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Adenovirus $E1A_{243}$ Disrupts the ATF/CREB-YY1 Complex at the Mouse c-*fos* Promoter

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The adenovirus $E1A_{243}$ protein can activate transcription of the mouse c-*fos* gene in a manner that depends on treatment of cells with inducers or analogs of cyclic AMP (cAMP). Activation requires conserved region 1 and the N-terminal domain of $E1A_{243}$ and is mediated by a 22-bp E1A response element containing a cAMP response element (CRE) at −67 and a binding site for transcription factor YY1 at −54. In the absence of $E1A_{243}$, YY1 represses CRE-dependent transcription of c-*fos* by physically interacting with ATF/CREB proteins bound to the −67 CRE. Here we present evidence that expression of $E1A_{243}$ leads to relief of YY1-mediated repression by a disruption of the ATF/CREB-YY1 complex. Addition of $E1A_{243}$ to in vitro binding assays prevented binding of ATF-2 to glutathione *S*-transferase–YY1. Similarly, expression of $E1A_{243}$ in HeLa cells prevented the association of a YY1-VP16 fusion protein with endogenous ATF/CREB proteins bound to the −67 CRE of a transfected c-fosCAT reporter plasmid. In each case, the N-terminal domain of $E1A_{243}$, which mediates a direct interaction with YY1, was responsible for disruption of the ATF/CREB-YY1 complex. On the basis of these and previously published results, we present a model for the synergistic transcriptional activation of the c-*fos* gene by $E1A_{243}$ and cAMP.

The adenovirus $E1A_{243}$ protein can cooperate with an activated Ha-Ras oncoprotein (44) or the adenovirus E1B proteins (4, 10) to induce cellular transformation. Three domains of $E1A_{243}$, termed conserved region 1 (CR1), CR2, and the N-terminal domain, are required for induction of transformation (23, 54). Much is known about the function of CR2, which in conjunction with CR1 mediates the association of $E1A_{243}$ with the tumor suppressor retinoblastoma protein (Rb) and the related proteins p107 and p130 (12). Binding of $E1A_{243}$ to Rb or p107 abolishes the interaction between each of these proteins and transcription factor E2F, thereby altering the effects of E2F on normal cell cycle regulation (22, 26, 39).

Considerably less is known about the role of the N-terminal domain in transformation. Together with CR1, the N-terminal domain binds to the cellular protein p300 (13, 55). $E1A_{243}$ has been shown to block transcriptional activation by p300, as well as the related CREB-binding protein (CBP), with which it can also associate (2, 34). This finding strongly suggests that p300 and CBP are functional targets for $E1A_{243}$ in transformation. In addition, the N-terminal domain binds to the cellular protein p400 and to Dr1, an inhibitor of TATA box-binding protein (TBP) (7, 25).

The N-terminal domain has also been found to mediate the association of $E1A_{243}$ with transcription factor YY1 (30). YY1 functions in various promoter contexts as either a transcriptional activator, a transcriptional repressor, or an initiator protein (5, 6, 8, 11, 17–19, 29, 32, 33, 35–38, 40, 41, 43, 45–53, 58). The ability of $E1A_{243}$ to bind YY1 suggests that YY1 also plays an important role in the regulation of cell growth. This idea is strengthened by the fact that the N-terminal domain of $E1A_{243}$ is required both for binding to YY1 (30) and for triggering cellular transformation. Furthermore, two of the genes known to be controlled by YY1 are the proto-oncogenes c-*myc* (43, 51) and c-*fos* (17, 19, 38).

We have reported previously that the mouse c-*fos* gene can be transcriptionally activated by $E1A_{243}$ in a manner that depends on treatment of cells with analogs or inducers of cyclic AMP (cAMP) (14, 15, 17). Domains of $E1A_{243}$ required for activation of the c-*fos* promoter include the N-terminal domain and CR1 (15, 17). Analysis of the c-*fos* promoter revealed that activation by $E1A_{243}$ is mediated by a 22-bp E1A response element (ERE), consisting of a cAMP-response element (CRE) located at −67 and a neighboring binding site for YY1 at −54 (17). Cellular proteins that directly recognize the c-*fos* ERE include YY1, which represses c-*fos* transcription (17, 38), and members of the ATF/CREB family, which bind to the CRE and activate transcription (9, 28). Recently we demonstrated that ATF/CREB proteins can physically interact with YY1 both in vitro and in cells (57). We have proposed that this interaction can account for the ability of YY1 to repress transcription from the c-*fos* promoter, since we and others have shown that such repression depends on a functional CRE (17, 38).

The observed interaction between ATF/CREB and YY1 suggested that the ATF/CREB-YY1 complex might be a molecular target of $E1A_{243}$ in activation of the c-*fos* gene. This seemed an attractive possibility because binding sites for these two factors function together in constituting the c-*fos* ERE (17). Here we present the results of in vitro and in vivo experiments which demonstrate that $E1A_{243}$ functions to prevent the interaction between YY1 and ATF/CREB. In vitro, ATF-2 showed strongly reduced ability to bind to immobilized glutathione *S*-transferase (GST)–YY1 in the presence of $E1A_{243}$. In vivo, $E1A_{243}$ was able to specifically block ATF/CREB-dependent tethering of a YY1-VP16 fusion protein to the c-*fos* promoter in transfected HeLa cells. The in vitro and in vivo effects of $E1A_{243}$ required residues 2 to 36 of the protein, which overlap significantly with a domain required for activation of the c-*fos* promoter (15, 17) and which are important in the interaction between $E1A_{243}$ and YY1 (reference 30 and this work). These data support a model in which $E1A_{243}$ activates c-*fos* transcription by dissociating the ATF/CREB-YY1 com-

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plex, thereby reversing YY1-mediated transcriptional repression.

MATERIALS AND METHODS

Cell culture. Monolayer HeLa cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% newborn calf serum, penicillin, and streptomycin at 37°C in a 10% CO_2 incubator. All media, sera, and antibiotics were from GIBCO/BRL.

