JOURNAL OF VIROLOGY, May 1997, p. 3788–3798 Vol. 71, No. 5
0022-538X/97/$04.00+0

Regulation of p53 Levels by the E1B 55-Kilodalton Protein and E4orf6 in Adenovirus-Infected Cells

EMMANUELLE QUERIDO,[1] RICHARD C. MARCELLUS,[1] ALBERT LAI,[1] RACHEL CHARBONNEAU,[1] JOSE G. TEODORO,[1] GARY KETNER,[2] AND PHILIP E. BRANTON[1,3]*

Departments of Biochemistry[1] and Oncology,[3] McGill University, Montreal, Quebec, Canada H3G 1Y6, and Department of Molecular Microbiology and Immunology, Johns Hopkins University School of Hygiene and Public Health, Baltimore, Maryland 21205[2]

Received 11 November 1996/Accepted 29 January 1997

The adenovirus type 5 243R E1A protein induces p53-dependent apoptosis in the absence of the 19- and 55-kDa E1B polypeptides. This effect appears to result from an accumulation of p53 protein and is unrelated to expression of E1B products. We now report that in the presence of the E1B 55-kDa polypeptide, the 289R E1A protein does not induce such p53 accumulation and, in fact, is able to block that induced by E1A 243R. This inhibition also requires the 289R-dependent transactivation of E4orf6 expression. E4orf6 is known to form complexes with the E1B 55-kDa protein and to function both in the transport and stabilization of viral mRNA and in shutoff of host cell protein synthesis. We demonstrated that the block in p53 accumulation is not due to the generalized shutoff of host cell metabolism. Rather, it appears to result from a mechanism targeted specifically to p53, most likely involving a decrease in the stability of p53 protein. The E1B 55-kDa protein is known to interact with both E4orf6 and p53, and as demonstrated recently by others, we showed that E4orf6 also binds directly to p53. Thus, multiple interactions between all three proteins may regulate p53 stability, resulting in the maintenance of low levels of p53 following virus infection.

The most common targets of human adenoviruses are terminally differentiated epithelial cells of the bronchial tract. Adenoviruses possess relatively small genomes, and thus in order to produce progeny virus, they must first activate host cell DNA synthetic machinery which is then utilized to replicate viral DNA. This induction of DNA synthesis by the products of early region 1A (E1A) appears to underlie the oncogenic potential of human adenoviruses in rodent cells in which lytic infection fails to occur, and cells which exhibit unregulated proliferative capacities sometimes survive. Although E1A represents the major viral oncogene, products of early region 1B (E1B) are usually required to yield stable transformants (reviewed in reference 4). Both E1A and E1B encode multiple products due to alternative splicing. The two major adenovirus type 5 (Ad5) E1A proteins of 289 and 243 residues (289R and 243R, respectively) are identical apart from an internal region, termed CR3, which transactivates expression of early viral transcription units 3 and 4 (E3 and E4) and, to some extent, early region 2 (E2) and some cellular genes (55). The 289R and 243R E1A proteins can both induce DNA synthesis and transform rodent cells through the formation of complexes with two classes of cellular proteins, the retinoblastoma (RB) family of growth suppressors and the transcriptional modulator p300 and related proteins (reviewed in reference 4). However, expression of these E1A proteins is toxic, and cells die rapidly from apoptosis unless E1B products are also expressed. The E1B 19-kDa protein is able to block apoptosis induced by a wide variety of agents and seems to be functionally analogous to the cellular Bcl-2 protein (5, 40, 46). The 55-kDa protein binds to and inhibits p53 (28, 53, 64, 66), a cellular transcriptional regulator which induces growth arrest and/or apoptosis following the induction of unscheduled DNA synthesis or DNA damage (reviewed in reference 29). We have shown recently that the 55-kDa protein is able to block p53-dependent apoptosis induced by E1A (58) or by expression of p53 in p53-null Saos-2 human osteosarcoma cells (34). Thus, one role of E1B products in both productive infection and cell transformation appears to be to prevent untimely E1A-induced cell death, thus permitting production of high yields of progeny or survival of transformants. In productively infected human cells, the E1B 55-kDa polypeptide also interacts with the E4orf6 protein to form complexes which enhance accumulation and transport of late viral mRNAs and induce shutoff of host cell protein synthesis (1, 2, 8, 12, 21, 30, 43, 44, 50, 52, 63).

It is now clear that E1A induces apoptosis by two mechanisms, one requiring p53 (13, 33) and the other operating via a p53-independent pathway involving one or more viral E4 proteins (35, 57, 60). It has been known for some time that expression of E1A induces p53 protein accumulation (6, 17, 19, 20, 32). Recently, we have found that such increases in p53 levels are induced by complex formation with p300 or, in the case of human cells, with RB family members (45). The molecular basis of this p53 accumulation remains uncertain. One possibility is that E1A products somehow increase the stability of p53, which has a half-life of about 20 min or so in most normal cells. In stable E1A-transformed cell lines, p53 half-lives of over 2 h have been reported, especially in cells that also express the E1B 55-kDa product (17, 19, 20, 32). p53 is usually cytoplasmic in cells containing the E1B 55-kDa protein, whereas it is largely nuclear in the absence of this E1B product (61, 67). In some cell lines, two populations of p53 appear to exist, one with a normal short half-life and another containing more long-lived species (19, 20). In addition, the E1B 55-kDa protein or the equivalent 54-kDa product in serotype 12 (Ad12) has been shown to stabilize p53 in the absence of E1A (19, 61). However, transformants in which little change in p53 half-life is apparent also exist (18, 19). A second possibility is that E1A products increase expression of p53 mRNA. Braithwaite et al. have reported such an induction of p53 transcription following adenovirus infection (6). More recently, it has

* Corresponding author. Phone: (514) 398-8350. Fax: (514) 398-7384. E-mail: branton@medcor.mcgill.ca.

also been suggested that p53 expression may be regulated at the translational level (15, 39).

In the present study, we report that although the E1A 243R protein induces the accumulation p53, such is not the case with E1A 289R. We show that this suppression of p53 accumulation requires the E4orf6 protein, which we confirm is able to bind directly to p53 in vitro. Furthermore, the E1B 55-kDa species is also required, suggesting that multiple interactions involving p53, the E1B 55-kDa protein, and E4orf6 might play a role in preventing the E1A-induced accumulation of p53.

MATERIALS AND METHODS

Cells and viruses. Normal MRC-5 (ATCC CCL 175) and IMR-90 (ATCC CCL 186) diploid human embryonic lung fibroblasts were cultured on 60-mm-diameter dishes (Corning Glass Works, Corning, N.Y.) in α-modified minimal essential medium (α-MEM) supplemented with 10% fetal calf serum. The MRC-5 cell line was shown to contain wild-type p53 and is not immortalized (11). The cells used for the present studies were from passages 6 to 11. Human 293 cells were derived from human embryonic kidney cells and express Ad5 E1A and E1B proteins (16). Human HeLa and KB cells, as well as mouse 10T1/2 cells (47), were also cultured as described above. Cells were infected with wild-type (wt) or mutant virus at a multiplicity of 35 PFU per cell, as was described previously (48). For double infections, the viruses were premixed before infection at a multiplicity of 35 PFU per cell for each virus. The wt Ad5 used in most of the present studies was described by Harrison et al. (24), although in some cases, including for the preparation of all E1A and E1B mutants, *dl*309 (27) was used as wt. Some experiments required mutant *pm*975 (37), which expresses E1A 289R but not 243R, or mutant *dl*520 (22), which produces E1A 243R but not 289R. The mutant 12S/E1B$^-$ (originally *dl*520T) produces only the E1A 243R protein and no E1B products (56). The mutant E1B$^-$ expresses both major E1A products but no E1B species (60). Mutant E1B/55K$^-$ (originally *pm*2019/2250) fails to express the E1B 55-kDa protein, and mutant E1B/19K$^-$ (originally *pm*1716/2072) does not express the E1B 19-kDa polypeptide (36). Additional E1B 55-kDa protein mutants included *pm*490/1A and *pm*490/1/5A, which contain alanine residues in place of the Ser-490, Ser-491, and Thr-495 phosphorylation sites, thus reducing transforming and transcriptional repression activities and the ability to inhibit p53 activity but not affecting complex formation with p53 (58, 59), and mutants A262 and R309, which contain insertions next to residues 262 and 309 which block interactions with p53 (28, 65). E1A and E1B mutant viruses were propagated on 293 cells. Figure 4 illustrates a series of Ad5 E4 deletion mutant viruses used for mapping studies, including *dl*1010, *dl*1011, *dl*1013, *dl*1014, *dl*1015, and *dl*1019, as described previously (8, 9). These viruses, which express all E1A and E1B products, were propagated on W162 monkey cells, as described previously (62). The AdLacZ adenovirus vector in which the E1A and E1B regions have been replaced by the *Escherichia coli lacZ* gene under the control of the cytomegalovirus (CMV) promoter (3) was provided by Frank Graham. The AdHis55K vector, expressing the Ad5 E1B 55-kDa protein under the CMV promoter in an adenovirus vector that lacks both E1A and E1B, was described previously (34). The AdE4orf6 vector expressing adenovirus type 2 (Ad2) E4orf6 under the control of the CMV promoter was made by introducing an appropriate cDNA (42) into the same adenovirus vector.

