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# Adenovirus E1A Proteins Interact with the Cellular YY1 Transcription Factor

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**The adenovirus 12S and 13S E1A proteins have been shown to relieve repression mediated by the cellular transcription factor YY1. The 13S E1A protein not only relieves repression but also activates transcription through YY1 binding sites. In this study, using a variety of in vivo and in vitro assays, we demonstrate that both E1A proteins can bind to YY1, although the 13S E1A protein binds more efficiently than the 12S E1A protein. Two domains on the E1A proteins interact with YY1: an amino-terminal sequence (residues 15 to 35) that is present in both E1A proteins and a domain that includes at least a portion of conserved region 3 (residues 140 to 188) that is present in the 13S but not the 12S E1A protein. Two domains on YY1 interact with E1A proteins: one is contained within residues 54 to 260, and the other is contained within the carboxy-terminal domain of YY1 (residues 332 to 414). Cotransfection of a plasmid expressing carboxy-terminal amino acids 332 to 414 of YY1 fused to the GAL4 DNA-binding domain can inhibit expression from a reporter construct with GAL4 DNA binding sites in its promoter, and inclusion of a third plasmid expressing E1A proteins can relieve the repression. Thus, we find a correlation between the ability of E1A to interact with the carboxy-terminal domain of YY1 and its ability to relieve repression caused by the carboxy-terminal domain of YY1. We propose that E1A proteins normally relieve YY1-mediated transcriptional repression by binding directly to the cellular transcription factor.**

The adenovirus E1A oncoproteins (reviewed in references 7, 39, 45, and 46) can induce resting human cells to enter the cell cycle, providing an optimal environment for viral replication. As a consequence of their ability to manipulate the cell cycle, E1A proteins can cooperate with other oncoproteins to transform rodent cells. The E1A proteins activate transcription, and their ability to modulate gene expression is probably the basis for their effect on cell cycle regulation and their role in oncogenic transformation.

E1A proteins can activate a wide variety of viral and cellular promoters, functioning through various *cis*-acting elements which include the binding sites for the TATA-binding protein, E2F, ATF, and AP-1. E1A proteins don't exhibit sequence-specific DNA binding activity. Rather, they bind to a variety of cellular proteins (15, 21, 24, 53) and modulate their function.

The 13S E1A protein can bind directly to the TATA-binding protein (23, 31), the DNA-binding subunit of TFIID, and binding correlates with transcriptional activation through the TATA motif. Both the 12S and the 13S E1A proteins bind to the retinoblastoma susceptibility protein, pRB (9, 51, 52). One function of pRB is to form a complex with the cellular transcription factor E2F, apparently inhibiting the ability of E2F to activate transcription. When the E1A protein binds to pRB, E2F is released from the complex (1, 4, 5), and the displacement correlates with transcriptional activation through E2F sites (22). The 13S E1A protein also binds to the ATF-2 transcription factor (3, 34, 35) and might, in this case, serve as an adapter, bridging from ATF-2 to TBP. Both E1A proteins can cooperate with cyclic AMP to activate transcription through AP-1 binding sites (10, 11). This activation is indirect, resulting from the induction of the cellular *junB* and c-*fos* genes (38), which leads to a marked increase of AP-1 within infected cells.

It has previously been shown that the adeno-associated virus P5 promoter contains a motif centered at −60 relative to its initiation site that mediates transactivation by the 13S E1A protein (2). A cellular factor, YY1, that binds to the motif was identified, and its cDNA was cloned (47). This factor has also been cloned as a result of its binding to transcriptional control regions of the immunoglobulin κ gene (40), ribosomal L30 and L32 protein genes (20), and the Moloney leukemia virus long terminal repeat (14). YY1 is a 414-amino-acid zinc finger protein that represses transcription when bound upstream of heterologous basal promoters; E1A proteins relieve the repression and activate transcription through YY1 (47).

In this study, we demonstrate that both 12S and 13S E1A proteins bind to YY1. Two sites on the E1A proteins mediate the interaction, an amino-terminal domain (within residues 15 to 35) and a domain that includes at least a portion of conserved region 3 (residues 140 to 188). Either site alone is sufficient for binding to intact YY1, but the 13S E1A protein that contains both sites binds to YY1 more efficiently than E1A proteins with only one site. E1A proteins bind to two sites on YY1, within amino acids 54 to 260 and 332 to 414. Analysis of a variety of mutant E1A proteins demonstrated that relief of YY1-mediated repression by E1A correlates with the ability of E1A protein to bind to YY1 within its carboxy-terminal domain (amino acids 332 to 414).

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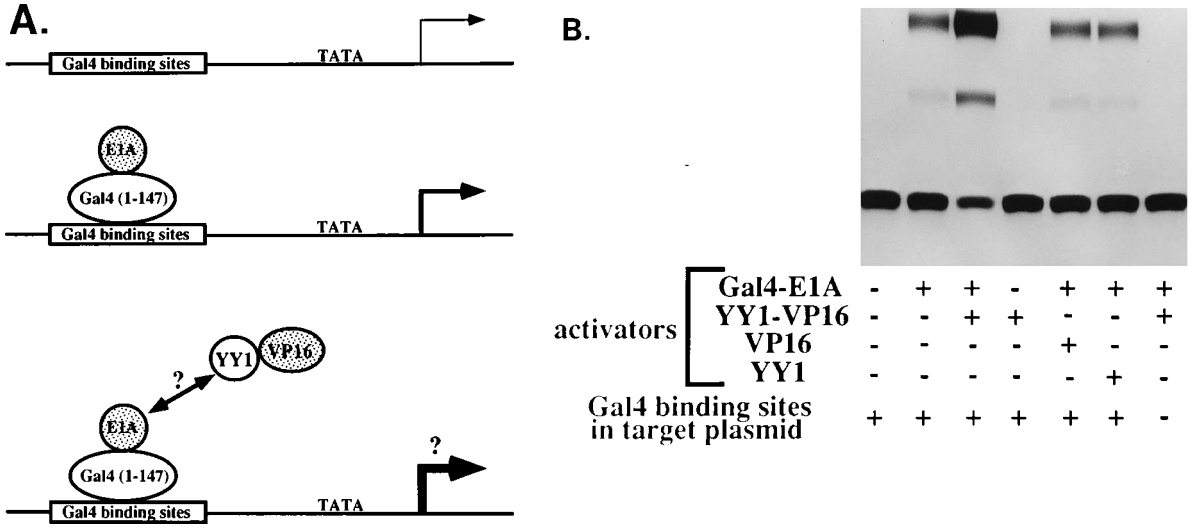


FIG. 1. YY1-VP16 induces CAT expression in the presence of GAL4-E1A. HeLa cells were cotransfected with a CAT reporter construct containing GAL4 DNA binding sites plus various activator plasmids. (A) Experimental design. (B) Results of a representative CAT assay. Three independent experiments yielded consistent results. Plasmids included in each lane are indicated (+).

