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p300 Binding by E1A Cosegregates with p53 Induction but Is Dispensable for Apoptosis

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E1A expression during adenovirus infection induces apoptosis. E1A expression causes accumulation of the p53 tumor suppressor protein, and E1A-induced apoptosis is p53 mediated in primary rodent cells, implying that p53 induction may be linked to apoptosis induction by E1A. Adenoviruses containing mutations in the *E1A* gene were tested for the ability to trigger both p53 accumulation and the appearance of enhanced cytopathy (*cyt* phenotype) and degradation of DNA (*deg* phenotype), indicative of apoptosis in infected HeLa cells. The adenoviruses had mutations which disrupted the pRb- and/or p300-binding activities of E1A so that the relationship between p53 induction and apoptosis and binding to these cellular proteins by E1A could be determined. An E1A mutation that specifically disrupted the p300-binding activity failed to induce p53 accumulation, whereas mutations in E1A which affected pRb binding induced p53 accumulation. Thus, p300 binding was required and pRb binding was dispensable for E1A-mediated accumulation of p53 in HeLa cells. All the E1A mutant viruses, regardless of the ability to induce p53 accumulation, induced the *cyt* and *deg* phenotypes, suggesting that p53 induction in infected HeLa cells was not essential for apoptosis, nor was binding of E1A to the pRb and/or p300 protein. The possibility that E1A induced a p53-independent apoptosis pathway was tested by analyzing the appearance of the *cyt* and *deg* phenotypes in Saos-2 cells, which were null for both alleles of p53, upon adenovirus infection. An adenovirus expressing wild-type 12S E1A induced both the *cyt* and *deg* phenotypes in Saos-2 cells, as did all the E1A mutant viruses. Thus, E1A expression during infection of human cells may trigger redundant p53-independent and -dependent apoptotic pathways.

Apoptosis is essential for normal development and plays a role in the regulation of viral pathogenesis (12, 19, 28, 43). Viral infection and viral gene expression can trigger apoptosis, and some viruses encode inhibitors of cell death to escape this host response and maximize the production of viral progeny. Virus infection models have been used for studying the mechanism of apoptosis induction and have provided insight into the cellular pathways which regulate apoptosis.

One useful viral model for studying apoptosis is adenovirus infection. Adenoviruses encode genes in early region 1 (E1) that can both activate and suppress apoptosis (38, 43). E1A expression in quiescent primary baby rat kidney (BRK) cells induces proliferation and transformation (29). However, the transformed foci degenerate due to apoptosis (29). Coexpression of E1B with E1A in primary BRK cells leads to efficient transformation by inhibition of E1A-induced apoptosis (29, 41). The *E1B* gene encodes 55-kDa (55K) and 19K proteins, both of which can independently block E1A-induced apoptosis (29, 38, 43, 45). However, the E1B 19K protein is much more effective than the 55K protein in inhibiting apoptosis (29, 46).

During productive infection of human cells, E1A expression also triggers apoptosis while E1B 19K expression efficiently inhibits apoptosis (41). Infection by mutant adenoviruses lacking a functional E1B 19K results in the appearance of extensive nuclear and viral DNA degradation (*deg* phenotype) and enhanced cytopathic effect (*cyt* phenotype) (39, 41, 44, 47), both of which are indicative of apoptosis. The ability of mutant E1B 19K adenoviruses to trigger the *cyt* and *deg* phenotypes (apoptosis) requires E1A expression, since apoptosis does not occur upon substitution of a nonfunctional *E1A* into the E1B 19K mutant virus (47). Also, apoptosis is not observed in the absence of E1A proteins at high multiplicities of infection (MOI) when efficient viral replication occurs (47), suggesting that apoptosis induction is specifically a function of E1A and not a general consequence of viral DNA replication. Thus, analysis of how E1A induces apoptosis may yield further insight into the mechanism of apoptosis.

The *E1A* gene encodes several different transcripts due to alternative splicing. However, all the known biological functions of E1A have been attributed to 13S and 12S transcripts encoding 289- and 243-amino-acid products, respectively (reviewed in reference 24). The 13S E1A product contains a region of high sequence homology, conserved region 3 (CR3), which is unique to this E1A gene product and which is not required for deregulation of cell growth (reviewed in reference 3) or E1A-induced cytotoxicity (41). The CR3 region of E1A is mainly involved in transcriptional transactivation of viral early promoters during productive adenovirus infection (5, 23, 30). Expression of the 12S gene product is sufficient for induction of cell proliferation and apoptosis (41). The regions in the 12S E1A protein that are closely linked to the deregulation of cell growth function have been mapped by mutagenesis studies to the extreme amino terminus of the E1A protein as well as to two regions of high conservation among 12S E1A proteins from different adenovirus serotypes, designated conserved regions 1 and 2 (CR1 and CR2) (37). These regions contain binding sites for two classes of cellular proteins. One class contains the retinoblastoma tumor suppressor protein (pRb) and the pRb-related p107 and p130 proteins (37). The other class contains the p300 cellular protein (37) and the closely related CREB-binding protein, both of which belong to a family of transcriptional coactivators (1, 2).

The ability of E1A to bind these cellular proteins is required

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for growth deregulation by E1A. Binding of E1A to pRb interferes with pRb, which functions as a negative growth regulator in the active hypophosphorylated form (4, 9). Inactivation of pRb is closely associated with entry into the S phase of the cell cycle, as pRb binding is mapped to the regions of E1A also involved in the stimulation of DNA synthesis (37). The p300 protein is believed to be involved in the induction of cell cycle progression through transcriptional activation (reviewed in reference 2). Binding of E1A to p300 also maps to the amino-terminal region responsible for the stimulation of cell cycle progression (37).

Induction of apoptosis has previously been mapped to the amino-terminal region of E1A. A mutational analysis with E1A mutant viruses lacking a functional E1B 19K protein shows that both the amino terminus and the CR1 regions of E1A are involved in induction of apoptosis by E1A (41). This suggests that the p300/CBP- and the pRb-binding functions of E1A are required for apoptosis induction. To examine the relationship between the induction of apoptosis and cellular protein binding by E1A, we tested the ability of adenovirus mutants defective for binding to pRb and/or p300 to induce apoptosis. The results showed that binding of E1A to pRb and/or p300 was not required for induction of apoptosis, indicating that E1A-induced apoptosis occurred through functions unrelated to those associated with pRb and p300 binding or that redundant pathways controlled E1A-induced cell death.

Expression of E1A causes accumulation of the p53 tumor suppressor protein to high levels (22), and E1A-induced apoptosis is mediated by the p53 protein in BRK cells (7). Thus, accumulation of p53 protein to high levels may be associated with activation of apoptosis by E1A. Since p53 accumulation is also observed upon E1A expression during adenovirus infection of HeLa cells (6), the question arises whether p53 induction is linked to the induction of apoptosis by E1A during productive adenovirus infection. This hypothesis was tested by mutational analysis of the p53 induction function of E1A. Furthermore, the relationship between the p53 induction and the pRb- and p300-binding functions of E1A was examined.

