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Adenovirus E1A Directly Targets the E2F/DP-1 Complex▽

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Deregulation of the cell cycle is of paramount importance during adenovirus infection. Adenovirus normally infects quiescent cells and must initiate the cell cycle in order to propagate itself. The pRb family of proteins controls entry into the cell cycle by interacting with and repressing transcriptional activation by the E2F transcription factors. The viral E1A proteins indirectly activate E2F-dependent transcription and cell cycle entry, in part, by interacting with pRb and family members to free the E2Fs. We report here that an E1A 13S isoform can unexpectedly activate E2F-responsive gene expression independently of binding to the pRb family of proteins. We demonstrate that E1A binds to E2F/DP-1 complexes through a direct interaction with DP-1. E1A appears to utilize this binding to recruit itself to E2F-regulated promoters, and this allows the E1A 13S protein, but not the E1A 12S protein, to activate transcription independently of interaction with pRb. Importantly, expression of E1A 13S, but not E1A 12S, led to significant enhancement of E2F4 occupancy of E2F sites of two E2F-regulated promoters. These observations identify a novel mechanism by which adenovirus deregulates the cell cycle and suggest that E1A 13S may selectively activate a subset of E2F-regulated cellular genes during infection.

Cells have multiple checkpoints to ensure that aberrant DNA replication does not occur and to ascertain that the necessary requirements have been met before entering the cell cycle. Efficient viral replication within a cell typically requires that all relevant pathways that could lead to abortive infection have been closed. Indeed, to ensure a productive replicative cycle, many viruses encode one or more proteins that target and block such cellular checkpoints.

Adenovirus normally infects noncycling cells, which are poor hosts for viral replication. Consequently, these viruses have evolved proteins that force the host cell into the cell cycle and induce the expression of the cellular biosynthetic machinery and substrates that are required for efficient production of viral progeny. The initiators and the primary executors of cell cycle modulation in adenovirus-infected cells are the E1A proteins. These proteins are the first viral gene products expressed during adenovirus infection. Alternative splicing generates five E1A mRNAs during infection by human adenovirus 5. Two major products are the 13S and 12S mRNAs, which encode proteins of 289 and 243 amino acids, respectively (2, 3). There are four regions within the 13S-encoded E1A protein that are highly conserved among the different serotypes of human and simian adenoviruses, and these are referred to as conserved region 1 (CR1) to CR4 (1, 3).

Immunoprecipitation of E1A-bound proteins with anti-E1A antibodies detected a variety of coprecipitating cellular polypeptides ranging in size from 30 to 400 kDa (2). The first of these proteins to be identified was pRb, a key regulator of exit from the G_1 phase of the cell cycle (33). E1A similarly targets p107 and p130, two other pRb family members (2). Overexpression of pRb induces cells to arrest in G_1 (15), and this is most closely correlated with its ability to repress activation from E2F-responsive elements (6). E2F is a heterodimeric transcription factor containing one of six E2Fs and one of three DP family members (11, 17). Two other members of the E2F family, E2F7 and E2F8, are somewhat different in that they do not dimerize with DP proteins (17). Individual E2F family members may function primarily as activators or repressors of transcription, and they control the expression of genes required for cell cycle progression (4, 8, 11, 20, 31). The main regulator of pRb binding to E2F is phosphorylation of pRb (13). Hyperphosphorylation of pRb inhibits its binding to E2F and leads to stimulation of cell proliferation. The region of E1A that is responsible for binding to pRb and inhibiting its interaction with E2Fs is the CR2 domain, with a portion of the CR1 domain stabilizing the interaction and displacing E2Fs (2). E1A 12S overcomes the repression of E2F-regulated genes to force quiescent cells to enter the cell cycle by eliminating corepressor complexes consisting of p130-E2F4 and HDAC1/2-mSin3B from the promoters of E2F-regulated genes (29). Once these repressive complexes are removed, the promoters become occupied by activating E2F family members, which stimulate transcription (29). In summary, E1A interacts with several pRb family members in order to inhibit their interaction with E2Fs and induce cell cycle entry, as well as to activate

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a variety of cellular and viral genes. Interference with pRb function is only one of several mechanisms by which E1A activates the cell cycle (2), and it is possible that not all means by which E1A initiates the cell cycle are known or well understood.

Recently, it was shown that HPV E7 and adenovirus E1A can interact with E2F6 (21). We were interested in determining whether other classical E2Fs can bind E1A and how this influences E2F function independently of the interaction of E1A with pRb. Furthermore, as the influence of E1A on E2F function has largely been elucidated by using E1A 12S, we wanted to determine whether there are differences when E1A 13S is present. In the present study, we show that E1A 13S directly binds to E2F/DP-1 complexes, likely via association with DP-1. This binding results in activation of E2F-regulated genes, with E2F4 and E2F5 being activated the most strongly. Activation is restricted largely to E1A 13S, while E1A 12S has a minimal effect. We also show that E1A is directly recruited to E2F-regulated promoters during viral infection and that E1A 13S, but not E1A 12S, can enhance E2F4 promoter occupancy. These results add to our understanding of how E1A can deregulate the cell cycle and highlight important differences between how E1A 12S and E1A 13S function.

MATERIALS AND METHODS

Cell lines, tissue culture, and viruses. IMR-90, U2OS, and HeLa cells were grown in Dulbecco's modified Eagle medium (DMEM; Invitrogen) supplemented with 10% fetal bovine serum (FBS; Invitrogen), streptomycin, and penicillin.

HeLa cells were infected with a multiplicity of infection (MOI) of 10 with the indicated viruses. *dl*309 expresses both E1A 13S and 12S, *pm*975 expresses only E1A 13S, and *dl*520 expresses only E1A 12S. Roughly 5×10^6 cells were infected with each virus. Infections were carried out for 1 h in serum-free medium, after which 10 ml of complete medium was added and the cells were incubated for an additional 16 h. We arrested the growth of IMR-90 cells by allowing them to become confluent for at least 72 h and serum starving them in 0.2% FBS DMEM.

Stable cell lines with the integrated E2F reporter were made by cotransfection of U2OS cells with 1 μg of pGL-E2F-Luc reporter plasmid and 9 μg of a fragment of the pcDNA3.1-Hygro plasmid encoding the hygromycin resistance marker. Cells were selected in hygromycin, and a stable pool was used for subsequent experiments in order to avoid clonal variation.

E1A, E2F, and DP-1 expression vectors and protein purification. E1A12S and E1A13S cDNAs were cloned into the pcDNA3 mammalian expression vector (Invitrogen), while the *dl*1100 series of 13S and 12S E1A deletion mutants were cloned into pcDNA3.1 (Invitrogen). Enhanced green fluorescent protein (EGFP) fusions of E1A fragments were generated by cloning the specific fragments into the pEGFP-C1 expression vector (Clontech) in frame with the N-terminal EGFP. Hemagglutinin (HA)-tagged DP-1 expression vectors were described previously (34). Fusion of DP-1 with glutathione *S*-transferase (GST) was done by subcloning the cDNA into pGEX-4T1 (GE Healthcare Life Sciences) in frame with the N-terminal GST tag. His-tagged E1A 13S and E2F1 were made by subcloning the entire E1A 13S cDNA or E2F1 cDNA into the pET42 vector (Novagen) in frame with a C-terminal 6×His tag. Proteins were expressed in *Escherichia coli* strain BL21 and purified on their respective resins according to the manufacturer's specifications. GST pulldown assays were carried out as previously described (25).

Transfection and reporter assay. Twenty-four hours prior to transfection, U2OS cells were seeded into six-well dishes (Sarstedt) at a density of 100,000 per well in DMEM. U2OS cells were transfected with 1 μg of the reporter plasmid (pGal6-Luc) and 0.5 μg of the transactivator pCMV-E2F and pCMV-DP-1. Transfections were carried out using the Superfect reagent (Qiagen) according to the manufacturer's instructions. Luciferase activity was assayed 24 h after transfection in U2OS cells using the Promega Luciferase Reporter kit according to the manufacturer's instructions. Luciferase activity was normalized to both transfection efficiency using beta-galactosidase activity and protein levels.

