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Functional Dissection of a Human Dr1-DRAP1 Repressor Complex

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The heterotetrameric Dr1-DRAP1 transcriptional repressor complex was functionally dissected. Dr1 was found to contain two domains required for repression of transcription. The tethering domain interacts with the TATA box binding protein and directs the repressor complex to the promoter. This tethering domain can be replaced by a domain conferring sequence-specific recognition to the repressor complex. In the absence of the tethering domain, Dr1 interacts with its corepressor DRAP1, but this interaction is not functional. The enhancement of Dr1-mediated repression of transcription by DRAP1 requires the tethering domain. The second domain of Dr1 is the repression domain, which is glutamine-alanine rich. A 65-amino-acid polypeptide containing the repression domain fused to the Gal4 DNA binding domain repressed transcription when directed to TATA-containing and TATA-less promoters. This repression domain was also found to functionally and directly interact with the TATA box binding protein.

Accurate initiation of transcription from eukaryotic protein-encoding genes requires the assembly of a large multiprotein complex consisting of approximately 42 polypeptides onto promoter DNA. To date, most of these polypeptides have been characterized and include the general transcription factors (GTFs) TFIIA, TFIIB, TFIID, TFIIE, TFIIF, and TFIIH, as well as the subunits of RNA polymerase II (RNAPII) (reviewed in references 40, 68, and 71). Of the six GTFs, TFIID, which has an affinity for the TATA elements, is the only one presently known to exhibit sequence-specific DNA binding activity (46, 62). Although TFIID exists as a large multisubunit complex (15, 51), its DNA binding activity is intrinsic to a single polypeptide of 38 kDa named the TATA box binding protein, or TBP (20, 26a, 49). The remaining components of the TFIID complex are referred to as TBP-associated factors (TAFs) (13, 60, 72). TAFs are important for activation of transcription yet are apparently dispensable for basal transcription of TATA-containing class II promoters (13, 60, 72). Recently, it has also been shown that TAFs can serve as basal promoter selectivity factors by making contact with sequences overlapping the transcription start site as well as downstream regions of certain promoters (reviewed in reference 64a).

The binding of TFIID to the promoter DNA is facilitated by TFIIA (7, 39), which is thought to play an important role in transcription activation as well as in antirepression (35, 37, 48, 59, 67). Once bound to the TATA motif, TFIID serves as a scaffold for the entry of TFIIB and subsequent loading of the remaining GTFs and RNAPII (7, 39). The complete DBPolFEH complex is then competent to initiate RNA synthesis upon addition of ribonucleoside triphosphates (reviewed in reference 70). Recent studies have suggested an alternative pathway for preinitiation complex formation involving the recruitment of a preassembled RNAII holoenzyme to the promoter (8, 28, 41, 47).

The initiation of transcription from class II genes is subject to multiple levels of regulation (1, 32, 34, 50, 53). The characterization and cloning of the various genes encoding the general transcription factors have allowed the identification of regulatory proteins that directly interact with the GTFs. One such class of regulators modulates the rate of transcription initiation by interacting directly with components of the basal transcription machinery (70). These include negative regulators such as Dr1, MOT1, p53, and Dr2, (2a, 25, 42–44, 57); positive cofactors-activators including ACF, PC1, PC2, PC4, and HMG2 (14, 30, 42–44, 58); viral activators such as E1A, Zta, and VP16; and others (reviewed in reference 18). The molecular mechanism whereby such regulators affect transcription initiation has recently been described for one of the negative regulators, Dr1. It was found that Dr1 represses RNAPII transcription by precluding the entry of TFIIA and TFIIB into the preinitiation complex. This prevents the formation of an active transcription complex (25). Biochemical fractionation and coimmunoprecipitation with Dr1 antibodies have recently shown that native Dr1 can exist in a heterotetrameric complex with a novel 28-kDa protein called DRAP1 (Dr1-associated protein 1). The Dr1-DRAP1 repressor complex is also called NC2 (15a). In that nomenclature, Dr1 is referred to as NC2β and DRAP1 is referred to as NC2α. cDNA clones encoding DRAP1 were isolated, and it was found that the recombinant DRAP1 polypeptide enhances the repressing ability of Dr1 (45). Structural-functional studies have indicated that Dr1 has at least three different domains, a TBP binding domain, a glutamine-alanine (QA)-rich region, and a region with homology to the histone fold motif (45, 66). Previous studies have demonstrated that both the TBP binding and QA-rich regions are essential for Dr1-mediated repression of transcription in vitro and in vivo (66). Subsequent studies with recombinant DRAP1 revealed that the histone fold motif of Dr1 was required for the interaction with DRAP1 and also essential for the DRAP1-mediated enhancement of Dr1-mediated repression of transcription (45).

In this study, we analyzed the function of each component of the Dr1-DRAP1 complex in mediating repression of class II transcription. The effects of the individual subunits of the repression complex on transcription initiation when tethered to the promoter were examined by construction of chimeric fu-

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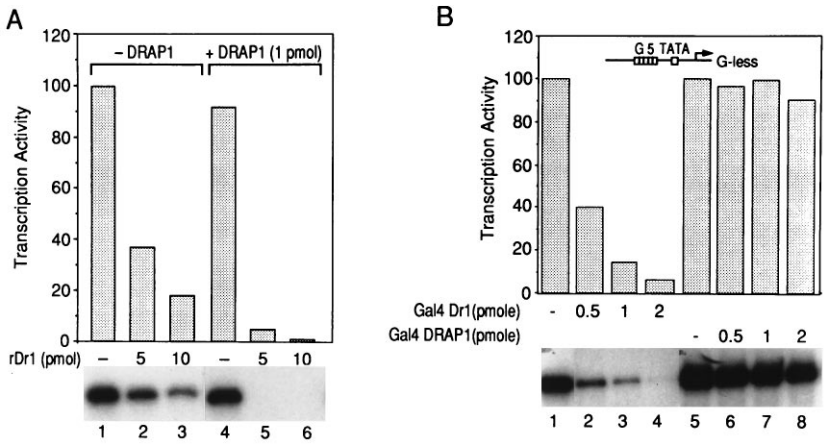

FIG. 1. Dr1 represses transcription by two mechanisms. (A) DRAP1-independent Dr1-mediated repression of transcription. (Top) Bar graph representing transcription activity of transcription reaction mixtures containing the Ad-MLP reconstituted with purified or recombinant GTFs, RNAPII, and different amounts of rDr1 as indicated (see Materials and Methods for details). The assay was done in the absence (lanes 1 to 3) or in the presence (lanes 4 to 6) of rDRAP1. Transcription activity was quantified on a phosphorimager (Bio-Rad). (Bottom) Autoradiogram of RNA transcripts from transcription reactions quantified in the bar graph above. The transcription activity of lane 1 is arbitrarily set as 100. (B) Dr1 contains a repressor domain. (Top) Bar graph representing transcription activity of reaction mixtures containing the Ad-MLP with Gal4 binding sites reconstituted with purified or recombinant general transcription factors, RNAPII, and different amounts of Gal4-Dr1 or Gal4-DRAP1 as indicated. (Bottom) Autoradiogram of RNA transcripts from transcription reactions quantified in the bar graph above.

sion proteins between the DNA binding domain of Gal4 ($Gal4_{1–94}$) and Dr1 or DRAP1. We show that the TBP binding region of Dr1 is analogous to a DNA tethering domain that anchors the protein at the promoter. We also demonstrate that the QA-rich region possesses features of a repressor domain when directed to the promoter. In addition, we identify the functional target of the QA-rich region.