Plasmids. pm27fosCAT, pm28fosCAT, pm27.28fosCAT, and Gal4-VP16 have been described previously (57). pCMVYY1/VP16 (27) was provided by Yang Shi. In vitro transcription/translation plasmids encoding wild-type E1A$_{243}$, *dl*2-36, *dl*38-67, and *dl*73-120 (42) were provided by Joe Nevins. Expression of E1A$_{243}$ in HeLa cells was from pCMVE1A12S (provided by Joe Nevins) or from plasmid pCMVE1A$_{243}$. These plasmids gave identical results. pCMVE1A$_{243}$ was constructed by PCR amplification of a 12S E1A cDNA, using oligonucleotide primer ESP2 (5′-CCACACACGCAATCACAGGT-3′), which contains an artificial *Hin*dIII site, and primer SH3 (5′-GTCAAGCTTATGAGACATATTAT CTGC-3′), which contains an artificial *Sma*I site. The amplified fragments were digested with *Hin*dIII and *Sma*I, isolated by agarose gel electrophoresis, and ligated to *Hin*dIII- and *Sma*I-digested pCMV5 vector (1). pCMV*dl*2-36, pCMV *dl*38-67, pCMV*dl*73-120, and pCMVpm928/961 were created by subcloning the appropriate *Bam*HI-*Hin*dIII fragment from GSTE1A-*dl*2-36, GSTE1A-*dl*38-67, GSTE1A-*dl*73-120, and GSTE1A-pm928/961 (25) (provided by Joe Nevins) into the *Bgl*II-*Hin*dIII site of pCMV4 (1). Plasmid pm928/961 was created by subcloning the appropriate *Bam*HI-*Eco*RI fragment from GSTE1Apm928/961 into *Bam*HI- and *Eco*RI-digested Bluescript KS$^-$ (Stratagene). pCMVE1A$_{243}$-VP16 was constructed by subcloning the appropriate *Xba*I fragment from plasmid E1A13SVP16 (provided by Michael Green) into the *Xba*I site of pCMVE1A$_{243}$. pfos/Gal4CAT was constructed by mutagenesis of pm27fosCAT, using the Altered Sites mutagenesis system (Promega) according to the manufacturer's protocol. A GAL4 DNA-binding site, 5′-CGGAAGACTCTCCTCCG-3′, was inserted at position −47. The oligonucleotide used for the mutagenesis was 5′-AT AGAAGCGCTGTGACGGAGGAGAGTCTTCCGTTTTTTTTTACTTC CT-3′. The mutant was confirmed by sequencing.

Production of GST fusion proteins and in vitro binding and competition reactions. Production of purified GST fusion proteins has been described previously (57). The amount of purified GST protein was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by staining with Coomassie blue and comparison with protein standards. ^{35}S-labeled proteins were produced by using 1 μg of circular DNA for in vitro transcription and translation, using the Promega TNT Coupled Reticulocyte Lysate System according to the manufacturer's protocol. Binding reactions were performed as described previously (57).

Pelleted beads corresponding to 0.5 to 1.0 μg of GST fusion protein were incubated with 300 μl of incubation buffer (50 mM KCl, 40 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES; pH 7.5], 5 mM 2-mercaptoethanol, 0.1% Tween 20, 0.5% nonfat dry milk, 0.2 mM $ZnCl_2$) and 2 μl of in vitro translation product for 1 h at 4°C. The beads were then pelleted and washed twice with 1 ml of wash buffer (100 mM KCl, 40 mM HEPES [pH 7.5], 5 mM 2-mercaptoethanol, 0.1% Tween 20, 0.5% nonfat dry milk). Bound proteins were then subjected to SDS-PAGE and fluorography. Competition reactions were performed in an identical manner, but with addition of in vitro-translated competitor protein as indicated in the figures.

Transfections and CAT assays. Monolayer HeLa cells in 10-cm-diameter dishes were transiently transfected by the calcium phosphate method exactly as described previously (3). The microgram amounts of DNA for each experiment are indicated in the figure legends. For all transfections, salmon sperm DNA was used to supplement the DNA up to a total of 25 μg per plate. Expression plasmids for YY1-VP16, Gal4-VP16, E1A$_{243}$, and E1A$_{243}$-VP16 (described above) were under the control of the cytomegalovirus (CMV) enhancer/promoter. Cells were harvested 48 h after transfection, and chloramphenicol acetyltransferase (CAT) assays were performed as described previously (57). Each experiment was performed a minimum of three times, and values are expressed as average counts per minute ± standard deviation. In addition, all transfections were performed in duplicate to confirm reproducibility within the experiment.

Immunoprecipitation and Western blot (immunoblot) analysis. Immunoprecipitations were performed essentially as described previously (21). HeLa cells were transfected with the wild-type or mutant E1A expression plasmids as indicated in the figures. Forty-eight hours after transfection, the cells were harvested, washed twice with ice-cold phosphate-buffered saline, and lysed in 0.5 ml of ice-cold lysis buffer (50 mM HEPES, 250 mM NaCl, 0.1% Nonidet P-40, 0.5 mM phenylmethylsulfonyl fluoride, 5 mM dithiothreitol, and 5 mM EDTA). Lysates (400 μl) were transferred to a tube containing 100 μl of M73 (20) and incubated at 4°C for 1 h with rocking. Seventy-five microliters of a 10% slurry of protein A-Sepharose beads was then added, and the lysate was rocked for an additional hour. The beads were pelleted by centrifugation and washed three times with 1 ml of ice-cold lysis buffer. Western blot analysis of the bound proteins was performed essentially as described previously (21), with modifications. Proteins were subjected to SDS-PAGE, transferred to nitrocellulose, and probed with M73. Filters were incubated with horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (Boehringer Mannheim). Bands were visualized with an enhanced chemiluminescence kit (Amersham).