Immunoprecipitations. For immunoprecipitation, transfected HeLa cells were lysed in NP-40 lysis buffer (0.5% NP-40, 50 mM Tris [pH 7.8], 150 mM NaCl) supplemented with protease inhibitor cocktail. One milligram of the cell lysate was used for immunoprecipitation with anti-E1A monoclonal antibody M73 (33). E1A was detected using the M73 monoclonal antibody, while E2F and DP-1 were detected using anti-HA rat monoclonal antibody (clone 3F10; Roche). Endogenous DP-1 was detected using WTH24 mouse hybridoma supernatant. Endogenous E2F4 was detected using a custom rabbit antibody.

ChIP and reprecipitation. Chromatin immunoprecipitation (ChIP) was carried out as previously described (25). Briefly, contact-inhibited, arrested IMR-90 cells were infected with the indicated adenoviruses and harvested 24 h after infection for ChIP analysis. For immunoprecipitation of E1A, the M73 and M58 monoclonal antibody cocktail was used. For E2F4 ChIPs, a custom polyclonal rabbit anti-E2F4 antibody was utilized. Mouse anti-rabbit antibody was used as a negative IgG control (Sigma). Re-ChIPs were carried out by eluting the primary ChIP product with 10 mM dithiothreitol for 15 min three times, pooling the eluates, and diluting them 10-fold in the original dilution buffer.

PCRs were carried out using 1× iQ-SYBR green SuperMix (Bio-Rad) according to the manufacturer's directions using 2% of the total ChIP DNA as the template according to the manufacturer's instructions and a MyiQ real-time PCR instrument (Bio-Rad) or a Life Technologies ABI 7900HT sequence detector. The annealing temperature used was 60°C, and 40 cycles were run.

Real-time expression analysis. Contact-inhibited and arrested IMR-90 cells were infected with *dl*309, *dl*520, *pm*975, E1A 13S Δ2-11, or the virus with E1 deleted, and 36 h after infection, total cellular RNA was extracted using the TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Five micrograms of total RNA was used in a reverse transcriptase reaction with SuperScript II reverse transcriptase (Invitrogen) according to the manufacturer's guidelines, using random hexanucleotides for priming. The cDNA was subsequently used for real-time expression analysis using an Applied Biosystems ABI 7900HT sequence detector. Fold changes in expression were determined by comparing expression levels with those obtained in infections with the virus with E1 deleted and analyzing the expression data using Data Assist software (Life Technologies).

BrdU incorporation assay. Arrested IMR-90 human primary fibroblasts were pulsed with 30 μM bromodeoxyuridine (BrdU; Sigma) for 1 h at 16 or 24 h after virus infection. Cells were fixed in 4% paraformaldehyde for 10 min at room temperature. Cells were permeabilized with 0.2% Triton X-100 in phosphate-buffered saline (PBS), washed three times in PBS-Tween, and blocked for 1 h in blocking buffer (1% normal goat serum, 1% bovine serum albumin, and 0.2% Tween 20 in PBS). Primary anti-BrdU mouse monoclonal antibody (Sigma) was diluted in blocking buffer containing 5 mM $MgCl_2$ and 50 U/ml DNase I and incubated on cells at 37°C for 80 min. The cells were then washed three times in PBS-Tween and incubated in an anti-mouse–Alexa Fluor 488 secondary antibody (Invitrogen) for 30 min at 37°C. Cells were washed three times in PBS-Tween and mounted using ProLong Gold mounting medium with 4′,6-diamidino-2-phenylindole (DAPI; Invitrogen). Images were acquired on a Zeiss LSM510 laser scanning confocal microscope. Three random low-power fields of view were obtained (20× objective lens), and total nuclei were determined using DAPI staining.

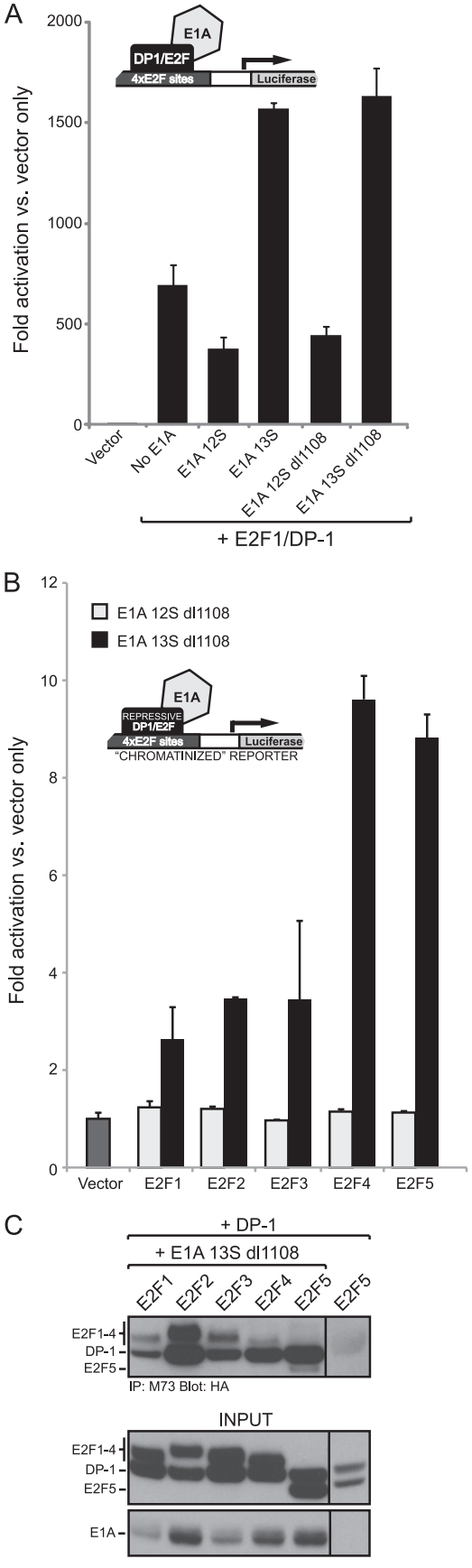
Virus generation. Mutant viruses encoding E1A 13S Δ2-11 or E1A 12S Δ2-11 Y47H/C124G were generated by subcloning a fragment of E1A from the ATG codon to the XbaI site in exon 2 into pXC1-ΔE1A at the EcoRI/XbaI sites. Either plasmid was then cotransfected with pJM17 into low-passage 293 cells. After a complete cytopathic effect was observed on the plate, virus was harvested by freeze-thawing and plaque purified to obtain pure mutant virus, which was subsequently used in infection experiments.

Virus growth assay. IMR-90 fibroblasts were growth arrested by contact inhibition and infected with *pm*975 or *dl*520 at an MOI of 5. Virus was adsorbed for 1 h at 37°C in 5% CO_2, after which the medium was removed and cells were washed five times with PBS. Cells were bathed in new medium and reincubated at 37°C in 5% CO_2. Supernatants were collected 48 and 120 h after infection, and plaque assays were performed on 293 cells by serial dilution.

E2F4 antibody generation. To generate a polyclonal anti-E2F4 antibody, a peptide corresponding to the 15 C-terminal residues of human E2F4 (SEGVC DLFDVPVLNL) was used to immunize rabbits. The antibody was subsequently affinity purified on a peptide column.

