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Accumulation of p53 Induced by the Adenovirus E1A Protein Requires Regions Involved in the Stimulation of DNA Synthesis

EMMANUELLE QUERIDO,[1] JOSE G. TEODORO,[1] AND PHILIP E. BRANTON[1,2]*

Departments of Biochemistry[1] and Oncology,[2] McGill University, Montreal, Quebec, Canada H3G 1Y6

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It has been known for some time that expression of the 243-residue (243R) human adenovirus type 5 (Ad5) early region 1A (E1A) protein causes an increase in the level of the cellular tumor suppressor p53 and induction of p53-dependent apoptosis. Deletion of a portion of conserved region 1 (CR1) had been shown to prevent apoptosis, suggesting that binding of p300 and/or the pRB retinoblastoma tumor suppressor and related proteins might be implicated. To examine the mechanism of the E1A-induced accumulation of p53, cells were infected with viruses expressing E1A-243R containing various deletions which have well-characterized effects on p300 and pRB binding. It was found that in human HeLa cells and rodent cells, complex formation with p300 but not pRB was required for the rise in p53 levels. However, in other human cell lines, including MRC-5 cells, E1A proteins which were able to form complexes with either p300 or pRB induced a significant increase in p53 levels. Only E1A mutants defective in binding both classes of proteins were unable to stimulate p53 accumulation. This same pattern was also apparent in p53-null mouse cells coinfected by Ad5 mutants and an adenovirus vector expressing either wild-type or mutant human p53 under a cytomegalovirus promoter, indicating that the difference in importance of pRB binding may relate to differences between rodent and human p53 expression. The increase in p53 levels correlated well with the induction of apoptosis and, as shown previously, with the stimulation of cellular DNA synthesis. Thus, it is possible that the accumulation of p53 is induced by the induction of unscheduled DNA synthesis by E1A proteins and that increased levels of p53 then activate cell death pathways.

The products of early region 1A (E1A) of human adenoviruses play several critical roles in viral replication. The largest E1A product, 289 residues (289R), which is encoded by the 13S mRNA, is important in the activation of transcription of all early viral genes, largely through the action of the 46-residue conserved region 3 (CR3) domain (19). Adenoviruses normally replicate in terminally differentiated epithelial cells and thus have evolved mechanisms to induce DNA synthesis in order to permit replication of the viral DNA genome. Work by our group (31) and others (4, 60) showed that E1A proteins induce DNA synthesis by two probably independent mechanisms involving interactions with two classes of cellular proteins. Complex formation with the pRB tumor suppressor and related proteins p107 and p130 was shown to stimulate DNA synthesis in serum-starved baby rat kidney (BRK) cells (31). Such interactions result in the release of transcription factor E2F heterodimers which regulate the expression of genes involved in DNA synthesis (45). However, binding of E1A proteins to the transcriptional modulator p300 (15) and/or related proteins p400 (3) and the CREB-binding protein (1) also results in the activation of the S phase in quiescent cells (31). Figure 1 shows that complex formation with the pRB family of proteins involves a primary binding site within conserved region 2 (CR2) and a secondary but functionally important site in conserved region 1 (CR1) (9, 17, 58). Binding of p300 requires both CR1 and the extreme amino terminus of E1A proteins (3, 17, 58). The 243-residue (243R) product of the 12S E1A mRNA is identical to 289R except that it lacks CR3, and thus both 289R and 243R are capable of inducing unscheduled DNA synthesis. It is likely that the stimulation of DNA synthesis via these two mechanisms constitutes the basis for the transforming activity of these two E1A products.

E1A alone transforms cells very poorly because E1A proteins induce apoptosis and cell death. Stable E1A-dependent transformation occurs most efficiently in the presence of products of early region 1B (E1B), which encodes two major polypeptides of 19 and 55 kDa. These proteins play important roles in both productive infection and cell transformation, most probably by preventing rapid cell death due to E1A-induced apoptosis. The 19-kDa product is functionally homologous to the cellular Bcl-2 protein, which blocks apoptosis, at least in part, through complex formation with the Bax protein regulator of terminal death pathways (27). The 55-kDa E1B protein binds to and inactivates the p53 tumor suppressor (36, 50, 59), which has been linked to the regulation of growth arrest and apoptosis (37). Expression of either E1B product allows E1A-dependent cell transformation to occur, although the expression of both proteins results in much higher transforming efficiencies.

It is now known that E1A induces both p53-dependent and p53-independent apoptosis. The latter is produced only by the 289R E1A product and requires expression of an early region 4 (E4) protein (40, 55). The 243R E1A polypeptide is unable to induce apoptosis in 53-null cells (55). However, in the presence of wild-type p53 protein, either expressed endogenously or introduced ectopically into p53-null cells, E1A-243R induces the full range of apoptotic events including cell death, degradation of DNA to nucleosome-sized fragments, and all of the classic morphological hallmarks (55). Expression of E1A-243R leads to a large increase in the levels of p53, and it is likely that this increase causes apoptosis (5, 8, 12, 23–25, 39,