The results showed that p53 induction mapped to the amino terminus of E1A and required the p300- but not the pRb-binding function of E1A. Also, p53 induction was inhibited by the expression of E1A containing the CR3 region. The requirement of p300 binding for p53 induction and the inhibition of p53 accumulation by E1A containing CR3 suggested possible mechanisms for p53 stabilization by E1A. The results also showed that high levels of p53 protein accumulation were not required for induction of apoptosis by E1A, indicating that E1A may induce apoptosis through a p53-independent mechanism. Induction of apoptosis by adenovirus infection in the absence of p53 confirmed that E1A expression can induce apoptosis through p53-independent means during productive infection of human cells.

MATERIALS AND METHODS

Cell lines. HeLa and 293 cells were maintained in Dulbecco's modified Eagle's medium fortified with 10% fetal bovine serum. Saos-2 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 15% fetal bovine serum.

Viruses and viral infections. The viruses used in the experiments (see Fig. 3) were propagated in 293 cells. The Ad5*dl*309, Ad5*dl*337, 12S, 12SE1B$^-$, CX, 928, NC, PS, and 9S viruses were described previously (6, 18, 33, 41). The following viruses were generously provided by Elizabeth Moran (Temple University, Philadelphia, Pa.): 12S.15.35, 12S.RG2, 12S.HN3, 12S.LS20, 12S.DA21, 12S.YH47, 12S.928, 12S.RG2.928, 12S.YH47.928, and 12S.952 (37).

The viruses 12S.RG2/E1B$^-$, 12S.928/E1B$^-$, 12S.DA21/E1B$^-$, 12S.RG2.928/E1B$^-$, 12S.YH47.928/E1B$^-$, which contain missense mutations in the E1A 12S sequence and a deletion in the E1B gene, were constructed as follows. *Eco*RI-*Xba*I restriction fragments from plasmids p12S.RG2, p12S.928, p12S.DA21, p12S.YH47, p12S.RG2.928, and p12S.YH47.928 (37) were ligated to the large fragment of *Xba*I-digested 12SE1B$^-$ virus, which lacked the *E1B* coding sequence (6). The 12S E1A mutant/E1B$^-$ viruses were propagated in 293 cells, and mutant viral DNA was isolated by a modified Hirt DNA preparation method (13, 44) and screened for the correct point mutations by sequencing.

Cells were infected at a MOI of 200 with CX, 9S, 12S, and the 12S E1A mutant viruses, all of which lacked the E1A CR3 region and were therefore replication impaired. The cells were infected at a MOI of 20 with the E1A CR3-containing and replication-competent Ad5*dl*309, Ad5*dl*337, 928, NC, and PS viruses. The cells were harvested for preparation of protein extracts at 24 and 48 h postinfection. They were infected with the 12S E1A/E1B$^-$ viruses at a MOI of 500 for Hirt DNA isolation at 48 h postinfection, concomitant with apparent enhanced cytopathic effects. Infections with the E1B-containing viruses were repeated three times, and those with the E1B-deleted viruses were repeated eight times; the results were reproducible.

Northern blot analysis. Cytoplasmic RNA was extracted from mock-infected and 12SE1B$^-$ virus-infected cells at the indicated time points postinfection (see Fig. 1a) by methods described previously (31, 42). A 10-μg portion of cytoplasmic RNA from each infection was analyzed by Northern blotting as described previously (31). The probes used for Northern blot analysis were a cDNA corresponding to human p53 (generously provided by A. Levine, Princeton University, Princeton, N.J.) and a cDNA corresponding to human p21/WAF-1/cip-1 (generously provided by G. Hannon, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.).

Western blot analysis. Following infection, protein extracts were prepared and p53 protein levels were analyzed by Western blot analysis. The pAb1801 monoclonal antibody against p53 (Oncogene Science) was used to detect human p53. Immune complexes were visualized by enhanced chemiluminescence (Amersham). Expression of the E1A proteins after infection was also detected with the M73 monoclonal antibody (Oncogene Science) against E1A proteins. The Western blot analyses were repeated several times concomitant with the infections. Because of variability in the magnitude of p53 induction from blot to blot and experiment to experiment, the p53 induction data was not quantitated in detail. The ability of each E1A mutant to induce p53 accumulation was reproducible, although the magnitude of the induction did vary from experiment to experiment. The Western blots which best represented this data are presented.

Transfection, indirect immunofluorescence, and cell viability. Plasmids containing the E1A, 12S E1A, 12S.928, 12S.RG2, 12S.RG2.928, and 12S.YH47.928 coding sequences were transfected into HeLa cells by the standard method of calcium phosphate precipitation of DNA. At 24 h posttransfection, the cells were fixed in methanol for immunostaining (40). p53 and E1A proteins were visualized with monoclonal antibody pAb421 against p53 and monoclonal antibody M73 against E1A and a rhodamine-conjugated secondary antibody. Cells staining positively were photographed and quantified.

For the E1A cytotoxicity assay, E1A plasmids and a plasmid containing the β-galactosidase gene driven by a cytomegalovirus promoter were cotransfected into HeLa cells at a ratio of 5:1, respectively. At 48 h posttransfection, the cells were fixed in 1% glutaraldehyde solution and incubated in 0.2% 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) for 4 h at 37°C (21). Cell viability was measured by determining the percentage of blue (transfected) cells in a population of 10,000 total cells.

Immunoprecipitations. HeLa cells were plated at a density of 1.3×10^6 cells per 60-mm plate 24 h before infection and infected with 12SE1B$^-$ virus or mock infected. At 36 h postinfection, each plate was pulse-labeled for 2 h with 200 μCi of [^{35}S]methionine (NEN Life Science Products) and then chased for 0, 1, 2, 4, 6, or 8 h. p53 protein was immunoprecipitated with pAb421 antibody and visualized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. 293 cells were simultaneously plated at a density of 1.3×10^6 cells per 60-mm plate, labeled with [^{35}S]methionine, and chased with medium containing cold methionine.

RESULTS

Expression of 12S E1A during adenovirus infection of HeLa cells causes p53 stabilization. Lowe and Ruley (22) reported that E1A expression caused p53 accumulation in REF 52 cells, which was associated with the induction of apoptosis (22). Expression of E1A in primary BRK cells also caused p53 accumulation to high levels (29a). We have also observed that adenovirus infection of HeLa cells causes the accumulation of p53 protein (6). This elevation in p53 protein levels may be due to upregulation of *p53* expression or stabilization of p53 protein by E1A. In REF 52 cells, E1A expression extends the half-life of the p53 protein (22). Accumulation of p53 by E1A in infected HeLa cells may also be regulated at the level of the p53 protein half-life. However, p53 protein levels are low in HeLa cells due to the presence of the human papillomavirus (HPV) E6 protein. Binding of HPV E6 to p53 has been shown

