Gal4, Dr_1 repressed the promoter >70% (Fig. 6). To determine whether the inability of GAL4–Dr_1 to repress transcription is attributable to the presence of the Gal4 DNA-binding moiety, we studied the effect of GAL4–Dr_1 on an AdML promoter without the Gal4 DNA-binding sites. GAL4–Dr_1 repressed the AdML promoter >80%, a level similar to that with unfused Dr_1 (Fig. 6, inset). Taken together, these results indicate that Dr_1 is a general repressor of class II promoters and that binding to TBP is a prerequisite for Dr_1-mediated repression. Furthermore, the TBP-binding domain of Dr_1 cannot be replaced by a

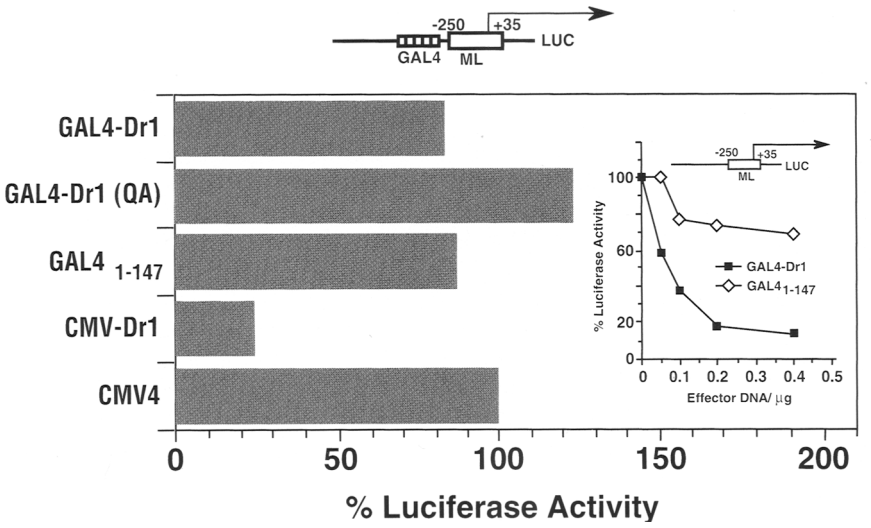

Figure 6. TBP-binding domain of Dr_1 cannot be replaced by a heterologous DNA-binding domain. Full-length or amino acids 124–168 (QA) of Dr_1 were fused in-frame to the first 147 amino acids of Gal4 and expressed under the SV40 early promoter. Each construct (0.5 µg), GAL4–Dr_1, GAL4–Dr_1–QA, $GAL4_{1-147}$, CMV–Dr_1, or CMV4, was cotransfected into HeLa cells with 2 µg of 5× Gal4–ML–luciferase reporter DNA. Forty-eight hours after transfections, cells were harvested and the cell extracts were assayed for luciferase activities. Luciferase activity of extract from cells transfected with CMV4 alone was arbitrarily set as 100%. (*Inset*) Repression of transcription of the AdML promoter by GAL4–Dr_1. Different amounts of the GAL4–Dr_1 or $GAL4_{1-147}$ expression plasmids were cotransfected into HeLa cells with 2 µg of the AdML promoter-driven luciferase reporter gene. Forty-eight hr after transfections, cells were harvested, and cell extracts were assayed for luciferase activities.

heterologous DNA-binding domain, and the presence of Dr_1 at the promoter is not sufficient for repression.