## MATERIALS AND METHODS

**In vivo assay for E1A-YY1 interaction within transfected HeLa cells.** The target plasmids, pG5BCAT and pBCAT, and effector plasmids, pGal4-E1A and pMSV-VP16, have been described elsewhere (33). pYY1/VP16 and pYY1 were constructed by cloning the YY1/VP16 coding region (43) and the YY1 cDNA (47) into the expression vector pGS5 (18), in which they are expressed under control of the simian virus 40 early promoter. HeLa cells were transfected by the calcium phosphate method with 1 μg of pG5BCAT or pBCAT, 1 μg of pGal4-E1A or pGal4, and 5 μg of pYY1/VP16, pMSV-VP16, or pYY1. Cells were harvested at 48 h after transfection, and chloramphenicol acetyltransferase (CAT) activity was determined in 30-min reactions (16).

**In vitro assays for E1A-YY1 complex formation.** Sedimentation velocity centrifugation of the E1A-YY1 complex was done as previously described for the E1A-TBP complex (31) except that 0.1 M KCl was included in the sample buffer and gradient. Small aliquots of each fraction were subjected to electrophoresis in a sodium dodecyl sulfate (SDS)-containing 8% polyacrylamide gel, and bands corresponding to $^{35}$S-labeled E1A proteins were visualized by autoradiography.

Far Western blot (immunoblot) assays were performed by the procedure of Horikoshi et al. (23), using $^{32}$P-labeled 12S and 13S E1A proteins as probes. They were prepared by treating purified recombinant proteins produced in *Escherichia coli* with purified calf thymus casein kinase II in a reaction mixture containing [γ-$^{32}$P]ATP. Labeled proteins were tested for their ability to bind recombinant TFIID (expressed from pEthIID [29]), pRB (expressed from pET-Rb [23]), or YY1 (expressed from pHIS-YY1 [47]). Extracts of *E. coli* containing recombinant proteins were subjected to electrophoresis in an SDS-containing 10% polyacrylamide gel and transferred to a nitrocellulose membrane (BA 85; Schleicher & Schuell). After being blocked with HB buffer (20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid] [pH 7.9], 50 mM KCl, 5 mM $MgCl_2$, 0.5 mM dithiothreitol, 10 μM $ZnCl_2$) containing 5% nonfat dried milk, the filter was incubated with $^{32}$P-labeled probe in HB buffer containing 1% milk, 0.25% polyvinylpyrrolidone, 0.25% Ficoll 400, 0.5 mM ATP, and 0.1 mM sodium $PP_i$ at 37°C for 4 h. The filter was washed three times with buffer F (20 mM HEPES [pH 7.9], 0.1 M KCl, 1 mM EDTA, 1 mM dithiothreitol, 10 mM $ZnCl_2$, 0.1% Triton X-100) for 10 min each and exposed to X-ray film (Kodak XAR-5).

For capture experiments using glutathione *S*-transferase (GST) fusion proteins, an expression vector producing GST-YY1 fusion protein, pGEX-2T-YY1, was constructed by cloning an *Apa*I-*Kpn*I fragment from the cDNA clone of human YY1 in pGEM-72f (47) into pGEX-2T so that the YY1 open reading frame is in frame with the open reading frame of GST. Additional nucleotides from the polylinker of pGEM-72f are inserted between the GST and YY1 coding sequences. Some experiments used pGEX-2TK-YY1, in which YY1 was cloned into pGEX-2TK (30). Derivatives of pGEX-2TK-YY1 that contain deletions within the YY1 coding region were used to produce GST fusion proteins that contain different portions of YY1. To generate $^{35}$S-labeled E1A proteins, plasmids pGEM-13S and pGEM-12S (31) were transcribed in vitro by using SP6 RNA polymerase, and the resulting RNA was translated in a rabbit reticulocyte lysate. In some experiments, $^{35}$S-labeled E1A proteins were generated from pOS7-13S and pOS7-12S by using a coupled transcription-translation rabbit reticulocyte lysate system. 12S E1A mutants (42) were transcribed by using SP6 RNA polymerase. 13S*dl*2-36 was constructed by cloning a *Dra*III fragment, including conserved region 3, from pGEM-13S into the *Dra*III site of 12S*dl*2-36.

To capture $^{35}$S-labeled 12S or 13S E1A proteins, approximately equal molar quantities, as judged by Coomassie blue staining, of either GST or GST-YY1 fusion protein on glutathione-Sepharose beads were incubated with an appropriate amount of rabbit reticulocyte lysate in 150 to 300 μl of incubation buffer (50 mM KCl, 40 mM Tris-HCl [pH 8.0], 0.1% Tween 20, 0.5% nonfat dry milk, 5 mM β-mercaptoethanol) for 1 h at 4°C. The glutathione-Sepharose beads were then rinsed three times in incubation buffer containing an additional 0.1 to 0.5 M KCl or NaCl. To assay for the amount of $^{35}$S-labeled E1A that remained bound, the beads were boiled in sample buffer containing 1% SDS and released protein was analyzed by electrophoresis in a 10% polyacrylamide gel containing SDS. After electrophoresis, the gel was soaked in fluor (Resolution; DuPont) and dried, and radioactive protein bands were detected by autoradiography.

**YY1 repression assays.** HeLa cells were transfected with 10 μg of p5GAL4TKCAT, 5 μg of effector plasmid, and, where appropriate, 10 μg of RP1 (E1A genomic region expressed under control of its own promoter; kindly provided by Ed Harlow). The total amount of DNA in each transfection mixture was brought to 30 μg with either salmon sperm DNA or pBluescript plasmid DNA. Transfections employed the calcium phosphate precipitation method, and the precipitate was left in the culture medium for 18 h, after which cells were rinsed and fed with fresh culture medium. After 48 h, cells were harvested and CAT assays were performed (16). Most assays were normalized to secreted growth hormone levels by including in the transfection mixture 1 μg of pTKGH (Nichols Institute Diagnostics, San Juan Capistrano, Calif.). Assays done with and without growth hormone normalization yielded similar results.