Western blotting analysis. Cell extracts were prepared on ice in lysis buffer (50 mM HEPES, pH 7.9, containing 400 mM KCl; 0.1% [vol/vol] Nonidet P-40; 4 mM NaF; 4 mM $NaVO_4$; 0.2 mM EDTA; 0.2 mM EGTA; 1 μg [each] of aprotinin, leupeptin, and pepstatin per ml; 0.5 mM phenylmethylsulfonyl fluoride; and 1 mM dithiothreitol). Total protein was measured by a Bio-Rad protein assay according to the manufacturer's specification, and 50 mg of total cell protein was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), using either 10 or 12% polyacrylamide gels. The material was transferred to nitrocellulose membranes for 1 h in a semidry transfer apparatus, and the membranes were probed with primary antibodies which recognize p53, E1A proteins, the E1B 55-kDa product, or certain E4 proteins. Anti-p53 mouse monoclonal antibody Ab1801 (Ab-2; Oncogene Science) was purified by precipitation with ammonium sulfate from supernatants obtained from hybridoma cell cultures grown in Dulbecco's minimal essential medium (DMEM) supplemented with 10% low immunoglobulin G fetal calf serum (BRL). Anti-p53 mouse monoclonal antibody Ab421 was obtained from Oncogene Science or, as in the cases of mouse monoclonal antibodies M73 against E1A proteins (23) and 2A6 against the E1B 55-kDa protein (54), was collected as ascites fluid generated in mice, using appropriate hybridoma cells. The M45 mouse monoclonal antibody against the amino-terminal portion of the Ad2 E4orf6 and E4orf6/7 proteins (41) was generously provided by Pat Hearing. Binding patterns were determined by addition of a secondary antibody, horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (Jackson Immuno Research Laboratories), and the signal was detected by an enhanced chemiluminescence (ECL) reagent (Dupont NEN).

Measurement of p53 half-life. 293 cells were grown on 60-mm-diameter plates and mock infected or infected with AdLacZ virus, and at 7.5 h postinfection (hpi), the cells were labeled for 15 min with 100 μCi of [^{35}S]methionine (1,175 Ci/mmol; Dupont NEN) per plate and then incubated further for various times with α-MEM supplemented with 15 mg of L-methionine (ICN Biochemicals) per ml. The cells were then lysed in radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl, pH 7.2, containing 150 mM NaCl, 1% [vol/vol] Triton X-100, 0.1% SDS, 1% sodium deoxycholate, 1 mM EDTA, 500 mM sodium vanadate, 50 mM NaF, 1 mM β-glycerol phosphate, and 100 Kallikrein international units of aprotinin) and immunoprecipitated with a mixture of Ab1801 and Ab421. The labeled p53 was detected on a Fuji BAS-III imaging plate, and the amount of radioactivity was quantified with a Fujix Bas 2000 phosphorimager.

Host cell shutoff and p53 translation. Cells were grown on 60-mm-diameter plates and infected with wt or mutant Ad5. At the indicated times following infection, the cells were labeled for 1 h with 100 μCi of [^{35}S]methionine (1,175 Ci/mmol; Dupont NEN) per plate and harvested. To analyze host cell shutoff, cell lysates were prepared in the same lysis buffer as that used for Western blotting, and 50 μg of total cell protein, as determined by Bio-Rad assays, was analyzed by SDS-PAGE (10% polyacrylamide). To analyze synthesis of p53, cell lysates were prepared in RIPA buffer and immunoprecipitated with a mixture of Ab1801 and Ab421 antibodies. In all cases, protein patterns were visualized by autoradiography with Kodak X-Omat AR film. In addition, the labeled p53 was detected as described above on a Fuji BAS-III imaging plate, and the radioactivity was quantified with a Fujix Bas 2000 phosphorimager.

Immunoprecipitation and E4orf6 protein binding assays. For in vitro E4orf6 binding assays, cell extracts were prepared in 500 μl of RIPA buffer from 293 cells grown on 100-mm-diameter plates and harvested at 7 and 16 hpi with wt Ad5. E4orf6 and E4orf6/7 proteins were immunoprecipitated with M45 antibody in the presence of a mixture of protein A and protein G-Sepharose (Pharmacia). Following extensive washing with RIPA buffer, the precipitates were incubated in buffer G (20 mM Tris-HCl, pH 6.8, containing 137 mM NaCl; 0.1% [vol/vol] Nonidet P-40; 1 mM EDTA; 2 mg of bovine serum albumin per ml; 1 μg [each] of aprotinin, leupeptin, and pepstatin per ml; and 0.5 mM phenylmethylsulfonyl fluoride) containing only nonionic detergent, in the presence of [^{35}S]methionine-labeled proteins synthesized by an in vitro transcription-translation system. DNA from expression plasmids P53pSP65 containing the murine p53 cDNA (28) and pGEM-55K containing the Ad5 E1B 55-kDa protein open reading frame under the T7 promoter (59) was linearized, and transcription was performed with 2 μg of DNA and SP6 or T7 RNA polymerase (Pharmacia). Translation of the resulting mRNAs was performed in a 50-μl translation reaction volume containing 35 μl of pretreated rabbit reticulocyte lysate (Promega) in the presence of 40 μCi of translation-grade [^{35}S]methionine (1,175 Ci/mmol; Dupont NEN). The precipitates were incubated for 2 h in the presence of [^{35}S]methionine-labeled p53 and E1B 55-kDa proteins before extensive washing in buffer G. Bands were visualized by autoradiography after separation by SDS-PAGE on a 10% polyacrylamide gel.

RESULTS

Expression of the 243R but not the 289R E1A protein induces an accumulation of p53. It has previously been reported that expression of human adenovirus E1A protein results in the accumulation of p53 (6, 10, 13, 17, 19, 20, 32, 49). Figure 1A shows the results of an experiment in which extracts of human MRC-5 cells infected with various Ad5 viruses or mock infected were analyzed following SDS-PAGE by Western blotting with an anti-p53 antibody. After infection with mutant *dl*520, which produces the E1A 243R protein but not E1A 289R, the level of p53 was seen to increase about 8- to 10-fold relative to that in mock-infected cells (Fig. 1A, compare lanes 2 and 1, respectively). A similar increase was observed with mutant 12S/E1B$^-$, which produces only E1A 243R and no E1B products (Fig. 1A, lane 3). Such was not the case in cells infected with *pm*975, which produces the E1A 289R protein but not 243R. In these cells, little increase in p53 was observed (Fig. 1A, lane 4) and levels were similar to those in mock-infected cells (Fig. 1A, lane 1). Analysis of the levels of E1A proteins synthesized by these (and all other) mutants in Fig. 1D, by Western blotting with anti-E1A M73 monoclonal antibody, indicated that *pm*975 actually yielded slightly smaller amounts than did *dl*520 (Fig. 1D, compare lanes 4 and 2). Thus, the failure to accumulate p53 could possibly have been due to the presence of smaller amounts of E1A protein; however, such did not appear to be the explanation, as in cells doubly infected with *pm*975 and *dl*520, the accumulation of p53 induced by E1A-243R of *dl*520 was completely blocked by expression of even lower levels of 289R by *pm*975 (Fig. 1A,

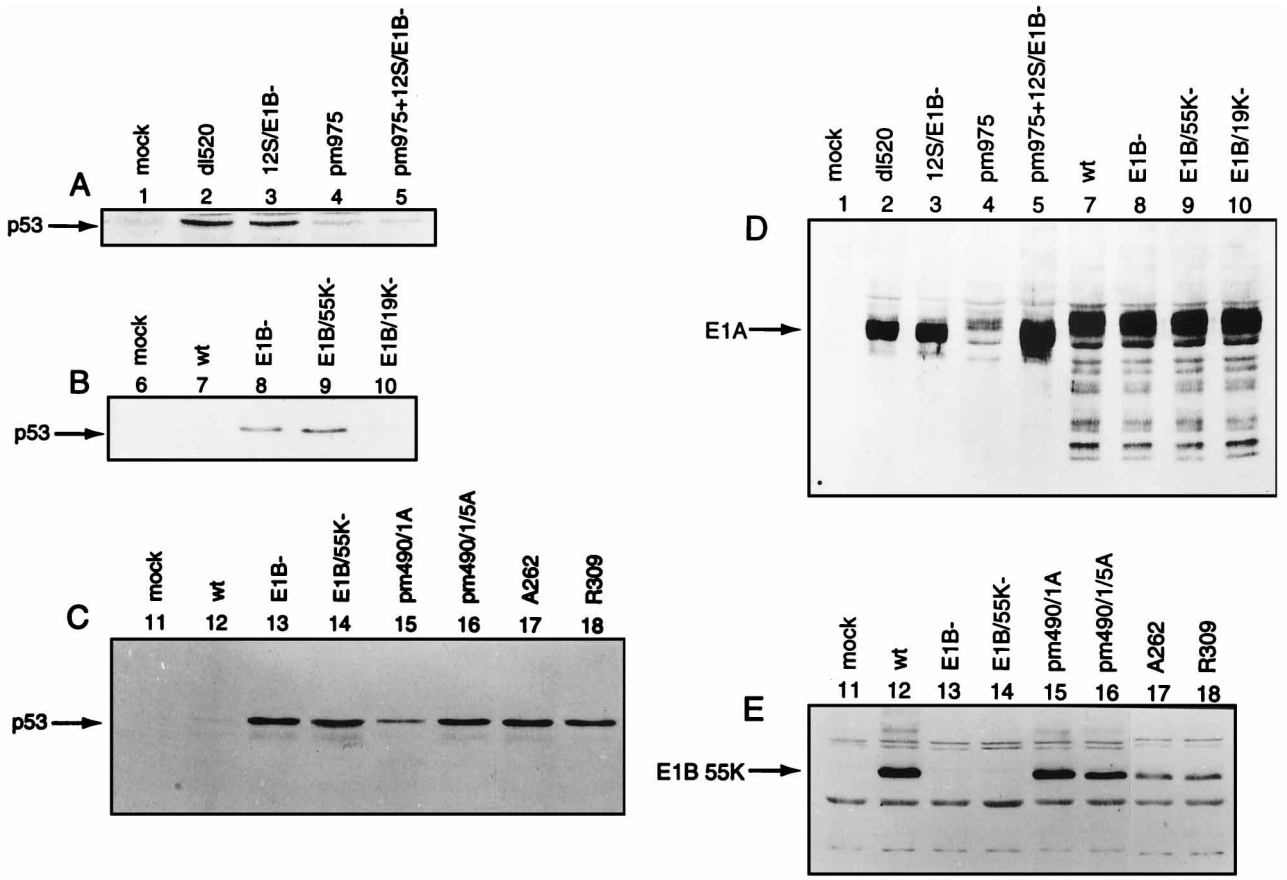

FIG. 1. Effects of E1A and E1B products on the accumulation of p53. MRC-5 cells were infected with wt or mutant Ad5, and cell extracts were prepared at 24 hpi and separated by SDS-PAGE. (A through C) Analysis of p53 levels. p53 levels were determined by Western blotting with Ab1801 antibodies, and binding patterns were determined by ECL, as described in Materials and Methods. Lanes are as indicated. (D) Analysis of E1A protein levels. The amounts of E1A proteins present were determined by immunoblotting, as described above, with E1A-specific M73 monoclonal antibody. Lanes are as indicated. (E) Analysis of E1B 55-kDa protein levels. Levels of the 55-kDa protein were determined by immunoblotting, as described above, with 2A6 monoclonal antibody. Lanes are as indicated.