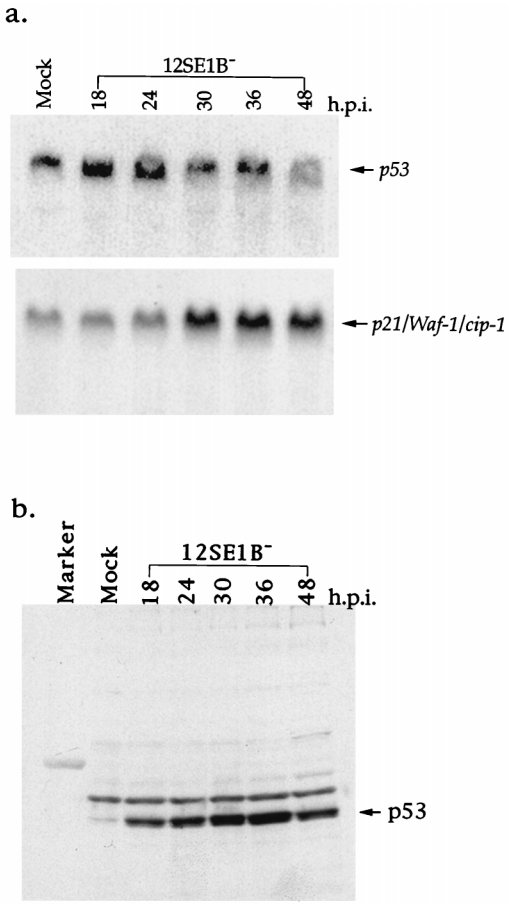

FIG. 1. E1A expression during adenovirus infection of HeLa cells induces p53 protein accumulation. (a) Northern blot analysis of *p53* and *p21/WAF-1/cip-1* expression in 12SE1B$^-$ virus-infected cells. HeLa cells were mock infected or infected with the 12SE1B$^-$ virus, and mRNA levels were monitored through the course of infection. The hours postinfection (h.p.i.) at which mRNA samples were taken are indicated above the lanes. The positions of the *p53* and *p21/WAF-1/cip-1* mRNAs are indicated. (b) Western blot analysis of p53 protein levels in cells infected with the 12SE1B$^-$ virus. Cells infected with 12SE1B$^-$ virus were analyzed for p53 levels by Western blot analysis in parallel with the Northern blot analysis. The position of p53 is indicated, and lanes are labeled above as mock infected or infected with virus for the indicated lengths of time.

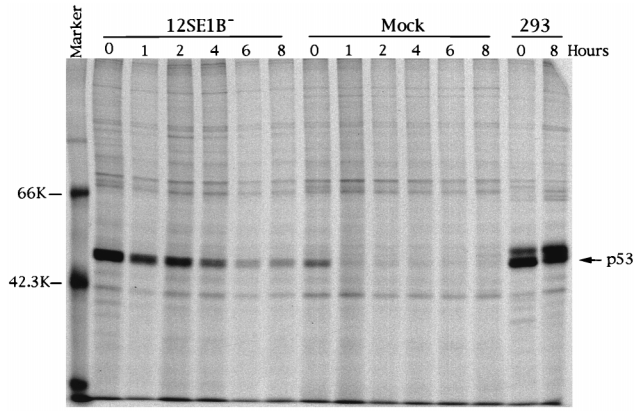

FIG. 2. E1A expression during adenovirus infection extends the half-life of the p53 protein. HeLa cells were mock infected or infected with 12SE1B$^-$ virus. At 36 h postinfection, the infected cells were pulse-labeled with [^{35}S]methionine and chased for the times indicated at the top of the gel. At the times indicated, p53 was immunoprecipitated with a monoclonal antibody directed against p53. Infections are also indicated at the top of the gel. As a positive control for p53 immunoprecipitation, 293 cells were pulse-labeled and chased for the indicated lengths of time, and the p53 protein was immunoprecipitated.

to promote p53 degradation via the ubiquitin-dependent proteolysis pathway (34). Therefore, induction of p53 accumulation by E1A in HeLa cells would require that E1A defeat the E6 mechanism for promotion of p53 degradation. However, since E1A can induce p53 accumulation in cells that lack E6 (22), induction of p53 by E1A does not occur through regulation of E6 levels or activity.

To determine if p53 induction by E1A was regulated transcriptionally or at the level of p53 protein half-life, *p53* mRNA levels and p53 protein turnover were examined. Northern blot analysis indicated that p53 mRNA levels were unchanged and were similar to those in the mock-infected cells during the course of adenovirus infection (Fig. 1). Thus, E1A expression during adenovirus infection apparently did not result in the upregulation of *p53* gene expression. Expression of a known p53-inducible gene, *p21/WAF-1/cip-1* (8), was monitored simultaneously as a control for functional induction of p53. Northern blot analysis revealed that *p21/WAF-1/cip-1* mRNA levels increased upon infection of HeLa cells by the 12SE1B$^-$ virus (Fig. 1), suggesting that upregulation of p53 occurred upon adenovirus infection. Western blot analysis performed simultaneously showed that p53 protein levels increased upon adenovirus infection (Fig. 1), suggesting that regulation of p53 during infection occurred at the protein level.

To address the mechanism of p53 accumulation by E1A, the half-life of p53 was analyzed in infected or mock-infected cells by pulse-chase analysis. p53 levels in the E1A- and E1B-transformed 293 cells were unchanged and remained high even at 8 h of chase, indicating that p53 was stabilized by extending its half-life as expected. p53 was detected in mock-infected cells in the presence of [^{35}S]methionine but was rapidly chased away to undetectable levels within 1 h of removal of the label (Fig. 2). In contrast, labeled p53 protein was detectable for up to 8 h in cells infected by the 12SE1B$^-$ virus (Fig. 2), which suggested that E1A overcame the HPV E6-associated proteolysis pathway to stabilize p53 in infected HeLa cells.

p53 induction cosegregates with the p300-binding but not the pRb-binding activity of E1A. The function of adenovirus E1A has been extensively studied by mutational analysis. A series of mutant adenoviruses exist whose E1A genes carry mutations that interfere with specific known E1A functions (diagrammed in Fig. 3) (37, 41). Thus, the relationship between p53 induction and other E1A functions may be identified by mapping the p53 accumulation activity of E1A by using the existing mutant E1A viruses. HeLa cells were infected with these E1A mutant viruses, and p53 protein levels in infected cells were analyzed by Western blot analysis. Mock-infected cells contained low p53 levels, as expected since HPV E6 promotes p53 degradation in HeLa cells. Surprisingly, infection by the Ad5*dl*309 virus, which was used as a wild-type control for p53 accumulation, did not result in p53 accumulation to high levels in the cell (Fig. 4). Infection by the Ad5*dl*337 virus, which did not express functional E1B 19K but expressed wild-type E1A, also failed to cause p53 accumulation to high levels in the cell (Fig. 4). Infection by the 12S virus, which expressed only the 12S E1A gene product, caused p53 accumulation to high levels in the cell (Fig. 4). Thus, the 12S gene product of E1A was sufficient for p53 induction. Since the Ad5*dl*309 and Ad5*dl*337 viruses contained the CR3 region, which was lacking in the 12S virus, it appeared that presence of the CR3 region exerted a negative effect on p53 accumulation