RESULTS

Effect of E1A$_{243}$ on the in vitro association between YY1 and ATF-2. The hypothesis that the ATF/CREB-YY1 complex can be a direct target of E1A$_{243}$ was first tested in an in vitro binding assay. Previously we demonstrated specific binding of several ATF/CREB proteins (including ATF-2) to immobilized GST-YY1 (57). To test the effect of E1A$_{243}$ on the ability of ATF-2 and GST-YY1 to interact, beads containing immobilized GST-YY1 were incubated with in vitro-translated, ^{35}S-labeled ATF-2 in the presence or absence of increasing amounts of in vitro-translated, ^{35}S-labeled E1A$_{243}$. After the beads were washed to remove nonspecifically bound protein, the products of the binding reactions were subjected to SDS-PAGE. In preliminary experiments, the amount of ^{35}S-ATF-2 required to saturate the GST-YY1 beads was determined (data not shown). The binding reactions including ^{35}S-E1A$_{243}$ were carried out under these conditions, allowing us to determine if the binding of E1A$_{243}$ and ATF-2 to the GST-YY1 beads was mutually exclusive. As shown in Fig. 1A, addition of increasing amounts of ^{35}S-E1A$_{243}$ to the binding reactions resulted in retention of ^{35}S-E1A$_{243}$ on the beads and a corresponding decrease in binding of ^{35}S-ATF-2. In contrast, addition of increasing amounts of ^{35}S-CAT protein had no effect on the interaction between ATF-2 and YY1 and did not result in retention of ^{35}S-CAT on the GST-YY1 beads (the input translated ^{35}S-E1A$_{243}$ and ^{35}S-CAT proteins are shown in Fig. 1B, lanes 1 and 3, respectively). Since binding of E1A$_{243}$ was inversely related to binding of ATF-2, these data strongly suggest that E1A$_{243}$ and ATF-2 competed for a limiting amount of YY1.

We considered the possibility that the effect of E1A$_{243}$ on retention of ATF-2 might be due to a direct interaction between E1A$_{243}$ and ATF-2. E1A$_{243}$ has been shown previously to be unable to interact with ATF-2 in vitro (31). As shown in Fig. 1B, ^{35}S-E1A$_{243}$ did not interact with immobilized GST–ATF-2 under reaction conditions identical to those used in the competition experiments (lane 4). Therefore the effect of E1A$_{243}$ on retention of ATF-2 to the GST-YY1 beads was clearly not due to an interaction between E1A$_{243}$ and ATF-2. As a positive control for this experiment, ^{35}S-E1A$_{289}$, which has been demonstrated to interact with ATF-2 (31), bound efficiently to the GST–ATF-2 beads (lane 5). Furthermore, binding of E1A$_{243}$ to GST-YY1 could take place in the absence of ATF-2 (lane 7), demonstrating that retention of ^{35}S-E1A$_{243}$ to the beads did not depend on the presence of ATF-2. Taken together, these data demonstrate that E1A$_{243}$ can effectively compete with ATF-2 for binding to a limiting amount of YY1, leading to a decrease in ATF-2 binding to GST-YY1.

E1A$_{243}$ reverses tethering of YY1 to the c-*fos* promoter in cells. One implication of our in vitro experiments is that expression of E1A$_{243}$ in cells might lead to decreased association between ATF/CREB proteins and YY1 at the c-*fos* promoter by a similar competition mechanism. Since YY1 normally functions as a repressor of c-*fos* transcription, and since E1A$_{243}$ binds directly to YY1 and also activates transcription of the c-*fos* gene, a reasonable hypothesis is that E1A$_{243}$ acts to disrupt the ATF/CREB-YY1 interaction, thus relieving transcriptional repression of the c-*fos* gene by YY1.

We therefore tested the effect of E1A$_{243}$ on the interaction between YY1 and the c-*fos* promoter in living cells. This was accomplished by way of a promoter tethering assay, used previously to demonstrate the in vivo interaction between YY1

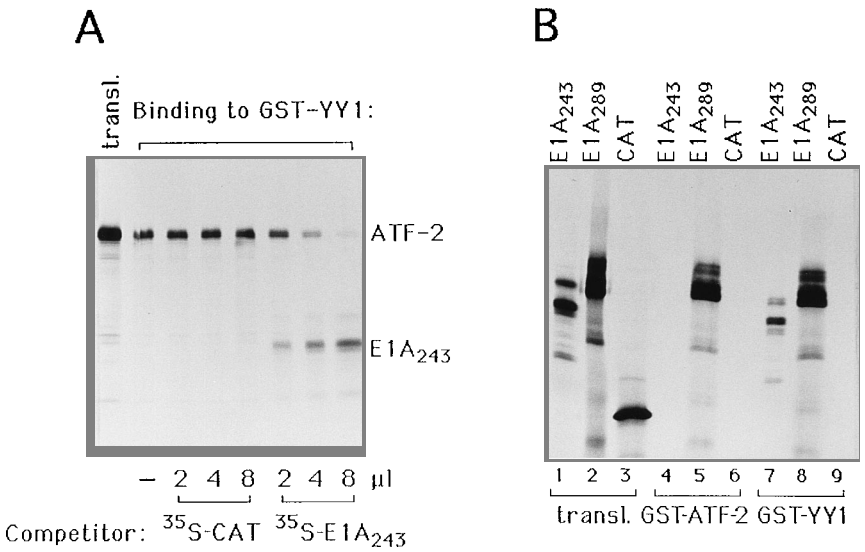

FIG. 1. $E1A_{243}$ competes with ATF-2 for binding to GST-YY1. (A) Immobilized GST-YY1 was incubated with 2 μl of ^{35}S-ATF-2 in the absence or presence of the indicated amounts of either ^{35}S-CAT or ^{35}S-$E1A_{243}$. The reaction products were washed and then analyzed by SDS-PAGE. (B) Immobilized GST–ATF-2 or GST-YY1 was incubated with either ^{35}S-$E1A_{243}$ (2 μl), ^{35}S-$E1A_{289}$ (1 μl), or ^{35}S-CAT (2 μl). The reaction products were washed and then analyzed by SDS-PAGE. transl., in vitro translation products of the indicated proteins, in half the amount added to the binding reactions.

and the c-*fos* promoter (57). Previous studies demonstrated that YY1 interacts with the c-*fos* promoter in two ways. First, YY1 can bind directly to two overlapping YY1 DNA-binding sites located at −54 and −50 (38, 57). Second, YY1 can associate with the promoter by a direct interaction with ATF/CREB proteins that are bound to the promoter at the nearby −67 CRE (57). In the promoter tethering assay, HeLa cells are transiently transfected with two plasmids. One is a CAT reporter plasmid (pfosCAT) containing the −76 to +10 region of the murine c-*fos* gene and carrying the closely spaced CRE and YY1 DNA-binding sites, the TATA element, and the transcription initiation site at +1. The other is an expression plasmid encoding full-length YY1 fused to the transcriptional activation domain of the herpes simplex virus VP16 protein. In this system, transcriptional activation mediated by the VP16 activation domain occurs when YY1-VP16 is tethered to the promoter by protein-protein and/or protein-DNA interactions. In the absence of such tethering, activation by the VP16 activation domain does not occur.