lane 5). Figure 1B shows that infection of MRC-5 cells by wt Ad5, which expresses both E1A 289R and 243R, also does not result in any increase in p53 compared with the case for mock-infected cells (Fig. 1B, compare lanes 7 and 6, respectively). However, infection with mutant E1B$^-$ (Fig. 1B, lane 8), which produces both major E1A products but no E1B proteins, resulted in the accumulation of p53, suggesting that one or more E1B proteins were required for the inhibition of p53 accumulation. To determine which E1B product was responsible for this effect, cells were infected with mutants that expressed both major E1A products but only one of the major E1B proteins. Expression of the E1B 19-kDa protein but not the E1B 55-kDa product, using mutant E1B/55K$^-$, resulted in the accumulation of p53 (Fig. 1B, lane 9), whereas in the presence of the 55-kDa protein but not the 19-kDa polypeptide with mutant E1B/19K$^-$, this accumulation did not occur (Fig. 1B, lane 10). Identical results were also obtained with the IMR-90 human cell line (data not shown). Thus, these results indicated that expression of E1A 289R prevented the accumulation of p53 induced by the E1A 243R protein alone and that this inhibition required expression of the E1B 55-kDa protein.

Inhibition of E1A-induced accumulation of p53 requires a functional E1B 55-kDa protein. The 55-kDa E1B product possesses intrinsic repression activity which is believed to block the action of the p53 activation domain following complex formation with p53 (58, 66). To examine the role of this E1B protein in the inhibition of E1A-induced accumulation of p53, the levels of p53 protein were examined in extracts from E1B mutant-infected cells by Western blotting. Figure 1C shows again that in MRC-5 cells infected with wt Ad5 (lane 12), the levels of p53 were similar to those in mock-infected cells (lane 11), whereas with mutants E1B$^-$ and E1B/55K$^-$, which express no E1B 55-kDa protein (lanes 13 and 14, respectively), increased levels of p53 were evident. Studies were also conducted with mutants *pm*490/1A and *pm*490/1/5A, which contain Ala residues in place of the Ser-490, Ser-491, and Thr-495 phosphorylation sites of the E1B 55-kDa species. These alterations have been found to diminish cell transformation dependent on the 55-kDa protein, reduce its ability to inhibit p53 activation activity, and eliminate its transcriptional repression activity without affecting the efficiency of binding to p53 or the induction of host cell shutoff (58, 59). The precise role of phosphorylation in the regulation of these functions has still not been established. Figure 1C (lanes 15 and 16) shows that, in both cases, significant increases in p53 levels were observed. The increase was greater with *pm*490/1/5A, which encodes a 55-kDa protein that is also more defective than that produced by *pm*490/1A, both in endogenous repression activity and in cooperation with E1A in cell transformation (58). Thus, complex formation with p53 is not sufficient for inhibition of accumulation of p53, which appears to require a functional 55-kDa protein repression domain. To analyze the role of the 55-kDa

protein further, two additional mutants A262 and R309, which contain insertions in the p53 binding region, thus blocking complex formation with p53 but not affecting transcriptional repression, were examined (28, 65). Figure 1C (lanes 17 and 18) shows that, with both of these mutants, accumulation of p53 occurred at high levels, suggesting that binding to p53 may be required. Levels of expression of 55-kDa proteins by all the E1B 55-kDa mutants were measured by immunoblotting with 2A6 monoclonal antibody. Figure 1E shows that mutants yielded qualities approaching those of the wt (lane 12), although as shown previously (65), the levels with both A262 and R309 (lanes 17 and 18) were somewhat lower, due possibly to reduced protein stability. Thus, inhibition of E1A-induced accumulation of p53 requires an E1B 55-kDa protein that possesses intrinsic repression activity and, most likely, that can bind to p53.

Inhibition of E1A-induced accumulation of p53 requires an additional viral protein and a shortening of the half-life of p53. The data just presented indicated that expression of the E1A 289R protein and the E1B 55-kDa product resulted in the inhibition of E1A-induced accumulation of p53. Of some interest in this context are 293 cells which express Ad5 E1A 289R and 243R proteins and E1B products yet accumulate very high endogenous levels of p53 protein (18). It was possible that this accumulation in the presence of E1A 289R (and that found in other E1A-E1B transformed cells) could occur either because 293 cells lack a cellular protein required for the inhibition of E1A-induced p53 accumulation or because such inhibition requires an additional viral protein encoded by a region other than E1A and E1B. To address this question, 293 cells were infected with the adenovirus vector AdLacZ, which contains the β-galactosidase gene in place of E1A and E1B, and the levels of p53 were determined by Western blotting with extracts prepared at various times following infection. Figure 2A shows that high levels of p53 were evident in uninfected 293 cells; however, following infection, the amount began to diminish by as early as 10 hpi (lane 3) and continued to decrease up to 25 hpi (lane 6). Thus, a reduction in the accumulation of p53 occurred in 293 cells after introduction of the viral genome, suggesting that diminished levels of p53 resulted from the expression of one or more viral proteins in addition to E1A and E1B products.

It was possible that infection of 293 cells by AdLacZ provided some factor that rendered p53 proteins less stable, thus reducing p53 levels. Figure 2C shows the results of a representative pulse-chase experiment in which the half-life of p53 was determined in both AdLacZ-infected and mock-infected 293 cells. p53 half-life in mock-infected 293 cells was found to be about 2.5 h, while in AdLacZ-infected cells, the half-life, as measured at 7.5 hpi was about 20 min. Thus, expression of additional viral genes is responsible for a decrease in the stability of the p53 protein. This decreased stability could therefore account for some or all of the reduction in p53 levels. It was also possible that some of the decrease in p53 levels following infection with AdLacZ could be linked to generalized host cell shutoff, a process requiring both the E1B 55-kDa protein and E4orf6. To examine the relationship between host cell shutoff and the observed decreases in p53 levels, whole-cell extracts from 293 cells which had been labeled for 1 h with [^{35}S]methionine at various times after infection with AdLacZ were analyzed by SDS-PAGE. Figure 3A shows that shutoff of host protein synthesis began between 12.5 and 15 hpi, as was evident by the decrease in labeling of most cellular proteins (see lanes 5 to 7). Thus, this effect did not correlate with decreases in p53 levels which, as shown previously in Fig. 2A, began several hours earlier, between 5 and 10 hpi. In addition

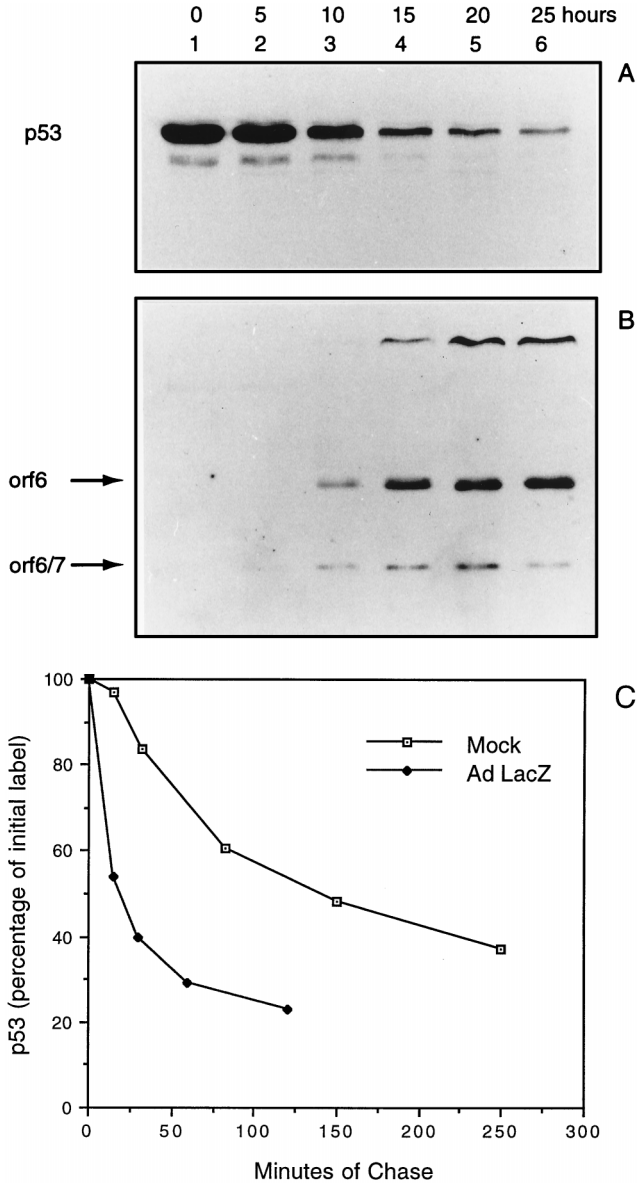

FIG. 2. Analysis of p53 and E4orf6 levels in 293 cells infected by AdLacZ vector. Human 293 cells were infected with AdLacZ. In some cases, cells were harvested at various times after infection and whole-cell extracts were separated by SDS-PAGE. Following transfer to nitrocellulose, the levels of p53 and E4orf6 were determined by Western blotting. In other cases, cells were labeled at 7.5 hpi for 15 min with [^{35}S]methionine, and the half-life of p53 proteins was measured in pulse-chase experiments, as described in Materials and Methods. (A) Analysis of p53 levels. Amounts of p53 were determined by Western blotting with anti-p53 antibody Ab1801 and ECL. The position of migration of p53 is shown on the left. (B) Analysis of E4orf6 levels. Amounts of E4orf6 were determined by Western blotting with M45 antibody and ECL. The positions of migration of E4orf6 and E4orf6/7 are shown on the left. (C) Determination of p53 half-life. Mock- and AdLacZ-infected 293 cells were analyzed in a pulse-chase experiment commencing at 7.5 hpi. Labeled p53 was immunoprecipitated with a mixture of Ab1801 and Ab421, and the amount of labeled p53 was measured with a phosphorimager and was plotted as a percentage of the value at time zero, as described in Materials and Methods. The data presented are from one representative experiment of four independent analyses.