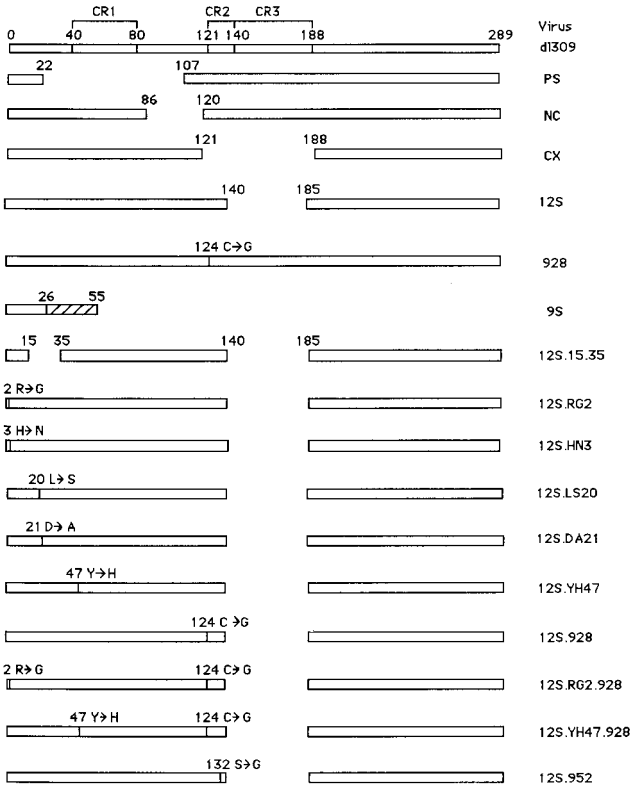

FIG. 3. Diagrammatic representation of wild-type and mutant E1A proteins. Long boxed regions indicate E1A coding sequences present. Discontinuous regions represent deleted amino acids. Amino acid substitutions and their positions are indicated by vertical lines. Regions shifted into another reading frame are indicated by hatching. The positions of the conserved regions CR1, CR2, and CR3 are also indicated. The viruses that express the E1A proteins are indicated on the right.

by 12S E1A. However, the presence of CR3 did not completely abolish the p53 accumulation function of 12S E1A, since low levels of p53 accumulation were observed after infection by the Ad5*dl*309 and Ad5*dl*337 viruses (Fig. 4). The CR3 region of E1A is involved in the transcriptional transactivation of viral early genes. Therefore, interference of p53 induction by E1A

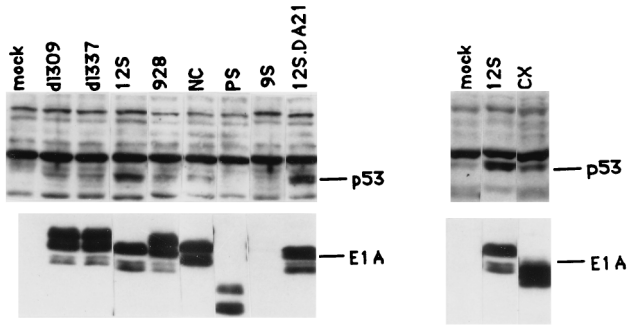

FIG. 4. Mapping of p53 induction by E1A expression during adenovirus infection. HeLa cells were mock infected or infected with 12S, CX, or 9S virus at a MOI of 200 or with Ad5*dl*309, Ad5*dl*337, 928, NC, or PS virus at a MOI of 20. p53 and E1A protein levels were visualized by Western blot analysis at 48 h postinfection with monoclonal antibodies pAb1801 and M73 for human p53 and E1A, respectively. Each lane represents 20 μg of total cell protein extract. The positions of p53 and E1A proteins are indicated. Viruses used in the infections are indicated above each lane.

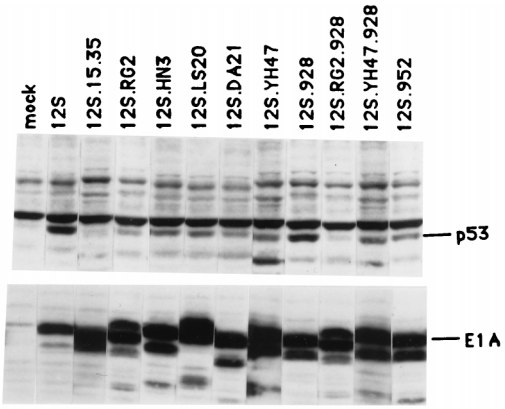

FIG. 5. Mapping of p53 induction by 12S E1A during adenovirus infection. HeLa cells were mock infected or infected with 12S virus or with viruses containing mutations in the 12S sequence. A MOI of 200 was used for all the viruses. Protein levels were examined by Western blot analysis at 48 h postinfection with the pAb1801 anti-p53 monoclonal antibody and the M73 anti-E1A antibody. The positions of the p53 and E1A proteins are indicated. The viruses used for the infections are also indicated above each lane.

containing CR3 was possibly due to the expression of viral genes downstream of E1A. Alternatively, CR3 may directly prevent p53 accumulation in the infected cell.

Analysis of p53 levels in cells infected with mutant E1A viruses revealed that p53 induction was associated with the extreme amino terminus and the CR1, but not the CR2, of E1A. Infection with the 928 virus, which contained CR3 and had a point mutation (a cysteine-to-glycine change) at amino acid 124 in CR2 of E1A (Fig. 3), resulted in p53 accumulation in cells to levels lower than that of the 12S infection but comparable to that of the Ad5*dl*309 infection (Fig. 4). This suggested that the E1A functions associated with amino acid 124 were not required for p53 induction. This observation was confirmed by infection with the 12S.928 virus, which lacked CR3 but contained the identical point mutation in CR2, which caused p53 to increase to levels comparable to that of the 12S infection (Fig. 5). Infection with the CX virus, which has a deletion in CR2 and CR3 of E1A, resulted in a dramatic increase in p53 levels similar to that of 12S virus infection (Fig. 4). This indicated that CR2 and CR3 were not required but the amino-terminal portion of E1A including CR1 was sufficient for induction of p53. Infection by the NC virus, which carried a deletion in the region between CR1 and CR2, resulted in p53 accumulation in the cell comparable to that of Ad5*dl*309 infection (Fig. 4). This suggested that the region between CR1 and CR2 was not required for induction of p53 by E1A. Cells infected by the PS virus, which had a deletion in the CR1 and surrounding regions but contained an intact CR3 region (Fig. 3), contained undetectable levels of p53 protein (Fig. 4). This suggested that CR1 and the surrounding region was important for p53 induction by E1A. Infection with the 9S virus, which expressed a truncated nonfunctional E1A transcript (Fig. 3), also failed to induce p53 accumulation (Fig. 4). Thus, expression of the extreme amino terminus of the *E1A* gene alone was insufficient for induction of p53 accumulation.

As an indication of the efficiency of viral infection, E1A levels from each of the infections were monitored by Western blot analysis. E1A protein was not detected in mock-infected cells but was present at high levels in cells infected by each virus (Fig. 4). The similarity in E1A protein levels in all the infections suggested that any differences in the level of p53 accumulation was not likely to be due to differences in the level

of E1A production but, rather, was due to the specific mutations found in the *E1A* genes.