Transcriptional activators can overcome Dr_1-mediated repression in vivo

In light of the above observations, we analyzed whether activators known to interact with TBP could displace Dr_1 from the TFIID complex or induce a conformational change within the complex that would overcome Dr_1-mediated repression. Four activators were examined: a cysteine zinc finger-containing activator ($E1A_{13S}$), an acidic activator (VP16), a glutamine-rich activator (Sp1), and a proline-rich activator (CTF). To simplify the analysis, the activation domains of VP16, Sp1, and CTF were fused to the DNA-binding domain of the yeast transcriptional activator, Gal4, and these constructs were cotransfected with an AdML promoter containing five Gal4 DNA-binding sites driving expression of the luciferase gene. For the cysteine zinc finger-containing activator, full-length $E1A_{13S}$ and its natural variant $E1A_{12S}$ were analyzed. As expected, Dr_1 repressed transcription from the AdML promoter by >80% (Fig. 7A, cf. lanes 1 and 2, 6 and 7, 11 and 12). Cotransfection of increasing amounts of either $E1A_{13S}$ or GAL4–VP16 completely restored promotor activity (Fig. 7A, lanes 8–10, 13–15). The effect is specific as the Gal4 DNA-binding domain alone (data not shown), or the $E1A_{12S}$, in which the activation domain is missing because of alternative splicing, were not able to overcome repression (Fig. 7A, lanes 1–4). Contrary to the effect of $E1A_{13S}$ and GAL4–VP16, transfection of GAL4–Sp1 could only restore ~50% of the promoter activity and GAL4–CTF had no effect on Dr_1-mediated repression (Fig. 7B). The failure of GAL4–Sp1 to efficiently overcome repression is not because of failure of the chimeric protein to activate the AdML promoter, as GAL4–Sp1 activated the promoter to a similar extent as $E1A_{13S}$ (Fig. 7C, cf. lanes 1–4 with 5–8). Although, consistent with previous studies, GAL4–CTF is a weaker activator, it activated the AdML promoter sixfold at its highest concentration (Fig. 7C, lane 12). Taken together, these results indicate that the Dr_1-mediated repression can be overcome by a defined class of activators.

Discussion

The studies described here extend our analysis of Dr_1, a repressor of transcription. We established that Dr_1 functions in vivo and that repression of transcription is mediated in part by a direct interaction with TBP.

By use of diverse approaches, including coimmunoprecipitation, the yeast two-hybrid system, and transient transfection techniques, we have demonstrated a functional and direct interaction between Dr_1 and TBP/TFIID. We extended this observation and showed that it is a functional interaction both in vitro and in vivo. Importantly, overexpression of Dr_1 in cells containing a class II promoter driving luciferase, revealed that Dr_1 represses TATA-containing viral (SV40 and adenovirus) and cellular promoters (heat shock), as well as a TATA-less (β-DNA polymerase) promoter. Consistent with our previous in vitro studies, Dr_1-mediated repression in vivo could be overcome by increasing the intracellular concentration of hTBP. Conversely, no effect on Dr_1-mediated repression was observed when the intracellular concentration of TFIIB or the two largest subunits of TFIIA were overexpressed. The interaction between Dr_1 and TBP appears to be highly specific as no interaction between Dr_1 and TFIIB, RAP30, and the activation domain of VP16 could be demonstrated under the same assay conditions. Thus, our results demonstrate that the association of Dr_1 with TFIID (TBP) is biologically rele-