## RESULTS

**YY1 and E1A proteins interact.** To test for an interaction between YY1 and E1A, we initially utilized the approach of Liu and Green (34) in which a protein-protein interaction within transfected HeLa cells activates transcription of a CAT reporter gene (Fig. 1A). Three plasmids were employed: a CAT reporter construct with a series of five GAL4 DNA binding sites upstream of the adenovirus E1B TATA motif and start site (33), a construct encoding a GAL4-E1A fusion protein including amino acids 121 to 223 of the 13S E1A polypeptide (33), and a construct encoding a YY1-VP16 fusion protein. Transfection of HeLa cells with the reporter plasmid and the plasmid expressing the GAL4-E1A protein led to the induction of CAT expression (Fig. 1B), as demonstrated previously (33, 36). If there is an interaction between E1A and YY1, then cotransfection of HeLa cells with the reporter construct plus plasmids encoding GAL4-E1A and YY1-VP16 fusion proteins might be expected to further induce CAT expression, as

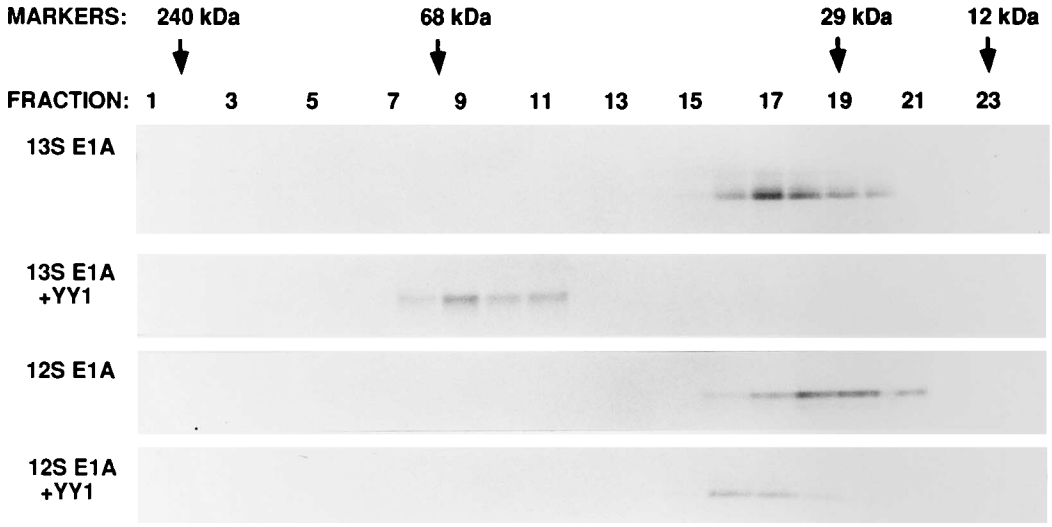


FIG. 2. Sedimentation velocity centrifugation of E1A-YY1 complex. $^{35}$S-labeled 12S or 13S E1A proteins with or without unlabeled recombinant YY1 protein purified from *E. coli* were subjected to centrifugation in sucrose gradients. Fractions were collected, and the E1A proteins were located by subjecting portions of each fraction to electrophoresis in an SDS-containing polyacrylamide gel followed by autoradiography. Molecular mass markers sedimented at the indicated positions.

the strong VP16 transcription-activating domain is brought to the promoter. This proved to be the case (Fig. 1B, third lane from the left). In a control experiment, expression of the YY1-VP16 protein without the GAL4-E1A protein failed to induce CAT expression (Fig. 1B). Moreover, VP16 and YY1 proteins failed to further stimulate CAT levels produced by the GAL4-E1A plasmid, and CAT expression in response to the GAL4-E1A plus YY1-VP16 fusion proteins required GAL4 binding sites in the reporter plasmid (Fig. 1B). These data argue that a physical interaction occurs between YY1 and amino acids 121 to 223 of the 13S E1A protein within transfected HeLa cells.

We used three in vitro biochemical assays to test more directly for a physical interaction between YY1 and E1A. In our first assay (Fig. 2), in vitro-translated, $^{35}$S-labeled E1A protein was subjected to velocity sedimentation in a sucrose gradient either alone or in the presence of excess recombinant YY1 protein (250 ng) that was produced and purified from *E. coli*. The 12S or 13S E1A protein alone sedimented predominantly in fractions 16 to 20. The addition of YY1 resulted in a shift of the labeled 13S E1A protein into fractions 8 to 11, indicating that the two proteins interacted to produce a more rapidly sedimenting complex. The sedimentation of the complex, relative to that of marker proteins, was most consistent with the formation of a YY1-13S E1A heterodimer. Addition of YY1 to the 12S E1A protein shifted its position in the gradient only slightly, suggesting that the two proteins did not bind tightly enough to cosediment.

Our second assay for a YY1-E1A interaction (Fig. 3) was a far Western protein blot, a procedure used previously to demonstrate an interaction between E1A and the TATA-binding protein (23, 31). In this assay, 12S and 13S E1A proteins were produced in *E. coli*, purified, and labeled with $^{32}$P by using casein kinase II (23). Labeled E1A proteins were then used as probes to detect interacting proteins present in bacterial cell extracts that were separated by electrophoresis on an SDS-containing polyacrylamide gel and transferred to a nitrocellulose membrane. The 13S E1A probe bound to two control proteins (Fig. 3, lanes 1 and 2), a fragment of the retinoblastoma susceptibility protein, pRB (51), and the TATA-binding protein (23, 31). In addition, the 13S protein bound to bacterially expressed YY1, but it did not bind to any proteins in a bacterial extract from cells that contained the expression vector with no inserted coding region (Fig. 3, compare lanes 3 and 4). This assay not only shows that the E1A protein can interact with YY1 but also demonstrates that the interaction is most likely direct. No contaminating animal cell proteins that might bridge between the E1A probe and the YY1 on nitrocellulose could be present in the purified E1A protein that was produced in *E. coli* and used as the probe. In contrast to the results of the sedimentation analysis (Fig. 2), the 12S E1A probe also bound to bacterially expressed YY1 in the protein blot analysis (Fig. 3, compare lanes 5 and 6). This apparent contradiction could be explained if the sedimentation analysis is a more stringent assay for the protein-protein interaction and the 13S E1A

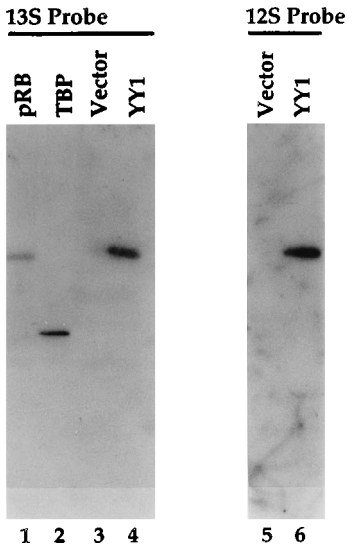


FIG. 3. 12S and 13S E1A proteins bind to YY1 in a far Western protein blot assay. *E. coli* lysates containing recombinant TATA-binding protein (TBP), pRB, or YY1 were subjected to electrophoresis in an SDS-containing polyacrylamide gel and transferred to a nitrocellulose filter. The filter was incubated with $^{32}$P-labeled 12S or 13S E1A protein and washed, and then radioactively labeled bands were identified by autoradiography.