The amino-terminal portion of E1A is associated with the ability of E1A to interact with cellular proteins, such as pRb and p300 (37). Therefore, the relationship between the cellular protein-binding and p53 induction activities of E1A was investigated in our system. Several missense mutations in 12S E1A which result in disruption of the pRb- and/or p300-binding functions of 12S E1A have been identified (37). Adenoviruses containing these mutations in 12S E1A were examined for their p53 induction capability by infection of HeLa cells and Western blot analysis of p53 protein levels. E1A levels were also monitored by Western blot analysis. As expected, mock-infected cells contained undetectable levels of E1A and barely detectable levels of p53 (Fig. 5), whereas infection with the 12S virus, which expressed abundant wild-type 12S E1A protein, resulted in high levels of p53 (Fig. 5). The 12S.DA21 infection, which efficiently produced an E1A mutant protein that retained pRb and p300-binding activities, caused p53 accumulation to high levels (Fig. 5). High p53 levels were also observed from infections with the 12S.YH47, 12S.928, and 12S.YH47.928 viruses (Fig. 5), all of which efficiently expressed 12S E1A mutant proteins (Fig. 5) that were defective in binding to pRb but retained p300-binding activity (37). These results indicated that pRb binding was not required for p53 accumulation by E1A. In contrast, infections with the 12S.RG2 and 12S.15.35 viruses, which produced high levels of mutant E1A proteins that had lost the p300-binding activity but bound pRb (37), did not result in p53 accumulation (Fig. 5). This demonstrated that p53 induction cosegregated with the p300-binding activity but not the pRb-binding activity of E1A. Infection with a virus which expressed a mutant 12S E1A defective for both pRb and p300 binding (37), the 12S.RG2.928 virus, also did not result in p53 accumulation in the cell (Fig. 5), further supporting the observation that p300 binding was essential for p53 induction. Furthermore, infections by the 12S.HN3, 12S LS20, and 12S.952 viruses, which produced 12S E1A mutants that retained partial p300-binding activity, resulted in p53 accumulation in the cell to slightly reduced levels compared to those resulting from wild-type 12S virus infection (Fig. 5). Thus, there was a close linkage between the p300-binding and the p53 induction activities of E1A.

Induction of apoptosis by E1A does not require the accumulation of p53 to high levels in adenovirus-infected HeLa cells. Previous genetic analyses with adenoviruses containing deletion mutations in E1A have demonstrated that induction of the *cyt* and *deg* phenotypes, indicative of apoptosis, is associated with both the pRb- and p300-binding activities since the only E1A mutant that loses the ability to induce *cyt* and *deg* is PS*dl*, which is impaired for binding to both pRb and p300 and for induction of cellular DNA synthesis (41). Using the same E1A mutant viruses, we showed that the p300-binding activity of E1A was also associated with p53 induction. Thus, it was possible that p53 induction was related to the induction of apoptosis by E1A. To address this question, the p53 induction data reported above was compared with the published data on apoptosis induction by E1A. E1A expression from the CX, Ad5*dl*337, NC, and 928 viruses, regardless of the ability to induce high levels of p53 accumulation (Fig. 4), is able to induce the *cyt* and *deg* phenotypes (41). This suggests that the induction of apoptosis by E1A does not require high p53 levels, but it does not eliminate the possibility that low levels of p53 accumulation are sufficient for induction of apoptosis. E1A expression from the PS and 9S viruses, which failed to induce p53 accumulation (Fig. 4), also does not induce the apoptosis phenotypes, indicating that the induction of apoptosis and the accumulation of p53 are both associated with the amino-terminal portion of E1A deleted in these mutant proteins.

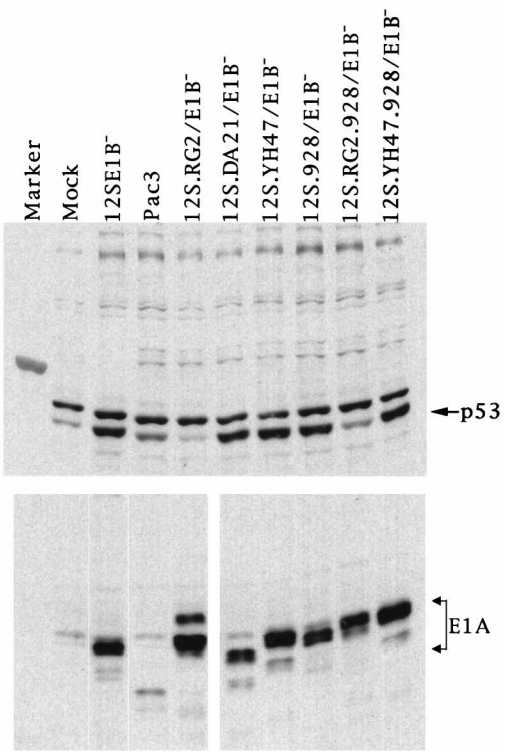

FIG. 6. Induction of p53 protein by infection with 12S E1A/E1B$^-$ mutant viruses. HeLa cells were mock infected or infected with 12SE1B$^-$ or mutant 12S E1A/E1B$^-$ viruses at a MOI of 200. Human p53 and E1A protein levels were examined by Western blot analysis at 48 h postinfection with pAb1801 and M73 antibodies, respectively. The positions of p53 and E1A proteins are indicated. The viruses used in the infections are also indicated above each lane.

To address the questions whether p53 induction cosegregates with activation of apoptosis and whether pRb and p300 binding by E1A is related to apoptosis induction by E1A during infection, the mutant 12S E1A coding sequences from the 12S.RG2, 12S.DA21, 12S.YH47, 12S.928, 12S.YH47.928, and 12S.RG2.928 viruses were rebuilt into an adenovirus backbone which lacked a functional *E1B*. The resulting mutant 12S E1A/E1B$^-$ viruses were tested for their ability to induce both p53 protein accumulation and the *cyt* and *deg* (apoptosis) phenotypes upon infection of HeLa cells.

As expected, mock-infected cells contained extremely low levels of p53 (Fig. 6), while cells infected by the 12SE1B$^-$ virus, which expressed wild-type 12S E1A but not functional E1B, contained high levels of p53 (Fig. 6). Infection of HeLa cells with the Pac3 virus, which contained a deletion in *E1A* and *E1B*, resulted in undetectable E1A expression and slight p53 accumulation in the cell (Fig. 6). The increase in p53 levels in the cell as a result of Pac3 virus infection was much lower than the increase as a result of 12SE1B$^-$ virus infection, which suggested that viral infection in the absence of E1A expression could also trigger p53 accumulation to low levels but that E1A expression was necessary for accumulation of p53 to high levels. Infections with the 12S.DA21/E1B$^-$, 12S.YH47/E1B$^-$, 12S.928/E1B$^-$, and 12S.YH47.928/E1B$^-$ viruses also caused accumulation of p53 to high levels (Fig. 6). In contrast, infections with the 12S.RG2/E1B$^-$ and 12S.RG2.928/E1B$^-$ viruses resulted in dramatic reductions in p53 accumulation (Fig. 6). These results again showed that p53 induction cosegregates