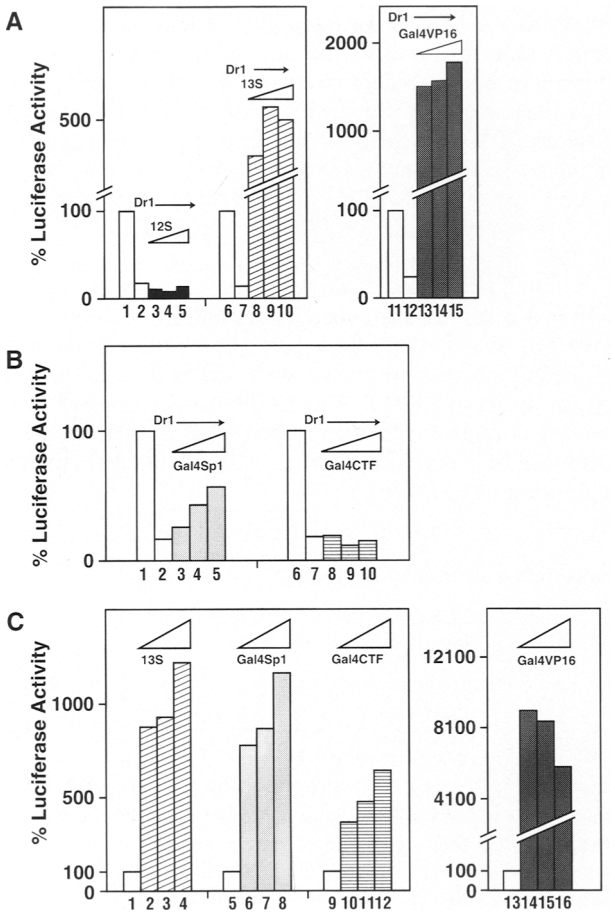

Figure 7. The effects of different activators on Dr_1-mediated repression of AdML promoter. (*A*) Dr_1-mediated repression of AdML promoter is reversed by both $E1A_{13S}$ and GAL4–VP16. HeLa cells were cotransfected with 1.5 μg of 5× Gal–ML (lanes *11–15*) or ML (lanes *1–10*) luciferase reporters and 0.5 μg of Dr_1 DNA without (lanes *2,7,12*) or with increasing amount (0.1, 0.2, and 0.4 μg) of either CMV–$E1A_{12S}$ (lanes *3–4*), CMV–$E1A_{13S}$ (lanes *8–10*), or GAL4–VP16 (lanes *13–15*) expression vector DNA. Forty-eight hours after transfection, cells were harvested and the cell extracts assayed for luciferase activity. Luciferase activity of extract from cells transfected with promoters alone was arbitrarily set as 100% (lanes *1,6,11*). (*B*) Effects of GAL–Sp1 and GAL–CTF on Dr_1-mediated repression of the AdML promoter. HeLa cells were cotransfected with 1.5 μg of 5× Gal–ML luciferase reporters and 0.5 μg of Dr_1 DNA without (lanes *2,7*) or with increasing amount (0.1, 0.2, and 0.4 μg) of either CMV–GAL4–Sp1 (lanes *3–5*) or CMV–GAL4–CTF (lanes *8–10*) expression vector DNAs. Forty-eight hours after transfection, cells were harvested and the cell extracts were assayed for luciferase activity. Luciferase activity of extract from cells transfected with promoter alone was arbitrarily set as 100% (lanes *1,6*). (*C*) Activation of AdML or 5× Gal–ML promoters by different classes of activators. HeLa cells were cotransfected with 1.5 μg of 5× Gal–ML (lanes *5–16*) or ML (lanes *1–4*) luciferase reporters and an increasing amount (0.1, 0.2, and 0.4 μg) of either CMV–$E1A_{13S}$, GAL4–VP16, CMV–GAL4–Sp1, or CMV–GAL4–CTF expression vector DNAs. Luciferase activity of extract from cells transfected with promoter alone was arbitrarily set as 100% (lanes *1,5,9,13*).The effects of different activators on AdML promoters are presented by percent luciferase activity.

vant and that repression of class II genes by Dr_1 is dependent on this interaction.

The mutational analysis of Dr_1 revealed a 20-amino-acid region (80–100) that is sufficient for its association with hTBP. A column containing this peptide as a GST fusion protein retained hTBP. Computer analysis indicated that this region could form an α-helical amphipathic structure. In accord with the in vitro biochemical studies, this same region is required for LexA–Dr_1 to associate with hTBP in yeast.

Our studies also demonstrate that Dr_1 repression of class II promoters can be reversed in vivo by activators such as $E1A_{13S}$ and VP16. This finding may be related to the fact that these two activators are known to interact directly with hTBP (Stringer et al. 1990; Horikoshi et al. 1991; Lee et al. 1991), and thus they may displace Dr_1 from the transcription complex. Interestingly, we found that Sp1 was inefficient at overcoming Dr_1-mediated repression and that CTF is completely unable to overcome repression. It is possible that the graded effect observed with the different activators is related to the strength of the activator or that the activators contact different components of the TFIID complex. Recent studies have suggested that Sp1 (Emili et al. 1994) and CTF (J. Greenblatt, unpubl.) interact directly with TBP. It is possible that the TBP residues that mediate the interaction with CTF and Sp1 are different from those that accommodate interactions with $E1A_{13S}$ and VP16. The effect of activators on inhibitor-mediated repression has also been addressed by Ptashne and co-workers. They have isolated two chimeric repressors (from random sequences from *E. coli* DNA) that respond differently to activators. These investigators concluded that there is a direct correlation between the strength of an activator and its ability to counteract repression (Saha et al. 1993). It is important, however, to indicate that in our studies, both $E1A_{13S}$- and Sp1-activated transcription of the AdML promoter to approximately the same extent, yet $E1A_{13S}$, but not Sp1, could efficiently overcome repression by Dr_1. Thus, our results suggest that contact by the activator with TFIID, as well as the strength of the activator, is important to antirepress the effects of Dr_1.