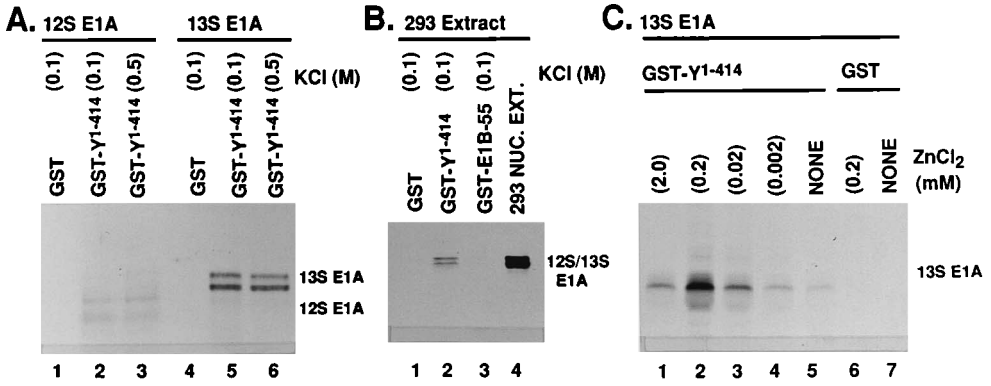


FIG. 4. A GST-YY1 fusion protein can bind to E1A proteins. (A) GST-YY1 protein binds to both 12S and 13S E1A proteins. GST-YY1 protein was bound to glutathione-Sepharose beads and incubated with equal amounts (60,000 cpm) of $^{35}$S-labeled 12S or 13S E1A protein. The GST-YY1 beads were washed with buffer containing either 100 or 500 mM KCl and boiled in sample buffer containing 1% SDS, eluted proteins were subjected to electrophoresis in an SDS-containing polyacrylamide gel, and protein bands were located by autoradiography. (B) GST-YY1 protein binds to E1A proteins within extracts of 293 cells. GST, GST-YY1, or GST-E1B protein was bound to glutathione-Sepharose beads and incubated with a 293 cell extract. The beads were washed in buffer containing 100 mM KCl and boiled in sample buffer containing 1% SDS. Eluted proteins were subjected to electrophoresis in an SDS-polyacrylamide gel, transferred to nitrocellulose, and assayed for the presence of E1A proteins by Western blot analysis using the E1A-specific M73 monoclonal antibody (21). Lane 293 NUC. EXT. contains 5 μg of a 293 cell nuclear extract. (C) $ZnCl_2$ enhances the interaction of E1A protein with the GST-YY1 fusion protein. $^{35}$S-labeled 13S E1A protein was captured with the GST-YY1 fusion protein in buffer containing the indicated concentrations of added $ZnCl_2$.

protein binds to YY1 more efficiently than the 12S E1A protein. The experiments described below indicate that this is indeed the case.

The third assay for a protein-protein interaction employed a YY1 affinity matrix to capture E1A proteins (Fig. 4A). A bacterially expressed GST-YY1 fusion protein (GST-$Y^{1-414}$) was bound to glutathione-Sepharose beads and incubated with $^{35}$S-labeled 12S or 13S E1A protein produced by in vitro translation in a reticulocyte lysate. The beads were washed under various salt conditions, boiled in sample buffer with detergent (1% SDS), and analyzed by electrophoresis in an SDS-containing polyacrylamide gel. GST-$Y^{1-414}$ bound both 12S and 13S E1A proteins, but the 13S E1A protein was captured more efficiently than the 12S E1A species. The difference in binding efficiency is consistent with the results presented above in which the 12S E1A-YY1 interaction was detected in the protein blot assay (Fig. 3) but not in the sedimentation assay (Fig. 2), which is presumably more stringent. The 12S and 13S E1A-YY1 complexes were stable to high salt concentrations. In the experiment whose results are displayed in Fig. 4A, complexes were washed in 0.1 or 0.5 M KCl; additional experiments (data not shown) revealed that the complexes were only partially disrupted by 1 M KCl. In addition, the binding of E1A proteins and GST-$Y^{1-414}$ was insensitive to RQ1 DNase, indicating that the interaction was not due to simultaneous binding of YY1 and E1A to contaminating DNA (data not shown). GST protein lacking the YY1 fusion partner failed to capture either E1A protein. The GST-$Y^{1-414}$ fusion protein was also tested for its ability to capture E1A proteins from the complex mixtures of proteins present in extracts of 293 cells (Fig. 4B), which express the viral oncoprotein (17). 293 whole-cell extracts were incubated with beads containing GST-$Y^{1-414}$ protein (Fig. 4B, lane 2) or, as controls, the GST protein without a fusion partner or a GST-E1B 55-kDa fusion protein (Fig. 4B, lanes 1 and 3); beads were washed in buffer containing 100 mM KCl; and captured E1A proteins were assayed by Western blot. E1A proteins were bound by the GST-$Y^{1-414}$ protein but not by the control proteins. Finally, 200 μM $ZnCl_2$ was found to substantially enhance YY1-E1A binding in the GST capture assays (Fig. 4C). The reduction in E1A-YY1 binding observed in the presence of 2 mM $ZnCl_2$ is likely due to the precipitation of proteins from the rabbit reticulocyte lysate. All subsequent assays were performed in the presence of added $ZnCl_2$ (200 μM).

In sum, the results of a series of in vivo (Fig. 1) and in vitro (Fig. 2 to 4) assays demonstrate that the adenovirus E1A proteins associate with YY1, and they show that the interaction is direct. Furthermore, the 13S E1A protein appears to bind YY1 more efficiently than the 12S species.

**Two YY1 domains interact with two domains on E1A.** A series of GST-YY1 fusion proteins that carried portions of the YY1 protein were used to identify the domains on YY1 necessary for its interaction with in vitro-translated, $^{35}$S-labeled 13S E1A (Fig. 5A) or 12S E1A (Fig. 5B) protein. Two YY1 domains were identified, either of which could bind independently to both 12S and 13S E1A proteins. One YY1 domain was located within amino acids 54 to 260, and the other was within amino acids 333 to 414. Both YY1 domains bound more efficiently to the 13S E1A protein than to the 12S protein.

The E1A-binding domain within residues 54 to 260 of YY1 may itself be composed of multiple interacting sites. A GST-YY1 fusion protein containing only the first 101 amino acids of YY1 (GST-$Y^{1-101}$) was sufficient to bind both 13S and 12S E1A proteins (Fig. 5, lanes 6), whereas GST-$Y^{1-53}$ did not bind either E1A protein better than GST protein with no fusion partner (Fig. 5A and B, compare lanes 2 and 7), suggesting that a region including at least a portion of amino acids 54 to 100 is required for binding. Interestingly, a stretch of 11 consecutive histidine residues is found within this region (amino acids 70 to 80). Maximal E1A binding, however, was not observed until the YY1 portion of the GST-YY1 fusion protein was extended from 142 to 260 amino acids (Fig. 5A, compare lanes 4 and 5). Either a strong E1A binding site resides within amino acids 143 to 259 or this region enhances the binding of E1A to amino acids 54 to 100. A more detailed mapping of the binding of E1A to this region is required to distinguish between these possibilities.