MATERIALS AND METHODS

Construction of GST-DRAP1, Gal4-DRAP1, Gal4-Dr1, Gal4-Dr1$_{\Delta 85–99}$, Gal4-Dr1$_{\Delta 144–157}$, and Gal4-QA. Gal4-QA was constructed with the following pair of oligonucleotides: 5′-CGCCGGATCCACCATGCTTGGCATTCCTGAAGAA GAG-3′ and 5′-TATATATCTAGATCATCCCGCCTGATTAGATGC-3′. These primers were used to amplify a Dr1 cDNA fragment encoding a peptide of 65 amino acids (amino acids 101 to 165 of Dr1). The PCR product was digested with *Xba*I and *Bam*HI and cloned in frame with histidine-tagged $Gal4_{1–94}$ in the plasmid vector pRJR1 (a gift of Mark Ptashne). Gal4-Dr1 and mutated derivatives were generated by inserting the Dr1 cDNA between the *Bam*HI-*Xba*I sites of pRJR1. Gal4-DRAP1 and glutathione *S*-transferase (GST)-DRAP1 were constructed by ligating the DRAP1 cDNA between the *Bam*HI-*Xba*I sites of pRJR1 or GEX-2T (Pharmacia), respectively. All DNA constructs were verified by DNA sequencing.

Expression of Gal4 proteins in *Escherichia coli*. *E. coli* BL21(DE3) (Novagen) containing different Gal4 constructs was grown in Luria-Bertani medium supplemented with ampicillin (100 μg/ml) at 37°C. Cells were grown until the optical density at 600 nm reached 0.6 and then induced with 1 mM isopropylthiogalactoside (IPTG). After 3 h, the cells were pelleted, and the histidine fusion proteins were purified as described by Hoffmann and Roeder (19).

Protein binding assays using GST fusion proteins, Gal4-Dr1, Gal4-Dr1$_{\Delta 85–99}$, and Gal4-Dr1$_{\Delta 144–157}$. GST fusion proteins were expressed in *E. coli* cells and purified as previously described (66). Approximately 40 μl of glutathione-agarose beads containing 1 μg of either GST or GST-DRAP1 was incubated at 4°C in 0.5 ml of buffer containing 20 mM Tris-HCl (pH 7.5), 0.1 M NaCl, 0.2 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 0.2% Nonidet P-40 (NP-40). Purified recombinant proteins (0.2 μg), Gal4-Dr1, or its mutated derivatives, were added, and binding was allowed to proceed for 1 h. The beads were washed four times with the same buffer as described above, except 0.4% NP-40 was added. The bound proteins were eluted with 30 μl of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer and resolved by electrophoresis. The bound protein fractions as well as 1/10 of the input proteins were analyzed by Western blotting with $Gal4_{1–147}$ polyclonal antibodies (Upstate Biotechnology).

Coimmunoprecipitation and Western blot analysis. Antibodies were incubated with protein A-Sepharose (Repligen) in lysis buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 1% NP-40) for 30 min at 23°C. $Gal4_{1–94}$ or different Gal4 fusion proteins and immunopurified hemagglutinin (HA)-tagged TFIID or *E. coli* whole-cell extract containing HA-tagged human (h)TBP were added and incubated for an additional 2 h at 4°C with mixing. Immunoprecipitates were washed three times with ice-cold lysis buffer. Samples were then eluted from the protein A resin antibody complexes using 200 mM glycine–HCl, pH 2.6. The samples were then boiled for 5 min in SDS-PAGE sample buffer, resolved by SDS–13% PAGE, and transferred to a nitrocellulose membrane. The blot was blocked with 0.3% gelatin in Tris-buffered saline–0.05% Tween 20 for 15 min and incubated with either monoclonal anti-TBP antibody or anti-Gal4 antibodies for 1 h at room temperature. After extensive washing, the immunoblots were incubated with secondary antibodies conjugated to alkaline phosphatase for 45 min. Alkaline phosphatase-coupled material was visualized with nitroblue tetrazolium and BCIP (5-bromo-4-chloro-3-indolylphosphate toluidinium).

In vitro transcription assays. Transcription assays were performed as previously described (66). Each transcription assay was performed at least three times with similar results. Transcription factors and RNAPII were purified as described previously (24). The transcription factors used in the assays were recombinant TFIIB (rTFIIB) (10 ng), hTBP (5 ng), or epitope-tagged TFIID (eTFIID) (10 ng of TBP determined by quantitative Western blotting), rTFIIE (15 ng), rTFIIF (23 ng), HeLa cell-purified TFIIH (phenyl-Superose column fractions, 26 ng), and anti-CTD affinity-purified RNAPII (50 ng). Reaction mixtures were incubated at 30°C for 1 h. The RNA products were separated by electrophoresis on denaturing gels. Quantitation was performed with a phosphorimager (Bio-Rad). The promoter construct, dihydrofolate reductase (DHFR) G-less cassette, has been described elsewhere (2), and the construction of the G5-HSP G-less cassette will be described elsewhere.

RESULTS

Two modes of transcription repression by Dr1. Our previous studies have demonstrated that Dr1 interacts with DRAP1 and that this heterotetrameric complex represses transcription more efficiently than Dr1 alone (45). We have previously shown that Dr1, in the absence of DRAP1, associates with the TBP-TATA complex and prevents the entry of TFIIB into the preinitiation complex (25). The Dr1-DRAP1 heterotetramer also associates with the TBP-TATA complex, but in this case there is an apparent change in the conformation of the DNA-protein complex (DRAP1-Dr1-TBP-TATA) which precludes the association of TFIIA and TFIIB with the TBP-TATA complex (25, 45). The disparate modes of interaction between Dr1 and the TBP-TATA complex have profound effects on the ability of Dr1 to repress transcription. As exemplified in Fig. 1A, Dr1 in the absence of DRAP1 represses transcription. However, under these conditions a 15-fold molar excess of Dr1 to that of TBP was necessary to reach approximately 60% inhibition of transcription. In the presence of DRAP1, the same amount of Dr1 completely inhibited transcription.

DRAP1 has no effect on transcription in the absence of Dr1 (45) (data not shown) (see below).