RESULTS

E1A 13S activates E2F genes in a pRb-independent manner. Previously, it was reported that E1A can interact with E2F6 (21). We were intrigued by this observation and wanted to determine whether an interaction between E1A and E2Fs had



a direct effect on E2F-mediated transactivation independently of E1A binding to pRb. It was necessary to discriminate between E1A's association with E2Fs and its association with pRb, because the latter indirectly affects E2F-mediated transcriptional activation. In order to eliminate pRb binding to E1A, we utilized an E1A deletion mutation (*dl*1108) that disrupts the highly conserved LxCxE pRb-binding motif. This mutation largely abrogates E1A binding to pRb (and other pocket proteins) (14). Intriguingly, we observed that E1A 13S and E1A 13S *dl*1108 were both equally efficient in inducing expression from a transiently transfected E2F-regulated luciferase reporter in asynchronously dividing U2OS cells (Fig. 1A), whereas E1A 12S and E1A 12S *dl*1108 did not induce activation of this reporter. A similar result was observed with the other E2Fs tested (E2F1 to E2F5). In each case, E1A 13S potently activated expression, whereas E1A 12S moderately repressed this reporter (data not shown). These results clearly demonstrate that E1A 13S, but not E1A 12S, can stimulate E2F-mediated transcriptional activation independently of binding to the pocket proteins.

In order to determine whether the effect observed with a plasmid reporter system occurred in a more physiological setting, we engineered a cell line (U2OS-E2F) harboring an integrated pGL-E2F reporter in the cellular chromatin. For these experiments, a drug-resistant pool was used in order to minimize the effects of clonal variation. To determine the level of activation from individual E2Fs, cells were cotransfected with the indicated E2Fs, DP-1, and the indicated E1A constructs (Fig. 1B). In this context, E1A 13S *dl*1108, but not E1A 12S *dl*1108, activated the integrated reporter when an E2F was present. This suggests that E1A 13S, but not 12S, can activate E2F-responsive promoters independently of its role in interference with pRb function. Intriguingly, when the two repressive E2Fs (E2F4 and E2F5) were individually cotransfected with E1A, a "superactivation" was observed that was greater than double that observed using E2F1, E2F2, or E2F3. This shows, for the first time, that E1A 13S can directly and specifically influence the expression of genes that are being transcriptionally silenced by repressive E2F4 or E2F5. In effect,

FIG. 1. E1A 13S induces pRb-independent activation of E2F-mediated transcription and binds E2F/DP-1 complexes. (A) U2OS cells were cotransfected with the reporter plasmid pGL-E2F (having four synthetic consensus E2F binding sites) together with E2F1 and DP-1 and the indicated E1A constructs or the vector alone. Luciferase assays were performed 48 h after transfection, and results were normalized to the protein concentration. Data are plotted as the *n*-fold increase over the reporter alone. (B) A U2OS cell line harboring an integrated ("chromatinized") pGL-E2F reporter was cotransfected with the indicated E2Fs together with DP-1 and either E1A 12S *dl*1108 or E1A 13S *dl*1108. Luciferase activity was measured 48 h after transfection and is represented as *n*-fold activation versus that in cells transfected only with empty plasmids (vector = 1). (C) HeLa cells were transfected with plasmids expressing HA-tagged E2F1 to E2F5, HA-tagged DP-1, and E1A 13S *dl*1108 as indicated. Forty-eight hours after transfection, cells were lysed and immunoprecipitations for E1A were carried out using M73 anti-E1A monoclonal antibody. Immunoprecipitates were resolved on SDS-PAGE, and associated E2F/DP-1 complexes were detected by Western blotting using anti-HA antibody (3F10). Inputs of E2Fs, DP-1, and E1A are shown.

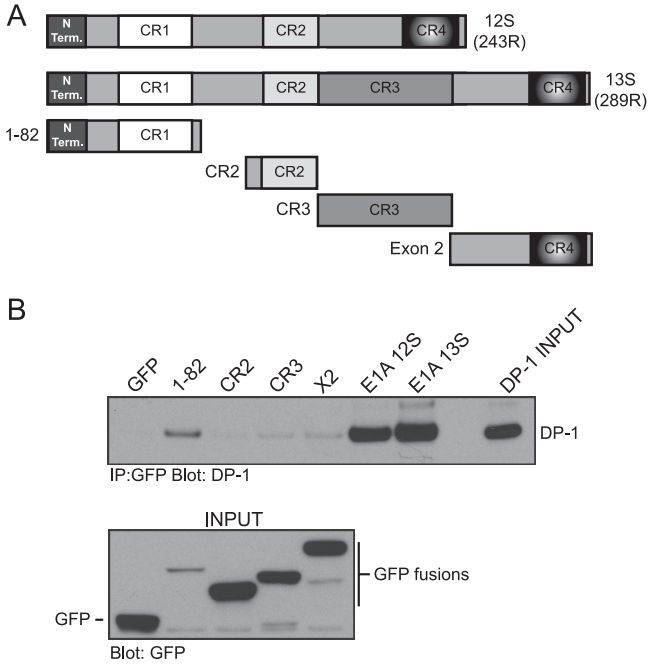


FIG. 2. E1A N terminus binds to DP-1. (A) Schematic representation of E1A-GFP fusion fragments used in the experiment. (B) HeLa cells were cotransfected with the indicated GFP-E1A fragment fusions or with E1A 12S or E1A 13S together with HA-tagged DP-1. Either GFP or E1A (in the cases of E1A 12S and E1A 13S) was immunoprecipitated (IP) from cell lysates and resolved by SDS-PAGE, and associated DP-1 was detected using anti-HA antibody (3F10). Inputs of DP-1 and GFP fusion proteins are shown.

E1A 13S appears to be turning a transcriptional repressor into an activator in this specific context.

Because of the observed differences in the level of transcriptional activation between "activating" E2F1 to E2F3 and "repressive" E2F4 and E2F5, we wanted to determine whether E1A could bind to the different E2F complexes and whether the observed differences were due to different affinities for distinct E2F/DP-1 heterodimers. To test this, we transfected HeLa cells with E2F1 to E2F5, DP-1, and E1A 13S *dl*1108 and carried out coimmunoprecipitations 48 h after transfection (Fig. 1C). E1A was found to bind to all E2F/DP-1 complexes. Unexpectedly, binding to E2F4/DP-1 and E2F5/DP-1 was the weakest and binding to DP-1 was surprisingly strong. In fact, DP-1 was consistently bound by E1A at an apparent higher stoichiometry than the corresponding E2F, suggesting that E1A interacts with DP-1 independently of the E2F subunit.

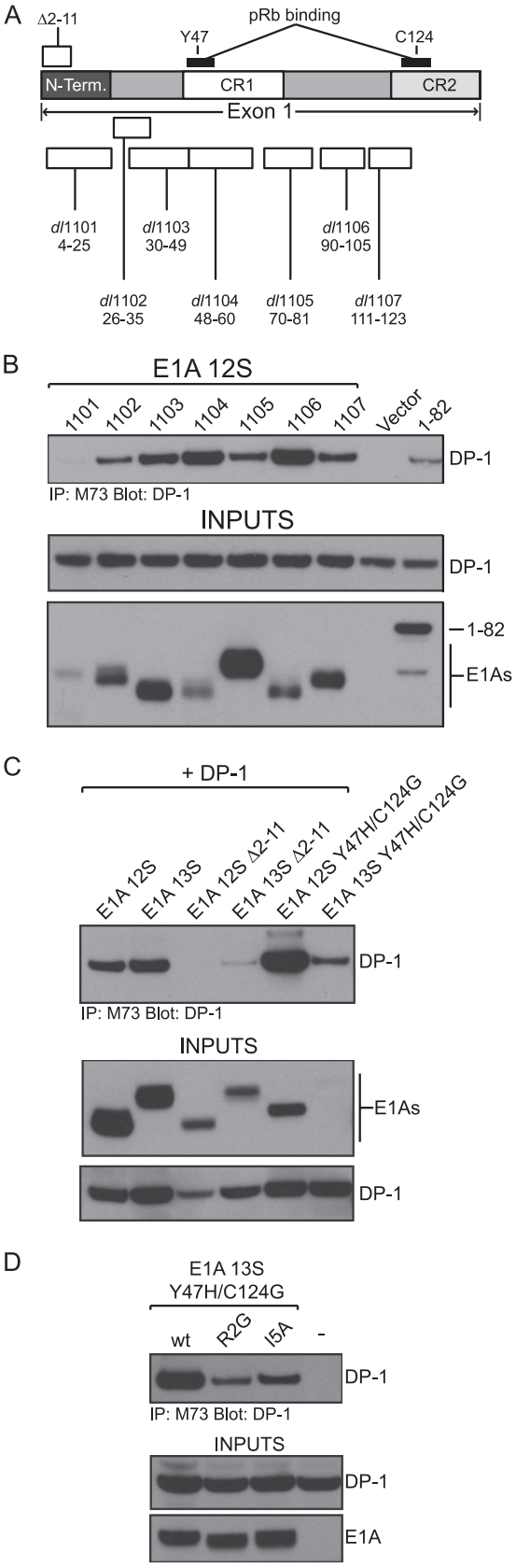
Together, these results show that E1A 13S, but not E1A 12S, can activate transcription from E2F-regulated genes in a pRb-independent manner. Furthermore, these results suggest that E1A binds to DP-1 better than any of the E2Fs.