The difference in efficiency with which the two E1A proteins bind to YY1 could be explained by proposing that 12S E1A protein, which binds less efficiently, contains a single binding site that is present in both E1A proteins, while the 13S E1A protein, which binds more efficiently, contains a second bind-

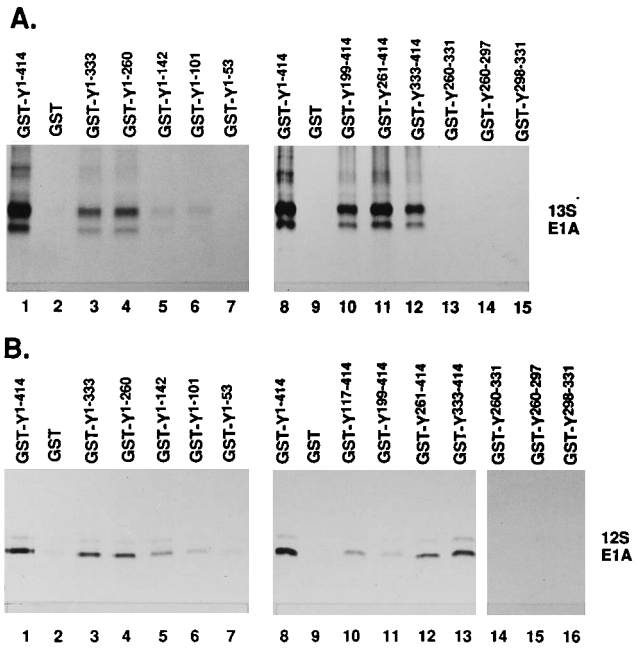


FIG. 5. Two YY1 domains interact with either 12S or 13S E1A protein. Approximately equal molar amounts of GST, GST-$YY1^{1-414}$, or derivatives of GST-$YY1^{1-414}$ that contain various portions of YY1 fused to GST were bound to glutathione-Sepharose beads and incubated with either $^{35}$S-labeled 13S E1A protein (A) or $^{35}$S-labeled 12S E1A protein (B). The beads were rinsed three times in buffer containing 0.5 M NaCl and boiled in sample buffer containing 1% SDS. Eluted proteins were subjected to electrophoresis in an SDS-containing polyacrylamide gel, and E1A proteins were visualized by fluorography. The GST-YY1 fusion proteins are numbered according to the amino acids from YY1 that are present in the fusion protein.

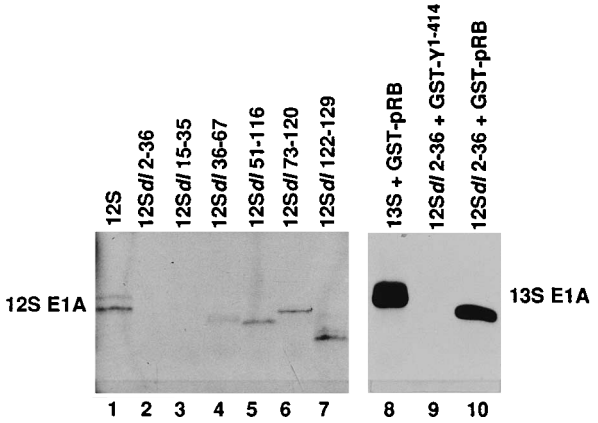


FIG. 6. Amino acids 15 to 35 of E1A proteins interact with YY1 independently of E1A conserved region 3. GST-$Y^{1-414}$ protein (lanes 1 to 7 and 9) or GST-pRB (lanes 8 and 10) was bound to glutathione-Sepharose beads and incubated with $^{35}$S-labeled 12S E1A protein, deleted derivatives of 12S E1A (numbers above lanes indicate the residues deleted), or 13S*dl*2-36. The GST-YY1 beads were washed with buffer containing 100 mM KCl and boiled in sample buffer containing 1% SDS, eluted proteins were subjected to electrophoresis in an SDS-containing polyacrylamide gel, and protein bands were located by autoradiography.

ing site involving its unique 46-amino-acid domain, a domain that is termed conserved region 3. An E1A-YY1 interaction was observed within transfected HeLa cells when a YY1-VP16 fusion protein with the complete YY1 sequence and a GAL4-E1A fusion protein that contained only amino acids 121 to 223 from E1A were used (Fig. 1). The E1A domain in the fusion protein includes conserved region 3 (amino acids 140 to 188). It is highly likely that conserved region 3 is involved in the binding documented in Fig. 1, given the more efficient interaction of the 13S than the 12S E1A protein with YY1 (Fig. 4A and 5) and the observation that 12S E1A variants lacking an amino-terminal YY1 binding site exhibit no residual E1A binding (see Fig. 6).

To search for a region outside conserved region 3 that was able to bind to YY1, we assayed a panel of 12S E1A deletion mutants (42) for the ability to be captured with a GST fusion protein containing the entire YY1 sequence (GST-$Y^{1-414}$). 12S E1A variants lacking amino acids 2 to 36 or 15 to 35 did not bind to the YY1 fusion protein (Fig. 6, lanes 2, 3, and 10), while 12S E1A derivatives lacking amino acids 36 to 67, 51 to 116, 73 to 120, or 122 to 129 were able to bind YY1 (Fig. 6, lanes 4 to 7). These experiments located an E1A domain needed for the interaction with YY1 between amino acids 15 and 35. To rule out the possibility that the amino-terminal deletions generated denatured, nonfunctional E1A proteins, we assayed 12S*dl*2-36 for binding to a GST-pRB fusion protein (28). The E1A-binding domains for pRB are located between residues 111 and 127 (25, 42, 52) and residues 30 and 60 (52). The 12S E1A variant with an amino-terminal deletion between residues 2 and 36 was indeed functional; it bound as efficiently as wild-type E1A proteins to GST-pRB (Fig. 6, lane 10).

We next assayed the ability of each of the two E1A domains to interact with each of the two YY1 domains. GST-YY1 fusion proteins that contained either the intact YY1 protein (GST-$Y^{1-414}$) or derivatives that separated the two YY1 domains that interact with E1A (GST-$Y^{1-260}$ and GST-$Y^{261-414}$) were used to capture in vitro-translated $^{35}$S-labeled 13S E1A (which contains both interacting domains), 12S E1A (which contains the domain from amino acids 15 to 35 but lacks conserved region 3), or 13S*dl*2-36 E1A (which lacks amino acids 2 to 36 but contains conserved region 3). Furthermore, the capture experiments were performed in the presence or absence of 200 μM $ZnCl_2$ to ascertain whether the divalent cation was important for some YY1-E1A interactions but not others. The 13S E1A protein interacted equally well with intact YY1 or either of the individual YY1 domains (Fig. 7A). The same was true for the 12S E1A protein, although, as expected, it was captured less efficiently than the 13S protein (Fig. 7B). In contrast, the 13S*dl*2-36 E1A protein was captured at a very low efficiency by GST-$Y^{1-414}$ or GST-$Y^{1-260}$, while it was not detectably captured by GST-$Y^{260-414}$ (Fig. 7C). Added $ZnCl_2$ enhanced all of the interactions.