To analyze the function of each subunit of the Dr1-DRAP1 heterotetrameric complex, we constructed Gal4-DRAP1 and Gal4-Dr1 chimeric proteins. The fusion proteins were expressed with histidine tags in bacteria and purified by nickel affinity chromatography to apparent homogeneity. Recombinant Gal4-Dr1 or Gal4-DRAP1 fusion proteins were added to transcription reaction mixtures reconstituted with GTFs, RNAPII, and the adenovirus major late promoter (Ad-MLP) with or without Gal4 binding sites. In agreement with results shown in Fig. 1A, recombinant Gal4-Dr1 represses transcription from the wild-type Ad-MLP minimally (data not shown). In contrast, Gal4-Dr1 efficiently repressed transcription from an Ad-MLP containing Gal4 DNA binding sites (Fig. 1B, lanes 1 to 4). At the highest concentration of Gal4-Dr1 (2 pmol), transcription was inhibited by approximately 90%. In contrast, Gal4-DRAP1, in the absence of Dr1, had no effect on transcription (Fig. 1B, lanes 5 to 8). The inability of Gal4-DRAP1 to repress transcription was not due to a defective protein, since Gal4-DRAP1 efficiently enhanced Dr1-mediated repression of transcription (data not shown). Taken together, our results indicate that the repression by the Dr1-DRAP1 complex is mediated through the Dr1 subunit.

The TBP binding domain of Dr1 tethers Dr1 to the promoter and is necessary for DRAP1 corepressor function. Our previous studies demonstrated that Dr1-mediated repression of transcription requires the TBP binding domain as well as a glutamine-alanine (QA)-rich domain. These motifs were mapped to residues 85 to 99 and 144 to 157, respectively (66).

To further examine the function of these two regions within the context of Dr1 repression and DRAP1 enhancement of Dr1-mediated repression of transcription, Dr1 derivatives with deletions in either the TBP binding ($Dr1_{\Delta 85–99}$) or the QA-rich ($Dr1_{\Delta 144–157}$) domain were fused to the DNA binding domain of the *Saccharomyces cerevisiae* transcriptional factor Gal4. The chimeric proteins were expressed with histidine tags in bacteria and purified by nickel affinity chromatography to apparent homogeneity. Recombinant Gal4-Dr1 mutant proteins were added to transcription reaction mixtures in a system reconstituted with GTFs, RNAPII, and the Ad-MLP with or without Gal4 binding sites.

Similar to its wild-type counterpart, Gal4-$Dr1_{\Delta 85–99}$ repressed transcription from the Gal4-responsive promoter template in a concentration-dependent manner (Fig. 2A). At the highest concentration of Gal4-$Dr1_{\Delta 85–99}$ (4 pmol), approximately 70% of the transcription activity from the Ad-MLP was repressed (Fig. 2A, lane 4). The Gal4-$Dr1_{\Delta 85–99}$ polypeptide failed to repress transcription from a promoter lacking Gal4 binding sites (Fig. 2A, lanes 6 to 8). Therefore, Dr1 can repress transcription in the absence of the TBP binding domain, provided that Dr1 is directed to the promoter via a DNA binding domain. These results indicate that the interaction between TBP and Dr1 functions in part to tether Dr1 to the promoter during the process of Dr1-mediated repression of transcription.

The Gal4-$Dr1_{\Delta 85–99}$ chimeric protein provided an excellent reagent to investigate whether the presence of the TBP binding domain of Dr1 is required for DRAP1 activity. We assayed the effect of DRAP1 on Gal4-$Dr1_{\Delta 85–99}$-mediated repression of transcription in the reconstituted system described above. As expected, DRAP1 enhanced the repression activity of Gal4-Dr1 (Fig. 2B, compare lane 6 with lanes 7 to 9). However, unlike its wild-type counterpart, the repression activity of Gal4-$Dr1_{\Delta 85–99}$ was not affected by the presence of DRAP1 (Fig. 2B, lanes 10 to 13). The unresponsiveness of Gal4-$Dr1_{\Delta 85–99}$ to DRAP1 was not due to the presence of the Gal4 moiety, since both Dr1 and Gal4-Dr1 repressed transcription to the same extent in the presence of DRAP1 (Fig. 2B, compare lanes 3 to 5 with lanes 7 to 9).

It is also possible that the inability of DRAP1 to function through Gal4-$Dr1_{\Delta 85–99}$ was due to a defective interaction between Dr1 and DRAP1. To address this possibility, we analyzed binding of Gal4-$Dr1_{\Delta 85–99}$ to DRAP1 in a GST pull-down assay. As shown, Gal4-Dr1 and Gal4-$Dr1_{\Delta 85–99}$ were retained by the GST-DRAP1, but not the control GST, column (Fig. 2C). These results indicate that the TBP binding region of Dr1 is functionally required to mediate the corepression activity of DRAP1, yet it is not required for Dr1-DRAP1 interaction.

The glutamine-alanine-rich domain of Dr1 represses transcription when recruited to a class II promoter. In light of the results with Gal4-$Dr1_{\Delta 85–99}$, we proceeded to determine if Dr1 with a deletion of the QA-rich domain (residues 144 to 157) functions when tethered to a promoter. Our previous results have demonstrated that Dr1 with such a deletion is not capable of repressing transcription (66). Similarly, we observed that $Dr1_{\Delta 144–157}$ protein, when tethered to the promoter via the Gal4 DNA binding domain (Gal4-$Dr1_{\Delta 144–157}$), had no effect on transcription (Fig. 3A) and was also unable to respond to DRAP1 (data not shown). We have previously shown that Gal4-$Dr1_{\Delta 144–157}$ interacts with TBP, albeit to a lesser extent than the wild-type polypeptide (66). However, to eliminate the possibility that the inability of Gal4-$Dr1_{\Delta 144–157}$ to repress transcription and respond to DRAP1 was due to a defective protein, we analyzed its ability to interact with DRAP1. GST and GST-DRAP1 fusion proteins were purified to apparent homogeneity, and equal amounts of each polypeptide were immobilized on glutathione-agarose columns. As shown in Fig. 3B, Gal4-Dr1 and Gal4-$Dr1_{\Delta 144–157}$ were retained by the GST-DRAP1, but not the control GST, column. These results confirm that the QA-rich domain of Dr1 is essential for repression of transcription.

While our previous studies indicated that Dr1-mediated repression of transcription is manifested by the inhibition of the association of TFIIA and TFIIB with the TBP-TATA complex, the findings described above demonstrate that association of Dr1 with TBP, through the TBP binding domain of Dr1, is not sufficient for repression. These studies revealed that the QA-rich domain is essential for Dr1 function. Therefore, we further analyzed the function of the QA-rich domain of Dr1 by fusing amino acid residues 101 to 165 of Dr1 to the Gal4 DNA binding domain (amino acid residues 1 to 94) (Gal4-QA). The fusion protein was expressed with a histidine tag in bacteria and purified by nickel affinity chromatography (Fig. 4A). The addition of the Gal4-QA fusion protein to reconstituted transcription reaction mixtures resulted in repression of transcription in a dose-dependent manner (Fig. 4B). At the highest concentration of Gal4-QA (6 pmol), approximately 80% of the transcription activity from the Ad-MLP was inhibited (Fig. 4B, compares lanes 5 to 7 with lane 1). QA-mediated repression of transcription was also observed in the presence of TAFs, that is, when TFIID replaced TBP (Fig. 4C). Repression of transcription was dependent on the presence of the Gal4 binding site, since Gal4-QA had no significant effect from a promoter without Gal4 sites (Fig. 4B, compare lanes 9 to 11 with lane 8). Moreover, the Gal4 DNA binding domain without the QA moiety had no appreciable effect on transcription from a promoter with Gal4 binding sites (Fig. 4B, lanes 1 to 4).