E1A binds to DP-1 via the N terminus. To identify the area of E1A required for binding to DP-1, we carried out coimmunoprecipitation experiments with GFP fusions of E1A fragments corresponding to the N terminus (residues 1 to 82), CR2 (residues 93 to 139), CR3 (residues 139 to 204), and the exon 2-encoded region containing CR4 (residues 187 to 289). Only the N-terminal portion of E1A was capable of interacting with DP-1 strongly (Fig. 2B). Indeed, DP-1 was readily detected in immunoprecipitations despite the relatively low levels of GFP–E1A-1-82 expression. In addition, a strong association of full-length E1A 12S and E1A 13S with DP-1 was detected. As expected, no interaction between GFP alone and DP-1 was detected (Fig. 2B).

In order to further define the minimal region required for DP-1 binding, a panel of N-terminal E1A deletion mutant forms (16) was used in coimmunoprecipitation experiments (Fig. 3A). Binding to DP-1 was abrogated only when E1A residues 4 to 25 were deleted (Fig. 3B). All other mutant forms showed largely normal DP-1 binding (Fig. 3B). As *dl*1101 deletes a relatively large portion of E1A (residues 4 to 25), we wanted to further narrow down the binding region. For this purpose, we constructed a mutant form by removing residues 2 to 11. E1A 12S Δ2-11 lost all ability to bind to DP-1. Similarly E1A 13S Δ2-11 was also largely deficient in binding (Fig. 3C). Importantly, a double point mutant form of E1A (Y47H and C124G) that is completely devoid of pRb binding (32) was still able to efficiently and detectably coimmunoprecipitate DP-1 (Fig. 3C). Deletion of residues 2 to 11 also impacts p300/CBP binding; therefore, it was necessary to distinguish DP-1 binding from the p300/CBP interaction. Two point mutant forms of E1A (R2G and I5A) that no longer bind to p300 *in vivo* were still found to detectably interact with DP-1 in the context of the Y47H and C124G background (Fig. 3D). These results demonstrate that E1A interacts with DP-1 via the N terminus, with residues 2 to 11 being essential for binding, and further show that this interaction occurs independently of the ability of E1A to associate with the pRb family of proteins or p300/CBP.

Residues 1 to 102 of DP-1 are required for binding to E1A. In order to determine the region of DP-1 bound by E1A, we used a series of deletion mutant forms of DP-1 (Fig. 4A) (34). Deletions within the DNA-binding (residues 103 to 126) or dimerization (residues 232 to 272) domain had no effect on the interaction between DP-1 and E1A 12S or E1A 13S (Fig. 4B, left panel). Deletion of the C terminus of DP-1 up to residue 233 did not reduce binding compared to that of full-length DP-1. Indeed, several deletion mutant forms bound E1A substantially better (Fig. 4B, right panel). However, the deletion of N-terminal amino acids 1 to 127 completely abolished the interaction between DP-1 and E1A and any further deletions from the N terminus also produced a failure to bind. It should be noted that the nature of the prominent band above the main DP-1 band in the coimmunoprecipitation between E1A and DP-1 amino acids 1 to 346 is unknown, but the lack of this band in any other immunoprecipitation lanes suggests that it is an artifact of this truncated mutant form of DP-1. Nevertheless, these results indicate that the N terminus of DP-1, comprising the first 102 amino acids, is required for binding with E1A.

To determine whether E1A bound to endogenous DP-1 during viral infection, normal human lung fibroblast IMR-90 cells were infected with viruses expressing wild-type E1A 12S, E1A 12S Δ2-11 Y47H/C124G, or E1A 13S (Fig. 5A). E1A 12S was readily able to coprecipitate associated DP-1 (Fig. 5A, lanes 3 and 4), whereas the triple mutant form E1A 12S Δ2-11 Y47H/C124G failed to bind to DP-1 (Fig. 5A, lanes 5 and 6). This result agrees with our earlier observations showing that the N terminus of E1A was critical for DP-1 binding (Fig. 2 and 3). E1A 13S also coprecipitated DP-1; however, the amount was less than that obtained with E1A 12S. This was most likely



due to the lower level of expression of E1A 13S during infection (Fig. 5A, compare E1A input lanes 3 and 4 with lanes 7 and 8). Furthermore, E1A was able to bind to endogenous E2F4 in the same cells following viral infection (Fig. 5B) but this binding was completely lost when residues 2 to 11 were deleted along with mutations in the pRb-binding regions (Y47H and C124G). To our knowledge, this represents the first *in vivo* demonstration that E1A can stably interact with E2F4 or DP-1.

Together, these results show that E1A bound to the N terminus of DP-1 and this occurred during normal viral infection.

E1A 13S binds directly to the DP-1/E2F1 complex and DP-1 alone. E2F/DP-1 heterodimers are intricately regulated and form complexes with many other binding partners (11), which could be mediating the interaction with the N terminus of E1A. Therefore, it was important to determine whether E1A directly targets DP-1 and/or the E2F/DP-1 complex. This is particularly important from the viewpoint of E1A, since the N terminus of E1A associates with many transcriptional coregulators, some of which also bind to E2F/DP-1 complexes (24). Our use of the *dl*1107, *dl*1108, and Y47H/C124G mutants in the work presented here allowed us to rule out the pRb family of proteins (pRb, p107, and p130) as potential mediators of this interaction. Furthermore, p300 and CBP were ruled out as potential mediators of the interaction between E1A and DP-1 by the use of R2G and I5A point mutant E1As. To determine whether E1A directly associates with DP-1, we used a GST pulldown approach with purified recombinant proteins (Fig. 6). GST, GST–DP-1/E2F1-6×His complex, and GST–DP-1 were used to determine whether they could pull down purified recombinant E1A 13S using glutathione Sepharose beads. As expected, GST alone was not capable of detectable interaction with E1A 13S *in vitro*. However, the recombinant DP-1/E2F1 complex or DP-1 alone detectably pulled down E1A 13S, demonstrating that this interaction is direct. Interestingly, there was a substantial difference between the level of E1A 13S pulled down by the DP-1/E2F1 heterodimer and that pulled down by DP-1 alone. Nevertheless, these results clearly show that E1A 13S directly interacts with the DP-1/E2F1 complex and DP-1 alone *in vitro*.