In sum, the amino-terminal E1A domain interacts equally well with either of the YY1 domains. The conserved region 3 E1A domain enhances the YY1 binding of E1A protein containing the amino-terminal domain, but it binds very poorly to YY1 when present in an E1A protein that lacks the N-terminal domain.

**Functional domains of YY1.** It has previously been shown that YY1 can repress transcription when bound upstream of a basal promoter and that 12S or 13S E1A protein can alleviate the repression (47). To determine whether either of the E1A binding sites on YY1 correlates with a domain responsible for repression, we prepared three plasmids that express GAL4-YY1 fusion proteins: pGAL-$Y^{1-414}$, pGAL-$Y^{1-331}$, and pGAL-$Y^{332-414}$. The fusion proteins with portions of YY1 each contain one of the two sites on YY1 that interact with E1A proteins. To assay for repression, HeLa cells were cotransfected with plasmids that express the fusion proteins and the CAT reporter that contains GAL4 DNA binding sites upstream of a basal promoter (Table 1). It has previously been

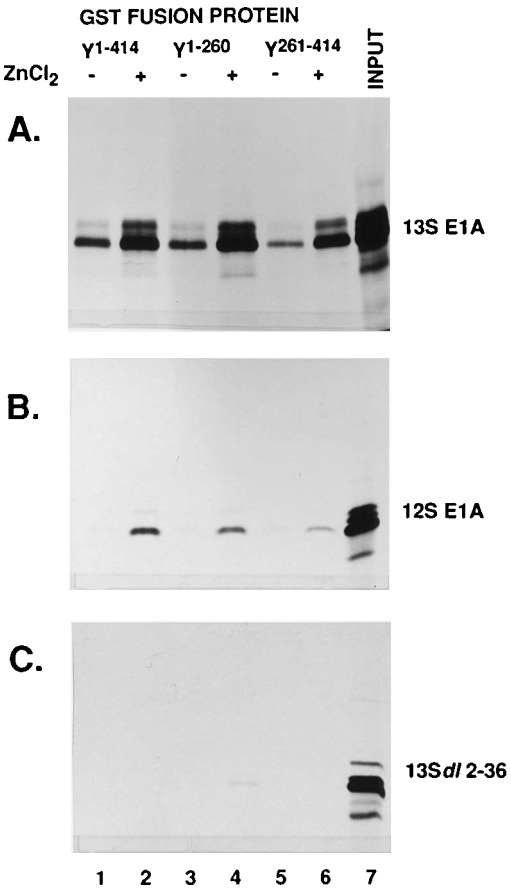


FIG. 7. Interactions between E1A and YY1 subdomains. GST fusion proteins GST-YY1$^{1-414}$ (lanes 1 and 2), GST-YY1$^{1-260}$ (lanes 3 and 4), and GST-YY1$^{261-414}$ (lanes 5 and 6) bound to glutathione-Sepharose beads were incubated with an approximately equal molar quantity of either 13S E1A protein (A), 12S E1A protein (B), or 13S*dl*2-36 E1A protein (C). Binding reactions were carried out in either the presence (lanes 2, 4, and 6) or the absence (lanes 1, 3, and 5) of added $ZnCl_2$ (200 mM). Lane 7 contained the amount of E1A protein added to each reaction mixture (INPUT) subjected to electrophoresis without capture by a GST fusion protein. The beads were rinsed three times in buffer containing 0.5 M NaCl and boiled in sample buffer containing 1% SDS. Eluted proteins were subjected to electrophoresis in an SDS-containing polyacrylamide gel, and E1A proteins were visualized by fluorography.

TABLE 1. E1A proteins relieve repression mediated by the carboxy-terminal domain of YY1[a]

| Expt and GAL4-YY1 fusion protein | CAT activity relative to reporter alone[b] | |
|---|---|---|
| | Without E1A | With E1A |
| 1 | | |
| pGAL-Y$^{1-414}$ | <0.05 | ND[c] |
| pGAL-Y$^{1-331}$ | 1.10 | ND |
| pGAL-Y$^{332-414}$ | <0.05 | ND |
| pGAL-Y$^{1-201}$ | 0.10 | ND |
| 2 | | |
| pGAL-Y$^{1-414}$ | 0.10 | 0.75[d] |
| pGAL-Y$^{1-331}$ | 1.15 | 1.80 |
| pGAL-Y$^{332-414}$ | 0.20 | 0.90 |
| pGAL-Y$^{1-201}$ | 0.20 | 0.20 |
| 3 | | |
| pGAL-Y$^{1-414}$ | 0.05 | 0.70 |
| pGAL-Y$^{1-331}$ | 0.95 | 1.80 |
| pGAL-Y$^{332-414}$ | 0.05 | 0.70 |
| pGAL-Y$^{1-201}$ | 0.05 | 0.05 |

[a] HeLa cells were transfected with a reporter plasmid containing the CAT coding region under control of a basal promoter with upstream GAL4 DNA binding sites. As appropriate, effector plasmids were included in the transfection mixture: a plasmid expressing a GAL4-YY1 fusion protein and, where indicated, a plasmid expressing both E1A proteins (with E1A). At 48 h after transfection, cell lysates were prepared and CAT activity was quantified. The data are from three independent CAT assays that were performed in duplicate.

[b] After chromatography, radioactivity in spots corresponding to mono- and diacetylated chloramphenicol was quantified by using a PhosphorImager. The level of CAT activity produced in the presence of a GAL4-YY1 effector plasmid was divided by the activity produced in the absence of an effector plasmid to produce the relative values that are shown (rounded to the nearest 0.05 U).

[c] ND, not determined.