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

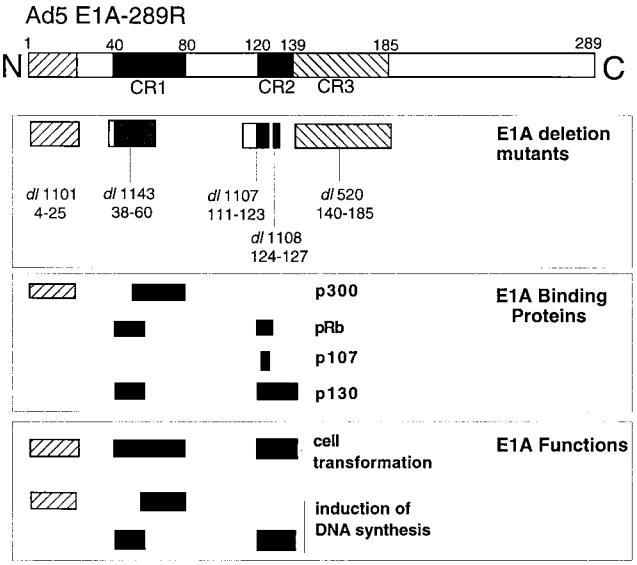

FIG. 1. Ad5 E1A functions and mutants. The Ad5 E1A protein containing CR1, CR2, and CR3 is illustrated, along with the sequences deleted in a series of E1A mutants. The sequences required for the binding of p300- and pRb-related proteins (3) are indicated, as are those shown to play a role in cell transformation (16, 17, 38, 42, 52, 54, 57) and induction of DNA synthesis (4, 31, 60).

49). Previous studies have shown that induction of apoptosis by E1A-243R is prevented by deletions within CR1 and the amino terminus (44, 56), thus implicating complex formation with the p300 or pRB families of proteins or both.

In the present study, we have taken a genetic approach to investigate further the mechanism by which E1A-243R induces the accumulation of p53. The data indicated that complex formation with the pRB family and/or p300 appears to be responsible and that such accumulation induces apoptosis.

MATERIALS AND METHODS

Cells and viruses. Normal MRC-5 diploid human embryonic lung fibroblasts (ATCC CCL 175), IMR-90 (ATCC CCL 186), human HeLa cells, baby rat kidney (BRK) cells, mouse 10T1/2 cells (47), and mouse 10(1) cells which fail to express p53 (30) were cultured on 60-mm-diameter dishes (Corning Glass Works, Corning, N.Y.) in α-modified minimal essential medium supplemented with 10% fetal calf serum. Human 293 cells derived from human embryonic kidney cells and expressing adenovirus type 5 (Ad5) E1A and E1B proteins (22) were also used in some experiments and for preparation and titer determination of all virus stocks. Cells were infected with wild-type (*wt*) or mutant virus at a multiplicity of 35 PFU per cell, as described previously (48). Almost all viruses used in the present studies were derived from *dl*520, which produces the E1A 12S mRNA but not the 13S mRNA and thus produces E1A 243R but not 289R (26). Construction of the series of Ad5 deletion mutants containing in-frame deletions within the E1A coding sequence of exon 1 of *dl*520 has been described previously (17, 31, 34, 35). In addition, most of the E1A mutants used have been introduced into a background of mutant 12S/E1B$^-$ (originally *dl*520T), which produces only E1A-243R and no E1B products (53). As illustrated in Fig. 1, these include 01/12S/E1B$^-$ (amino acids 4 to 25 deleted), 43/12S/E1B$^-$ (38 to 60 deleted), 07/12S/E1B$^-$ (111 to 123 deleted), 08/12S/E1B$^-$ (124 to 127 deleted), and 01/08/12S/E1B$^-$ (4 to 25 and 124 to 127 deleted). In one experiment (see Fig. 7), two viruses which express both 12S and 13S E1A mRNAs were used. These included *wt* Ad5 (29) and mutant E1B$^-$, which expresses neither the 19-kDa nor the 55-kDa E1B product (55). The Ad5 vectors used in this study included Adp53wt, in which the E1 (E1A and E1B) region of Ad5 was replaced by the *wt* human p53 gene under the cytomegalovirus (CMV) promoter; Adp53mut, which is identical except that the p53 sequence contains dominant negative mutations affecting the codons for Pro-72 and Cys-135; and AdLacZ, which is a similar construct but expresses *Escherichia coli lacZ* (2).

Western blotting analysis. Whole-cell extracts were prepared from mock- or adenovirus-infected cells at 24 h postinfection. Cells were lysed in 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 mg each of aprotinin, leupeptin, and pepstatin per ml, 0.5 mM phenylmethylsulfonyl fluoride, and 1 mM dithiothreitol). Total protein was measured by the Bio-Rad protein assay as specified by the manufacturer, and 50 μg of total protein was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with a 10% polyacrylamide gel. 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 or E1A proteins. Anti-p53 mouse monoclonal antibodies Ab1801 (Ab-2; Oncogene Science), Ab240 (Ab-3), and Ab421 (Ab-1) were concentrated by precipitation with ammonium sulfate from supernatants obtained from hybridoma cell cultures grown in α-minimal essential medium. Ab1801 was used for immunoblotting of human p53, and a mixture of Ab240 and Ab421 was used for detection of rodent p53. Mouse monoclonal antibody M73 against E1A proteins (28) was collected as an ascites fluid. Binding patterns were determined by the addition of a secondary antibody, either horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (Jackson Immuno Research Laboratories), which was detected by enhanced chemiluminescence (chemiluminescence reagent; Dupont NEN), or ^{125}I-labeled anti-mouse antibody from sheep (Amersham), which was detected by a Fuji BAS-III imaging plate. The amount of activity was quantified with a Fujix Bas 2000 PhosphorImager.