E1A is recruited to E2F-regulated promoters and selectively activates gene expression. Our data suggest that E1A 13S, but

FIG. 3. E1A residues 2 to 11 are required for pRb-independent association with DP-1. (A) Schematic representation of *E1A* exon 1 showing the mutant forms used in the study. N-Term., N terminus. (B) HeLa cells were cotransfected with vectors expressing the indicated E1A 12S deletion variants and HA–DP-1. Cells were lysed 48 h after transfection, and immunoprecipitations (IP) were carried out using anti-E1A antibody (M73). Associated DP-1 was detected using anti-HA antibody (3F10). Input levels are indicated. (C) HeLa cells were cotransfected with vectors expressing the indicated E1A proteins and HA–DP-1. Cells were lysed 48 h after transfection, and E1A immunoprecipitations were carried out using anti-E1A antibody (M73). Associated DP-1 was detected using anti-HA antibody (3F10). Input levels are indicated. (D) HeLa cells were cotransfected with vectors expressing the indicated E1A proteins and HA–DP-1. Cells were lysed 48 h after transfection, and E1A was immunoprecipitated with anti-E1A antibody (M73). Associated DP-1 was detected using anti-HA antibody (3F10). Input levels are shown. wt, wild type.

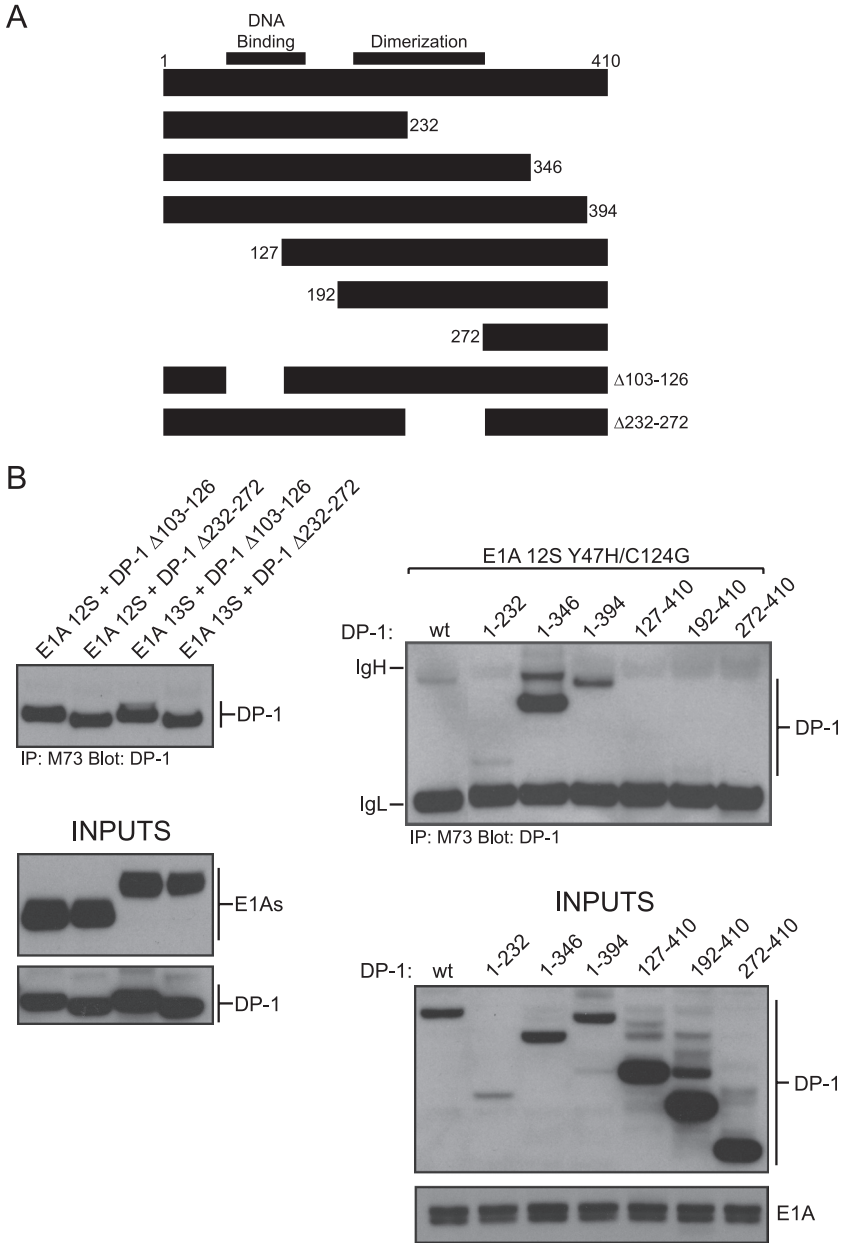


FIG. 4. DP-1 N terminus is required for association with E1A. (A) Schematic representation of DP-1 and deletion mutant forms used in this study. (B, left) HeLa cells were cotransfected with either E1A 12S or E1A 13S and the indicated deletion mutant forms of DP-1. Cells were lysed 48 h after transfection, and E1A was immunoprecipitated (IP) using anti-E1A antibody (M73). Associated DP-1 was detected using anti-HA antibody (3F10). (B, right) HeLa cells were cotransfected with the indicated deletion mutant forms of DP-1 and the Y47H/C124G double mutant form of E1A 12S that is unable to bind to pRb. Cells were lysed 48 h after transfection, and E1A was immunoprecipitated using anti-E1A antibody (M73). Associated DP-1 was detected using anti-HA antibody (3F10). Inputs are shown for all immunoprecipitations. wt, wild type.

not E1A 12S, could specifically activate E2F-responsive promoters (Fig. 1A). Interestingly, when the E2F reporter was integrated into cellular chromatin, E1A 13S led to enhanced reporter activation with the two repressive E2Fs, E2F4 and E2F5 (Fig. 1B). This observation suggests that E1A 13S may specifically enhance expression from E2F-regulated promoters, particularly those that are being repressed by the silencing E2F complexes. To address this, we performed ChIP, followed by real-time PCR quantification, in order to determine the occupancy of E1A and E2F4 on the *PCNA* and *MCM4* promoters during viral infection of arrested IMR-90 cells. The *PCNA* and *MCM4* promoters were chosen because both are regulated by E2Fs and it was previously reported that the *PCNA* promoter is occupied by repressive E2F4 (7, 9, 27) whereas *MCM4* is not. We infected G_0/G_1-arrested IMR-90 cells with viruses that expressed no E1A (E1 deleted), E1A 12S only (*dl*520), E1A 13S only (*pm*975), or wild-type genomic E1A (expresses all isoforms; *dl*309) and performed ChIPs and re-ChIPs 24 h after infection (Fig. 7A and B). Both E1A isoforms were found to occupy the *PCNA* promoter, with E1A 13S

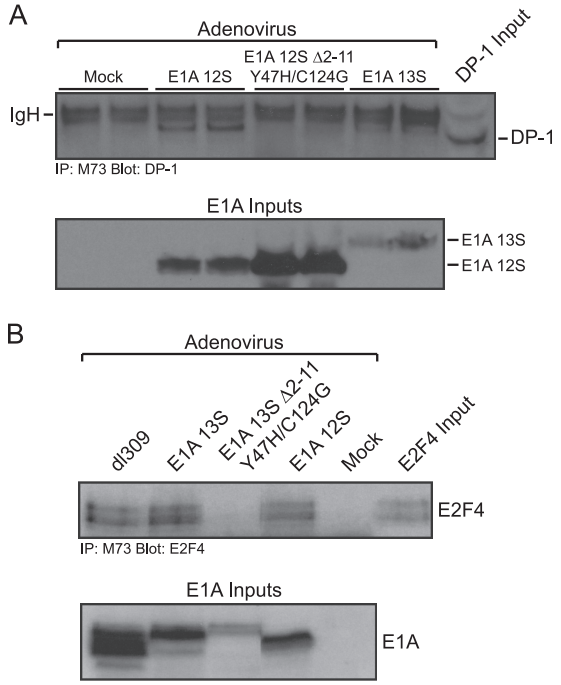


FIG. 5. E1A binds endogenous DP-1 during viral infection. (A) IMR-90 cells were infected with adenoviruses expressing the indicated E1A isoforms (no E1A [Mock], E1A 12S, E1A 12S Δ2-11 Y47H/C124G, or E1A 13S). Twenty-four hours after infection, cells were lysed and E1A was immunoprecipitated (IP) using anti-E1A (M73) antibody. Associated DP-1 was detected using anti-DP-1 antibody (WTH24). Each experiment was done twice from two independent infections. Inputs of DP-1 and E1As are shown. (B) IMR-90 cells were infected with adenoviruses expressing the indicated E1A isoforms (no E1A [Mock], wild-type genomic E1A [*dl*309], wild-type E1A 13S, or E1A 13S Y47H/C124G Δ2-11). Twenty-four hours after infection, the cells were lysed and E1A was immunoprecipitated using anti-E1A (M73) antibody. Associated endogenous E2F4 was detected using a C-terminal E2F4 antibody. Inputs of E2F4 and E1A are shown.