To study the promoter specificity of QA-mediated repression of transcription, we examined its effect on two other Gal4 binding sites containing cellular promoters, the TATA-con-

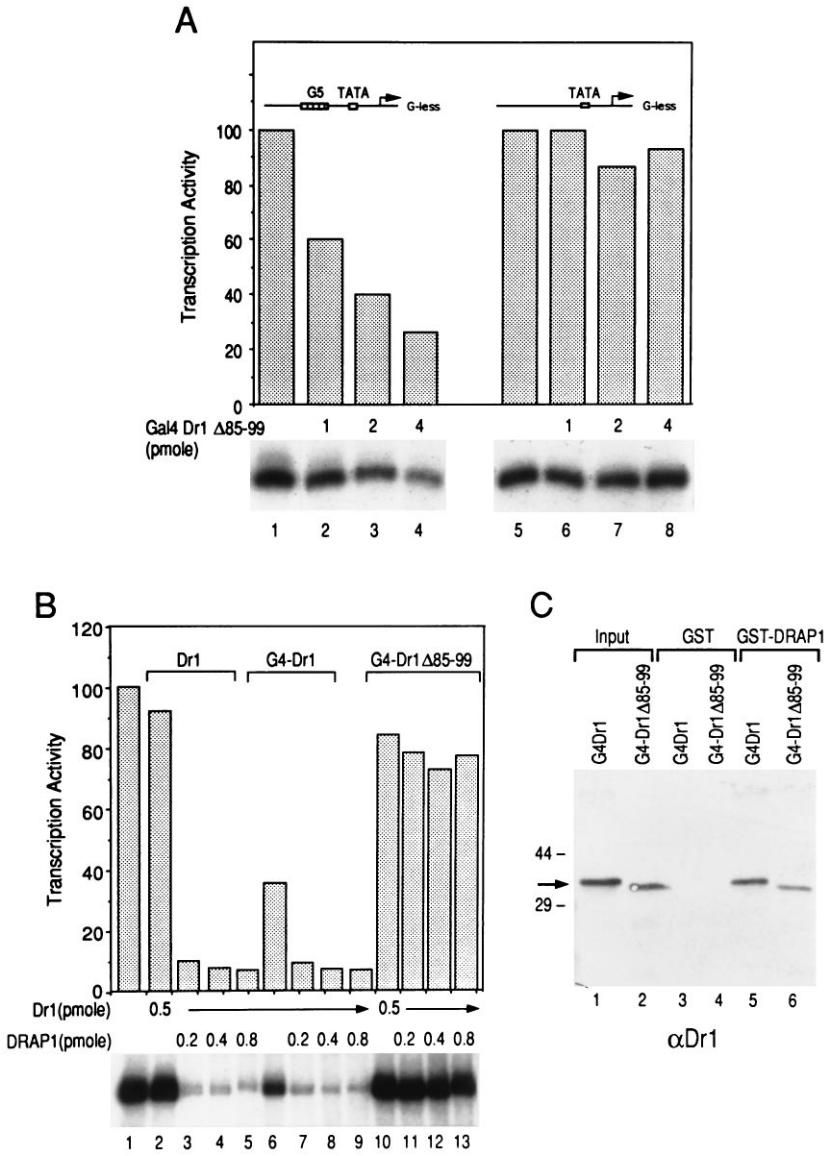

FIG. 2. A mutant Dr1 that lacks the TBP binding domain can repress transcription when tethered to the promoter. (A) (Top) Bar graph representing transcription activity of transcription reaction mixtures reconstituted with the Ad-MLP with or without Gal4 binding sites and increasing amounts of Gal4 $Dr1_{\Delta 85-99}$ as indicated. Reactions were performed as described in Materials and Methods. Transcription activity was quantified on a phosphorimager (Bio-Rad). (Bottom) Autoradiogram of RNA transcripts from transcription reactions quantified in the bar graph above. (B) The TBP binding domain of Dr1 mediates corepression of transcription by DRAP1. (Top) Bar graph representing transcription activity of transcription reaction mixtures containing G5-MLP reconstituted with purified or recombinant GTFs and RNAPII as described in Materials and Methods. Reaction mixtures also contained Dr1, Gal4-Dr1, different amounts of rDRAP1, and Gal4-$Dr1_{\Delta 85-99}$ as indicated. Transcription activity was quantified on a phosphorimager (Bio-Rad). (Bottom) Autoradiogram of RNA transcripts from transcription reactions quantified in the bar graph above. (C) Western blot probed with anti-Dr1 antibodies. One-half microgram of either Gal4-Dr1 or Gal4-$Dr1_{\Delta 85-99}$ was incubated with 1 μg of GST or GST-DRAP1 linked to glutathione-agarose beads, as indicated. Binding conditions were as described in Materials and Methods. The bound proteins were eluted in SDS-PAGE buffer, resolved by electrophoresis, and analyzed by Western blotting with Dr1 antibodies. The arrow indicates positions of Gal4-Dr1 proteins. Molecular mass markers (in kilodaltons) are shown on the left.

taining heat shock protein (HSP) and TATA-less DHFR promoters. As with the Ad-MLP, Gal4-QA repressed transcription in a concentration-dependent manner from both promoters. At the highest concentration of Gal4-QA, more than 60 and 80% of the transcription activity from the HSP and DHFR promoters, respectively, was repressed (Fig. 5, compare lanes 3 to 5 with lane 1 and lanes 8 to 10 with lane 6). The repression by Gal4-QA was specific and requires the QA-rich domain, since the $Gal4_{1-94}$ DNA binding domain on its own had no appreciable effect on transcription (Fig. 5, compare lanes 1 and 6 with lanes 2 and 7, respectively).