HeLa cells were transfected with pfosCAT and pCMVYY1-VP16, in the presence or absence of a plasmid expressing full-length $E1A_{243}$. Results of CAT assays of extracts from the transfected cells are shown in Fig. 2A. Consistent with our previous results, expression of YY1-VP16 gave a 44-fold activation of the reporter plasmid, a clear demonstration of tethering to the promoter. We have previously shown that a Gal4-VP16 fusion protein does not activate transcription of pfos CAT, indicating that activation by YY1-VP16 specifically depends on the YY1 portion on the protein and that mere expression of an irrelevant VP16 fusion protein does not result in activation of the promoter (57). YY1 itself normally represses c-*fos* transcription through the −54 YY1 site (17, 38). Therefore, activation of pfosCAT by YY1-VP16 in this assay required both the YY1 and the VP16 portions of the fusion protein, and the function of the VP16 activation domain was epistatic to repression by YY1. Additionally, we have reported that maximal activation of the c-*fos* promoter by YY1-VP16 requires both the YY1-DNA binding site and the neighboring CRE, which is bound directly by ATF/CREB proteins (57).

Importantly, expression of $E1A_{243}$ in cells transfected with the pfosCAT and YY1-VP16 expression plasmids resulted in a sharp decrease in activation by YY1-VP16 (7.9-fold compared with 44-fold), indicating that $E1A_{243}$ could effectively prevent the association between ATF/CREB and YY1-VP16 at the promoter. Transfection of the pCMV4 parental plasmid lacking $E1A_{243}$ coding sequences had no effect on activation by YY1-VP16, indicating that this effect was indeed due to expression of $E1A_{243}$.

It was important to determine if the decrease in transcription caused by $E1A_{243}$ was due to an effect on the YY1 portion of the fusion protein, or if perhaps $E1A_{243}$ was acting on the VP16 activation domain itself. To distinguish between these possibilities, we constructed a variant of pfosCAT that lacked the natural binding sites for YY1 but contained an artificial binding site for the yeast GAL4 protein. This construct was transfected into HeLa cells along with a plasmid encoding the DNA-binding domain of GAL4 fused to the VP16 activation domain (Gal4-VP16). Figure 2B shows that, as expected, expression of Gal4-VP16 resulted in a large transcriptional activation of the pfos/Gal4CAT reporter plasmid. When $E1A_{243}$ was coexpressed along with Gal4-VP16, little or no effect on activation by Gal4-VP16 was observed. We conclude from these experiments that $E1A_{243}$ could effectively decrease the association between YY1-VP16 and the wild-type c-*fos* promoter and that this action was due to a specific effect on the YY1 portion of the fusion protein. The specificity of $E1A_{243}$ for YY1 makes good sense, given the known ability of $E1A_{243}$ to interact directly with YY1 (30).

$E1A_{243}$ reverses tethering of YY1-VP16 to cellular ATF/CREB. YY1 interacts with the c-*fos* promoter through protein-protein and protein-DNA interactions (57). To investigate the molecular basis for the effect of $E1A_{243}$ on the association of YY1 with the promoter, we analyzed each of these interactions in isolation. Reporter plasmid pm27fosCAT contains an eight-nucleotide substitution in the −54 to −47 region which abolishes DNA-binding to YY1 (57). The −67 CRE in pm27fos CAT is intact, however. Previously we have shown that this reporter is strongly activated by YY1-VP16 in HeLa cells, in a

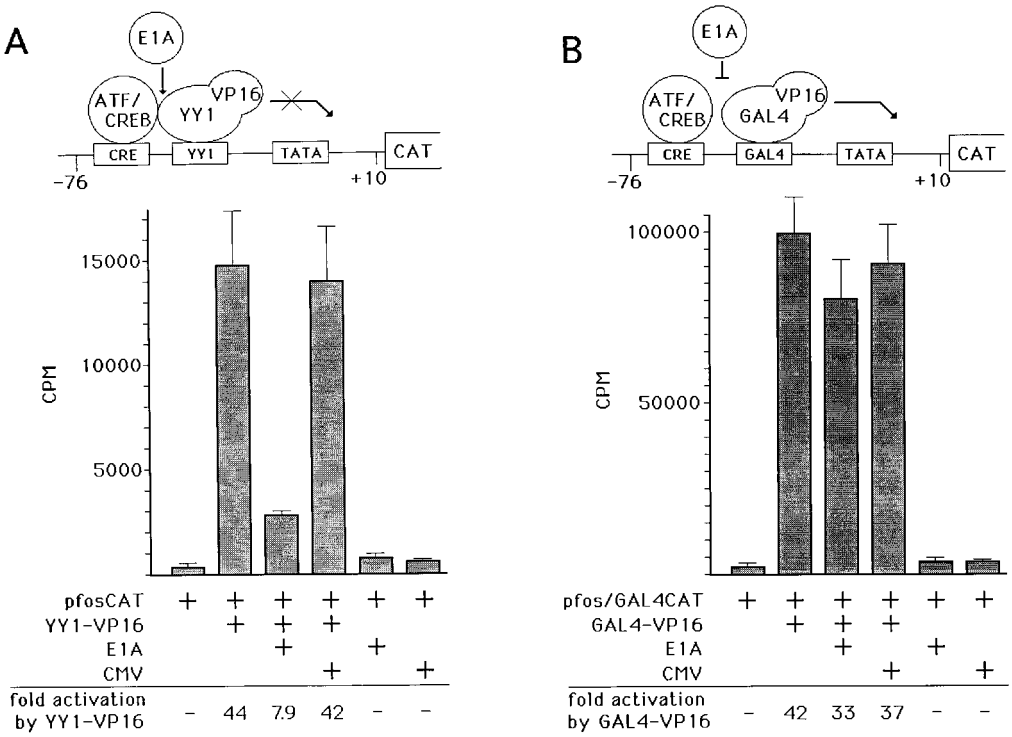

FIG. 2. E1A$_{243}$ reverses tethering of YY1-VP16 to the wild-type c-*fos* promoter. HeLa cells were cotransfected with 5 μg of the pfosCAT (A) or pfos/Gal4CAT (B) reporter, along with expression plasmids for YY1-VP16 (2.5 μg), Gal4-VP16 (2.5 μg), E1A$_{243}$ (5 μg), and/or the CMV parental expression plasmid (5 μg), as indicated. After 48 h, cells were harvested and assayed by the liquid scintillation CAT assay. Fold activation was calculated as CAT activity (in counts per minute) from cells expressing YY1-VP16 (A) or Gal4-VP16 (B) divided by CAT activity from cells transfected only with the indicated reporter and carrier DNA. Schematic representations of pfosCAT (A) and pfos/Gal4CAT (B), and associated proteins, are shown at the top.