EMSA. Electrophoretic mobility shift assay (EMSA) for p53 was performed with whole-cell extracts from mock-infected and adenovirus-infected cells at 24 h postinfection. The oligonucleotide used for these experiments containing the consensus binding site for p53, 5′-tcgagAGGCATGTCTAGACATGCCTc-3′, has been described previously (18). The double-stranded oligonucleotides were labeled with [γ-^{32}P]dCTP (Amersham Corp.) and Klenow polymerase (Pharmacia). EMSAs were performed in 20-μl volumes containing 10 μg of whole-cell extract in 20 mM HEPES-KOH (pH 7.9), containing 40 mM KCl_2, 0.1 mM EGTA, 0.4 mM dithiothreitol, 4 μg of bovine serum albumin, 2.5% (vol/vol) Ficoll, 3 μg of sonicated salmon sperm DNA (Pharmacia), 100 μg of oligo(dT) and labeled probe containing 20,000 to 50,000 cpm. To activate p53 DNA binding activity, 100 ng of the anti-p53 monoclonal antibody pAb421 (Oncogene Science) was used, as described previously (32). The EMSA reaction mixtures were incubated for 30 min at room temperature and then analyzed on a 4% polyacrylamide gel (30:1, acrylamide-to-bisacrylamide) in 0.25× TBE (Tris-borate-EDTA) and subjected to electrophoresis at 200 V for 2 h. The gels were dried and analyzed by autoradiography with Kodak X-Omatic film.

Cell viability assays. Cells were infected with *wt* or mutant Ad5 in 24-well plates containing HeLa cells at about 80% confluence. At various times following infection, adherent and nonadherent cells were pooled and viability was assessed by Trypan Blue exclusion. At least 300 cells were counted at each time point.

Electron microscopy. Mock and 12S/E1B-infected cells were prefixed in situ with 2% (vol/vol) glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2 to 7.4) at 48 h postinfection and then postfixed with 1% (wt/vol) osmium tetroxide in cacodylate buffer. The cells were stained with uranyl acetate, and photographs of representative fields were taken with a Philips 410 electron microscope.

RESULTS

Expression of E1A-243R induces the accumulation of p53. Previous studies had already indicated that the expression of adenovirus E1A protein results in the production of increased levels of the p53 cellular tumor suppressor protein (5, 8, 12, 23–25, 39, 49). To analyze this phenomenon further, HeLa cells, which are derived from a human cervical cancer, were studied. These cells express human papillomavirus (HPV) proteins E6 and E7 and were known to contain very low levels of p53, at least in part because of the effects of HPV-E6, which enhances its degradation via ubiquitin pathways (51). HeLa cells were mock infected or infected with mutant *dl*520, which expresses the E1A-243R protein but not 289R, or 12S/E1B$^-$, which also expresses only 243R and in addition fails to produce E1B polypeptides. Because of the absence of E1A-289R, such cells fail to express significant levels of products of early regions 3 and 4 (E3 and E4). Cell extracts were collected, separated by SDS-PAGE, and, following transfer to nitrocellulose, analyzed for p53 content by Western blotting with a p53-specific monoclonal antibody. Figure 2 shows that infection with *dl*520 (lane 2) results in a large increase in p53 relative to mock-infected cells (lane 1), approaching the levels observed in Ad5-transformed 293 cells (lane 4). Quantitative studies indicated that the increase in the p53 level in HeLa cells is a minimum of 8- to 10-fold (data not shown). A similar increase was also observed with mutant 12S/E1B$^-$ (lane 3), suggesting that expression of neither the E1B 19-kDa protein nor the

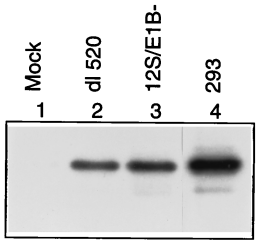

FIG. 2. Analysis of E1A-induced accumulation of p53 in HeLa cells by Western blotting. HeLa cells were infected with *dl*520 or 12S/E1B$^-$ or mock infected, and cell extracts were prepared at 24 h postinfection and separated by SDS-PAGE. Following transfer to nitrocellulose, p53 was detected with anti-p53 Ab1801 and ^{125}I-labeled anti-mouse antibody, as described in Materials and Methods. An extract from Ad5-transformed 293 cells was included as a control.

55-kDa polypeptide, known to interact with p53 (50, 59), is important for the rise in p53 levels.

Identification of the domains of E1A products involved in the accumulation of p53. To determine the molecular basis for the E1A-induced accumulation of p53, HeLa cells were infected with a series of mutants which express E1A-243R proteins containing various deletions (Fig. 1) and which fail to express E1B products. Previous detailed studies on these mutants had established the binding patterns with p300 and the related p400 proteins and with pRB and the related p107 and p130 polypeptides (3, 44). Mutant 01/12S/E1B$^-$ lacks an amino-terminal region required for p300/p400 binding but interacts with pRB and related proteins at fairly normal levels. Mutants 07/12S/E1B$^-$ and 08/12S/E1B$^-$ contain deletions in the primary pRB-binding site in CR2 and thus form complexes with these proteins very poorly; however, they interact with p300 and p400 normally. Mutant 01/08/12S/E1B$^-$ lacks binding sites for both p300 and pRB and thus binds to neither. Mutant 43/12S/E1B$^-$ contains a deletion within CR1 and thus is at least partially defective for binding p300, pRB, and related proteins (3). Figure 3D shows the results of a Western blotting analysis with E1A-specific M73 antibody and indicates that the synthesis of E1A proteins by all of these mutants occurred at quite comparable levels. Cell extracts were prepared from HeLa cells infected by these viruses and analyzed for p53 content by Western blotting as in Fig. 2. Figure 3A shows again that infection with 12S/E1B$^-$ (lane 2) induced a large increase in p53 relative to mock-infected cells (lane 1). Infection with mutant 01/12S/E1B$^-$ (lane 3) resulted in no increase in p53 levels. After infection with mutants 07/12S/E1B$^-$ and 08/12S/E1B$^-$ (lanes 4 and 5, respectively), increased levels of p53 were noted. Quantitative analysis of these samples with a PhosphorImager indicated that these mutants increased the p53 levels to about 70% of those obtained with *wt* 12S/E1B$^-$ (Fig. 3B). Infection with mutants 01/08/12S/E1B$^-$ (Fig. 3A, lane 7) and 43/12S/E1B$^-$ (lane 6), which produce E1A proteins that fail to bind or bind poorly to both p300 and pRB, did not alter the p53 levels. Thus, these results suggested that in HeLa cells, complex formation with p300 but not pRB correlated with E1A-induced accumulation of p53.