Gal4-QA represses transcription by targeting TBP during formation of the preinitiation complex. We have previously demonstrated that Dr1 represses transcription by interfering with an early step of preinitiation complex formation (25). It was of interest to determine whether Gal4-QA represses transcription by a similar mechanism. To address this question, Gal4-QA was added to a reconstituted transcription system either before or after preinitiation complex assembly. In agreement with the results presented above, Gal4-QA efficiently repressed transcription when added together with the other GTFs (Fig. 6A, lanes 1 and 2). Gal4-QA also repressed tran-

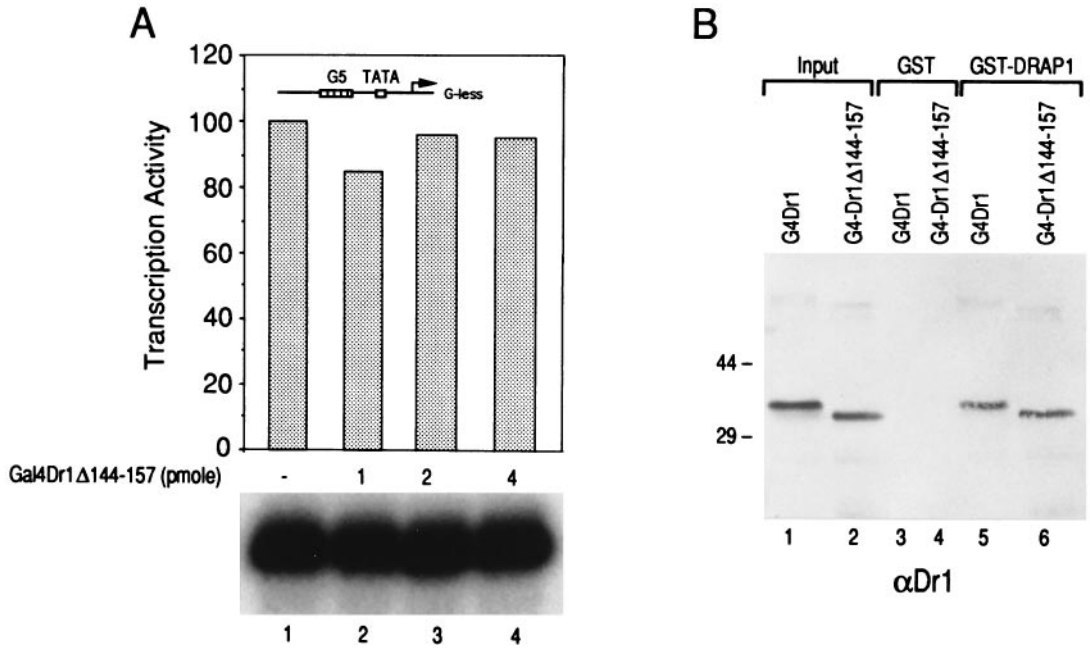

FIG. 3. A Dr1 mutant with a deletion of the glutamine-alanine-rich region cannot repress transcription when tethered to the promoter. (A) (Top) Bar graph representing transcription activity of transcription reaction mixtures reconstituted with the Ad-MLP with Gal4 binding sites and increasing amounts of Gal4 $Dr1_{\Delta 144-157}$ as indicated. Reactions were performed as described in Materials and Methods. Transcription activity was quantified on a phosphorimager (Bio-Rad). (Bottom) Autoradiogram of RNA transcripts from transcription reactions quantified in the bar graph above. (B) Western blot probed with anti-Dr1 antibodies. One-half microgram of either Gal4-Dr1 or Gal4-$Dr1_{\Delta 144-157}$ was incubated with 1 μg of either GST or GST-DRAP1 linked to glutathione-agarose beads, as indicated. Binding conditions were as described in Materials and Methods. The bound proteins were eluted in SDS-PAGE loading buffer, resolved by SDS-PAGE, and analyzed by Western blotting with Dr1 antibodies. Molecular mass markers (in kilodaltons) are shown on the left.

scription, albeit to a lesser extent, when added after the formation of the preinitiation complex (Fig. 6A, compare lanes 6 and 1). We reasoned that the Gal4-QA-mediated repression observed after preinitiation complex formation could be a consequence of inhibiting the loading of RNAPII molecules subsequent to initiation and escape of the first RNAPII molecule. To address this possibility, heparin, which limits transcription to a single round (52), was added to the reaction mixture. Consistently, heparin reduced transcription by approximately 50% (Fig. 6A, compare lanes 1 and 3). Under single-round transcription conditions, addition of Gal4-QA before the formation of the preinitiation complex intermediates inhibited transcription but had no appreciable effect if added after preinitiation complex formation (Fig. 6A, lanes 3, 4, and 5). The most likely explanation for these results is that the QA-rich domain interferes with the formation of the preinitiation complex. If added after formation of the preinitiation complex, the presence of the QA motif at the promoter affected the reassociation of the transcription factors. This is consistent with results of previous studies demonstrating that the transcription complex, including TFIIB, dissociates during the transition from initiation to elongation (69).

Having established that the presence of Gal4-QA during the formation of the preinitiation complex is essential for repression of transcription, we sought to determine which step(s) during complex assembly was affected. We investigated the effect of Gal4-QA on transcription during the formation of the preinitiation complex intermediates TBP-TATA, TFIIA-TBP-TATA, and TFIIB-TBP-TATA, using the approach described in the legend to Fig. 6A. In agreement with results presented in Fig. 6A, the addition of Gal4-QA before the formation of the preinitiation complex intermediates inhibited transcription (Fig. 6B, lanes 2, 5, and 8). There was no appreciable effect, however, if Gal4-QA was added after the formation of the TFIIA-TBP-TATA or TFIIB-TBP-TATA preinitiation complex intermediates (Fig. 6B, lanes 6 and 9). The observed effect was specific for the TFIIA-TBP-TATA and TFIIB-TBP-TATA complexes, since the incubation of TBP with DNA alone (lane 3) or in the presence of TFIIF (lane 12) was not capable of preventing repression by the QA-rich domain.

After demonstrating that Gal4-QA-mediated repression of transcription can be bypassed by formation of the TFIIA-TBP-TATA or TFIIB-TBP-TATA DNA protein complexes, we analyzed whether repression could be overcome by increasing the concentration of TBP, TFIIB, TFIIA, or other GTFs. We found that repression was partially alleviated in a concentration-dependent manner when the concentration of TBP was increased (Fig. 6C, lanes 2 to 4). The observed effect was due to the presence of the QA-rich domain, since increasing the concentration of TBP in the absence of Gal4-QA was without effect (Fig. 6C, lanes 9 and 10). At the highest concentration of rhTBP, the inhibition of transcription was overcome, resulting in approximately 60% transcription activity (Fig. 6C, compare lane 4 with lanes 1 and 8). The effect of TBP on QA-mediated repression of transcription appears specific, because increasing the concentration of rhTFIIB (Fig. 6C, lanes 5 to 7) or rTFIIA or rTFIIE (data not shown) had no effect.