to examining the whole-cell protein pattern (Fig. 3A), extracts from these [^{35}S]-labeled AdLacZ-infected 293 cells were immunoprecipitated with a mixture of Ab1801 and Ab421 anti-p53 antibodies, and the precipitates were analyzed by SDS-

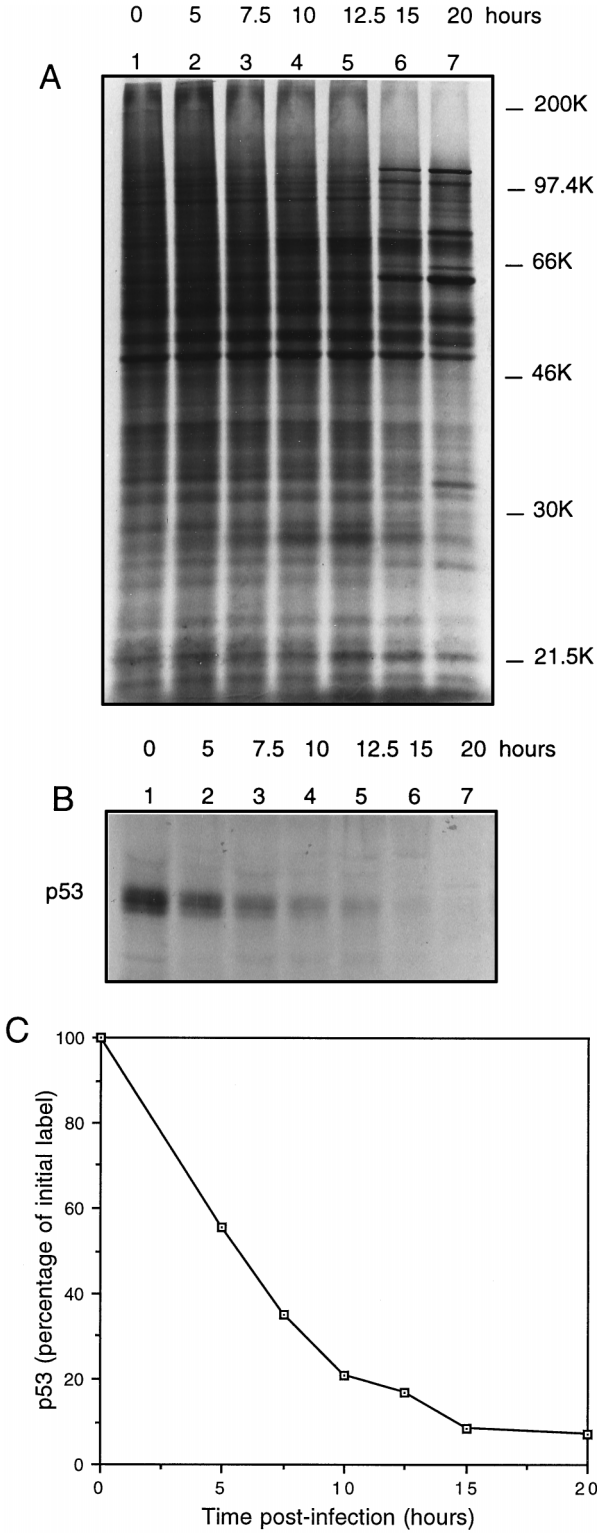

FIG. 3. Time course of host cell shutoff and p53 synthesis. 293 cells were infected with AdLacZ, and at various times they were labeled for 1 h with [^{35}S]methionine and cell extracts were prepared. (A) Analysis of whole-cell protein synthesis. Fifty micrograms of total cell protein of each sample was analyzed by SDS-PAGE followed by autoradiography. The positions of ^{14}C-labeled molecular mass markers are shown on the right. (B) Analysis of p53 synthesis. Equal aliquots of the cell extracts shown in panel A were immunoprecipitated with a mixture of Ab1801 and Ab421 anti-p53 antibodies, and the precipitates were examined by SDS-PAGE. The position of migration of p53 is shown on the left. (C) Quantitative analysis of p53 synthesis. The amount of labeled p53 was measured with a PhosphorImager and was plotted as a percentage of the value at time zero (uninfected cells).

PAGE. Fig. 3B and C show that synthesis and accumulation of p53 during the various 1-h intervals were reduced to approximately 50% of initial levels by about 5 to 6 hpi and continued to decrease up to about 15 to 20 hpi. Thus, following infection of 293 cells with AdLacZ, a specific decrease in the production of p53 appeared to take place, commencing several hours before the generalized shutoff of host cell protein synthesis in these productively infected cells. It remains possible, however, that host cell shutoff affects synthesis of p53 more acutely than that of other proteins. This possibility will be readdressed in separate experiments described below (see Fig. 9). Thus, while a decrease in the rate of translation of p53 could account for some of the drop in p53 production, it is quite possible that the seven- to eightfold reduction in p53 half-life reported above in Fig. 2C could account for all or most of this effect.

The E4orf6 protein is required to inhibit the E1A-induced accumulation of p53. Although it was possible that any of a number of proteins encoded by the E2, E3, and E4 regions, or even late viral products, could function in the prevention of accumulation of p53, the E4orf6 protein was a prime candidate, because it was already known to interact with the E1B 55-kDa protein and to play a cooperative role in host cell shutoff (7–9, 50, 51, 63). In addition, recent studies had argued that E4orf6-dependent host cell shutoff may be involved in the regulation of p53 levels (20). To analyze this possibility, we used a series of Ad5 deletion mutants which are defective in expression of various combinations of the seven E4 products. Figure 4 shows that *dl*1011 and *dl*1019 fail to produce any E4 products, whereas *dl*1013 produces E4orf4 and E4orf6, *dl*1014 yields E4orf4 alone, *dl*1015 synthesizes only E4orf3 and E4orf4, and *dl*1010 expresses all E4 products except E4orf6 (8, 9). Studies similar to those whose results are shown in Fig. 1 and 2 were conducted with human MRC-5 and 293 cells which were infected by wt Ad5 or various E4 deletion mutants. Cell extracts were analyzed for the amounts of p53 and E4orf6 proteins by Western blotting with anti-p53 monoclonal antibodies or a mouse monoclonal antibody M45 which recognizes the amino termini of the E4orf6 and E4orf6/7 proteins (41). The levels of E1A products were determined by Western blotting with M73 anti-E1A monoclonal antibody and were found to be comparable in all cases (data not shown). Figure 5A shows that no accumulation of p53 occurred following expression of wt Ad5 (lane 2) and that the amounts of p53 were small and similar to those in mock-infected cells (lane 1). The levels of p53 were similarly low in cells infected with mutant *dl*1013 (Fig. 5A, lane 4), which produces only E4orf4 and E4orf6. However, with all other E4 mutants, the accumulation of p53 to high levels was evident. This effect was also observed with *dl*1010 (Fig. 5A, lane 5), which produces all viral proteins with the exception of E4orf6.

These results indicated that E4orf6 is required for the inhibition of p53 accumulation. This conclusion was confirmed in experiments involving 293 cells infected by these E4 mutant viruses. Figure 5B shows that the high levels of p53 observed in mock-infected 293 cells (lane 1) were greatly reduced by infection with wt Ad5 (lane 2) and mutant *dl*1013 (lane 4) but not with the other mutants (lane 3 and 5 to 8). Figure 5C shows the pattern of expression of the E4orf6 and E4orf6/7 proteins, which share common amino-terminal sequences recognized by the M45 monoclonal antibody employed in this analysis. Reduction of p53 levels occurred only following infection with

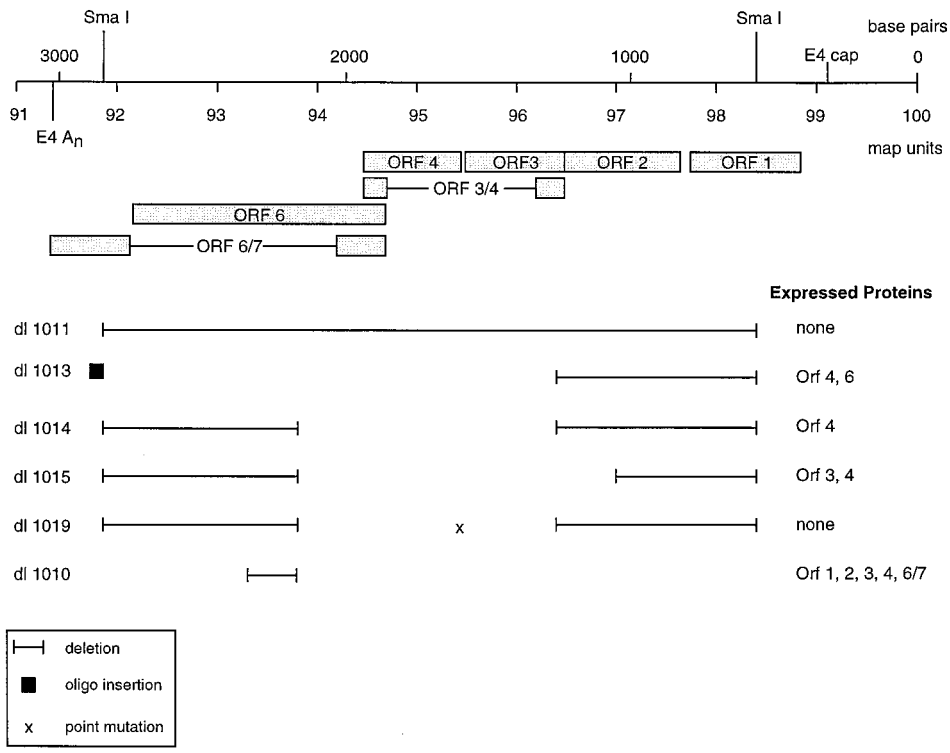

FIG. 4. E4 open reading frames and deletion mutants. The E4 region occupies about 3,000 bp at the right end of the adenovirus genome and contains open reading frames (Orfs) for at least seven proteins (12, 21). The Ad5 E4 deletion mutant viruses used in the present studies, including *dl*1010, *dl*1011, *dl*1013, *dl*1014, *dl*1015, and *dl*1019, have been described previously (8, 9).

viruses that express E4orf6, including wt Ad5 (Fig. 5C, lane 2) and *dl*1013 (Fig. 5C, lane 4). Further analysis of the time course of infection of 293 cells by AdLacZ shown previously in Fig. 2 also indicated that the reduction in p53 levels closely paralleled the appearance of the E4orf6 protein (compare Fig. 2B and A). Thus, E4orf6 appeared to be required, along with the E1B 55-kDa species, for the reduction in p53 levels.