To analyze this effect further, similar studies were conducted in other cell types which were known to express *wt* p53 and to lack endogenous HPV sequences. Figure 4A shows that a very similar pattern of p53 accumulation was observed following infection of BRK cells with the same series of E1A mutants. The levels of p53 in mock-infected BRK cells (lane 1) were higher than those found previously in HeLa cells (Fig. 2 and 3); nevertheless, following infection with *wt* 12S/E1B$^-$ (Fig. 4A, lane 2) an increase of about eightfold was observed. Similar increases were again found with mutants 07/12S/E1B$^-$ (lane 4) and 08/12S/E1B$^-$ (lane 5), suggesting that complex formation with pRB and related proteins was not critical for the effect. Levels similar to those in mock-infected cells were observed with 01/12S/E1B$^-$ (lane 3) and 01/08/12S/E1B$^-$ (lane 7), and only partially increased levels were seen with 43/12S/E1B$^-$ (lane 6), indicating that interactions with p300 were important. A virtually identical pattern was also observed in mouse 10T1/2 cells (Fig. 4B). As was the case in HeLa cells (Fig. 3D), the levels of E1A expression by all mutants in these and other cells discussed below were similar (data not shown). Thus, results

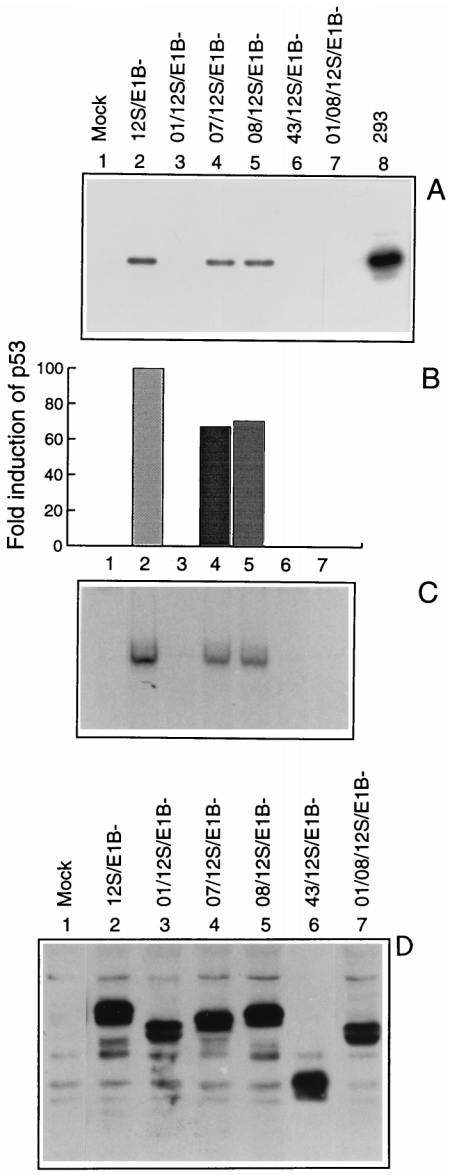

FIG. 3. Identification of the E1A regions required for the accumulation of p53 in HeLa cells. Extracts from HeLa cells infected with Ad5 12S/E1B$^-$ or a series of deletion mutants present in a 12S/E1B$^-$ background were prepared as in the experiment in Fig. 2. (A) Some of the extracts were analyzed by Western blotting with anti-p53 Ab1801 and ^{125}I-labeled anti-mouse antibody as in Fig. 2. (B) Such analyses were quantified with a PhosphorImager. (D) Some were analyzed by Western blotting with M73 anti-E1A monoclonal antibody and enhanced chemiluminescence. (C) Other aliquots were combined with Ab421 anti-p53 antibody, mixed with a ^{32}P-labeled p53-specific oligonucleotide, and then subjected to EMSA, as described in Materials and Methods.