The observation that the inhibition of transcription could be overcome by increasing the concentration of TBP prompted us to investigate whether the QA-rich domain could interact with TBP. *E. coli* extracts expressing HA-tagged TBP were mixed with purified Gal4-QA or $Gal4_{1-94}$ proteins, and putative complexes were immunoprecipitated with 12CA5 monoclonal antibodies which recognize the HA tag present in TBP. The

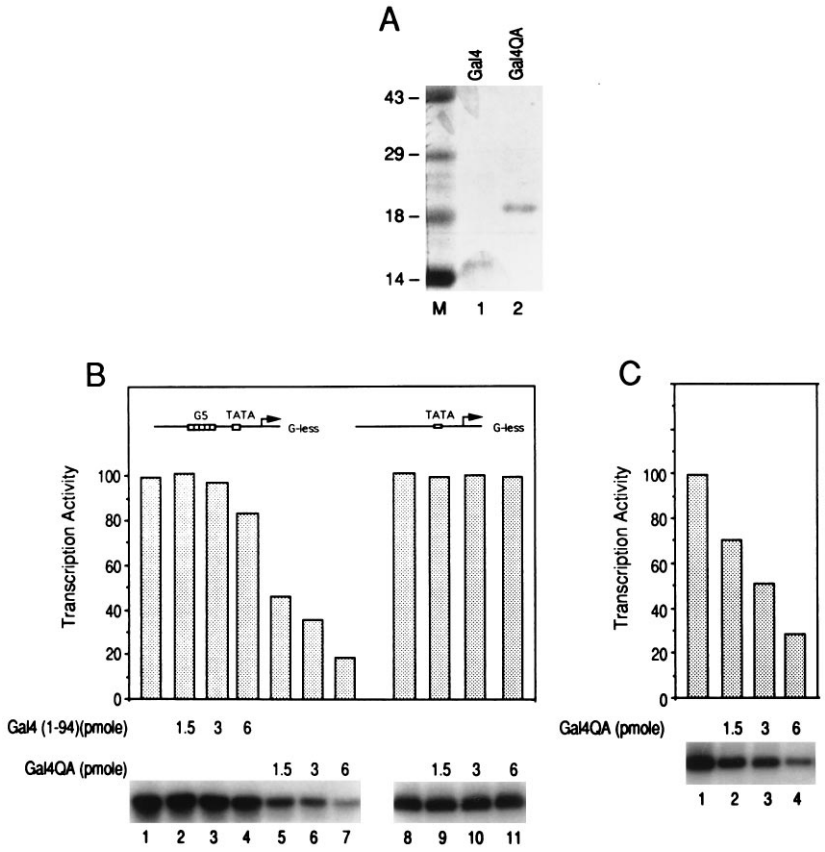

FIG. 4. The glutamine-alanine-rich region of Dr1 represses transcription. (A) An SDS-polyacrylamide gel stained with Coomassie blue showing the Gal4 and the Gal4-QA proteins. Molecular mass markers (in kilodaltons) are shown on the left (lane M). (B) (Top) Bar graph representing transcription activity of transcription reaction mixtures reconstituted with the Ad-MLP with or without Gal4 binding sites. Transcription reactions were reconstituted as indicated in Materials and Methods and contained increasing amounts of Gal4-QA or $Gal4_{1-94}$, as indicated. Transcription activity was quantified on a phosphorimager (Bio-Rad). (Bottom) Autoradiogram of RNA transcripts from transcription reactions quantified in the bar graph above. (C) (Top) Bar graph representing transcription activity of transcription reaction mixtures containing G5-MLP and reconstituted as described for panel B, except that eTFIID (10 ng) was used in place of TBP, in the presence of various amounts of Gal4-QA. Transcription activity was quantified on a phosphorimager (Bio-Rad). (Bottom) Autoradiogram of RNA transcripts from transcription reactions represented in the bar graph above.

immunoprecipitates were analyzed for the presence of Gal4-QA by Western blotting with anti-$Gal4_{1-147}$ antibodies. As shown, Gal4-QA but not the Gal4 DNA binding domain was coimmunoprecipitated in the presence of TBP (Fig. 6D, lanes 3 to 6).

It is known that in vivo TBP exists in a large multisubunit protein complex known as TFIID (51). Therefore, it was of interest to determine whether Gal4-QA could associate with TBP in the presence of TAF. This was analyzed with immunopurified HA-tagged TFIID, which was mixed with $Gal4_{1-94}$ or increasing amounts of purified Gal4-Dr1 or Gal4-QA proteins. Western blot analysis revealed that TFIID was immunoprecipitated, in a concentration-dependent manner, by the Gal4 antibodies from reaction mixtures containing Gal4-Dr1 and Gal4-QA but not from reaction mixtures containing Gal4 alone (Fig. 6E). The amount of TBP immunoprecipitated by Gal4-QA was smaller than that immunoprecipitated by Gal4-Dr1. These findings collectively indicate that there are two domains in Dr1 capable of interacting with TBP. These results are in agreement with our previous observations demonstrating that a mutated Dr1 protein which lacked the glutamine-alanine motif interacted with TBP more weakly than did the wild-type Dr1 protein (66).

DISCUSSION

In these studies, we have defined the function of each of the components of the Dr1-DRAP1 repressor complex through a combination of biochemical and molecular approaches. In agreement with our previous studies, we showed that the Dr1-DRAP1 heterotetramer is a highly efficient complex which represses class II transcription. In the absence of DRAP1, Dr1 can repress transcription, but repression is less efficient, as a much higher concentration of the factor was found to be required.

Fusion proteins consisting of the DNA binding domain of Gal4 and subunits of the Dr1-DRAP1 complex allowed us to analyze the role of each component in Dr1-DRAP1-mediated repression of transcription. We demonstrated that Dr1 could repress transcription when recruited to the promoter. Like DRAP1, Gal4-DRAP1 was found to have no effect on transcription from a promoter (Ad-MLP) containing Gal4 binding sites. The inability of Gal4-DRAP1 to repress transcription was not due to a defective protein, since Gal4-DRAP1 efficiently enhanced Dr1-mediated repression of transcription. This finding indicates that only the Dr1 subunit of the repressor complex contains a repressor domain.

Two distinct domains in Dr1, which are indispensable for

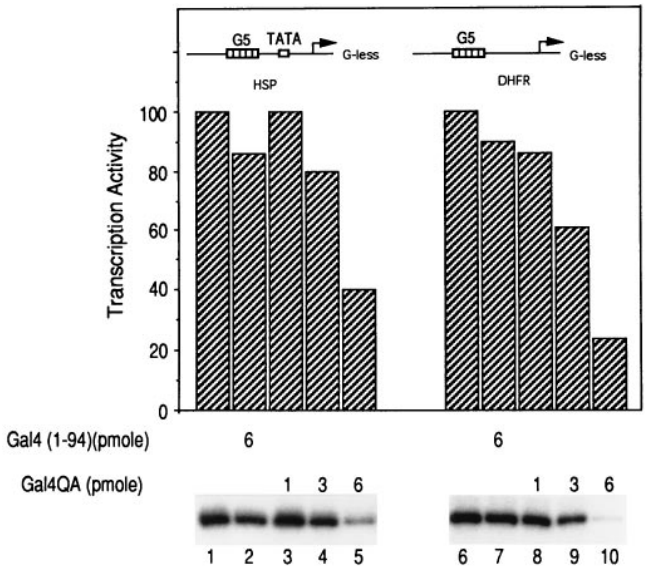

FIG. 5. Gal4-QA represses transcription from both TATA-containing and TATA-less promoters. (Top) Bar graph representing transcription activity of transcription reaction mixtures containing the HSP or DHFR promoter with Gal4 binding sites reconstituted as described in Materials and Methods. Reaction mixtures also contained $Gal4_{1-94}$ or increasing amounts of Gal4-QA, as indicated. Transcription activity was quantified on a phosphorimager (Bio-Rad). (Bottom) Autoradiogram of RNA transcripts from transcription reactions quantified in the bar graph above.