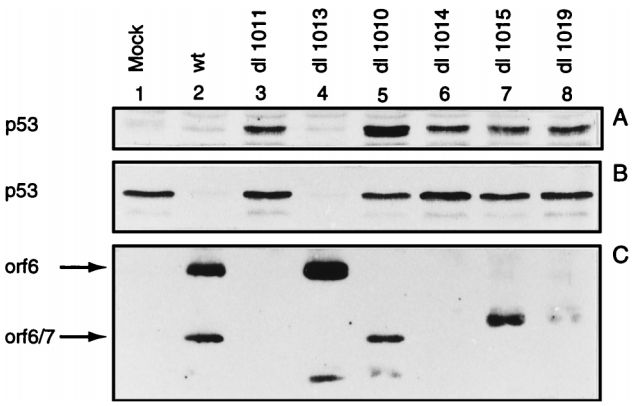

FIG. 5. Analysis of p53 accumulation and E4orf6 expression in wt and E4 mutant-infected cells. MRC-5 or 293 cells were infected with wt Ad5 or a series of Ad5 E4 mutants described in the legend to Fig. 4. At 24 hpi, cell extracts were prepared, proteins were separated by SDS-PAGE and transferred to nitrocellulose, and the amounts of p53 and E4orf6 proteins were determined by Western blotting as for Fig. 1 and 2 with ECL. (A) p53 in MRC-5 cells; (B) p53 in 293 cells; (C) E4orf6 (and E4orf6/7) in 293 cells. The membrane used for panel B was stripped and reprobed with anti-E4orf6 antibody M45. The positions of migration of p53, E4orf6, and E4orf6/7 are shown on the left.

Figure 5C also contains additional information. The small truncated form of the E4orf6/7 protein encoded by mutant *dl*1013 and containing amino-terminal sequences (see Fig. 4 for reference) is evident in lane 4 of Fig. 5C. Of more interest was the truncated form of E4orf6 produced by mutant *dl*1010 (Fig. 5C, lane 5). Figure 4 illustrates that the out-of-frame deletion present in *dl*1010 is predicted to yield an E4orf6 protein which contains only the first 96 amino-terminal residues of the 294-residue product of molecular mass of about 34 kDa (7). This truncated product appeared to be synthesized and to be relatively stable (Fig. 5C, lane 4), but it did not appear to retain the p53 inhibitory function of the full-length E4orf6.

E4orf6, p53, and the E1B 55-kDa protein each form complexes individually. It is known that the E1B 55-kDa protein interacts directly and independently with both p53 (53) and the E4orf6 product (52). To investigate the molecular basis of the inhibition of E1A-induced accumulation of p53 further, interactions between E4orf6 and p53 or the E1B 55-kDa protein were investigated by using both in vitro binding assays and coimmunoprecipitation from cell extracts. Extracts from mock-infected 293 cells or those infected with wt Ad5 and harvested at either 7 or 16 hpi were combined with M45 monoclonal antibody, which recognizes the amino terminus of the E4orf6 (and E4orf6/7) protein, and subjected to immunoprecipitation in the presence of ionic detergent, as described in Materials and Methods. Following extensive washing, precipitates were resuspended in buffer lacking ionic detergent and thus capable of maintaining protein-protein interactions, and the mixtures were incubated with [^{35}S]methionine-labeled p53 which had been transcribed and translated in vitro with a p53-specific cDNA. E4orf6 proteins were repurified by immunoprecipita-

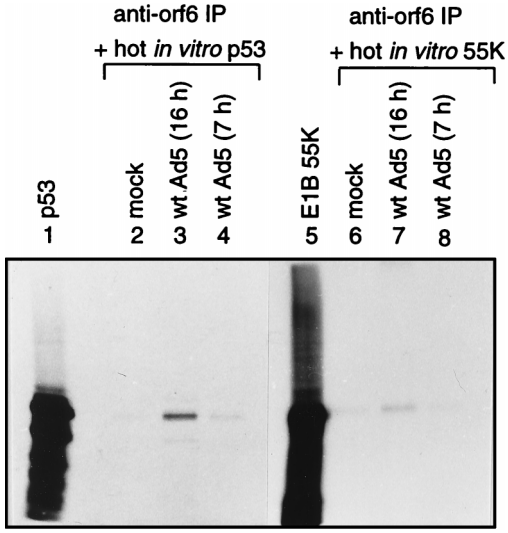

FIG. 6. In vitro binding of E4orf6 to p53 and the E1B 55-kDa protein. Mock- or Ad5-infected 293 cells were harvested at 7 and 16 hpi, and E4orf6 protein was purified by immunoprecipitation (IP) with M45 antibody. Following extensive washing in buffer containing ionic detergent, the precipitates were incubated with in vitro-synthesized [^{35}S]methionine-labeled p53 or E1B 55-kDa protein, and the amount of binding was assessed, as described in Materials and Methods. Lanes are as indicated.

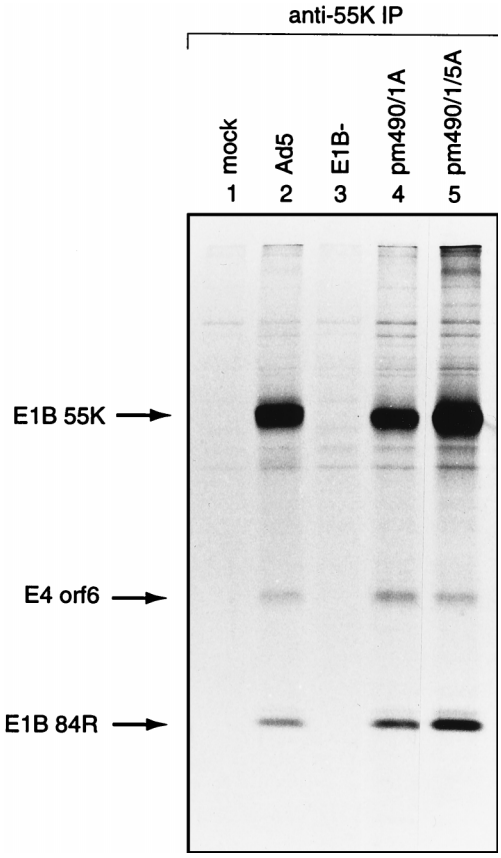

FIG. 7. Complex formation between E4orf6 and mutant and wt E1B 55-kDa protein. Mock-infected KB cells or those infected by wt Ad5 or E1B 55-kDa protein phosphorylation mutants were labeled at 16 hpi with [^{35}S]methionine for 1 h. Cell extracts were prepared under mild conditions, and immunoprecipitation (IP) was performed with 2A6 antibody, which recognizes the E1B 55-kDa protein and related products. Proteins were visualized by autoradiography. The positions of migration of the 55-kDa protein, 84R, and E4orf6 are shown on the left.

tion under mild conditions and analyzed by SDS-PAGE. Figure 6 shows that high levels of p53 as well as several truncated forms of this species were synthesized (lane 1). Addition of labeled p53 to precipitates from mock-infected cells was without effect, as no labeled species were apparent (Fig. 6, lane 2). However, in the case of precipitates from infected cells, material which comigrated with full-length p53 was present (Fig. 6, lanes 3 and 4). More p53 was present in the sample prepared with material isolated from cells at 16 hpi (Fig. 6, lane 3) than at 7 hpi (Fig. 6, lane 4), in proportion to the relative amounts of E4orf6 present at these times (data not shown). Parallel experiments were also performed with in vitro-transcribed and -translated E1B 55-kDa protein. Figure 6 shows that this protein also interacted with E4orf6 precipitates from infected cells (lanes 7 and 8) but not mock-infected cells (lane 6) in a similar fashion. (Note: the low levels present in the sample from mock-infected cells in Fig. 6, lane 6, were due to spillover from lane 5 and were not present in other analyses.) While the present work was being completed, a report was published which also established the existence of complex formation between p53 and E4orf6 and suggested that such interactions could lead to the inhibition of p53 transactivation activity (14).

Studies were also carried out to determine the role of complex formation between the E1B 55-kDa protein and E4orf6. The 55-kDa polypeptide was immunoprecipitated under mild conditions from [^{35}S]methionine-labeled wt Ad5-infected human KB cells with 2A6 monoclonal antibody, which recognizes the amino terminus of this E1B species as well as the related E1B 84R polypeptide. Figure 7 shows that in addition to the E1B 55-kDa protein and the 84R product, a species migrating at about 34 kDa was detected (lane 2). This protein appears to be E4orf6, as in other studies (data not shown) it was found to be recognized by immunoblotting with E4orf6-specific M45 serum and to be absent in extracts from cells infected with mutant *dl*1010, which fails to synthesize E4orf6. Neither the E1B species nor E4orf6 was detected with mock-infected cells (Fig. 7, lane 1) or cells infected with mutant E1B$^-$, which produces no E1B products (Fig. 7, lane 3). Figure 7, lanes 4 and 5, shows results of a similar experiment with the E1B mutants *pm*490/1A and *pm*490/1/5A, which were shown in Fig. 1B to be defective in the 289R-dependent block in accumulation of p53. Clearly, both mutant 55-kDa proteins were able to form complexes with E4orf6 at normal levels, in agreement with the finding that they induce host cell shutoff normally (59). These results also indicated that complex formation between E4orf6 and p53 is not sufficient to induce a reduction in p53 levels and that inhibition of p53 transactivation activity may be necessary.