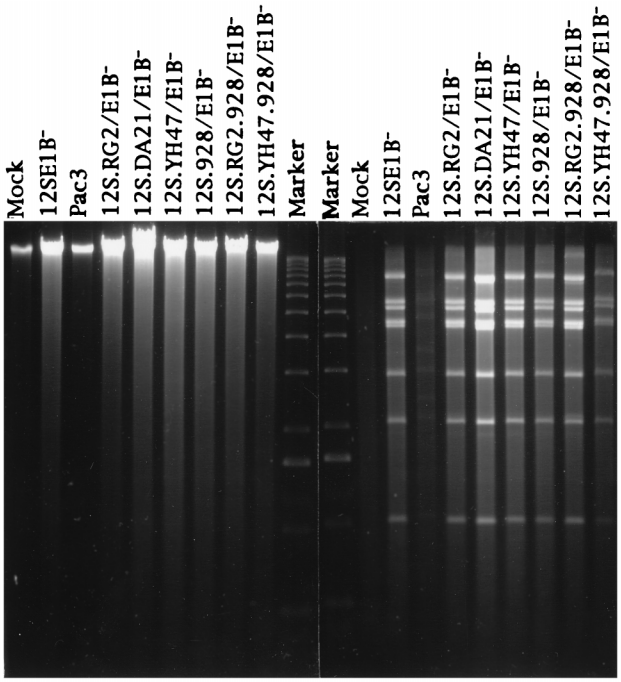

FIG. 7. Induction of DNA degradation by infection of HeLa cells with 12S E1A/E1B$^-$ mutant viruses. Infections with viruses were as described in the legend to Fig. 6. DNA fragmentation was monitored by a modified Hirt assay (13, 44). DNA from an equivalent number of infected cells were analyzed in each sample. The viruses used for the infections are indicated above each lane. The panel on the left represents degraded, undigested Hirt DNA, while the panel on the right represents *Hin*dIII-digested Hirt DNA from the indicated infections.

with p300 binding by E1A and were in accord with those obtained from infections with viruses expressing functional E1B.

The mutant 12S E1A/E1B$^-$ viruses were simultaneously tested by Western blot analysis for their ability to produce E1A proteins. E1A was undetectable in mock-infected cells, whereas high levels of E1A were observed in cells infected by the 12SE1B$^-$ virus and all the mutant 12S E1A/E1B$^-$ viruses (Fig. 6).

To test for the ability of the mutant 12S E1A/E1B$^-$ viruses to induce apoptosis, HeLa cells were infected with the mutant viruses and observed for the appearance of the *cyt* and *deg* phenotypes. Mock infection did not induce either *cyt* (data not shown) or *deg* (Fig. 7, left panel). The 12SE1B$^-$ virus infection was able to induce significant *cyt* (data not shown) and *deg* (Fig. 7, left panel) phenotypes. Although Pac3 virus infection induced slight p53 accumulation, it did not cause the *cyt* (data not shown) or *deg* (Fig. 7, left panel) phenotype, which was expected since the Pac3 virus did not express E1A. Surprisingly, the 12S.RG2/E1B$^-$, 12S.DA21/E1B$^-$, 12S.YH47/E1B$^-$, 12S.928/E1B$^-$, 12S.RG2.928/E1B$^-$, and 12S.YH47.928/E1B$^-$ viruses all induced the apoptosis phenotypes whether or not they induced accumulation of p53 to high levels (Fig. 7, left panel). All the viruses replicated efficiently except for Pac 3 (Fig. 7, right panel). Thus induction of apoptosis by the mutant 12S E1A/E1B$^-$ viruses appeared to be independent of high p53 levels. One explanation for these results is that a low level of p53 accumulation is sufficient for E1A-induced apoptosis. Another explanation is that 12S E1A is able to induce apoptosis through p53-independent means.

Transient expression of wild-type and mutant E1A induces p53 accumulation and is toxic to transfected HeLa cells. It has been shown that E1A expression alone is sufficient to induce p53 accumulation (22). To test whether expression of the mutant 12S E1A proteins alone is also able to induce p53 accumulation, HeLa cells were transiently transfected with plasmids carrying either wild-type or mutant *E1A* coding sequences and assayed for the expression of E1A and accumulation of p53 by indirect immunofluorescence. E1A was undetectable in cells transfected with carrier DNA alone but was detected at high levels in cells transfected with either wild-type or mutant 12S E1A-expressing plasmids (Fig. 8a). p53 was detected at background levels in cells transfected with carrier DNA alone, while p53 levels significantly higher than background were observed in cells transfected with plasmids expressing wild-type E1A, 12S, 12S.DA21, 12S.YH47, 12S.928, or 12S.YH47.928 (Fig. 8a). High levels of p53 were observed in 15 to 26% of cells relative to the population of cells in which E1A was expressed (Fig. 8b). Thus, transient expression of wild-type E1A or mutant E1A which retained p300-binding activity was sufficient to induce p53 accumulation. In contrast, transient expression of the 12S.RG2 and 12S.RG2.928 mutant E1A proteins failed to induce p53 to high levels (Fig. 8a). Higher-than-background p53 levels were observed in only 2 to 4% of p12S.RG2- or p12S.RG2.928-transfected cells relative to the E1A-containing population (Fig. 8b). Thus, transient expression of mutant E1A proteins which had lost p300-binding activity was insufficient to induce p53 accumulation. These results were in accord with the observations from the analysis of p53 induction during adenovirus infection of HeLa cells.

Note that transient expression of wild-type E1A resulted in the accumulation of p53 to levels similar to those induced by transient expression of 12S E1A in transfected cells. Thus, expression of E1A containing CR3 in the absence of other viral genes did not inhibit p53 induction by 12S E1A protein, which suggested that downstream viral genes are required for inhibition of p53 accumulation.

It has previously been shown that E1A expression during adenovirus infection (41, 47) or in transient-expression assays in HeLa cells induces apoptosis, as indicated by the morphological changes and the induction of DNA fragmentation (41). We tested whether transient expression of the 12S E1A mutant proteins alone was also able to induce cell death. To do this, we cotransfected HeLa cells with either wild-type or mutant *E1A*-coding plasmid expression vectors and a plasmid expressing β-galactosidase and assayed them for viability by a β-galactosidase assay described previously (10). The cell population cotransfected with carrier DNA and a plasmid expressing the β-galactosidase gene contained a significantly higher percentage of viable transfected cells (blue cells) than did the cell populations cotransfected with the β-galactosidase plasmid and the plasmids expressing either wild-type E1A or the E1A mutants (Fig. 9). Thus, transient expression of either wild-type E1A or mutant 12S E1A alone was sufficient to kill transfected cells.

The relationship of p53 and apoptosis induction to known biological functions of E1A in HeLa cells. To gain further insight into the functional significance of p53 and apoptosis induction by E1A, we analyzed the relationship of the p53 and apoptosis induction functions to other functions of E1A. Mapping of the known biological activities of E1A is summarized in Table 1. Previous reports have shown that mutations in E1A which interfere with either pRb binding (12S.928 and 12S.YH47.928) or p300 binding (12S.RG2) do not inhibit the induction of DNA synthesis by E1A, but the 12S.RG2.928 mutation, which eliminates all the cellular protein-binding activity of E1A, inhibits the DNA synthesis function of E1A (Table 1) (37). These results suggest that either pRb or p300