There are several possible mechanisms by which a repressor can repress initiation of transcription from class II promoters (for review, see Renkawitz 1990; Drapkin et al. 1993). Repressors can prevent an activator from binding to its cognate DNA site (Benezra et al. 1990; Zabel and Baeuerle 1990) or can block an activating domain (Ma and Ptashne 1988). Direct repressors such as Kr, eve, en, Wilms' tumor gene (WT1) product (K.C. Yeung et al., unpubl.), E1B-55K, p53, Ssn6–Tup1, and MOT1 (ADI, Auble and Hahn 1993; Davis et al. 1992) repress by acting directly on the general transcription factors (Licht et al. 1990; Keleher et al. 1992; Auble and Hahn 1993; Han and Manley 1993; Yew et al. 1994). Typically, a direct repressor is composed of two functionally distinct domains: a DNA-tethering domain that anchors the protein at the promoter, and a repression domain. Dr_1 appears to function as a direct transcriptional repressor in a manner similar, yet distinct from other direct repressors.

By use of an in vitro transcription assay, we identified two distinct domains in Dr_1 that are required for repression. The first domain is the TBP-binding domain (amino acids 80–100) as defined by in vitro biochemical assay, as well as by the yeast two-hybrid system. The second domain maps to amino acids 144–157. Inspection of the primary sequence shows a preponderance of alanine residues. Results from a recent genetic screen for repressor domains in yeast, together with studies on *Drosophila* repressors eve, en, and Kr, have defined a repression domain lacking serine, threonine, and acidic residues (Han and Manley 1993; Saha et al. 1993). Thus, the second domain of Dr_1 possesses features of a typical repressor domain. Furthermore, the repression domain of the *Drosophila* transcriptional repressor en is also rich in alanine residues, and, importantly, the alanine residues were shown to be critical for their function. Alanine is also one of the predominant residues in the transcriptional repressor eve. The mechanism of how the alanine-rich domain functions in repression remains unknown, but these residues most likely interfere with preinitiation complex formation. To date, Dr_1 does not exhibit DNA-binding activity. Our results indicate that Dr_1 is tethered to the promoter by interacting with TBP via the TBP-binding domain. Like Dr_1, neither MOT1, E1B-55K, nor Ssn6–Tup1 have a DNA-binding domain. Each of these repressors is recruited to promoters by different DNA-bound proteins (Keleher et al. 1992; Auble and Hahn 1993; Yew et al. 1994). However, replacement of the TBP-binding domain with a heterologous DNA-binding domain (Gal4) did not restore the repression activity of Dr_1. Thus, unlike other repressors, such as WT1, Kr, eve, en, and E1B–55K, the mere presence of Dr_1 at the promoter is not sufficient to repress transcription. Recruitment of GAL4–Dr_1 either close to (-10) (data not shown) or far away (-250) from the TATA element did not result in repression of transcription. Our data therefore suggest that direct interaction with TBP is required for the Dr_1-mediated repression, a requirement that has not yet been established for other direct repressors.

As expected, transient transfection experiments showed that the TBP-binding domain of Dr_1 is absolutely required for its repressing activity in HeLa cells. However, the alanine-rich domain of Dr_1 is dispensable for repression of the SV40 early promoter mutant. Dr_1 carrying an internal deletion of amino acids 144–157 repressed the SV40 early promoter almost to the same extent as the wild type, even though the same region is required for the repression of both AdML and Hsp70 promoters. Despite the fact that only one human TBP has been isolated and cloned, there have been reports of the existence of functionally distinct TATA elements (Struhl 1986; Simon et al. 1988). It has been reported that replacement of the Hsp70 TATAA element with the SV40 TATTTAT sequences abolished the response of Hsp70 promoter to $E1A_{12S}$ activation (Simon et al. 1988). Like the Hsp70 promoter, the AdML promoter also possesses a TATAA element at -30. It is thus possible that the requirement of the alanine-rich domain for repression of a TATA-containing promotor may depend on the type of TATA element present in the promoter. Perhaps, the SV40 TATA is recognized by a functionally distinct TFIID complex with components that obviate the requirement of the Dr_1 alanine-rich domain for repression. This idea is supported by the studies of Williams and co-workers (1990), who found that the muscle-specific enhancer of the human myoglobin gene was functional when placed upstream of a promoter containing TATAAA (myoglobin TATA), but not TATTTAT (SV40 TATA).