[d] Although the E1A proteins can relieve repression and strongly activate transcription through YY1 DNA binding sites, presumably acting through YY1, E1A proteins substantially relieve repression but do not activate transcription through GAL4-YY1 fusion proteins bound to a GAL4 DNA binding site (47). The inability to activate transcription through the fusion protein might result from the fact that YY1 is not directly bound to DNA, and its orientation to the DNA is presumably abnormal in the fusion protein, i.e., the amino terminus of YY1 is appended to the DNA-binding domain in the fusion protein, whereas the normal YY1 DNA-binding domain resides at the carboxy terminus of the protein.

shown that the inhibition of CAT expression in this assay results from YY1-mediated transcriptional repression (47). Three independent experiments are tabulated because the extent to which the fusion constructs repressed CAT expression varied from assay to assay by a factor of about 10, and we have been unable to identify the variable responsible for the different absolute levels of repression. Nevertheless, the quantitative results within any one experiment and the qualitative results between experiments were entirely consistent, enabling us to determine with confidence the relative abilities of various GAL4-YY1 fusion proteins to inhibit expression of the reporter gene. Whereas pGAL-Y$^{1-414}$ and pGAL-Y$^{332-414}$ repressed transcription with very similar efficiencies, pGAL-Y$^{1-331}$ did not repress transcription (Table 1). Expression of the two E1A proteins from a third plasmid included in the transfection mixture relieved repression mediated by pGAL-Y$^{1-414}$ and pGAL-Y$^{332-414}$, but it had little effect (less than twofold) on expression of the reporter in the presence of pGAL-Y$^{1-331}$, which did not repress transcription (Table 1). Thus, one of the two domains on YY1 to which E1A binds (amino acids 332 to 414) can mediate repression, and E1A proteins can block the repression; the other E1A-binding domain on YY1 (amino acids 54 to 260) does not repress when present in a larger domain (amino acids 1 to 331). E1A proteins can bind to this large domain (Fig. 5), but they don't significantly influence expression in the assay which employs GAL4-YY1 fusion proteins (Table 1).

Curiously, residues 1 to 201 inhibited CAT activity nearly as well as a fusion protein containing the complete YY1 sequence (Table 1). This is somewhat of a paradox, since pGAL-Y$^{1-331}$, which encodes all of the amino acid sequence expressed by pGAL-Y$^{1-201}$, showed no repression activity (Table 1) (47). It is conceivable that the protein encoded by pGAL-Y$^{1-331}$ is unstable and rapidly degraded within transfected cells. Alternatively, deletion of the segment comprising amino acids 202 to 331 might uncover a cryptic repression function within residues 1 to 201 that can be blocked by the downstream domain. Coexpression of E1A proteins did not relieve pGAL-Y$^{1-201}$-mediated repression (Table 1). This segment of the YY1 protein might not include a strong YY1 binding site (Fig. 5), and this could explain the failure of E1A to influence its activity.

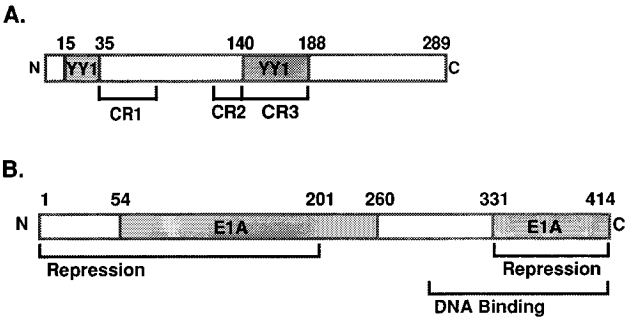


FIG. 8. Regions of E1A and YY1 required for E1A-YY1 binding. (A) Linear diagram of E1A, indicating portions of E1A required for binding to YY1 (shaded boxes). The positions of conserved regions 1 through 3 (CR1, CR2, and CR3, respectively) are indicated below the diagram. (B) Diagram of YY1. Portions of YY1 required for binding to E1A (shaded boxes) are indicated. Regions of YY1 that are able to repress transcription in *trans* or bind DNA are shown below the diagram.

## DISCUSSION

Our results show that the adenovirus 12S and 13S E1A oncoproteins physically interact with the cellular YY1 transcription factor. We assayed for the association because E1A proteins can modulate YY1 function within transfected HeLa cells (47). We demonstrated the interaction in vivo, by requiring that the two proteins interact to mediate transcriptional activation of a reporter gene within transfected HeLa cells (Fig. 1). We confirmed the interaction with a variety of in vitro assays, showing that the 13S E1A protein and YY1 cosediment (Fig. 2), determining that 12S or 13S E1A protein can bind to YY1 protein attached to a nitrocellulose membrane in a far Western blot assay (Fig. 3), and demonstrating that a GST-YY1 fusion protein can capture 12S and 13S E1A proteins (Fig. 4 to 7). These data add YY1 to a growing list of E1A-binding proteins that includes pRB family members that bind to and regulate the activity of the E2F transcription factor (pRB [51], p107 [12, 13], and p130 [19, 32]); p300, which regulates transcription and has sequence characteristics of a transcriptional adapter protein (8); and a variety of DNA-binding transcription factors (ATF-2 [3, 34, 35], the TATA-binding protein [23, 31], and Sp1 [35]). All of the cellular proteins known to interact directly with the E1A proteins control transcription, as does YY1.

Different domains on E1A are responsible for its interactions with various cellular proteins. E1A conserved region 2 (amino acids 111 to 127) with a stabilizing contribution from conserved region 1 (amino acids 30 to 60) is responsible for the interaction of E1A with pRB; conserved region 2 alone is essential for interaction with p107 and p130; an amino-terminal domain (residues 1 to 25) plus conserved region 1 mediates the interaction with p300; and conserved region 3 (amino acids 140 to 188, unique to the 13S E1A protein) interacts with ATF-2 and the TATA-binding protein (for a review, see references 41 and 45). The sites on E1A that mediate its interaction with YY1 (an amino-terminal sequence within residues 15 to 35 and conserved region 3 [Fig. 8A]) continue this pattern of diversity and reinforce the notion that the E1A proteins are composed of multiple domains that enable them to bind to a variety of cellular proteins.

Residues 15 to 35, which bind to YY1, partially overlap the E1A sequences involved in binding to p300 (amino-terminal domain plus conserved region 1 [50]), a cellular protein presumed to be involved in the induction of DNA synthesis, transformation, and transcriptional repression by E1A (25, 26, 37, 48, 52). Since the YY1 binding site partially overlaps the binding site for p300 on E1A, it is possible that YY1 binding contributes to the transformation-related functions of E1A. One might speculate that YY1 normally represses expression of one or more genes required for cellular growth and DNA synthesis, and E1A would promote cellular growth in part by relieving this repression.

When the two regions on the E1A protein that interact with YY1 are separated, the amino-terminal segment between residues 15 and 36 can bind to YY1 much more effectively than conserved region 3. 12S E1A protein, which contains the amino-terminal domain but lacks conserved region 3, binds to a GST-YY1 fusion protein much more efficiently than 13S*dl*2-36, which lacks the amino-terminal domain but contains conserved region 3 (Fig. 7). The weak interaction of conserved region 3 with YY1 was confirmed by the ability of a GAL4-E1A fusion protein containing a portion of E1A (residues 121 to 223), including conserved region 3 but lacking the amino-terminal domain, to interact with a fusion protein composed of YY1 plus a transcriptional activation domain within transfected HeLa cells (Fig. 1). Thus, either of the E1A domains can interact with YY1 independently. The interaction that involves at least a portion of conserved region 3 probably cooperates with the amino-terminal domain of E1A to stabilize the interaction with YY1, since the 13S E1A protein, which contains both domains, binds YY1 more efficiently than the 12S E1A protein, which lacks conserved region 3 (Fig. 2, 4, and 7).