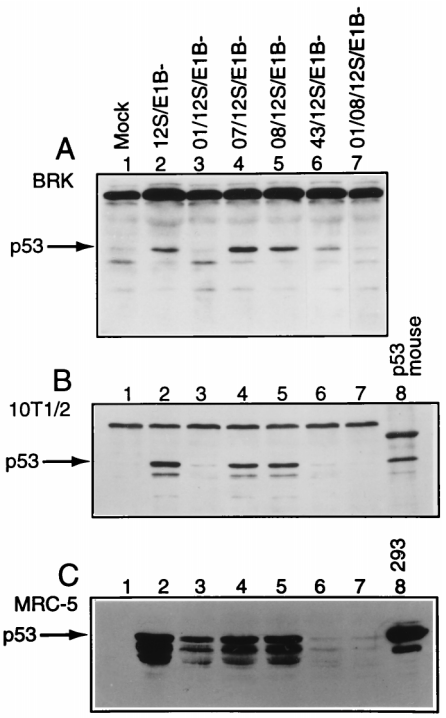

FIG. 4. Identification of the E1A regions required for the accumulation of p53 in rodent cells and in human MRC5 cells. An experiment similar to that described in the legend to Fig. 3 was performed with BRK cells, mouse 10T1/2 cells, and human MRC-5 cells. (A and B) p53 present in cell extracts was detected by Western blotting analysis with a mixture of Ab240 and Ab421; (C) Ab1801 was used to detect p53. Binding patterns were determined by enhanced chemiluminescence as described in Materials and Methods. (B) In vitro-transcribed/translated p53 from mouse cDNA was included in lane 8 as a control. (C) An extract from Ad5-transformed 293 cells was included in lane 8 as a control.

obtained with these rodent cells indicated that the E1A-induced increase in p53 levels appeared to require complex formation with p300 and related proteins but not with the pRB family. Similar results were obtained recently with E1A proteins expressed in PAM212 murine keratinocyte cells (49).

Analysis of additional human cell lines which are known to express *wt* p53 gave a somewhat different response than human HeLa cells (Fig. 3) and rodent cells (Fig. 4A and B). Figure 4C shows the results of an experiment with human MRC-5 cells in which the p53 detected by Western blotting analysis appeared as a triplet, presumably because of degradation, as is sometimes observed in these and other cells. Infection of MRC-5 cells with *wt* 12S/E1B$^-$ (Fig. 4C, lane 2) caused about a 10-fold increase in the amount of p53 relative to mock-infected cells (lane 1). Significant increases in p53 levels were also observed with mutants 01/12S/E1B$^-$ (lane 3), 07/12S/E1B$^-$ (lane 4), and 08/12S/E1B$^-$ (lane 5) relative to mock-infected cells. However, mutants 43/12S/E1B$^-$ (lane 6) and 01/08/12S/E1B$^-$ (lane 7), which fail to interact or interact poorly with both the p300 and pRB protein families, showed only a modest increase in p53 levels. An identical pattern was also observed in human IMR-90 cells (data not shown). These results suggested that in normal human cells, accumulation of p53 correlated with binding to either p300 or pRB. It appeared possible that the results obtained with human HeLa cells were complicated by the fact that these cells express HPV-E7 (33), which is known to interact with pRB (14) but has not been demonstrated to bind p300. We also considered the possibility that in HeLa cells E1A affects the expression of the HPV-E6 protein, thus altering the rate of p53 turnover; however, this cannot explain our similar results with several rodent and human lines which totally lack HPV sequences.

Effects of E1A-243R on ectopic expression of human p53 in p53-null mouse cells. To clarify further the ability of E1A-243R to induce the accumulation of p53, studies were carried out with mouse 10(1) cells which lack endogenous p53 (30). Such cells were shown to be resistant to the induction of apoptosis by E1A-243R unless p53 was expressed ectopically by using an adenovirus gene transfer vector (55). Experiments were conducted in which 10(1) cells were coinfected by the series of 12S/E1B$^-$ mutants and Adp53wt, which expresses *wt* p53 under the control of the CMV promoter (2). As in previous experiments, cell extracts were assessed for p53 content by Western blotting analysis with anti-p53 monoclonal antibody. Figure 5A shows that 10(1) cells express no p53 (lane 1); however, following infection with Adp53wt (lane 2), appreciable levels were apparent. Coinfection with 12S/E1B$^-$ (lane 3) led to a significant rise in p53, which, when quantified by PhosphorImager analysis (Fig. 5B), was about eightfold. These