In conclusion, our data represent the first evidence demonstrating that the direct interaction of a repressor with TBP is necessary for repression of transcription in vivo. The interaction of Dr_1 with TBP serves more than to simply recruit Dr_1 to the promoter because the TBP-binding domain of Dr_1 cannot be replaced with the Gal4 DNA-binding domain. This is a novel mechanism for repression of transcription.

Materials and methods

Construction of Dr_1 amino- and carboxy- terminal and in-frame internal deletion mutants

For amino-terminal deletion mutants, the following oligonucleotides were designed as amino-terminal primers for PCR: 5′-dGGATCCGTGGCCAACGATGCTCGA-3′ (Δ1–30); 5′-dGCGGATCCGGATTTGGCTCTTACATC-3′ (Δ1–70); 5′-dGCGGATCCAGAAGAAAGGCCAGTTCT-3′ (Δ1–100) (the underlined nucleotides indicate a *Bam*HI site). The above primers were used together with M13 reverse primer to amplify DNA fragments from the Dr_1 cDNA cloned in pBluescript II SK vector (SK–Dr_1) (Stratagene, Inc). After restriction digestion with *Bam*HI and *Xba*I, the fragments were inserted into a pGEX-2T vector (Pharmacia). For the carboxy-terminal deletion mutants, the following oligonucleotides were designed as carboxy-terminal primers for PCR: 5′-dTATATATCTAGATCATTTTAATGCTACTGTTTT-3′ (Δ101–176); 5′-dTATATATCTAGATCATCTAGCTTTTGCAAATAA-3′ (Δ130–176); 5′dTATATATCTAGATCATTGTTGGGCAGCTTGCTG-3′ (Δ151–176); 5′-dTATATATCTAGATCATCCCGCCTGATTAGATGC-3′ (Δ166–176) (the underlined nucleotides indicate a *Xba*I recognition site). Oligonucleotide 5′-dCCGGATCCACCATGGCTTCCTCGTCTGGC-3′ (the underlined nucleotides indicate a *Bam*HI site) was used as the amino-terminal primer. After PCR and restriction enzyme digestion with *Bam*HI and *Xba*I, the fragments were inserted into a pGEX-2T vector (Pharmacia). In-frame internal deletion mutants were constructed by PCR as described before with SK-Dr_1 as the DNA template (Yeung et al. 1993). The oligonucleotides used for the mutation were as follows: 5′-dCGCCGGATCCACCATGAGAGCTGCTATCAATAAAATG-3′ (Δ2–14); 5′-dCTCGAGCATCGTTGGGGATAGTGAGATC-3′ (Δ15-32); 5′-dCGGAAAAGAAGACCATCTCACCATTTGGCTCTTACATC-3′ (Δ69–79); 5′-dTTTGGCTCTTACATCAAAAGAAGAAAGGCC-3′ (Δ85–99); 5′-dACAGTAGCATTAAAACTTGGCATTCCTGAA-3′ (Δ101–110); 5′-dTTGTTGCTGTCTAGCGCCAAGGTTTTCCAA-3′ (Δ113–129); 5′-dCTGCATTTGAAGCCAAGCTTTTGCAAATAA-3′ (Δ130–140); 5′-dCTGATTAGATGCACTGGCTTGAAGCCATTCCTG-3′ (Δ144–157). The PCR products were digested with restriction enzymes *Bam*HI and *Xba*I and inserted into pGEX-2T (Pharmacia) or QE9 (Qiagen) vectors. All mutations were verified by DNA sequencing. GST–Dr_1 (79–150), (79–111), and (101–150) were constructed by PCR

with the following oligonucleotides:GST–Dr_1 (79–150), 5′-dCGCCGGATCCACCATGGGATTTGGCTCTTACATCAGT-3′, and 5′-dTATATATCTAGATCATTGTTGGGCAGCTTGCTG-3′; GST–Dr_1 (79–111), 5′-dCGCCGGATCCACCATGGGATTTGGCTCTTACATCAGT-3′, and 5′-dTATATATCTAGATCATTTTAATGCTACTGTTTT-3′; GST–Dr_1 (101–150), 5′-dCGCCGGATCCACCATGAGAAGAAAGGCCAGTTCTCGTTTG-3′, and 5′-dTATATATCTAGATCATTGTTGGGCAGCTTGCTG-3′. The PCR products were digested with restriction enzymes *Bam*HI and *Xba*I and inserted into a pGEX-2T (Pharmacia).