E1A proteins interact with two domains on YY1. One YY1 domain is located within residues 54 to 260, and the other is within residues 332 to 414 (Fig. 5 and 8B). Since two domains on each protein are involved in the E1A-YY1 interaction, one might imagine that two separate binding events occur, each involving one binding domain on each of the proteins. However, this view must be an oversimplification, since the amino-terminal E1A domain (residues 15 to 35) interacts with both YY1 domains (Fig. 7B). Alternatively, then, one might suggest that two subdomains on each protein could constitute a single binding domain in the three-dimensional structure of the proteins. The two proteins would then interact through binding domains composed of residues from regions spatially separated in the linear sequence of amino acids. This view also might be an oversimplification of the E1A-YY1 interaction, since the amino-terminal domain of E1A interacts with both YY1 domains (Fig. 7B). However, it is possible that the amino-terminal domain of E1A interacts with a domain that is composed of elements from each of the two YY1 domains, and, upon separation, each of the YY1 domains might fortuitously contain sufficient information for continued interaction with the E1A domain. Another interpretation of the data would suggest that YY1 contains two recognition sites for the amino-terminal domain of E1A (Fig. 7B), and, while conserved region 3 can interact with YY1 independently of the amino-terminal domain (Fig. 1 and 7C), it serves primarily to stabilize or enhance the interactions mediated by the amino termini of E1A proteins (Fig. 7A). Finally, one of the in vitro interactions we have measured using mutant proteins might be artifactual, perhaps because of the relatively large deletions used, and the biologically relevant interactions could be simpler than our assays suggest. More-detailed mapping experiments which more precisely delineate the E1A binding sites on YY1 should discriminate among these various possibilities.

The binding of both 12S and 13S E1A proteins to either domain of YY1 was enhanced by the addition of $ZnCl_2$ to the reaction mixture (Fig. 4 and 7). Although the 13S E1A protein binds zinc via a zinc finger motif within conserved region 3, the 12S protein does not bind zinc (6). Therefore, YY1 probably

requires zinc for optimal binding to 12S E1A. The carboxy-terminal domain of YY1, amino acids 332 to 414, includes four zinc finger motifs that are required for DNA binding (1a). Either YY1 is deficient in the amount of zinc bound when overexpressed in *E. coli* or some of the bound zinc was lost during purification. The addition of exogenous zinc presumably restores the proper folding and structure of the protein. Similarly, the DNA binding activity of Sp1 that has been stripped of zinc by dialysis against EDTA can be restored by the addition of exogenous zinc (27). Zinc was also required for the binding of E1A 12S protein to the amino-terminal half of YY1 (amino acids 1 to 260) (Fig. 7B). It is not clear how zinc facilitates this interaction. This region includes 11 consecutive histidine residues (amino acids 70 to 80), and it is conceivable that this sequence binds zinc.

The YY1 domain between amino acids 332 and 414 is able to repress transcription when appended to a GAL4 DNA-binding domain (Table 1), E1A can bind directly to this segment of YY1 (Fig. 5), and the presence of E1A proteins can block repression by either wild-type YY1 protein bound to its normal DNA recognition site (47) or a GAL4 fusion protein containing YY1 residues 332 to 414 bound to a GAL4 DNA binding site (Table 1). This correlation supports the view that the interaction of E1A with YY1 residues 332 to 414 is biologically relevant. Since both E1A 12S and 13S proteins bind amino acids 332 to 414 of YY1, either protein could relieve YY1-mediated repression by binding directly to its repression domain, although the 13S protein might be expected to relieve repression at a lower concentration than the 12S protein because it binds more efficiently than the 12S protein. The presence of E1A might occlude an active repression domain in the carboxy-terminal region of YY1, or E1A might block an interaction of YY1 with another cellular protein which is required for repression. In addition to relieving repression, the 13S E1A protein can strongly activate transcription through YY1 recognition sites (47). This could result from E1A serving as an adapter between YY1 and a transcription-activating protein, such as ATF-2, to which E1A can also bind. One can envision a model in which a cellular repressor is displaced from YY1 and replaced with 13S E1A protein plus a transcriptional activator. This would account for the ability of 13S E1A protein to simultaneously relieve repression and activate transcription through YY1 binding sites.

The possible consequences of the interaction of E1A proteins with a sequence within amino acids 54 to 260 of YY1 are not clear. There is no doubt that E1A proteins interact with this domain of YY1 in vitro. However, to accept an in vitro interaction as potentially meaningful, it is important to identify a biological correlate of the binding, as is the case for the association of E1A with amino acids 332 to 414 of YY1. However, the segment of YY1 from amino acids 1 to 331 does not repress transcription when fused to the GAL4 DNA-binding domain, and E1A does not activate transcription through the fusion protein (Table 1). The domain of YY1 from amino acids 1 to 201 did repress transcription as part of a GAL4 fusion protein, perhaps because a cryptic repression domain was uncovered by loss of the segment of YY1 from amino acids 202 to 331, but again, E1A proteins were not able to influence repression mediated by amino acids 1 to 201 (Table 1). Thus, as yet, we have not been able to identify a biological correlate of the in vitro association we have observed for E1A proteins with the segment of YY1 from amino acids 54 to 260. It is also possible, however, that the interaction does indeed mediate a biological effect, and we have so far failed to employ an assay that can reveal a biological consequence of E1A binding to the second site on YY1. YY1 binding sites can serve as transcriptional initiator elements (44), and YY1 helps to direct RNA polymerase II to the adeno-associated virus P5 promoter, which contains a YY1 initiator element (49). We have not yet tested whether E1A influences the initiator function of YY1. One or both of the E1A binding sites on YY1 could be involved if E1A proteins prove to modulate initiator function.

Sedimentation analysis of the E1A-YY1 complex suggests that the two proteins form a heterodimer when an excess of YY1 is mixed with limiting E1A protein (Fig. 2). Given the indication in our data that the two proteins interact through multiple independent protein-protein binding domains (Fig. 5 to 7), it is possible that a variety of complexes containing different molar ratios of E1A and YY1 can form as the ratio of the two proteins is changed, as would occur during the adenovirus infectious cycle. Perhaps as the concentration of E1A proteins increases with time after infection, two E1A molecules associate with individual molecules of YY1, and the different E1A-YY1 complexes could have different biological properties. E1A proteins might also serve to form complexes between YY1 and other cellular proteins that interact with different domains of E1A. For example, it is conceivable that YY1 and pRB simultaneously bind to the E1A proteins, since they appear to interact with distinct E1A domains (e.g., see Fig. 6B). Experiments to better delineate the composition and variety of E1A-YY1 complexes and ultimately how these associations influence YY1 function are in progress.

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B.A.L. and G.T. contributed equally to this work.

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