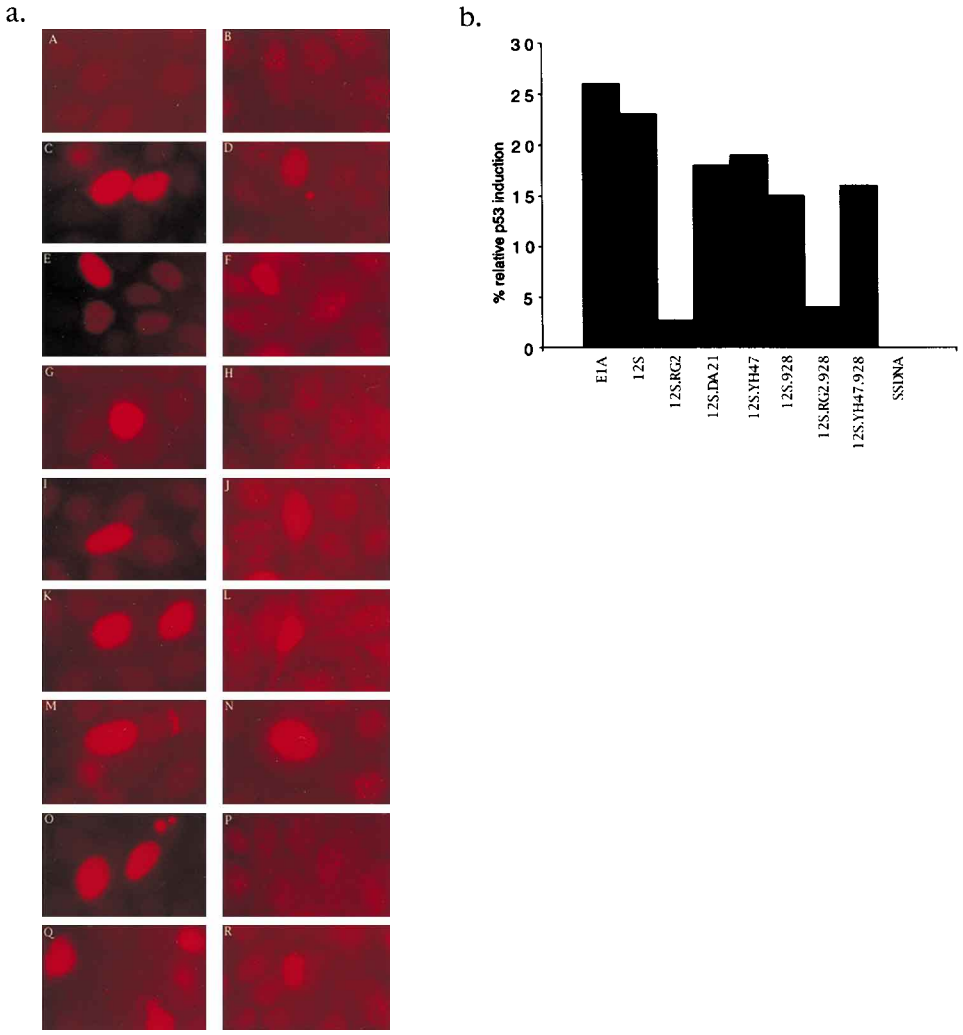

FIG. 8. Transient expression of wild-type and mutant E1A induces p53 accumulation and is toxic to HeLa cells. (a) p53 protein accumulation in HeLa cells by transient expression of E1A. HeLa cells were transfected with carrier DNA or plasmids expressing either wild-type E1A or mutant 12S E1A proteins. At 24 h posttransfection, the cells were treated with the pAb421 and M73 monoclonal antibodies against p53 and E1A, respectively, and labeled with rhodamine-conjugated secondary antibody. Fluorescent cells were visualized and photographed with the Nikon Microphot-FXA microscope. (A, C, E, G, I, K, M, O, and Q) E1A expression in cells transfected with carrier DNA alone and the E1A, 12S, 12S.RG2, 12S.DA21, 12S,YH47, 12S.928, 12S.RG2.928, and 12S.YH47.928 plasmids, respectively; (B, D, F, H, J, L, N, P, and R) p53 levels in the same cells. (b) Percentage of cells positive for high p53 levels relative to the population of E1A-positive cells. The percentage of cells staining positively for E1A and high levels of p53 were determined from the total cell population, and the percentage of cells containing high p53 levels relative to the percentage of E1A-positive cells was calculated. The corresponding E1A transfections are labeled on the *x* axis.

binding is sufficient for stimulation of DNA synthesis induction by E1A and that pRb and p300 binding control redundant pathways to stimulate the S phase of the cell cycle. Transformation by E1A also requires the p300- and pRb-binding activities, since mutations which cause the loss of pRb binding (12S.928) and p300 binding (12S.15.35 and 12S.RG2) also cause the loss of transformation capability (Table 1) (37). We have shown that p53 induction cosegregates with the p300-binding activity of E1A, suggesting that p53 accumulation is related to functions that correlate with p300 binding, such as induction of DNA synthesis and transformation (Table 1) (37).

In contrast, apoptosis induction by E1A during adenovirus infection of HeLa cells appears to be disassociated from the E1A functions listed in Table 1. Thus, induction of apoptosis by E1A during adenovirus infection seems to be a complex function which may not be localized to any specific region of E1A.

12S E1A protein can induce p53-independent apoptosis during adenovirus infection of human cells. Since the accumulation of p53 to high levels is not required for the induction of apoptosis by E1A, the question arises whether 12S E1A can induce p53-independent apoptosis in infected human cells. To address this question, human osteosarcoma Saos-2 cells, which are null for both p53 alleles, were infected with the mutant 12S E1A/E1B$^-$ viruses and the appearance of the *cyt* (data not shown) and *deg* apoptosis phenotypes was monitored. The efficiency of virus production was monitored by Western blot analysis of E1A protein expression, and viral DNA replication was analyzed by restriction analysis of Hirt DNA extracts from the infected cells. All the viruses expressed E1A and replicated efficiently in the Saos-2 cells (Fig. 10a). At 48 h postinfection, mock-infected cells showed no signs of either the *cyt* or the *deg* phenotype (Fig. 10b) while 12SE1B$^-$ virus-infected cells dramatic *cyt* or the *deg* phenotype (Fig. 10b). Pac3 virus-infected

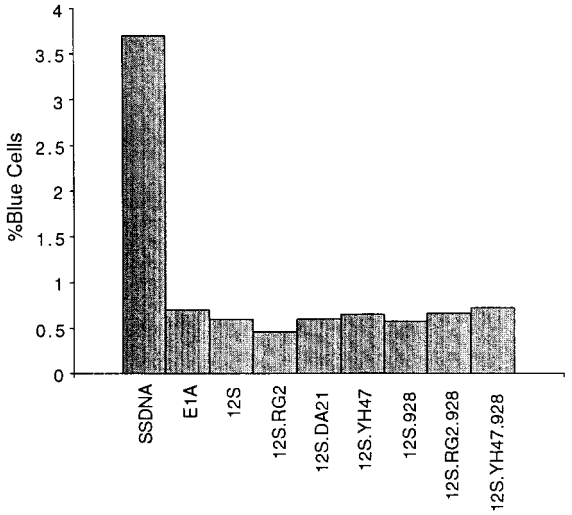

FIG. 9. Viability of HeLa cells transiently expressing wild-type or mutant E1A. The DNA used for each transfection is indicated on the *x* axis. The *y* axis represents the percentage of blue cells (viable transfected cells) from a total of 10,000 cells counted.

cells showed no signs of the *cyt* phenotype, although DNA degradation appeared at a reduced level compared to that of 12SE1B$^-$-infected cells (Fig. 10b). Emergence of both the *cyt* (data not shown) and the *deg* phenotypes was observed in cells infected by the 12S.RG2/E1B$^-$, 12S.DA21/E1B$^-$, 12S.YH47/E1B$^-$, 12S.928/E1B$^-$, 12S.RG2.928/E1B$^-$, and 12S.YH47.928/E1B$^-$ viruses (Fig. 10b). Apparently, both wild-type and mutant 12S E1A viruses were able to induce apoptosis in Saos-2 cells in the absence of p53, which suggested that 12S E1A can induce apoptosis via p53-independent means. There was also a lack of correlation between pRb and p300 binding and induction of apoptosis by E1A in infected Saos-2 cells, which suggested that E1A functions linked to these cellular protein-binding activities were not involved in the activation of p53-independent apoptosis.