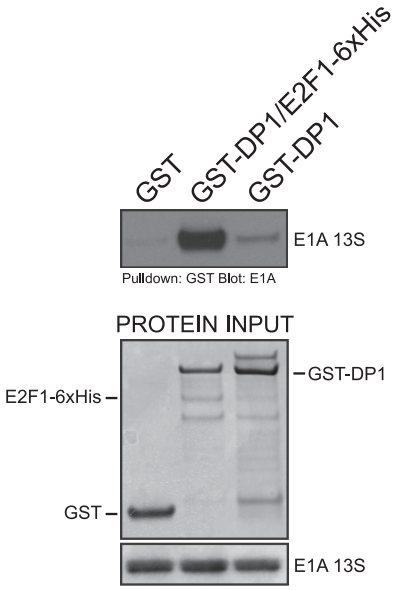


FIG. 6. E1A 13S binds directly to DP-1/E2F1 and DP-1 alone. GST pulldown assays were carried out with the purified DP-1/E2F1 complex and purified E1A 13S or DP-1 and E1A 13S alone. GST was used as a negative control. E1A pulled down by DP-1/E2F1 or DP-1 alone was detected using anti-E1A (M73) antibody. Inputs are shown.

having the highest level of occupancy (Fig. 7A, left panel). The occupancy of E1A at the *MCM4* promoter was quite different from what we observed with the *PCNA* promoter. Unlike at the *PCNA* promoter, E2F4 was not previously reported to be present at the *MCM4* promoter (7, 9, 27). During infection with virus expressing E1A 13S alone (*pm*975), E1A was present at the *MCM4* promoter at a relatively high level (~8% of the input; Fig. 7B, left panel). During infection with virus expressing E1A 12S alone (*dl*520), the level of occupancy by E1A was considerably lower, although it was well above the background level. Furthermore, there was low overall E1A occupancy at the *MCM4* promoter when E1A 12S and E1A 13S were coexpressed. Our results with E1A occupancy at the *PCNA* and *MCM4* promoters suggest that E1A 13S is efficiently gaining access to these promoters perhaps via the repressive E2Fs. To investigate this possibility, we performed ChIP for E2F4, followed by re-ChIP for E1A (Fig. 7A and B, middle and right panels). E2F4 was found to occupy both the *PCNA* and *MCM4* promoters, although occupancy at the *PCNA* promoter was approximately double that found at the *MCM4* promoter. Interestingly, we found that the presence of E1A 13S correlated with enhancement of E2F4 occupancy at these two promoters (Fig. 7A and B, middle panels). Furthermore, re-ChIP experiments with E1A indicate a preference for E1A 13S to be present along with E2F4 at the *PCNA* and *MCM4* promoters.

The ChIP experiments indicate that the different isoforms of E1A have different promoter occupancy patterns. However, these results do not tell us how these genes are transcriptionally regulated. For gene expression analysis, we infected arrested IMR-90 cells with each virus at a different MOI in order to produce similar levels of E1A expression, which are shown in Fig. 7C. To determine the transcriptional activity of E2F target genes, we performed real-time quantitative reverse transcriptase PCR (qRT-PCR) of *PCNA*, *BLM*, and *MCM4* transcripts following viral infection of arrested IMR-90 cells 36 h after infection with viruses that expressed no E1A (E1 deleted), E1A 12S only (*dl*520), E1A 13S only (*pm*975), E1A 13S Δ2-11, or wild-type genomic E1A (expresses all isoforms; *dl*309) (Fig. 7D). Results are expressed as the relative *n*-fold change in the expression level over that in the virus with E1 deleted. Transcriptional activation of these E2F-responsive genes was found to be the highest with the E1A 13S-only virus, while the E1A 12S and genomic viruses induced considerably lower expression levels, with the exception of *PCNA* (Fig. 7D). Importantly, deletion of the N-terminal DP-1-binding domain in the E1A 13S-only virus caused a severe reduction of gene activation with respect to *pm*975. Indeed, activation was below the levels seen with the E1A 12S virus and largely equivalent to those obtained with the virus with E1 deleted.

These results demonstrate that E2F4 is recruited to both the *PCNA* and *MCM4* promoters. Significantly, E1A 13S, but not E1A 12S, induced enhanced E2F4 promoter occupancy and largely only E1A 13S was found to co-occupy the *PCNA* and *MCM4* promoters together with E2F4 and presumably DP-1.