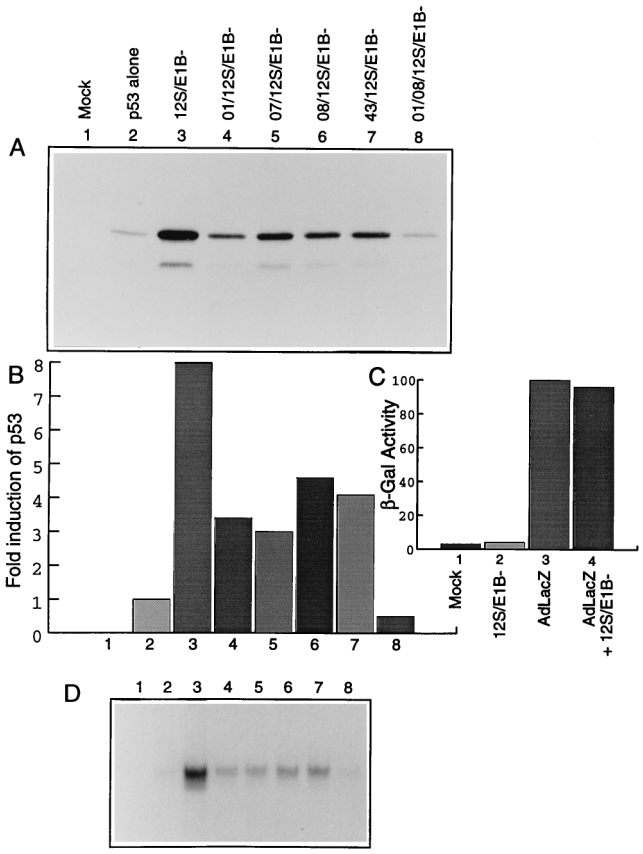

FIG. 5. Analysis of E1A-induced p53 accumulation in mouse 10(1) cells expressing human p53. Mouse 10(1) cells, which are p53 null, were coinfected by *wt* 12S/E1B$^-$ or various E1A mutants and the adenovirus vector Adp53wt, which expresses human p53 by using a CMV promoter. Cell extracts were prepared and analyzed for p53 content by Western blotting analysis and for p53-specific DNA-binding activity, as described in the legend to Fig. 3. As a control for expression from the CMV promoter, some cells were infected with the AdLacZ vector, which expresses bacterial LacZ instead of p53. In this case, cell extracts were analyzed for β-galactosidase activity. (A) Western blotting with anti-p53 Ab1801 and ^{125}I-labeled anti-mouse antibody. (B) Quantitation of results in panel A by PhosphorImager analysis. (C) Analysis of β-galactosidase expression in extracts from cells infected by the AdLacZ vector. (D) Analysis of p53 DNA-binding activity by EMSA. Lanes in panels A, B, and D are as indicated in panel A.

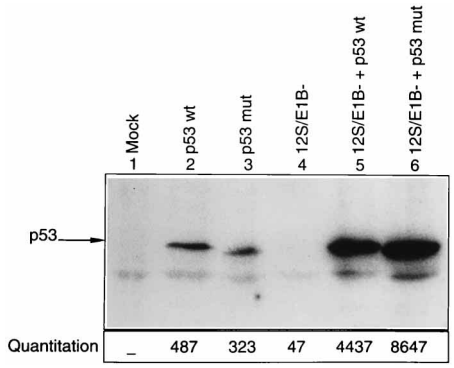

FIG. 6. Analysis of E1A-induced p53 accumulation in mouse 10(1) cells expressing human wild-type and mutant p53. An experiment similar to that described in the legend to Fig. 5 was performed, except that 10(1) cells were infected or coinfected with Ad5 12S/E1B$^-$ and either Adp53wt or Adp53mut. Cell extracts were analyzed for p53 content by Western blotting with anti-p53 Ab1801 and ^{125}I-labeled anti-mouse antibody as in the experiment in Fig. 2. The amount of p53 present was quantified by PhosphorImager analysis (shown below the blot).

results indicated that E1A-243R was able to induce the accumulation of human p53 which was expressed ectopically from the CMV promoter in these mouse cells. This increase was not due to enhanced transcription of p53 mRNA induced by the E1A protein, because no difference in expression of a CMV-driven *lacZ* gene in the AdLacZ vector was observed following coinfection with 12S/E1B$^-$ (Fig. 5C). Thus, accumulation must relate either to the stabilization of p53 or to some other function related to synthesis of the p53 protein. Induction of p53 accumulation was partially defective with mutant 01/12S/E1B$^-$ (Fig. 5A, lane 4), 07/12S/E1B$^-$ (lane 5), and 08/12S/E1B$^-$ (lane 6). Quantitative analyses indicated that only about a three- to fourfold increase was evident with all of these mutants (Fig. 5B). In the case of mutant 43/12S/E1B$^-$ (Fig. 5A, lane 7), which binds both p300 and pRB at reduced levels, a similar effect was observed. However, with 01/08/12S/E1B$^-$ (lane 8), which is completely defective for p300 and pRB binding, no increase whatsoever was evident. These results suggested again that induction of accumulation of human p53 occurred via complex formation with either p300- or pRB-related proteins, even if it occurred in mouse cells. Thus, the difference in the importance of interactions with the pRB family of tumor suppressors in these experiments (and those with human MRC-5 cells described in Fig. 4C), compared with those obtained by using rodent cells (Fig. 4A and B), related to the human origin of the cDNA, its transcript, or its protein product, and not to the cell type in which it is expressed.

An additional experiment was conducted with 10(1) cells in which both *wt* p53, expressed by Adp53wt, and a dominant negative mutant form of p53, expressed by Adp53mut, were tested in coinfection experiments with 12S/E1B$^-$. Figure 6 shows that the levels of both *wt* and mutant p53 (compare lanes 5 and 2 and lanes 6 and 3, respectively) increased after the expression of E1A-243R. Thus, the rise in p53 levels did not require a functional p53 protein.

E1A induces the accumulation of functional p53 protein. To determine if the increased quantities of p53 molecules induced by E1A protein are capable of interacting with target DNA sequences, EMSAs were carried out with a p53-specific oligonucleotide and extracts from 10(1) cells which had been coinfected with the Adp53wt vector and the series of 12S/E1B$^-$ E1A mutants. In these assays, as in many other similar studies, addition of the anti-p53 antibody Ab421 was required to obtain efficient DNA-binding activity. Figure 5D shows that no p53-DNA complexes were apparent in mock-infected 10(1) cells (lane 1). Low levels were evident in cells infected with Adp53wt alone (lane 2), and these were greatly increased upon coinfection with 12S/E1B$^-$ (lane 3). Detection of such p53-oligonucleotide complexes with various E1A mutants followed the same pattern as total p53 levels shown in Fig. 5A and B. A similar study was also carried out with endogenous p53, which was induced in HeLa cells by the expression of *wt* and mutant E1A-143R. Figure 3C shows that little p53-DNA complex was observed following infection by 01/12S/E1B$^-$ (lane 3), 01/08/12S/E1B$^-$ (lane 7), or 43/12S/E1B$^-$ (lane 6), which produce E1A proteins that bind poorly or not at all to p300. Conversely, high levels of p53-DNA complexes were detected with the *wt* (lane 2) and with mutants 07/12S/E1B$^-$ (lane 4) and 08/12S/E1B$^-$ (lane 5), which yield E1A products that do not interact efficiently with pRB and related proteins. Thus, in both cases, the increased quantities of p53 molecules induced by *wt* E1A-243R in mouse 10(1) cells expressing human p53 or in human HeLa cells expressing endogenous p53 could be induced to bind DNA in a normal fashion.