manner that strictly depends on the presence of the −67 CRE. Since YY1 can associate specifically with ATF/CREB proteins in vitro but cannot bind directly to the −67 CRE (38, 57), we concluded that in vivo activation of pm27fosCAT by YY1-VP16 is mediated by protein-protein interactions with promoter-bound ATF/CREB (57). Figure 3 depicts the ability of YY1-VP16 to activate pm27fosCAT in either the presence or absence of E1A$_{243}$. Consistent with our previous results, YY1-VP16 activated pm27fosCAT almost 17-fold (there was little or no activation by the parental CMV expression plasmid lacking YY1-VP16 sequences or by a plasmid encoding Gal4-VP16 [reference 57 and data not shown]).

As was the case for the wild-type reporter plasmid, expression of E1A$_{243}$ resulted in a significant decrease in activation of the pm27fosCAT reporter by YY1-VP16, down to only five-fold. These data indicate that E1A$_{243}$ can bind to YY1-VP16 in the cell and prevent its interaction with endogenous ATF/CREB proteins located at the c-*fos* promoter. They are consistent with the in vitro competition data presented in Fig. 1.

Lack of effect of E1A$_{243}$ on YY1-VP16 bound to DNA. We also investigated the effect of E1A$_{243}$ on DNA-bound YY1-VP16 in the context of the c-*fos* promoter in vivo. To do this, cells were transfected with the YY1-VP16 expression plasmid and the reporter plasmid pm28fosCAT, which contains both YY1 sites intact but lacks a functional CRE as a result of a C-to-G mutation at position −64 that completely abolishes ATF/CREB binding in vitro (16). Consistent with our earlier report, YY1-VP16 activated pm28fosCAT 5.4-fold (Fig. 4). We have demonstrated that this activation is due solely to the presence of the YY1 DNA-binding sites, since mutating the YY1 sites in this reporter results in a failure to respond to YY1-VP16 (57). Again, there was no activation by the parental expression plasmid lacking YY1-VP16 sequences and no activation by Gal4-VP16 (reference 57 and data not shown). In contrast to the results with the wild-type and pm27fosCAT reporters, expression of E1A$_{243}$ had little or no effect on activation of pm28fosCAT. Therefore, E1A$_{243}$ was unable to displace DNA-bound YY1-VP16 from this promoter, despite its strong ability to displace YY1-VP16 bound to the promoter through protein-protein interactions.

Despite its inability to block direct binding of YY1-VP16 to the DNA, we found that E1A$_{243}$ was still capable of interacting with DNA-bound YY1 to form a stable complex. We expressed an E1A$_{243}$-VP16 fusion protein in HeLa cells along with the pm28fosCAT reporter, which contains binding sites for YY1 but not for ATF/CREB. As shown in Fig. 4B, expression of E1A$_{243}$-VP16 was able to activate the reporter strictly in a YY1 site-dependent manner: strong activation was observed from pm28fosCAT, but no activation was recorded from pm27.28fos CAT, which is identical to pm28fosCAT except that it lacks binding sites for YY1. Furthermore, only E1A$_{243}$-VP16, but not Gal4-VP16 or E1A$_{243}$ alone, was able to activate pm28fos CAT. These data demonstrate that E1A$_{243}$, as expected, can interact with DNA-bound YY1 at the c-*fos* promoter and that it can form a stable complex. Our data clearly indicate, however, that E1A$_{243}$ does not disrupt the YY1-DNA complex in the context of the c-*fos* promoter in living cells.

The N-terminal domain of E1A$_{243}$ is responsible for disruption of the ATF/CREB-YY1 complex in vitro and in vivo. Previously we reported that the ability of E1A$_{243}$ to activate transcription of the c-*fos* promoter depends in part on amino acid residues within the N terminus of the protein (15, 17). We have

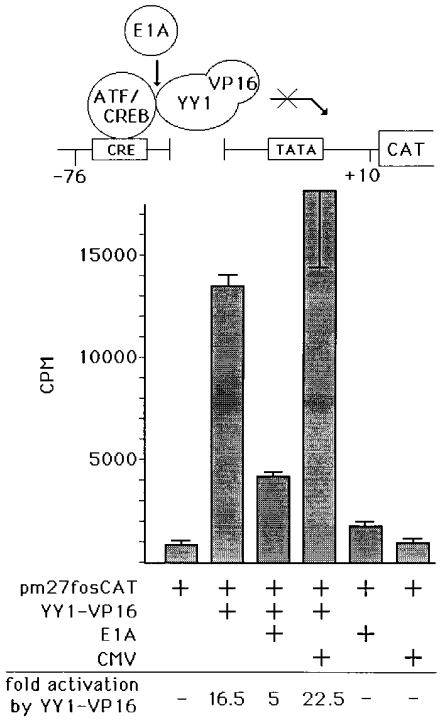

FIG. 3. $E1A_{243}$ reverses tethering of YY1-VP16 to the pm27fosCAT reporter. HeLa cells were cotransfected with 5 μg of the pm27fosCAT reporter, along with expression plasmids for YY1-VP16 (2.5 μg), $E1A_{243}$ (5 μg), and/or the CMV parental expression plasmid (5 μg), as indicated. After 48 h, cells were harvested and assayed by the liquid scintillation CAT assay. Fold activation was calculated as CAT activity (in counts per minute) from cells expressing YY1-VP16 divided by CAT activity from cells transfected only with pm27fosCAT and carrier DNA. A schematic representation of pm27fosCAT, and associated proteins, is shown at the top.

also shown that function of the ERE in the c-*fos* promoter requires a YY1 DNA-binding site along with the −67 CRE (17). These findings fit well with the recent report that physical interaction between $E1A_{243}$ and YY1 also depends on an N-terminal domain of $E1A_{243}$ (30). Together, they prompted us to examine whether disruption of the ATF/CREB-YY1 complex in vitro and in vivo also involves the N-terminal domain of $E1A_{243}$.