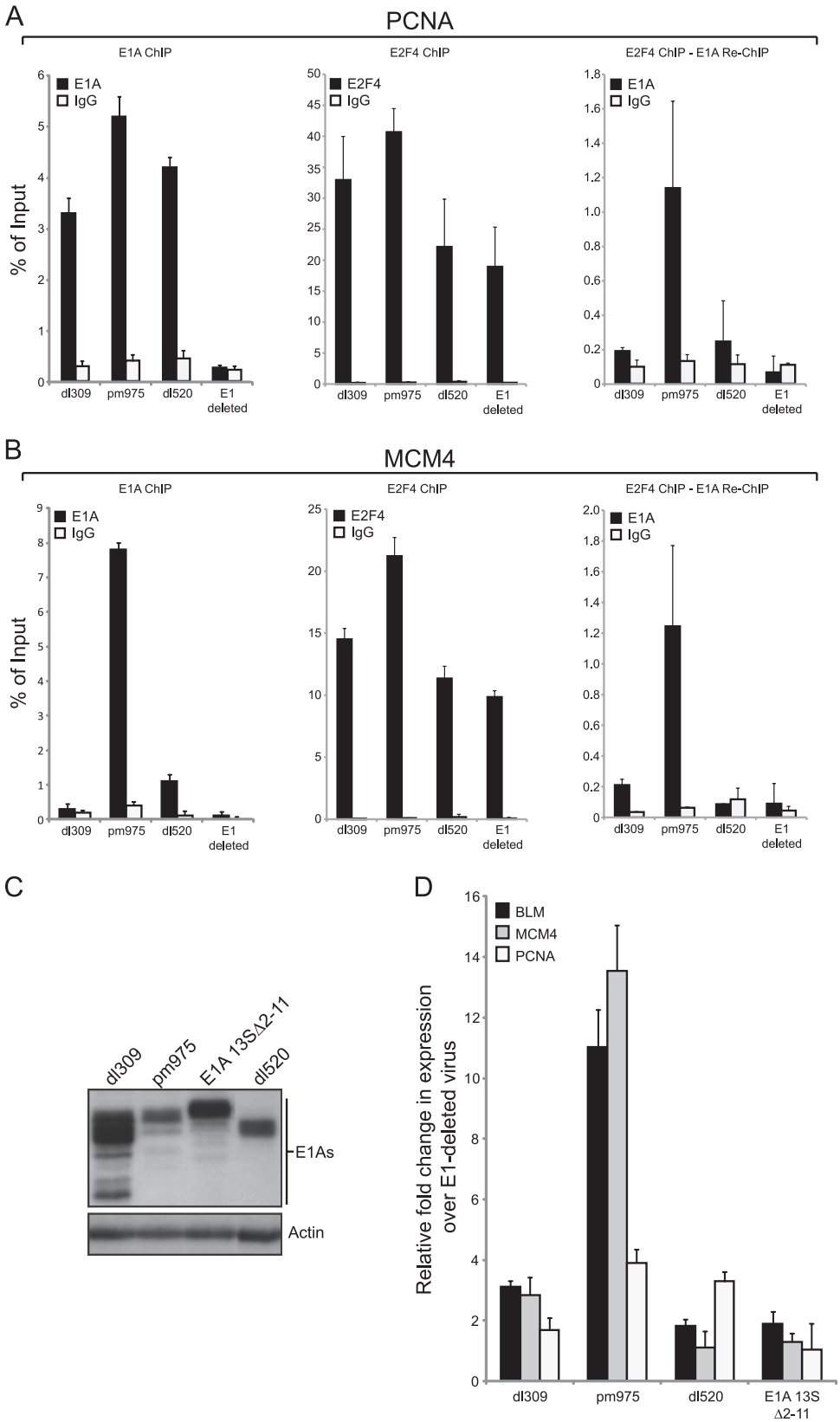


FIG. 7. E1A is recruited to E2F-regulated promoters, altering E2F4 occupancy and E2F target gene expression. (A) ChIPs and re-ChIPs were carried out for E1A (using M73 antibody), E2F4 C-terminal antibody, or IgG control antibody (mouse anti-rabbit) in arrested IMR-90 cells infected with adenovirus with E1 deleted, virus expressing only E1A 12S (*dl*520), virus expressing only E1A 13S (*pm*975), or virus expressing genomic E1A (both E1A 12S and 13S; *dl*309). Immunoprecipitations were carried out 24 h after infection. Occupancy of E1A and E2F4 was

Furthermore, these results show that there are differences in how E1A 12S and E1A 13S influence gene expression, which differ from how wild-type genomic E1A alters the cellular transcriptional program.

E1A residues 2 to 11 and CR3 are required for efficient S-phase induction and viral replication. We have observed that E1A 13S is preferentially targeted to E2F-regulated promoters, and this leads to induction of E2F target gene expression (Fig. 7). The activation of E2F-responsive genes was also dependent on the N terminus of E1A, which binds to DP-1. To test whether this had an impact on the induction of S phase and viral replication, we undertook a series of BrdU incorporation assays with arrested IMR-90 cells and a virus growth assay (Fig. 8). E1A 13S-expressing virus was found to be better at inducing S phase than E1A 12S-only-expressing virus. In fact, E1A 13S was almost equivalent to the wild-type genomic E1A virus at inducing cellular DNA replication (Fig. 8A). The ability of E1A 13S to induce S phase was diminished significantly when residues 2 to 11 were deleted; these amino acids are required for efficient binding of DP-1 to E1A. Finally, we have also observed that the E1A 13S-expressing virus grew an average of 100 times better than the E1A 12S-only-expressing virus (Fig. 8B). Together, these results demonstrate the importance of the N terminus targeting of DP-1 and a role for CR3 of E1A in inducing S phase and leading to efficient viral replication and growth.

DISCUSSION

To date, the study of how E1A deregulates the E2F family of transcription factors has been focused largely on the smaller isoform of E1A encoded by the 12S splice variant of human adenovirus 5 (3). This is paradoxical in a sense because E1A 12S is largely thought to be a transcriptional repressor (2, 16, 18), despite its ability to activate E2F target genes indirectly by interfering with pocket protein function. The recent observation that E1A, and the functionally equivalent HPV E7 protein, can bind to E2F6 (21) suggests that E1A could directly affect E2F-regulated transcription. Our previous work has shown that the larger E1A 13S isoform encoded by adenovirus 5 can utilize cellular factors for tethering directly to promoters in order to activate the transcription of viral (25) and cellular (24) genes, while others have shown that E1A 12S can be recruited to a large array of cellular promoters and alter their activity (12). Based on these observations, we have reevaluated the role that each major isoform of E1A has in altering E2F transcription factor function.

We report here a direct and hitherto unsuspected interaction between E1A and DP-1. Indeed, E1A may be universally

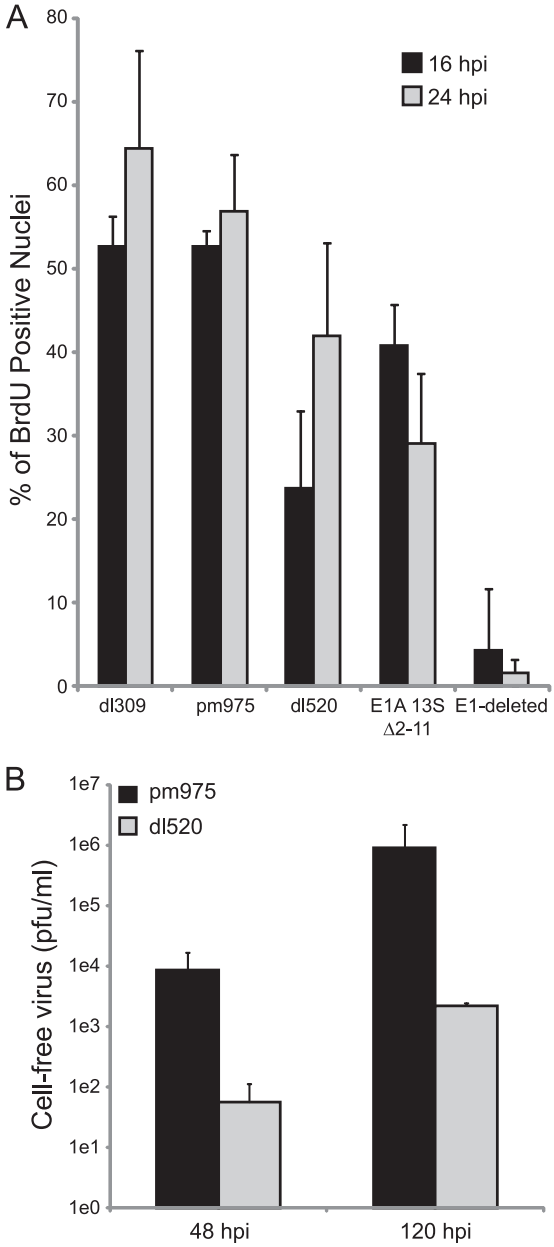


FIG. 8. CR3 and the N-terminal DP-1-binding domain are required for efficient induction of S phase and virus growth. (A) Arrested IMR-90 cells were infected with the indicated adenoviruses, pulsed with BrdU for 1 h at the indicated time points, fixed, and stained with anti-BrdU antibody. Results are expressed as a percentage of BrdU-positive nuclei and constitute a mean of three random fields of view at a low magnification. (B) Arrested IMR-90 cells were infected with *pm*975 or *dl*520 virus at an MOI of 5, and medium was assayed for cell-free virus 48 and 120 h after infection by a plaque assay on 293 cells.

assessed for the *PCNA* promoter around E2F sites by qRT-PCR and is expressed as a percentage of the input. (B) ChIPs and re-ChIPs were carried out for E1A (using M58 and M73 antibodies), E2F4 (using a custom rabbit E2F4 antibody), or IgG control antibody (mouse anti-rabbit) in arrested IMR-90 cells infected with either adenovirus with E1 deleted, virus expressing only E1A 12S (*dl*520), virus expressing only E1A 13S (*pm*975), or virus expressing genomic E1A (both E1A 12S and 13S; *dl*309). Immunoprecipitations were carried out 24 h after infection. Occupancy of E1A and E2F4 was assessed for the *MCM4* promoter around E2F sites using qRT-PCR and is expressed as a percentage of the input. (C) Expression levels of various E1A mutant adenoviruses in infected IMR-90 cells 24 h after infection. Arrested IMR-90 cells were infected with the indicated viruses and lysed, and E1A levels were detected using anti-E1A (M73) antibody. Actin was used as a loading control. (D) Real-time PCR quantification of expression levels of the *PCNA*, *MCM4*, and *BLM* gene products at 36 h after infection of arrested IMR-90 cells. The results are expressed as *n*-fold changes versus cells infected with adenovirus with E1 deleted.