Host cell shutoff is not responsible for the block in accumulation of p53 by E4orf6 and the E1B 55-kDa protein. Although the experiments described above suggested that the block in accumulation of p53 did not appear to result from generalized shutoff of host cell protein synthesis induced by the E1B 55-kDa protein and E4orf6, we conducted further experiments to examine this possibility, as others (20) had concluded that this process plays a role in the regulation of p53 levels. To establish the roles of these viral proteins more clearly, an adenovirus vector which expresses the Ad2 E4orf6 protein under the control of the CMV promoter was constructed. It was, therefore, possible to express E4orf6 in the absence of other viral proteins. HeLa cells were mock infected or infected with one or more Ad5 mutants and/or the AdE4orf6 vector, and extracts

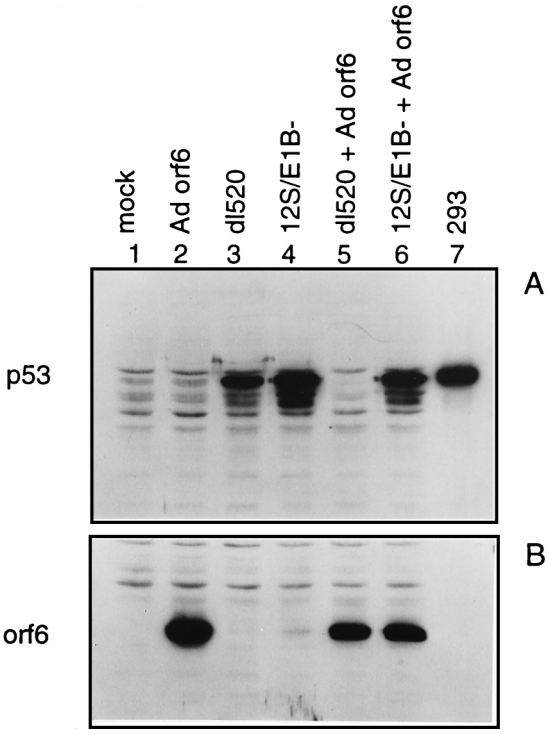

FIG. 8. Accumulation of p53 in HeLa cells infected with Ad5 mutants and Ad2E4orf6. HeLa cells were infected with various Ad5 mutants or the AdE4orf6 vector, and cell extracts were prepared at 24 hpi and separated by SDS-PAGE. Following transfer to nitrocellulose, p53 (A) and E4orf6 (B) were detected by Western blotting with Ab1801 and M45 antibodies, respectively, and ECL. The positions of migration of p53 and E4orf6 are shown on the left.

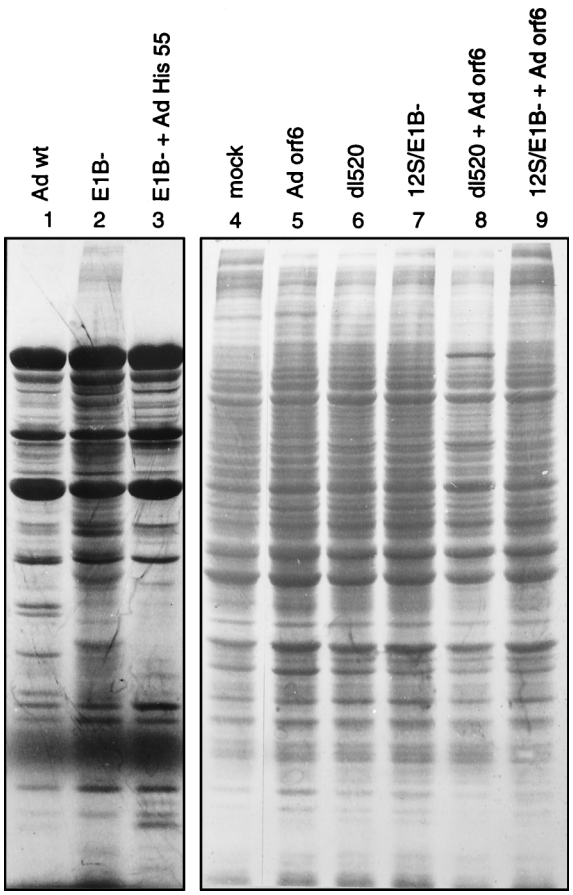

FIG. 9. Host cell shutoff in HeLa cells infected with Ad5 mutants and vectors. HeLa cells were infected with various Ad5 mutants or the adenovirus vectors AdE4orf6 or AdHis55K. At 36 hpi, the cells were labeled with [^{35}S]methionine for 1 h, cell extracts were separated by SDS-PAGE, and proteins were visualized by autoradiography. Lanes are as indicated.

were analyzed for p53 and E4orf6 protein levels by Western blotting, as in Fig. 1 and 2. Figure 8 shows that infection by the AdE4orf6 vector alone resulted in high levels of expression of E4orf6 (Fig. 8B, lane 2) but no effect on p53 levels (Fig. 8A, lane 2). Infection with mutant *dl*520 (Fig. 8A, lane 3), which expresses E1A 243R, and mutant 12S/E1B$^-$ (Fig. 8A, lane 4), which also expresses 243R but no E1B products, resulted in a large increase in p53 protein levels, as reported previously (10, 45) and as shown above in Fig. 1A. In these cases, only very low levels of E4orf6 were detected (see Fig. 8B, lanes 3 and 4, respectively), as E1A 243R alone does not induce either high levels of expression of E3 and E4 products or synthesis of late viral proteins. Coinfection by *dl*520 and the AdE4orf6 vector completely suppressed this accumulation of p53 (Fig. 8A, lane 5). However, such was not the case following coinfection with 12S/E1B$^-$ and AdE4orf6 (Fig. 8A, lane 6), as high levels of p53, which were at best only slightly reduced relative to 12S/E1B$^-$ alone, were present (Fig. 8A, lane 4). These results confirmed again that both E4orf6 and the E1B 55-kDa protein are necessary to block the E1A-induced accumulation of p53 efficiently. Similar results were also obtained with mouse 10T1/2 cells and human MRC-5 cells (data not shown).

Using the AdE4orf6 vector, it was also possible to examine more precisely the role of generalized host cell shutoff in the control of p53 levels. HeLa cells were infected by various combinations of Ad5 and adenovirus vectors, and after being labeled with [^{35}S]methionine for 1 h at 36 hpi, whole-cell extracts were analyzed by SDS-PAGE followed by autoradiography. Figure 9 shows that, in wt Ad5-infected cells (lane 1), a significant decrease in synthesis of cellular proteins was evident but that high levels of late viral polypeptides were being produced. This pattern was typical of the late host cell shutoff observed commonly in virus-infected cells. In the case of mutant E1B$^-$, which produces both major E1A products but no E1B proteins, late viral protein expression was evident, but synthesis of cellular proteins was not shut down significantly (Fig. 9, lane 2). This effect was reversed when the E1B 55-kDa protein was supplied by coinfection with adenovirus vector AdHis55K, which expresses a histidine-tagged version of this viral protein (Fig. 9, lane 3). Of considerable interest was the analysis of cells infected by *dl*520 and the AdE4orf6 vector. Synthesis of late viral proteins was poor in all cases (Fig. 9, lanes 4 to 9). In addition, no host cell shutoff was apparent relative to mock-infected cells (Fig. 9, lane 4) in cells infected by AdE4orf6 alone (lane 5), *dl*520 (lane 6), or 12S/E1B$^-$ (lane 7) or in those coinfected by *dl*520 or 12S/E1B$^-$ and AdE4orf6 (lanes 8 and 9, respectively). These results indicated that whereas it was possible to block accumulation of p53 induced by *dl*520 by supplying E4orf6 (Fig. 8A, lane 5), induction of generalized host cell shutoff appeared to require some additional viral protein, possibly one encoded by E3, E4, or a late product. Thus, the decrease in E1A-induced accumulation of p53 by E1B 55-kDa protein and E4orf6 is not due to host cell shutoff regulated by these proteins.

DISCUSSION

Expression of E1A proteins is known to induce the accumulation of p53, and other studies by our group have linked this increase to complex formation between E1A molecules and the p300 transcriptional modulator (and possibly related proteins p400 and the CREB binding protein) or the RB tumor suppressor and related polypeptides (45). Previous reports indicated that complex formation with either class of protein induces DNA synthesis in serum-starved primary rodent cells (25, 26, 56), and thus it is possible that in Ad5-infected cells, the rise in p53 levels results from stimulation of unscheduled DNA synthesis. The mechanism of accumulation of p53 has not been established. As discussed above, increased protein stability has been observed in the case of E1A-transformed human and rodent cells (17, 19, 20, 32); however, such is not always the case (18, 19). Increases in p53 levels induced by simian virus 40 large T antigen have been linked to increased stability of p53 as a result of complex formation with this viral protein (31). However, neither the 289R nor 243R E1A product is believed to bind directly to p53, and expression of the E1B 55-kDa protein, which does interact with p53, is not required for p53 accumulation. It has been suggested that p53 expression may also be regulated at the translational level (15, 39) and that the p53 protein may play some autoregulatory role (39). Further studies will be required to establish the molecular basis for E1A-induced accumulation of p53.

Of some interest was the observation that whereas infection with adenovirus encoding only the E1A 243R protein induced about an 8- to 10-fold rise in p53 levels, expression of the E1A 289R protein blocked this effect. Thus, infection with wt Ad5 or coinfection with viruses expressing each E1A protein individually resulted in no overt change in p53 quantities relative to the endogenous levels of uninfected cells. This inhibitory process required an active E1B 55-kDa protein, as in its absence, high levels of p53 were detected regardless of the E1A species expressed. Mutational analysis suggested that complex formation with p53 may be required, and in addition, the transcriptional repression activity resident in the 55-kDa species must be functional. These results could suggest that suppression of p53 transactivation activity is necessary. Of further interest was the fact that expression of E1A 289R and the E1B 55-kDa protein was not sufficient to prevent E1A induction of p53 accumulation. Human 293 cells contain high endogenous levels of p53, presumably due to the effects of E1A proteins, and such cells express both the E1A 289R and E1B 55-kDa proteins (16, 18). We showed that the reduction of p53 accumulation requires expression of the E4orf6 product. We were also able to show that expression of the E4orf6 and E1B 55-kDa proteins is sufficient to block the accumulation of p53 induced by E1A and that additional E3, E4, or late proteins do not appear to be required. In addition, expression of E4orf6 in the absence of the E1B 55-kDa product blocked p53 accumulation only very poorly, if at all.