Dr1-mediated repression, were previously identified by mutational analysis. These are the TBP binding domain (amino acids 85 to 99) and a region rich in glutamine and alanine residues (amino acids 144 to 157) (66). The Gal4-Dr1 protein with either of these two regions deleted was used to investigate the function of these two domains. We observed that while fusion of the DNA binding domain of Gal4 to a Dr1 molecule from which the QA-rich domain was deleted ($Dr1_{\Delta 144-157}$) had no noticeable effect, the Gal4 DNA binding domain could partially restore the repression activity of the Dr1 mutant lacking the TBP binding domain ($Gal4_{\Delta 85-99}$). These findings therefore confirmed our previous results which suggested that one of the functions of the TBP binding domain is to anchor Dr1 to the promoter by interacting with TBP. Studies of the effect of DRAP1 on $Dr1_{\Delta 85-99}$-mediated repression of transcription revealed that the TBP binding domain might have yet another function besides targeting Dr1 to the promoter. We have previously shown that DRAP1 enhanced Dr1-mediated repression of transcription and stabilized the Dr1-TBP-TATA complexes (45). Here, we show that the TBP binding domain of Dr1 is required for DRAP1-mediated enhancement of repression. This finding therefore infers that the TBP binding domain of Dr1 also plays an active role in the process of repression. This observation is in agreement with our previous studies demonstrating that DRAP1 affects the interaction of Dr1 with the TBP-TATA complex.

Results from a recent genetic screen for repressor domains in yeast, together with studies on the *Drosophila* repressors *even-skipped* (*eve*), *engrailed* (*en*), and *Krüppel* (*Kr*), have defined a repression domain as a hydrophobic unstructured region lacking acidic residues (17, 54). The QA-rich domain of Dr1 possesses features of such a typical repressor domain. Direct evidence demonstrating that the QA-rich region of Dr1 is a bona fide repressor domain was obtained from experiments utilizing chimeric proteins. A fusion protein between the DNA binding domain of Gal4 and a 64-amino-acid peptide containing the Dr1 QA-rich region was found to actively repress transcription from TATA-containing and TATA-less promoters when tethered to promoters via Gal4 binding sites.

It is important to note that once artificially tethered to the promoter, the ability of Gal4-QA or Gal4-Dr1 to repress transcription in vitro depends largely on the source of TBP. While Gal4-QA (or Gal4-Dr1) repressed transcription in a system reconstituted with rTBP or immuno-affinity-purified TFIID, it minimally repressed transcription in systems reconstituted with crude TFIID preparations (data not shown). These findings suggest the presence of a factor(s) in crude TFIID preparations that can overcome Dr1-mediated repression of transcription and may explain our early observations indicating that transient expression of Gal4-QA in HeLa cells had no effect on a reporter construct containing Gal4 binding sites (66).

The precise mechanism whereby the repressor domain functions in repression remains unknown. Biochemical and genetic studies of the *Drosophila* repressor proteins *eve* and *Kr* demonstrated that these proteins repress by contacting components of the general transcription machinery (53, 62). Specifically, the repressor domain of *eve* has been shown to interact with the TBP component of the TFIID complex (64). Unlike *eve*, *Kr* represses transcription by contacting the largest subunit of TFIIE (p56). The interaction between *Kr* and TFIIE occurs only after *Kr* dimerizes and binds to DNA (55).

How might the interaction with general transcription factors lead to transcription repression? In the case of *eve*, the interaction may either interfere with preinitiation complex formation or preclude the binding of TBP to the DNA. It is notable that the *eve*-TBP interaction may not be sufficient for *eve*-mediated repression, and additional models have been proposed (3, 61). In view of the functions of TFIIE in initiation complex formation and modulation of TFIIH activities (11, 36; reviewed in reference 12), interaction of *Kr* with TFIIE (p56) may result in the inhibition of any one of these functions. Two regions in Dr1 which interact with TBP, the TBP binding region and the QA-rich region, were identified. Apparently, the association of the QA-rich region with TBP is not adequately stable, and an additional interaction mediated by the TBP binding region of Dr1 is required to achieve repression. It is of interest to note that two domains on p53 which are required for p53-mediated repression of transcription were also found to interact with TBP (21).

At least three different types of transcriptional repressors exist: those that prevent the access of factors to DNA such as Id (6) and IkB (5); those that directly recognize promoter sequences such as *Kr* (33), *en*, *eve* (16, 17), and RBP2N-CBF1 (10, 23); and those that need to be tethered to the DNA and are part of a dynamic repressor complex (4, 9, 22, 27, 31, 56; see references 9a and 26 for recent reviews on different classes of repressor).

In general, repressor complexes are composed of two integral components, a specific DNA targeting subunit and a second component mediating the repression activity. For example, the Mad-Max–mSin3 is a transcriptional repressor complex, where the Mad-Max heterodimer confers the promoter binding specificity and mSin3 contains the ability to repress transcription (4, 56). In yeast, the Cyc8 (Ssn6)-Tup1 repressor complex has been shown to be recruited to promoters via different sequence-specific DNA binding proteins. For example, for repression of the a-specific genes, the Cyc8 (Ssn6)-Tup1 heterodimer interacts with the DNA-bound MCM1 and $\alpha 2$ heterodimer (27, 63). In the Ssn6-Tup1 complex, only Tup1 contains an identifiable repressor domain (63). In all identified cases, the repressor domain is confined to one