Induction of cell death by E1A deletion mutants. To correlate the accumulation of p53 with E1A-induced p53-dependent cell death, cell-killing assays were carried out with HeLa cells by using viruses which lack E1B expression and thus induce p53-dependent apoptosis. Figure 7 shows that in HeLa cells, very rapid cell death was produced by infection with mutant E1B$^-$, which expresses both major E1A products but no E1B proteins and induces both p53-dependent and p53-independent apoptosis (55). Mutant 12S/E1B$^-$, which yields only E1A-243R and no E1B polypeptides and is able to induce only p53-dependent apoptosis, produced cell death somewhat less rapidly but nevertheless impressively. Significant levels of killing were also observed with mutants 07/12S/E1B$^-$ and 08/12S/E1B$^-$, which cause the accumulation of p53. Little or no cell

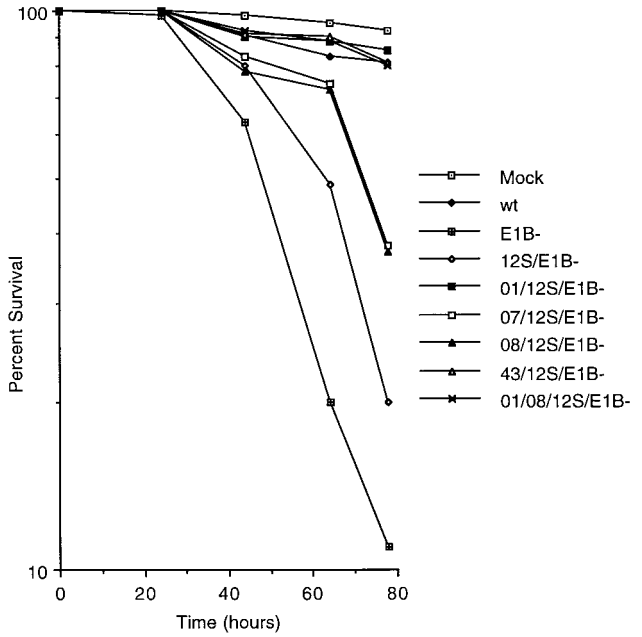

FIG. 7. Cell viability. HeLa cells were mock infected or infected by *wt* Ad5, E1B$^-$, 12S/E1B$^-$, or a series of mutants prepared in a 12S/E1B$^-$ background. The cells were tested for viability by a Trypan Blue exclusion assay at various times following infection, as described in Materials and Methods. The data are presented as the log percent viable cells.

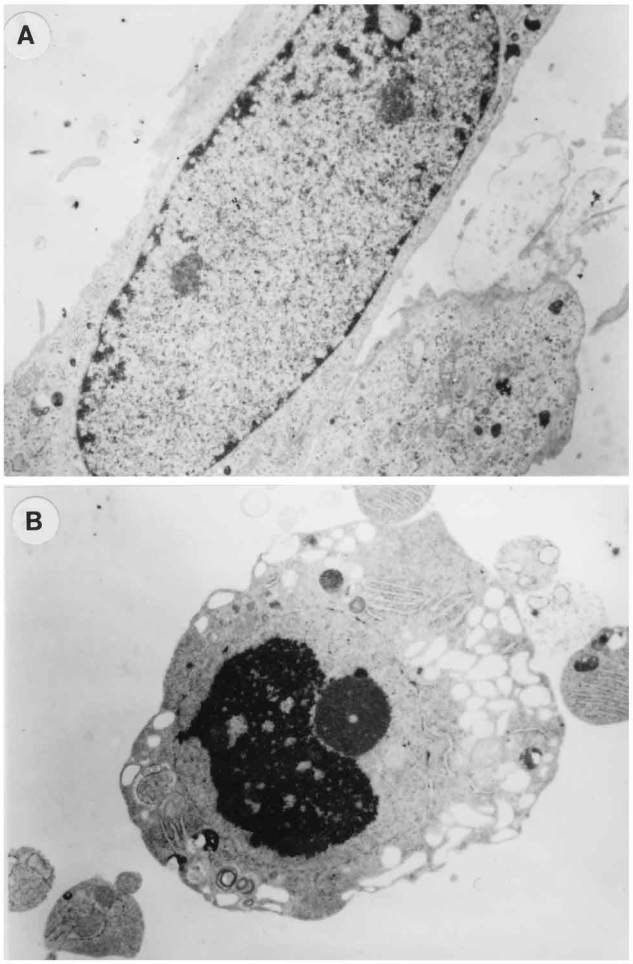

FIG. 8. Analysis of cell morphology by electron microscopy. Mock-infected (A) or 12S/E1B$^-$-infected (B) MRC-5 cells were harvested at 24 h postinfection and analyzed by electron microscopy, as described in Materials and Methods. Magnification, ×3,432.

death was seen in mock-infected cells or in cells infected by *wt* Ad5, 01/12S/E1B$^-$, 01/08/12S/E1B$^-$, or 43/12S/E1B$^-$, all of which fail to induce the accumulation of p53. These data indicated that induction of cell killing correlated with the accumulation of p53. To verify that induction of the p53 levels reported above in human MRC-5 cells also results in apoptosis, the morphologies of mock-infected MRC-5 cells and those infected with 12S/E1B$^-$ virus were examined by electron microscopy. Figure 8 shows that the infected cells (Fig. 8B) displayed highly condensed chromatin and vacuolization of the cytoplasm as is typical of apoptotic cells but absent in normal MRC-5 cells (Fig. 8A).