The mechanism by which this inhibition occurs remains to be established. As discussed above, it is possible that a decrease in the rate of translation plays a role in blocking the accumulation of p53. The present experiments were not designed to examine this question in detail. However, interactions between the E4orf6 and E1B 55-kDa proteins have been linked to shutoff of host cell protein synthesis and stabilization and transport of viral mRNA (1, 2, 12, 21, 30, 43, 44, 50, 52, 63). It was possible that such inhibition plays a role in the drop in p53 levels, as suggested in an earlier study (20). However, three observations argued strongly that generalized shutoff of host protein synthesis induced by complexes of E4orf6 and 55-kDa protein plays little or no role in the block in p53 accumulation. The first was that mutants *pm*490/1A and *pm*490/1/5A, which encode 55-kDa proteins lacking two or all three of the carboxy-terminal phosphorylation sites, fail to prevent the rise in p53 levels even though these mutant E1B products induce host cell shutoff effectively (59). Second, generalized host cell shutoff in infected 293 cells started over 7 h after the beginning of the decline in p53 levels. And third, expression of E4orf6 via an adenovirus vector in cells infected with *dl*520, which expresses only very small quantities of E3, E4, and late proteins, induced the block in p53 accumulation but not host cell shutoff. Thus, we believe that the effects on p53 are not the result of global inhibition of host cell protein synthesis but, rather, are caused by some other process that is targeted uniquely to p53.

The major cause of the block in E1A-induced accumulation of p53 is likely related to a change in the stability of p53 molecules. Results obtained with AdLacZ-infected 293 cells indicated that the extended half-life of about 2.5 h in uninfected cells diminished to about 20 min following virus infection and expression of E4orf6. Thus, the reduction in the rate of p53 production during 1-h intervals could possibly be explained entirely by this seven- to eightfold decrease in p53 stability. The presence of E4orf6 and the E1B 55-kDa product therefore may function to block increased p53 stability induced by E1A protein.

These effects probably involve the formation of multiprotein complexes. The E1B 55-kDa protein has been known for some time to bind to both p53 and E4orf6 (52, 53). We show here that E4orf6 is also capable of binding to p53, an observation reported recently by others who also demonstrated that such complex formation led to the inactivation of p53 transactivation activity (14). Thus, formation of trimeric complexes containing p53, E4orf6, and the E1B 55-kDa species could be responsible for preventing accumulation of p53 induced by E1A, although at present there is no direct evidence that such complexes are formed. Just how such complex formation could affect p53 stability remains to be established, although the present studies indicate that a functional E1B 55-kDa protein repression domain (and thus perhaps inhibition of the p53 transactivation activity) seems to be necessary.

As the present manuscript was being submitted, Moore *et al.* (38) reported that E4orf6 is able to cooperate with E1A in cell transformation, presumably through the inactivation of p53, as reported earlier (14). This study also reported that expression of E4orf6 in stable transformants induces both a decrease in p53 stability and a reduction in p53 levels. These data are in complete accord with the present results. However, our results differ in one significant way. Whereas in the stable transformants E4orf6 was able to produce these effects alone, in the present studies using virus-infected cells, the E1B 55-kDa protein was clearly also required. Further studies will be necessary to determine the reason for these differences and the molecular basis for these profound effects on p53.

ACKNOWLEDGMENTS

We thank Pat Hearing for providing M45 antibody, Göran Akusjärvi for the Ad2E4orf6 cDNA, Frank Graham for the gift of vector AdLacZ, Stan Bayley for mutant 12S/E1B$^-$, Arnie Berk for plasmid P53pSP65 and mutants A262 and R309, and Ed Harlow for M73 and 421 hybridoma cells. We also thank Dennis Takayesu for some technical assistance.

This work was supported by grants to P.E.B. from the Medical Research Council of Canada and the National Cancer Institute of Canada. E.Q. is supported by a Studentship from the Fonds FRSQ-FCAR-Santé du Québec, R.C.M. held a Student Research Award from the Glaxo/Burroughs-Wellcome Corporation, and J.G.T. was a Research Student of the National Cancer Institute of Canada.