First, a series of deletion mutants (kindly provided by Joe Nevins) was used to identify the domain of $E1A_{243}$ required for competing with ATF-2 for binding to immobilized GST-YY1 in vitro. These competition experiments were carried out exactly as those described above, this time using wild-type or mutant ^{35}S-labeled $E1A_{243}$ and ^{35}S-labeled ATF-2. As shown in Fig. 5, all of the mutant proteins except mutant *dl*2-36 were capable of binding to GST-YY1 and competing with ATF-2. This mutant could neither bind to GST-YY1 (lane 7) nor prevent ATF-2 binding (lane 14). All of the other mutants and the wild type bound efficiently to GST-YY1 in either the presence (lanes 13 and 15 to 17) or absence (lanes 6 and 8 to 10) of ATF-2. Therefore, the domain of $E1A_{243}$ required for binding in vitro to YY1 is identical to or significantly overlaps that required for competition with ATF-2.

To identify the domain of $E1A_{243}$ required to disrupt tethering of YY1-VP16 in transfected cells, a similar set of reagents was constructed by placing each of the deletion mutants shown in Fig. 5 into the eukaryotic expression vector pCMV4 (1). As presented earlier, expression of wild-type $E1A_{243}$ in HeLa cells had a strong effect on ATF/CREB-bound YY1-VP16 but not on DNA-bound YY1-VP16 (Fig. 3). Therefore, we used the pm27fosCAT reporter (lacking binding sites for YY1 but carrying the −67 CRE) to map the functional domain of $E1A_{243}$ responsible for disruption of tethering. As shown in

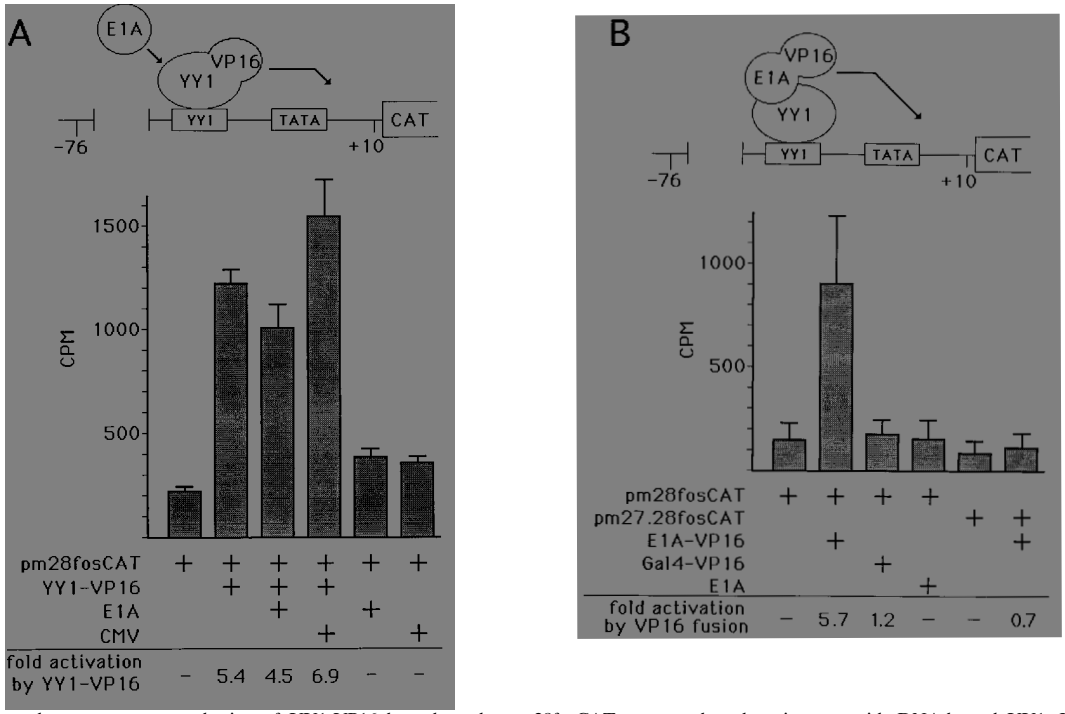

FIG. 4. $E1A_{243}$ does not reverse tethering of YY1-VP16 bound to the pm28fosCAT reporter but does interact with DNA-bound YY1. HeLa cells were cotransfected with 5 μg of either the pm28fosCAT (A and B) or pm27.28fosCAT reporter (B), along with expression plasmids for YY1-VP16 (2.5 μg), Gal4-VP16 (2.5 μg), $E1A_{243}$ (5 μg), and/or the CMV parental expression plasmid (5 μg), as indicated. After 48 h, cells were harvested and assayed by the liquid scintillation CAT assay. Fold activation was calculated as CAT activity (in counts per minute) from cells expressing YY1-VP16 (A), E1A-VP16 (B), or Gal4-VP16 (B) divided by CAT activity from cells transfected only with the appropriate reporter and carrier DNA. Schematic representations of pm28fosCAT, and associated proteins, are shown at the top.