targeting DP-1 in order to gain efficient access to all E2F-regulated promoters without having to individually target the different E2Fs themselves. *In vitro* binding experiments show that the interaction of E1A with DP-1 is direct (Fig. 6). However, there was an observable difference in the affinities of E1A for DP-1 alone and DP-1 in complex with E2F1. This suggests that E1A may preferentially target the DP-1 heterodimer, rather than the monomer, which usually is not found *in vivo*. Alternatively, DP-1 alone simply may not fold properly when it is not in a complex with an E2F and therefore lacks the optimal E1A-binding surface. Binding of E1A to DP-1 was found to occur via the N terminus of E1A targeting the N terminus of DP-1 (Fig. 2, 3, and 4). This region of E1A is present in both E1A 12S and 13S. It is not highly conserved between the E1A proteins of various human and simian adenoviruses but binds a large number of cellular proteins (24). The region of DP-1 bound by E1A encompasses neither the DNA-binding nor the dimerization domain, suggesting that these functions of DP-1 must remain intact while in complex with E1A. This agrees with our observations indicating that E1A can use E2F/DP-1 complexes to target itself to promoters, which would not occur if E1A interfered with either the DNA-binding or the dimerization function of DP-1.

Consistent with our identification of a direct interaction between E1A and DP-1, we determined that E1A 13S can activate expression from E2F-regulated promoters in a fashion that does not rely on E1A binding to pRb or other pocket proteins in asynchronously dividing cells (Fig. 1A and B). This occurred with all of the E2Fs tested (Fig. 1B), which is consistent with E1A targeting DP-1 directly and using it to localize E1A 13S at the promoter. The presence of the strong transactivation domain (CR3) in E1A was required for activation of the E2F reporter in this context, since E1A 12S was unable to induce transcription. This was somewhat unanticipated, as we would expect some degree of activation due to relief of pRb-mediated repression. It is likely that E1A 12S is unable to activate E2F-dependent transcription in asynchronously dividing cells because relief of pRb-mediated repression in the absence of CR3-dependent activation is not sufficient to efficiently drive expression above the basal activity of the reporter in these cells, where pRb function may be partially compromised. Conceivably, as both E1A 13S and 12S can bind DP-1 (Fig. 2B), either E1A protein could be recruited to E2F-responsive promoters. This could potentially establish a competition for occupancy that may serve as a means of fine-tuning transcriptional activation. Indeed, a previous study (12) showed that E1A 12S is still recruited to E2F-regulated promoters in the absence of pRb binding.

Activation of transcription by E1A 13S was significantly greater with E2F4 and E2F5 than with E2F1 to E2F3 (Fig. 1B). E2F4 and E2F5 are considered repressive members of the E2F family, whereas E2F1 to E2F3 are considered activators. This raises the intriguing possibility that E1A can preferentially target genes repressed by E2F for activation, rather than those already efficiently transcribed. This economy of action is reminiscent of the ability of E1A to bind hypophosphorylated active pRb but not hyperphosphorylated inactive pRb (22). Furthermore, some of the activating E2Fs (in particular, E2F1) can be strongly proapoptotic (30, 35). Presumably, such E2Fs are not superactivated by E1A, as this could lead to premature cell death and an abortive infection. It is still unclear how E1A achieves high activation levels through E2F4 and E2F5, but it is not simply due to higher affinity, as we observed a relatively weak association between E1A 13S and DP-1/E2F4 or DP-1/E2F5 (Fig. 1C). This ability of E1A 13S to transform a transcriptional repressor into an activator closely parallels what we have previously observed with CtBP and ZNF217 (5).

qRT-PCR and ChIP results shed an interesting light on how E1A deregulates transcription from E2F-responsive genes. Although previous reports (12, 29) showed that E1A 12S is recruited to E2F-regulated promoters and alters the composition of factors at these promoters, the role of E1A 13S in altering gene expression patterns has never been examined in detail. Here we report clear differences in the ways in which the different E1A isoforms regulate gene expression from E2F-responsive promoters. Our results indicate that E1A 13S is preferentially co-occupying *PCNA* and *MCM4* promoters together with E2F4/DP-1, whereas there is little E1A 12S present. Interestingly when both E1A 12S and E1A 13S are coexpressed, as is the case with the *dl*309 virus, we see a reduction of overall E1A occupancy and co-occupancy on these two promoters. This suggests that E1A 12S can somehow prevent E1A 13S from gaining full access to the promoter. The differences observed between E1A ChIP and re-ChIP following E2F4 ChIP suggest that there is more than one way in which E1A can access these promoters. This is not surprising, as many factors contribute to transcriptional activation and E1A itself binds a wide array of transcriptional coactivators (24). Intriguingly, we observed a considerable enhancement of E2F4 occupancy at the *PCNA* and *MCM4* promoters following infection where E1A 13S was present. A previous study showed that adenovirus E4 orf6/7 protein could induce nuclear translocation of E2F4 and enhanced promoter occupancy (28). This presents an intriguing axis of viral protein cooperation where E1A 13S induces expression of the E4 region, including E4 orf6/7, which then leads to enhanced occupancy by E2F4/DP-1 at target promoters that is subsequently utilized by E1A 13S to drive gene expression from otherwise repressed genes. Indeed, another E4 protein, E4 orf4, appears to play a role in regulating expression from E2F-responsive genes, highlighting the importance of activation of the E4 region by E1A 13S (19, 23, 26, 28). A recent study also demonstrated that a large proportion of the genes turned on in quiescent mouse 3T3 fibroblasts by E1A 12S were E2F4 targets (10), suggesting that this may be an important mechanism driving E2F target gene expression. In the present study, induction of cellular E2F target genes was also found to be the highest for those viruses that expressed E1A 13S. Importantly, we observed an almost total ablation of E2F gene activation when cells were infected with a deletion mutant form of E1A 13S that lacks the N-terminal DP-1-binding domain, signifying the importance of this region in driving E2F-responsive genes. Furthermore, while E1A 13S Δ2-11 was also impaired for induction of S phase, it was similar to E1A 12S. This may result from the ability of this mutant form to still bind to pRb and relieve E2F repression by displacing pRb or other pocket proteins. Interestingly, we observed large differences in the ways in which the different E1As were able to activate cellular gene expression. Unexpectedly, the wild-type E1A virus (*dl*309) was only slightly better than the E1A 12S virus at driving E2F-responsive genes, but it was

considerably better at inducing S phase. A fine control of the transcriptional program enabled by multiple variants (such as the different isoforms of E1A) may allow for optimal gene expression most suitable for virus growth. This hypothesis is further supported by transcriptional activation of E2F-regulated genes by the genomic E1A virus. The wild-type genomic virus showed only a moderately higher level of transcriptional activation than the E1A 12S-only virus, despite lower levels of promoter occupancy by E1A-expressing virus than by E1A 13S-expressing virus. However, it was still as efficient as E1A 13S in driving quiescent cells into S phase. This highlights the importance of having all E1A isoforms present and suggests that the wild-type virus has evolved complex mechanisms for optimally modulating gene expression.

In conclusion, we show for the first time that E1A directly associates with E2F/DP-1 complexes, likely through binding to DP-1. This potentially endows E1A with the ability to directly influence E2F-responsive genes without relying only on the deregulation of the pRb family of proteins. Interestingly, this activation of transcription may be restricted largely to E2F-repressed genes by E1A 13S and appears to be modulated in a competitive fashion by E1A 12S. The hitherto unappreciated tripartite complexity of functional interactions between pRb family of proteins, E2Fs, and various isoforms of E1A described here illustrates the extremely tight relationship between the infecting adenovirus and the host cell. Finally, our work suggests a novel functional cooperation axis whereby one viral gene (that for E1A 13S) induces the expression of another (that for E4 orf6/7), which then assists the first in carrying out its function, highlighting the tight relationships between all viral genes and the overriding importance of driving the infected cell into S phase.

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