REFERENCES

1. **Babiss, L. E., and H. S. Ginsberg.** 1984. Adenovirus type 5 early region 1b gene product is required for efficient shutoff of host protein synthesis. J. Virol. **50:**202–212.
2. **Babiss, L. E., H. S. Ginsberg, and J. J. Darnell.** 1985. Adenovirus E1B proteins are required for accumulation of late viral mRNA and for effects on cellular mRNA translation and transport. Mol. Cell. Biol. **5:**2552–2558.
3. **Bacchetti, S., and F. L. Graham.** 1993. Inhibition of cell proliferation by an adenovirus vector expressing the human wild type p53 protein. Int. J. Oncol. **3:**781–788.
4. **Bayley, S. T., and J. S. Mymryk.** 1994. Adenovirus E1A proteins and transformation Int. J. Oncol. **5:**425–444. (Review.)
5. **Boyd, J. M., S. Malstrom, T. Subramanian, L. K. Venkatesh, U. Schaeper, B. Elangovan, C. D'Sa-Eipper, and G. Chinnadurai.** 1994. Adenovirus E1B 19 kDa and Bcl-2 proteins interact with a common set of cellular proteins. Cell **79:**341–351. (Erratum, **79:**1120, 1994.)
6. **Braithwaite, A., C. Nelson, A. Skulimowski, J. McGovern, D. Pigott, and J. Jenkins.** 1990. Transactivation of the p53 oncogene by E1a gene products. Virology **177:**595–605.
7. **Bridge, E., and G. Ketner.** 1989. Redundant control of adenovirus late gene expression by early region 4. J. Virol. **63:**631–638.
8. **Bridge, E., and G. Ketner.** 1990. Interaction of adenoviral E4 and E1b products in late gene expression. Virology **174:**345–353.
9. **Bridge, E., S. Medghalchi, S. Ubol, M. Leesong, and G. Ketner.** 1993. Adenovirus early region 4 and viral DNA synthesis. Virology **193:**794–801.
10. **Chiou, S. K., C. C. Tseng, L. Rao, and E. White.** 1994. Functional complementation of the adenovirus E1B 19-kilodalton protein with Bcl-2 in the inhibition of apoptosis in infected cells. J. Virol. **68:**6553–6566.
11. **Crook, T., D. Wrede, and K. H. Vousden.** 1991. p53 point mutation in HPV negative human cervical carcinoma cell lines. Oncogene **6:**873–875.
12. **Cutt, J. R., T. Shenk, and P. Hearing.** 1987. Analysis of adenovirus early region 4-encoded polypeptides synthesized in productively infected cells. J. Virol. **61:**543–552.
13. **Debbas, M., and E. White.** 1993. Wild-type p53 mediates apoptosis by E1A, which is inhibited by E1B. Genes Dev. **7:**546–554.
14. **Dobner, T., N. Horikoshi, S. Rubenwolf, and T. Shenk.** 1996. Blockage by adenovirus E4orf6 of transcriptional activation by the p53 tumor suppressor. Science **272:**1470–1473.
15. **Fu, L. N., M. D. Minden, and S. Benchimol.** 1996. Translational regulation of human p53 gene expression. EMBO J. **15:**4392–4401.
16. **Graham, F. L., J. Smiley, W. C. Russel, and R. Nairn.** 1977. Characteristics of a human cell line transformed by DNA from human adenovirus type 5. J. Gen. Virol. **36:**59–72.
17. **Grand, R. J., M. L. Grant, and P. H. Gallimore.** 1994. Enhanced expression of p53 in human cells infected with mutant adenoviruses. Virology **203:**229–240.
18. **Grand, R. J., P. S. Lecane, D. Owen, M. L. Grant, S. Roberts, A. J. Levine, and P. H. Gallimore.** 1995. The high levels of p53 present in adenovirus early region 1-transformed human cells do not cause up-regulation of MDM2 expression. Virology **210:**323–334.
19. **Grand, R. J., P. S. Lecane, S. Roberts, M. L. Grant, D. P. Lane, L. S. Young, C. W. Dawson, and P. H. Gallimore.** 1993. Overexpression of wild-type p53 and c-Myc in human fetal cells transformed with adenovirus early region 1. Virology **193:**579–591.
20. **Grand, R. J., D. Owens, S. M. Rookes, and P. H. Gallimore.** 1996. Control of p53 expression by adenovirus 12 early region 1A and early region 1B 54K proteins. Virology **218:**23–34.
21. **Halbert, D. N., J. R. Cutt, and T. Shenk.** 1985. Adenovirus early region 4 encodes functions required for efficient DNA replication, late gene expression, and host cell shutoff. J. Virol. **56:**250–257.
22. **Haley, K. P., J. Overhauser, L. E. Babiss, H. S. Ginsberg, and N. C. Jones.** 1984. Transformation properties of type 5 adenovirus mutants that differentially express the E1A gene products. Proc. Natl. Acad. Sci. USA **81:**5734–5738.
23. **Harlow, E., B. J. Franza, and C. Schley.** 1985. Monoclonal antibodies specific for adenovirus early region 1A proteins: extensive heterogeneity in early region 1A products. J. Virol. **55:**533–546.
24. **Harrison, T., F. L. Graham, and J. Williams.** 1977. Host range mutants of adenovirus type 5 defective for growth in HeLa cells. Virology **77:**319–329.
25. **Howe, J. A., and S. T. Bayley.** 1992. Effects of Ad5 E1A mutant viruses on the cell cycle in relation to the binding of cellular proteins including the retinoblastoma protein and cyclin A. Virology **186:**15–24.
26. **Howe, J. A., J. S. Mymryk, C. Egan, P. E. Branton, and S. T. Bayley.** 1990. Retinoblastoma growth suppressor and a 300-kDa protein appear to regulate cellular DNA synthesis. Proc. Natl. Acad. Sci. USA **87:**5883–5887.
27. **Jones, N., and T. Shenk.** 1979. Isolation of adenovirus type 5 host-range deletion mutants defective for transformation of rat embryo cells. Cell **17:**683–689.
28. **Kao, C. C., P. R. Yew, and A. J. Berk.** 1990. Domains required for in vitro association between the cellular p53 and the adenovirus 2 E1B 55K proteins. Virology **179:**806–814.
29. **Ko, L. J., and C. Prives.** 1996. p53: puzzle and paradigm. Genes Dev. **10:**1054–1072.
30. **Leppard, K. N., and T. Shenk.** 1989. The adenovirus E1B 55 kd protein influences mRNA transport via an intranuclear effect on RNA metabolism. EMBO J. **8:**2329–2336.
31. **Linzer, D. I., and A. J. Levine.** 1979. Characterization of a 54K dalton cellular SV40 tumor antigen present in SV40-transformed cells and uninfected embryonal carcinoma cells. Cell **17:**43–52.
32. **Lowe, S. W., and H. E. Ruley.** 1993. Stabilization of the p53 tumor suppressor is induced by adenovirus 5 E1A and accompanies apoptosis. Genes Dev. **7:**535–545.
33. **Lowe, S. W., H. E. Ruley, T. Jacks, and D. E. Housman.** 1993. p53-dependent apoptosis modulates the cytotoxicity of anticancer agents. Cell **74:**957–967.
34. **Marcellus, R. C., J. G. Teodoro, R. Charbonneau, G. C. Shore, and P. E. Branton.** 1996. Expression of p53 in Saos-2 osteosarcoma cells induces apoptosis which can be inhibited by Bcl-2 or the adenovirus E1B-55 kilodalton protein. Cell Growth Differ. **7:**1643–1650.
35. **Marcellus, R. C., J. G. Teodoro, T. Wu, D. E. Brough, G. Ketner, G. C. Shore, and P. E. Branton.** 1996. Adenovirus type 5 early region 4 is responsible for E1A-induced p53-independent apoptosis. J. Virol. **70:**6207–6215.
36. **McLorie, W., C. J. McGlade, D. Takayesu, and P. E. Branton.** 1991. Individual adenovirus E1B proteins induce transformation independently but by additive pathways. J. Gen. Virol. **72:**1467–1471.
37. **Montell, C., T. Grodzicker, R. J. Roberts, M. B. Mathews, and B. Zerler.** 1982. Resolving the function of overlapping genes by site-specific mutagenesis at a mRNA splice site. Nature (London) **295:**380–384.
38. **Moore, M., N. Horikoshi, and T. Shenk.** 1996. Oncogenic potential of the adenovirus E4orf6 protein. Proc. Natl. Acad. Sci. USA **93:**11295–11301.
39. **Mosner, J., T. Mummenbrauer, C. Bauer, G. Scazakiel, F. Grosse, and W. Deppert.** 1995. Negative feedback regulation of wild-type p53 biosynthesis. EMBO J. **14:**4442–4449.
40. **Nguyen, M., P. E. Branton, P. A. Walton, Z. N. Oltvai, S. J. Korsmeyer, and G. C. Shore.** 1994. Role of membrane anchor domain of Bcl-2 in suppression of apoptosis caused by E1B-defective adenovirus. J. Biol. Chem. **269:**16521–16524.
41. **Obert, S., R. J. O'Connor, S. Schmid, and P. Hearing.** 1994. The adenovirus E4-6/7 protein transactivates the E2 promoter by inducing dimerization of a heteromeric E2F complex. Mol. Cell. Biol. **14:**1333–1346.
42. **Ohman, K., K. Nordqvist, and G. Akusjarvi.** 1993. Two adenovirus proteins with redundant activities in virus growth facilitate tripartite leader mRNA accumulation. Virology **194:**50–58.
43. **Ornelles, D. A., and T. Shenk.** 1991. Localization of the adenovirus early region 1B 55-kilodalton protein during lytic infection: association with nuclear viral inclusions requires the early region 4 34-kilodalton protein. J. Virol. **65:**424–429.
44. **Pilder, S., M. Moore, J. Logan, and T. Shenk.** 1986. The adenovirus E1B-55K transforming polypeptide modulates transport or cytoplasmic stabilization of viral and host cell mRNAs. Mol. Cell. Biol. **6:**470–476.
45. **Querido, E., J. G. Teodoro, and P. E. Branton.** 1997. Accumulation of p53 induced by the adenovirus E1A protein requires regions involved in the stimulation of DNA synthesis. J. Virol. **71:**3526–3533.
46. **Rao, L., M. Debbas, P. Sabbatini, D. Hockenbery, S. Korsmeyer, and E. White.** 1992. The adenovirus E1A proteins induce apoptosis, which is inhibited by the E1B 19-kDa and Bcl-2 proteins. Proc. Natl. Acad. Sci. USA **89:**7742–7746. (Erratum, **89:**9974, 1992.)
47. **Reznikoff, C. A., D. W. Brankow, and C. Heidelberger.** 1973. Establishment and characterization of a cloned cell line of C3H mouse embryo cells sensitive to postconfluence inhibition of cell division. Cancer Res. **33:**3231–3238.
48. **Rowe, D. T., P. E. Branton, and F. L. Graham.** 1984. The kinetics of synthesis of early viral proteins in KB cells infected with wild-type and transformation-defective host-range mutants of human adenovirus type 5. J. Gen. Virol. **65:**585–597.
49. **Sanchez-Prieto, R., M. Lleonart, and S. Ramón y Cajal.** 1995. Lack of correlation between p53 protein level and sensitivity of DNA-damaging agents in keratinocytes carrying adenovirus E1a mutants. Oncogene **11:**675–682.
50. **Sandler, A. B., and G. Ketner.** 1989. Adenovirus early region 4 is essential for normal stability of late nuclear RNAs. J. Virol. **63:**624–630.
51. **Sandler, A. B., and G. Ketner.** 1991. The metabolism of host RNAs in cells infected by an adenovirus E4 mutant. Virology **181:**319–326.
52. **Sarnow, P., P. Hearing, C. W. Anderson, D. N. Halbert, T. Shenk, and A. J. Levine.** 1984. Adenovirus early region 1B 58,000-dalton tumor antigen is physically associated with an early region 4 25,000-dalton protein in productively infected cells. J. Virol. **49:**692–700.
53. **Sarnow, P., Y. S. Ho, J. Williams, and A. J. Levine.** 1982. Adenovirus E1b-58kd tumor antigen and SV40 large tumor antigen are physically associated with the same 54 kd cellular protein in transformed cells. Cell **28:**387–394.
54. **Sarnow, P., C. A. Sullivan, and A. J. Levine.** 1982. A monoclonal antibody detecting the adenovirus type 5-E1b-58Kd tumor antigen: characterization of the E1b-58Kd tumor antigen in adenovirus-infected and -transformed cells. Virology **120:**510–517.

55. **Shenk, T., and J. Flint.** 1991. Transcriptional and transforming activities of the adenovirus E1A proteins. Adv. Cancer Res. **57:**47–85. (Review.)
56. **Shepherd, S. E., J. A. Howe, J. S. Mymryk, and S. T. Bayley.** 1993. Induction of the cell cycle in baby rat kidney cells by adenovirus type 5 E1A in the absence of E1B and a possible influence of p53. J. Virol. **67:**2944–2949.
57. **Subramanian, T., B. Tarodi, and G. Chinnadurai.** 1995. p53-independent apoptotic and necrotic cell deaths induced by adenovirus infection: suppression by E1B 19K and Bcl-2 proteins. Cell Growth Differ. **6:**131–137.
58. **Teodoro, J. G., and P. E. Branton.** 1997. Regulation of p53-dependent apoptosis, transcriptional repression, and cell transformation by phosphorylation of the 55-kilodalton E1B protein of human adenovirus type 5. J. Virol. **71:**3620–3627.
59. **Teodoro, J. G., T. Halliday, S. G. Whalen, D. Takayesu, F. L. Graham, and P. E. Branton.** 1994. Phosphorylation at the carboxy terminus of the 55-kilodalton adenovirus type 5 E1B protein regulates transforming activity. J. Virol. **68:**776–786.
60. **Teodoro, J. G., G. C. Shore, and P. E. Branton.** 1995. Adenovirus E1A proteins induce apoptosis by both p53-independent and p53-independent mechanisms. Oncogene **11:**467–474.
61. **van den Heuvel, S. J., T. van Laar, W. M. Kast, C. J. Melief, A. Zantema, and A. J. van der Eb.** 1990. Association between the cellular p53 and the adenovirus 5 E1B-55kd proteins reduces the oncogenicity of Ad-transformed cells. EMBO J. **9:**2621–2629.
62. **Weinberg, D. H., and G. Ketner.** 1983. A cell line that supports the growth of a defective early region 4 deletion mutant of human adenovirus type 2. Proc. Natl. Acad. Sci. USA **80:**5383–5386.
63. **Weinberg, D. H., and G. Ketner.** 1986. Adenoviral early region 4 is required for efficient viral DNA replication and for late gene expression. J. Virol. **57:**833–838.
64. **Yew, P. R., and A. J. Berk.** 1992. Inhibition of p53 transactivation required for transformation by adenovirus early 1B protein. Nature (London) **357:** 82–85.
65. **Yew, P. R., C. C. Kao, and A. J. Berk.** 1990. Dissection of functional domains in the adenovirus 2 early 1B 55K polypeptide by suppressor-linker insertional mutagenesis. Virology **179:**795–805.
66. **Yew, P. R., X. Liu, and A. J. Berk.** 1994. Adenovirus E1B oncoprotein tethers a transcriptional repression domain to p53. Genes Dev. **8:**190–202.
67. **Zantema, A., P. I. Schrier, O. A. Davis, T. van Laar, R. T. Vaessen, and A. J. van der Eb.** 1985. Adenovirus serotype determines association and localization of the large E1B tumor antigen with cellular tumor antigen p53 in transformed cells. Mol. Cell. Biol. **5:**3084–3091.