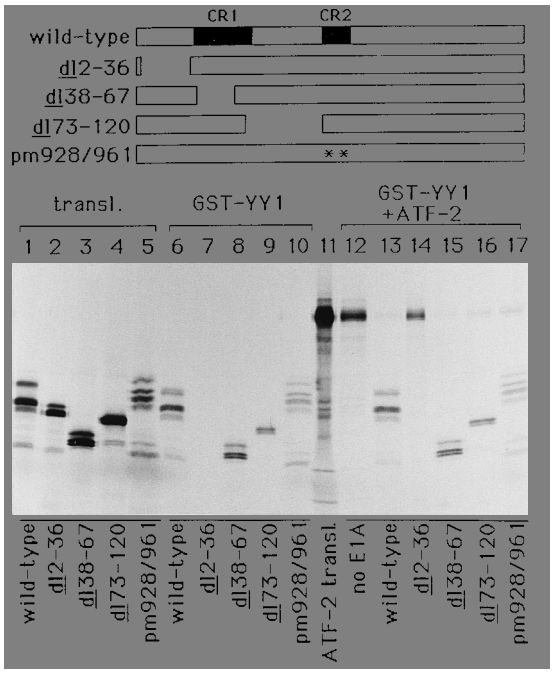

FIG. 5. The N-terminal domain of E1A$_{243}$ is required for binding to YY1 and for preventing binding of ATF-2 to YY1. Lanes: 1 to 5, 1 μl of wild-type and mutant ^{35}S-E1A$_{243}$ in vitro translation products (transl.); 6 to 10, binding of wild-type and mutant ^{35}S-E1A$_{243}$ (2 μl per reaction) to GST-YY1; 11, 2 μl of ^{35}S-ATF-2 in vitro translation products; 12 to 17, binding of ^{35}S-ATF-2 (2 μl per reaction) to GST-YY1 in the absence or presence of wild-type or mutant ^{35}S-E1A$_{243}$ (6 μl per reaction). The binding reaction products were washed and analyzed by SDS-PAGE. Schematic representations of wild-type and mutant E1A$_{243}$ proteins are shown at the top. Asterisks represent locations of the 928 and 961 point mutations that disrupt Rb and p107 binding.

Fig. 6A, only mutant *dl*2-36 was unable to prevent activation of the pm27fosCAT promoter by YY1-VP16, a result perfectly in line with the in vitro competition data presented in Fig. 5. For this experiment, expression of each of the E1A$_{243}$ derivatives was checked by Western blot analysis using the anti-E1A monoclonal antibody M73 (20). The level of each of the mutant proteins, including *dl*2-36, was comparable to that of wild-type E1A$_{243}$ (Fig. 6B). We conclude that the N-terminal 2–36 domain of E1A$_{243}$ prevents the interaction between YY1 and ATF/CREB at the c-*fos* promoter. Since the N-terminal domain is also required for activation of c-*fos* transcription (15, 17), we conclude that activation of c-*fos* transcription by E1A$_{243}$ involves a disruption of the ATF/CREB-YY1 interaction.

DISCUSSION

The ability of E1A$_{243}$ to prevent the ATF/CREB-YY1 interaction was found to depend on the same (or a significantly overlapping) N-terminal domain of E1A$_{243}$ that mediates direct binding to YY1. This finding, in conjunction with the fact that E1A$_{243}$ does not associate with ATF-2 in vitro (reference 31 and Fig. 1), leads us to conclude that prevention of ATF/CREB binding to YY1 is the result of a simple competition between E1A$_{243}$ and ATF/CREB for a limiting number of overlapping binding sites on YY1.

Two domains of YY1, spanning residues 54 to 260 and 331 to 414, are involved in binding to E1A$_{243}$ (30). We have shown previously that residues 331 to 414 of YY1 are sufficient for binding to ATFa2 in vitro (57), suggesting that this domain is the focal point for the competition between E1A$_{243}$ and ATF/CREB. Our previous studies also revealed that the bZIP domain of ATFa2 was sufficient for binding to YY1 in vitro (57). It remains to be determined if the bZIP domains of various ATF/CREB proteins share any structural features with the N-terminal domain of E1A$_{243}$ that might explain the ability of each protein to recognize the 331–414 region of YY1. Alternatively, ATF/CREB and E1A$_{243}$ may interact with overlapping, structurally distinct surfaces within the 331–414 domain of YY1.

The neighboring 282–330 region of YY1 is also capable of binding to ATFa2 in vitro, independently of the 331–414 domain (57). It is not known if this binding is blocked when E1A$_{243}$ is bound to the nearby 331–414 domain. Although we found substantially reduced binding of ATF-2 to GST-YY1 in the presence of E1A$_{243}$, we did not observe complete elimination of ATF-2 binding (Fig. 1 and 5 and data not shown). Assuming that the bZIP domains of ATF-2 and ATFa2 interact with YY1 in similar ways, this residual binding may be due to an interaction between ATF-2 and the 282–330 domain of YY1.

We have observed no evidence of trimolecular interactions among these proteins: if E1A$_{243}$, ATF-2, and YY1 formed a stable ternary complex, a decrease in ATF-2 association with the GST-YY1 beads would not be expected upon binding of ^{35}S-labeled E1A$_{243}$. Also, the presence of ATF-2 in the binding reactions was not required for E1A$_{243}$ binding to GST-YY1 (Fig. 1 and 5). This finding demonstrates that the ability of E1A$_{243}$ to enter into the complex with YY1 does not depend on an ATF/CREB protein and is consistent with an earlier report (30).

We also examined the effect of E1A$_{243}$ on the ATF/CREB-YY1 complex by the use of a promoter tethering assay, which we have used previously to demonstrate the interaction between YY1-VP16 and the c-*fos* promoter in living cells (57). The results with this assay were in striking agreement with

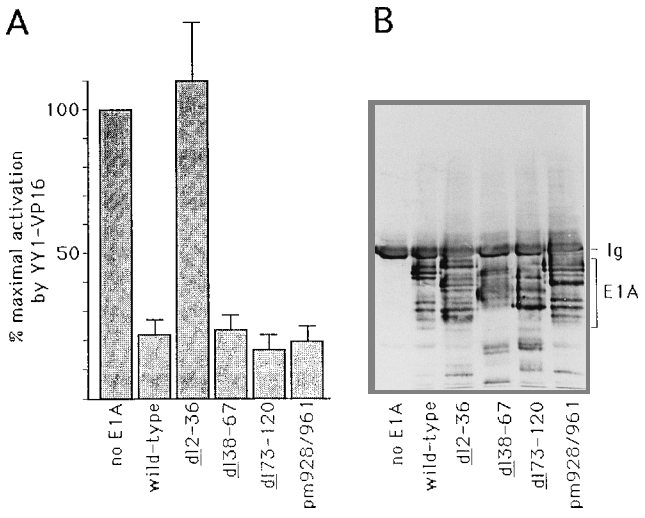

FIG. 6. The N-terminal domain of E1A$_{243}$ is required to reverse tethering of YY1-VP16 to the pm27fosCAT reporter. (A) HeLa cells were cotransfected with the pm27fosCAT reporter (5 μg), the YY1-VP16 expression plasmid (2.5 μg), and the indicated E1A$_{243}$ expression plasmids (5 μg) as described in Materials and Methods. After 48 h, cells were harvested and analyzed by the liquid scintillation CAT assay. Activation of pm27fosCAT by YY1-VP16 in the absence of E1A$_{243}$ is set at 100%. (B) Immunoprecipitation followed by Western blot analysis of wild-type and mutant E1A$_{243}$ proteins from extracts of transfected cells, using monoclonal antibody M73, was performed as described in Materials and Methods. Ig, immunoglobulin.