Yeast strains and expression vectors

Experiments with the two-hybrid system were carried in yeast strains EGY40 (*MAT***a**, *trp1*, *ura3*, *his3*, *leu2*) (a gift from R. Brent). The reporter is pJK103 (a gift from R. Brent, Massachusetts General Hospital, Boston), which has a *GAL–lacZ* gene with two high-affinity ColE1 LexA operators. pJK103 carries the Ura^+ marker and a 2 μ replicator. Construction of the LexA–Dr_1 fusion vectors was accomplished by cloning the *Bam*HI–*Nco*I Dr_1 cDNA fragment into the polylinker region of yeast expression vector pEG202-b, which is identical to pEG202 (a gift from R. Brent) except it contains a polylinker of a different reading frame. pEG202-b carries the His^+ marker and a 2 μ replicator. pGAD–hTBP, which carries a $Leu2^+$ marker and a 2 μ replicator, was a gift of R. Tjian (University of California, Berkeley).

Yeast transformation and β-galactosidase assay

Cells were grown in appropriate selective minimal medium and transformed by the method of Becker and Guarente (1991) with modification. A total of 5 μg of DNA together with 200 μg of herring sperm DNA was used to transform 100 μl of competent yeast cells. Before the heat shock, DMSO was added to a final concentration of 10%. Quantitation of β-galactosidase activity produced in transformed cells was performed by the method of Bartel et al. 1993). Yeast cell extracts were prepared from 10 ml of overnight culture. The cell pellets were resuspended in 0.5 ml of breaking buffer (0.1 M Tris at pH 8.0, 20% glycerol, 1 mM DTT, 2 mM PMSF). Acid-washed glass beads were then added and vortexed four times for 15 sec each. The cell debris was subsequently spun down and supernatants stored at −70°C. For the β-galactosidase assay, 0.1 ml of the extract was used.

Plasmid constructs for transfection

GAL4–Dr_1 was constructed by cloning the cDNA of Dr_1 into the mammalian expression vector pSG424 (Sadowski and Ptashne 1989). GAL4–Dr_1–QA was constructed by PCR with oligonucleotides 5′-CCGCTAGCGAATTATTTGCAAAAGCTAGA-3′ and 5′-CCGGATCCTCACTGAGAAGATCCCGCC-3′. The PCR product was cloned into pSG424. Hsp70–LUC was generated by insertion of a 1270-bp *Hin*dIII–*Sal*I DNA fragment from Hsp70–CAT, between the *Sac*I and *Xho*I sites of a luciferase reporter, GL2–basic (Promega). Enh–β-pol–LUC contains −20 to +60 of the β-polymerase promoter upstream of the luciferase reporter, GL2–Enh (Promega). SV40–LUC (GL2–control) was purchased from Promega. 5× GalML–LUC and ML–LUC were a gift of Dr. T. Hai, Ohio State University (Columbus). Dr_1 expression vectors, CMV–Dr_1 or its mutated derivatives were generated by insertion of the HA epitope-tagged Dr_1 cDNA between the *Kpn*I and *Xba*I sites of a CMV expression vector CMV4 (gift of M. Stinski, University of Iowa, Iowa City). CMV–TFIIA was constructed by insertion of the cDNA for the largest subunit (TFIIAα/β) of human TFIIA into the polylinker region of CMV5 (gift of M. Stinski). Expression vectors CMV–TBP and CMV–TFIIB have been described previously (Xu et al. 1993). Expression vectors GAL4–SP1, GAL4–CTF, GAL4–VP16, CMV_{12S}, and CMV_{13S} were a gift from M. Green (University of Massachusetts, Worcester) and J. Nevins (Duke University, Durham, NC).