DISCUSSION

It has been known for some time that the expression of adenovirus E1A protein results in an increase in the intracellular concentration of p53 protein and the induction of p53-dependent apoptosis (5, 8, 12, 23–25, 39, 49). It is likely that these effects relate to interactions with one or more cellular polypeptides, since the major function of E1A products seems to be to mediate biological responses through the formation of complexes with a variety of cellular proteins. Induction of apoptosis has been shown to be suppressed by deletion of a portion of the CR1 region of the E1A protein (44, 56) and thus may depend on complex formation with p300 and related proteins, the pRB family of tumor suppressors, or both. In the present studies, we have used a genetic approach to examine the accumulation of p53 induced by the 243R E1A protein and to relate this information to the induction of p53-dependent cell death and apoptosis. Our studies indicated clearly that in rodent cells, the accumulation of p53 corresponded to the formation of E1A-p300 complexes and interactions with the pRB family were not required. Similar results were obtained recently with murine PAM212 cells, in which the increase in p53 levels obtained by expressing E1A protein was much more dependent on interactions with p300 than with the pRB family (49). We also found a similar pattern with human HeLa cells to that found by another group in an accompanying paper (8a). However, we were concerned that the expression of HPV E6 and E7 proteins in HeLa cells may have affected this response. E7 proteins, which are expressed at least at low levels in HeLa cells, bind to pRB-related proteins (14) but have not been demonstrated to interact with p300. Thus, it is possible that pRB complexes are present constitutively in these cells. This concern may have been justified as analysis of other human cells indicated that interactions with either p300 or pRB induced the accumulation of p53, although higher levels were obtained when E1A molecules were able to interact with both. Thus, a difference exists in the role of the pRB family in mouse and human cells. Studies of mouse cells expressing human p53 encoded by an adenovirus gene transfer vector indicated that the difference was not caused by the cell type but, rather, derived from the origin of the p53 gene and its transcript. Thus, regardless of the species of the infected cell, accumulation of human p53 was at least partially induced by complex formation with pRB-related proteins whereas that of mouse p53 was not. At present, we do not know the basis for this difference. The pRB family plays a role in the regulation of the cell cycle and entry into the S phase in both human and rodent cells (21, 46). We (10, 11) and others (6, 7, 13, 41) have noted that the levels of p107 and p130 and of E2F complexes containing these proteins vary during the cell cycle. However, whereas pRB-E2F complexes were detected at high levels in human and monkey cells throughout the cell cycle, such complexes were only detected at significant levels in growth arrested or terminally differentiated mouse cells (10, 11). It is possible that this difference underlies a functional difference in the role of pRB in the two species. Further experiments are needed to understand the precise basis for this effect.

The mechanism by which p53 accumulates is unknown. It has been suggested that the E1A protein may alter the rate of transcription of the p53 gene (5). Although we have not studied this question directly, our results with p53-null mouse 10(1) cells infected with adenovirus vectors indicated that E1A-243R has little effect on the CMV promoter which controls p53 expression. Thus, at least in this case, the accumulation of p53 must be due to some other mechanism. Some increase in the half-life of p53 has been reported (25, 39), and it is possible that this effect accounts for the large (8- to 10-fold) increase in the levels of p53 reported here. In a recent report, it was suggested that translation of mouse p53 mRNA can be regulated via a sequence in the 5′ untranslated region of the p53 mRNA (43). Furthermore, these results suggested that the p53 protein itself may be involved in this translational regulation. Even more recently, it was suggested that the 3′ untranslated region may play a role in controlling human p53 mRNA translation (20). Our data would generally be consistent with such control, except for the fact that mutant p53, which is function-

ally inactive, also accumulated in response to E1A. Further studies are necessary to clarify this phenomenon. In addition, it should be noted that all the experiments carried out in the present study were performed with Ad5 strains that express the E1A-243R protein and not the 289R product. Thus, under these conditions of infection, little expression of the E3 and E4 genes takes place, since 243R lacks the CR3 transactivation domain. In recent experiments with *wt* Ad5 in our laboratory, we have found that the expression of E4orf6 and the E1B 55-kDa protein limits the accumulation of p53 induced by E1A protein (46a).

The p53 molecules accumulated by expression of E1A appear to be functional, at least in terms of their ability to be induced to interact with p53-specific binding sequences. We also found that induction of cell death correlates with the accumulation of p53. In mouse cells, cell killing was greatly reduced by mutations which blocked the E1A-induced increase in p53 levels. Previous studies indicated that E1A-243R is able to induce only p53-dependent apoptosis (55). Thus, it is likely that apoptosis results from the accumulation of active p53 molecules. It was of great interest that the pattern of induction of accumulation of p53 by E1A proteins resembles that observed for the stimulation of DNA synthesis. Previous studies indicated that E1A proteins able to bind either p300 or pRB were able to induce DNA synthesis in serum-starved cells (31). Thus, induction of unscheduled DNA synthesis through complex formation with either p300- or pRB-related proteins may represent the actual trigger for the accumulation of p53, and the resulting high levels of active p53 therefore induce apoptosis. Further experiments are under way to elucidate this mechanism further.

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