those from our in vitro experiments. YY1-VP16 that was bound to the promoter through the CRE (i.e., through an interaction with endogenous ATF/CREB) was displaced from the promoter by expression of $E1A_{243}$ (Fig. 2 and 3). As with the in vitro results, this effect depended exclusively on the N-terminal domain of $E1A_{243}$ (Fig. 6). Interestingly, DNA-bound YY1-VP16 was unaffected by expression of $E1A_{243}$ (Fig. 4A), despite the fact that an $E1A_{243}$-VP16 fusion protein could be shown to associate stably with DNA-bound YY1 (Fig. 4B). This finding strongly suggests that the large effect of $E1A_{243}$ on tethering of YY1-VP16 to the wild-type promoter, containing both the CRE and YY1 sites, was due solely to destabilization of the interaction between ATF/CREB and YY1-VP16 (Fig. 2A). The residual activation seen in this experiment (7.9-fold) was probably due to YY1-VP16 bound directly to the YY1 site, which is not affected by $E1A_{243}$. This interpretation is consistent with the fact that YY1-VP16 activates only weakly in the absence of the CRE (5.4-fold; Fig. 4A). This level of activation is similar to that seen with the wild-type promoter in the presence of $E1A_{243}$ (7.9-fold).

Together, these results allow us to propose a model for the role of the ATF/CREB-YY1 complex in $E1A_{243}$-dependent transcriptional activation of the c-*fos* gene. In the absence of $E1A_{243}$, YY1 represses transcription of the c-*fos* promoter in a manner that depends on an intact −67 CRE and the YY1 site at −54 (17, 38). Repression is effected, at least in part, by a specific interaction between YY1 and ATF/CREB (57). Binding of $E1A_{243}$ to YY1 leads to a stable association between these two proteins at the promoter and prevents the normal physical interaction between YY1 and ATF/CREB. This results in a relief of YY1-mediated transcriptional repression. It will be interesting to determine exactly which ATF/CREB family members in HeLa cells are capable of binding to the c-*fos* CRE in vitro and whether $E1A_{243}$ expression leads to an altered pattern of ATF/CREB and YY1 binding to the promoter. These issues are currently under investigation.

According to the model, disruption of the ATF/CREB-YY1 interaction is not sufficient to stimulate transcription, however. Activation also requires independent stimulation of the CRE-dependent pathway by cAMP. In support of this view, it is known that the −54 YY1 site acts as a negative transcriptional element only when the −67 CRE is intact (17, 38). In other words, removal of the −54 YY1 site does not result in relief of repression unless CRE-dependent transcription can also take place. This is consistent with our results regarding synergy between $E1A_{243}$ and cAMP in activation of the c-*fos* promoter in S49 cells (14, 15, 17) and in HeLa cells (56), in which $E1A_{243}$ has little or no effect on transcription of c-*fos* in the absence of cAMP signaling. Under this view, disruption of the ATF/CREB-YY1 complex by $E1A_{243}$ and stimulation of CRE-dependent transcription are distinct, independent events, and transcriptional activation can take place only if both events occur (i.e., there is synergy). This conclusion is also supported by the fact that $E1A_{243}$ is able to block tethering of YY1-VP16 to the c-*fos* promoter equally well in the absence or the presence of forskolin, an inducer of cAMP (56). If disruption of the ATF/CREB-YY1 complex and transcriptional activation were necessarily coupled, the ability of $E1A_{243}$ to disrupt the ATF/CREB-YY1 interaction might have been expected to depend on cAMP.

Results presented in Fig. 4B demonstrate that an $E1A_{243}$-VP16 fusion protein can associate with the pm28fosCAT reporter (functional YY1 site but no −67 CRE) in a manner that strictly depends on the YY1 DNA-binding site. The simplest interpretation of this experiment is that $E1A_{243}$-VP16 (and therefore native $E1A_{243}$) can form a stable complex with YY1 at this promoter. This raises the question of whether $E1A_{243}$, when bound to pm28fosCAT through an interaction with YY1, can alter transcription simply by virtue of its presence at the promoter. We demonstrated previously that in the absence of the −67 CRE, the −54 YY1 site is not sufficient to mediate a response to $E1A_{243}$, either in the presence or in the absence of cAMP (17). This finding suggests that whereas $E1A_{243}$ can form a stable complex with YY1 at this promoter, this is not sufficient to trigger transcriptional activation in the absence of the −67 CRE and associated factors.

There are additional aspects to the mechanism of activation of the c-*fos* gene by $E1A_{243}$. First, the N-terminal domain alone is not sufficient for triggering activation. Activation also requires amino acid residues within CR1, which together with the N-terminal domain are known to constitute a binding site for the cellular protein p300. Therefore, whereas disruption of the ATF/CREB-YY1 complex by the N-terminal domain is clearly involved in activation by $E1A_{243}$, it probably does not represent the entire story. Additionally, the fact that activation of the c-*fos* promoter by $E1A_{243}$ requires the −67 CRE (17) suggests a role for p300 and/or the related protein CBP, since both can function as coactivators of CREB-dependent transcription and both can bind to E1A (2, 34). Interestingly, whereas $E1A_{243}$ has been shown in some experiments to repress p300- and CBP-dependent transcription, $E1A_{243}$ activates transcription of the c-*fos* promoter in a CRE-dependent manner. Therefore, if p300 or CBP plays an important role in $E1A_{243}$-dependent activation of c-fos, it probably does so through a novel mechanism.

With this report, there are now three types of transcriptional repressor-activator complexes that are disrupted by the adenovirus $E1A_{243}$ protein. In addition to the ATF/CREB-YY1 complex described here, $E1A_{243}$ is known to disrupt the Rb (p107, p130)-E2F complex (12) and also the Dr1-TBP complex (24). Interestingly, the Dr1-TBP complex is also targeted by the N-terminal domain of $E1A_{243}$ (24). Thus, a common theme emerges in the mechanism of action of $E1A_{243}$, involving activation of transcription through disruption of activator-repressor complexes. It will be important to investigate further the role of the ATF/CREB-YY1 complex in cellular transformation by $E1A_{243}$ and in normal cellular regulation.

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