Transfection and luciferase assay

HeLa cells were grown in Dulbecco's minimal essential medium supplemented with 10% defined calf serum. Cells were plated on 35-mm dishes and transfected at 70–80% confluence with lipofectamine according to manufacturer specifications (BRL). A total of 2.5–3.25 μg of DNA and 2 μg of lipofectamine was used per plate. Each transfection was performed no less than three times. Cells were harvested 48 hr after transfection, and total cell extracts were assayed for luciferase with a kit purchased from Promega.

Immunoprecipitation reactions and Western blot analysis

Preparation of polyclonal antibodies against Dr_1 has been described (Inostroza et al. 1992). TBP monoclonal antibodies and antibodies against TAF150 were a gift of N. Hernandez (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY) and R. Tjian, respectively. In all immunoprecipitation reactions ~1 μg of antibody was used. Antibodies against Dr_1, TAF150, and TBP were incubated with protein A–Sepharose (Repligen) for 30 min at 23°C followed by the addition purified fractions and then incubated for an additional 2 hr at 4°C with mixing. Immunoprecipitates were washed with ice-cold lysis buffer containing 50 mM Tris-HCl buffer (pH 7.5), 50 mM NaF, 100 μM $NaVO_3$, 150 mM NaCl, 1 mM PMSF, and 1% NP-40 and one time with PBS. Samples were then eluted from the protein A–resin antibody complexes with 200 mM glycine–HCl (pH 2.6). Samples were then boiled for 5 min in SDS-PAGE sample buffer, resolved by 13% SDS-PAGE, and transferred to nitrocellulose membrane. The blot was blocked with 5% BSA in TBS, 0.05% Tween 20 for 2 hr and incubated with either anti-Dr_1, monoclonal TBP or TAF150 antibodies for 2 hr at room temperature, followed by secondary antibodies conjugated to either horseradish peroxidase (HRP) or alkaline phosphatase for 1 hr. HRP conjugates were visualized by use of the ECL system (Amersham), and alkaline phosphatase-coupled material was visualized with NBT and BCIP. The amount and localization of the various Dr_1 mutants in transient transfection experiments were studied by Western blot of nuclear extract prepared from HeLa cells transfected with the mutant DNA. The nuclear extract was prepared from a 100-mm plate of HeLa cells transfected with 6 μg of DNA as described by Han and Manley (1993).

Protein-binding assays using GST fusion proteins

The GST fusion proteins were expressed, and columns were prepared as described (Ha et al. 1993; Ma et al. 1993). Approximately 200 μl of glutathione–agarose beads containing the fusion protein was incubated at 4°C in 0.5 ml of buffer containing 20 mM Tris-HCl buffer (pH 7.5), 0.1 M NaCl, 0.2 mM EDTA, 1 mM DTT, 1 mM PMSF, and 0.2% NP-40. Equal amounts of bacterial lysate containing either rhTBP or rDr_1 was added, and binding was allowed to proceed for 1 hr. The bacterial hTBP expression vector has been described (Peterson et al. 1990). The beads were washed four times with the same buffer containing 0.4% NP-40, and the bound proteins were eluted with 30 μl of SDS-PAGE loading buffer and resolved by electrophoresis. Bacterially produced proteins were visualized by Western blotting.

Other methods

Transcription factors were purified as described by Flores et al. (1991). RNAPII was purified as described by Lu et al. (1991). Transcription assays were performed as described by Maldonado et al. (1990). His-tagged Dr_1 protein was purified as described by Hoffmann and Roeder (1991).

Acknowledgments

We thank R. Brent, M. Green, T. Hai, J. Nevins, M. Stinski, and R. Tjian for plasmids, Dr. R. Sternglanz for helpful advices on the two-hybrid system, and Dr. A. Berk for the hemagglutinin-tagged TBP cell line. We also thank N. Hernandez and R. Tjian for the monoclonal antibodies against hTBP and $hTAF_{II}150$, respectively. We are grateful to R. Tjian, R. Drapkin, and L. Zawel for reading the manuscript. This work was supported by grants from the National Institutes of Health (GM48518), and in part by grants from the National Science Foundation and the American Cancer Society. D.R. is a recipient of an American Cancer Society Faculty Research Award.

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Genes Dev. 1994, **8:**
Access the most recent version at doi:10.1101/gad.8.17.2097