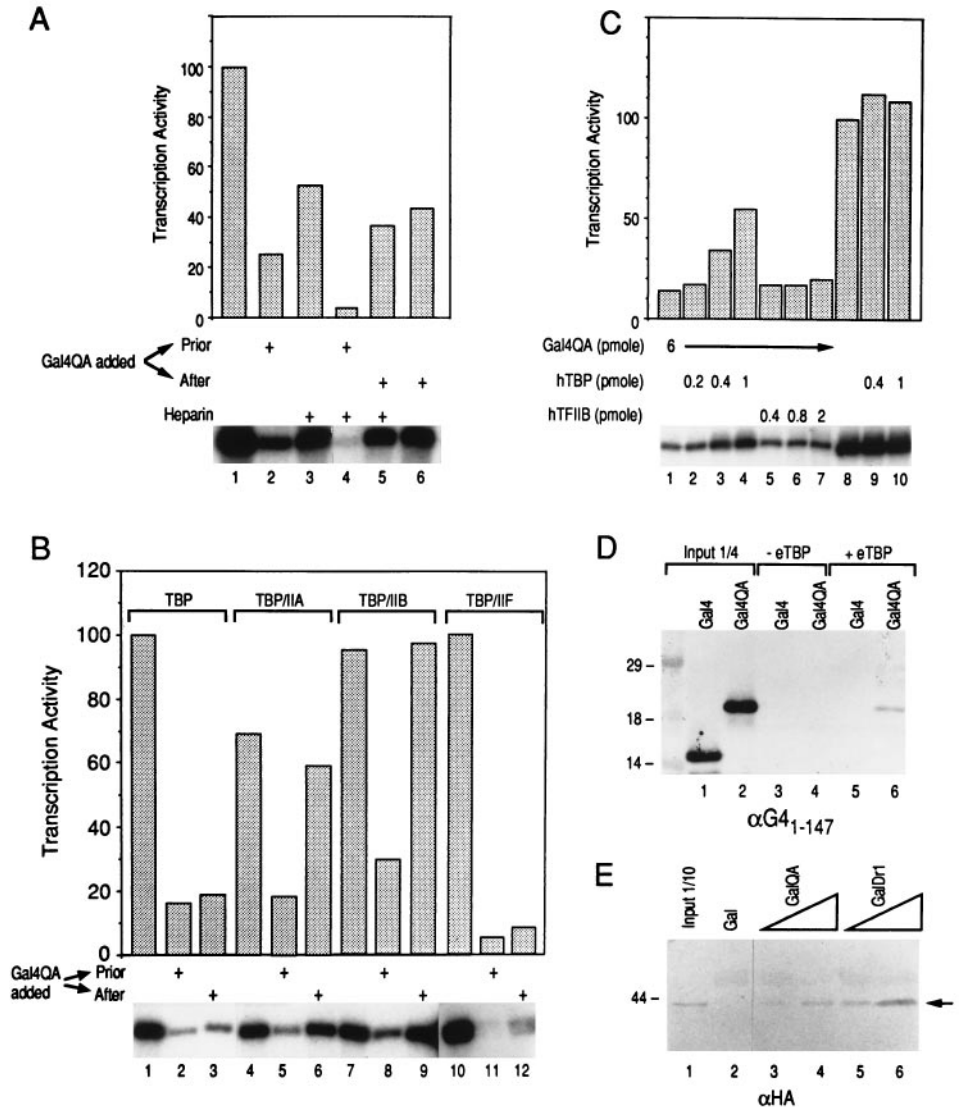

FIG. 6. Gal4-QA represses transcription by targeting TBP during the formation of the preinitiation complex. (A) (Top) Bar graph representing transcription activity of transcription reaction mixtures containing G5-MLP reconstituted with purified or recombinant GTFs, RNAPII, and Gal4-QA added prior to or after formation of the preinitiation complex in the absence or in the presence of heparin. When Gal4-QA was added after formation of the preinitiation complex (lanes 5 and 6), reaction mixtures were first incubated with GTFs, RNAPII, and G5-MLP DNA for 30 min, at which time 6 pmol of Gal4-QA, 0.6 mM ribonucleoside triphosphates (ATP and CTP), 1.5 μM UTP, and 0.5 μl of [α-^{32}P]UTP (25×10^3 cpm/pmol) were added. Reaction mixtures were incubated for an additional 30 min. In lanes 3 to 5, heparin (10 μg/ml) was added 3 min after the addition of ribonucleoside triphosphates to limit transcription to a single round. Transcription activity was quantified on a phosphorimager (Bio-Rad). (Bottom) Autoradiogram of RNA transcripts from transcription reactions quantified in the bar graph above. (B) (Top) Bar graph representing transcription activity of transcription reaction mixtures containing G5-MLP reconstituted with purified or recombinant GTFs, RNAPII, and Gal4-QA added prior to or after formation of the TBP-TATA-DNA (lanes 1 to 3), TFIIA-TBP-TATA-DNA (lanes 4 to 6), TFIIB-TBP-TATA-DNA (lanes 7 to 9), and TFIIF-TBP-TATA-DNA (lanes 10 to 12) preinitiation intermediate complexes. When Gal4-QA was added after formation of the preinitiation intermediate complexes (lanes 2, 5, 8, and 11), reaction mixtures were first incubated with GTFs as indicated and G5-MLP DNA for 4 min, after which 6 pmol of Gal4-QA, 0.6 mM ribonucleoside triphosphates (ATP and CTP), 1.5 μM UTP, and 0.5 μl of [α-^{32}P]UTP (25×10^3 cpm/pmol) were added. Reactions were continued for an additional 30 min. Heparin (10 μg/ml) was added 3 min after the addition of ribonucleoside triphosphates to limit transcription to a single round. Transcription activity was quantified on a phosphorimager (Bio-Rad). (Bottom) Autoradiogram of RNA transcripts from transcription reactions quantified in the bar graph above. (C) (Top) Bar graph representing transcription activity of transcription reaction mixtures containing G5-MLP reconstituted as described in Materials and Methods. Reaction mixtures also contained Gal4-QA (lanes 1 to 7). Extra amounts of hTBP (lanes 2 to 4 and 9 to 10) or hTFIIB (lanes 5 to 7), in addition to the amounts already present in the transcription reaction mixtures, were added at the beginning of the assays, as indicated. Transcription activity was quantified on a phosphorimager (Bio-Rad). (Bottom) Autoradiogram of RNA transcripts from transcription reactions quantified in the bar graph above. (D) Western blot probed with anti-Gal4 antibodies. Twenty picomoles of Gal4-QA (lane 5) or $Gal4_{1-94}$ (lane 6) was incubated with HA-tagged recombinant hTBP. Interaction was analyzed by immunoprecipitation with the anti-HA tag monoclonal antibody 12CA5, as described in Materials and Methods. The immunoprecipitated complexes were eluted with glycine-HCl (pH 2.6), resolved by SDS-PAGE, and analyzed by Western blotting with Gal4 antibodies. In the control, 20 pmol of Gal4-QA (lane 3) or $Gal4_{1-94}$ (lane 4) was incubated with the anti-HA tag monoclonal antibody 12CA5 without HA-tagged rhTBP. Molecular mass markers (in kilodaltons) are shown on the left. (E) Western blot probed with the anti-HA tag monoclonal antibody 12CA5. Two different concentrations (20 and 40 pmol) of Gal4-QA (lanes 3 and 4) or Gal4-Dr1 (lanes 5 and 6) were incubated with immunopurified HA-tagged TFIID (100 ng) for 2 h at 4°C with mixing. In the control, 40 pmol of $Gal4_{1-94}$ (lane 2) was used in place of Gal4-QA or Gal4-Dr1. Interactions were analyzed by immunoprecipitation with anti-Gal4 antibodies that were bound to protein A-Sepharose (Repligen). The immunoprecipitated complexes were washed and eluted with glycine-HCl as described in Materials and Methods. The eluted samples were then resolved by SDS-PAGE and analyzed by Western blotting with monoclonal antibody 12CA5.

component of the repressor complex. Our studies indicate that DRAP1 and Dr1 are members of this group of dual-component repressor complexes. However, an important functional difference exists between most of the repressor complexes studied and the Dr1-DRAP1 complex. This resides in the tethering factor. In the case of Dr1-DRAP1, the tethering factor is TBP, a global regulator of transcription that is required for transcription of all genes in the cell. This places Dr1-DRAP1 as a global repressor of transcription which must be highly regulated. In agreement with this, the adenovirus $E1A_{12S}$ protein (29), as well as an activity present in the partially purified TFIID protein fraction, can overcome Dr1-mediated repression of transcription. Furthermore, Dr1 also represses RNA polymerase III-transcribed genes (65).

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