DISCUSSION

It has been shown that E1A expression induces the accumulation of p53 by prolonging the half-life of p53 (22). We have previously observed that E1A expression also causes an elevation in p53 levels in adenovirus-infected HeLa cells (6). We investigated the mechanism of p53 accumulation by E1A during adenovirus infection and showed that this elevation in p53 levels was due to extension of the half-life of p53 protein by E1A, which supported the findings by Lowe and Ruley (22). Endogenous p53 levels in HeLa cells normally remain extremely low since the presence of the HPV E6 protein promotes p53 degradation (34). Binding of the HPV E6 protein to p53 promotes p53 degradation via the ubiquitin-dependent proteolysis pathway (34). This process is directed by the E6-associated protein (E6-AP), which is a ubiquitin-protein ligase (15–17). Thus, E1A was able to outcompete the HPV E6-associated p53 degradation in HeLa cells, suggesting that E1A may interfere directly with the E6–p53–E6-AP complex to inhibit p53 degradation or that E1A may downregulate E6-AP expression. It is unlikely that E1A affects E6 since p53 stabilization by E1A in cell lines lacking HPV E6 has been observed (22, 27, 29a). Further investigation is required to elucidate how E1A stabilizes the p53 protein.

The elevation in p53 levels accompanies the induction of apoptosis by E1A (22). To gain further insight into the relationship between p53 induction and apoptosis activation by E1A in adenovirus-infected HeLa cells, the p53 induction and apoptosis activation functions of E1A were mapped by mutational analysis. Adenoviruses containing mutations in *E1A* that disrupted pRb- and/or p300-binding functions of E1A were used in the analysis so that we may correlate the cellular protein-binding activities to the p53 and apoptosis induction functions of E1A. The results showed that the induction of p53 accumulation cosegregated with the p300-binding activity, which was also observed in human keratinocytes (32), but was not required for the apoptosis induction function of E1A in adenovirus-infected HeLa cells. The linkage between p53 induction and p300 binding by E1A in infected HeLa cells was simultaneously observed by others (27). This suggests that pRb induction is linked to functions of E1A closely associated with

TABLE 1. Mapping of p53 induction and apoptosis functions of E1A[a]

E1a protein[b]	p300	pRb	p107	p130	p53 induction	Apoptosis (*cyt*/*deg*)	DNA synthesis	Transformation
*dl*309	+	+	+	+	R	+	+	+
PS	−	−			−	−	−	−
NC	+	+			R	+	+	+
CX	+	−			+	+	+	−
12S	+	+	+	+	+	+	+	+
928	+	−			R	+	+	−
9S	−	−	−	−	−	−		−
12S.15.35	−	+			−			−
12S.RG2	−	+	+		−	+	+	−
12S.HN3	R	+			+			R
12S.LS20	R	R			+			R
12S.DA21	+	+			+	+		+
12S.YH47	+	−	+	−	+	+		+
12S.928	+	−	+	−	+	+	+	−
12S.RG2.928	−	−	−	−	−	+	−	
12S.YH47.928	+	−	−	−	+	+	+	
12S.952	+				+			

[a] +, positive; −, negative; R, reduced.
[b] E1A mutants used in this study for mapping the apoptosis induction function of E1A are in bold type.

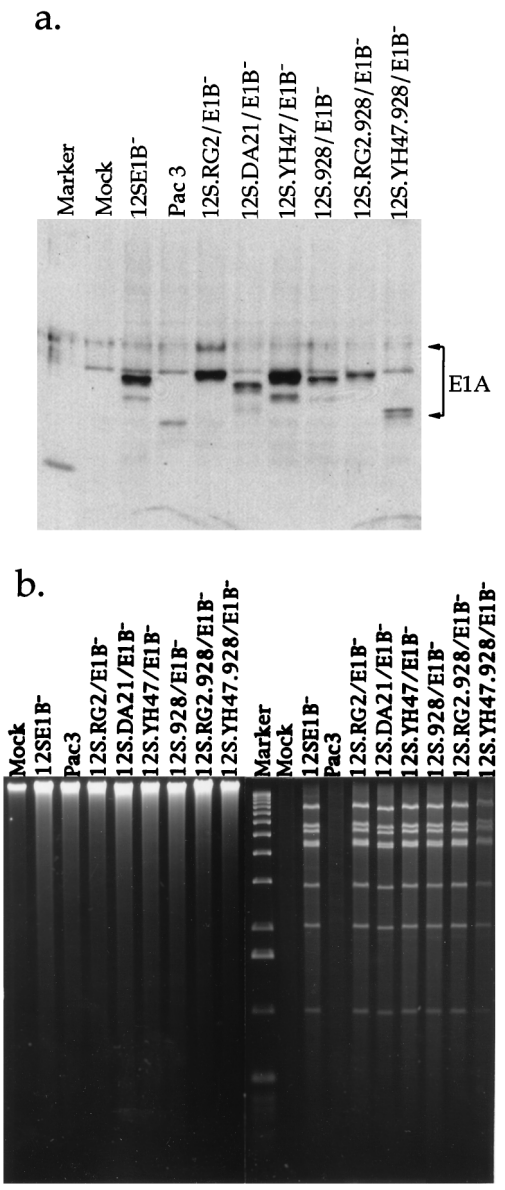

FIG. 10. E1A expression during adenovirus infection of Saos-2 cells induces the *deg* phenotype indicative of apoptosis. (a) E1A expression in infected Saos-2 cells. Saos-2 cells were mock infected, infected with wild-type 12S E1A virus, or infected with mutant 12S E1A/E1B$^-$ viruses. E1A expression was determined at 72 h postinfection, when the *cyt* and *deg* phenotypes were apparent. The positions of the E1A proteins are indicated. Virus infections are also indicated above the lanes. (b) Induction of DNA degradation by infection of Saos-2 cells with 12S E1A/E1B$^-$ mutant viruses. Infection with viruses was as described in the legend to Fig. 6. DNA fragmentation was monitored as described in the legend to Fig. 7. DNAs from an equivalent number of infected cells were analyzed in each sample. Viral infections are indicated above each lane. The left panel represents undigested Hirt DNA, while the right panel represents DNA from the indicated infections digested with *Hin*dIII.

p300 binding, such as stimulation of DNA synthesis (35) and transformation (37). Alternatively, p53 accumulation may be due to direct regulation of p53 turnover by p300. Binding of pRb is also associated with the induction of DNA synthesis and transformation by E1A (37). However, our results showed that pRb binding is not required for p53 accumulation in adenovirus-infected HeLa cells. Similar results in HeLa cells have also been observed by others, but p53 induction requires both pRb and p300 binding by E1A in other cell types (27). This is possible since pRb is nonfunctional in HeLa cells due to the presence of HPV E7, which interacts with pRb to inactivate its function (reviewed in reference 36). Thus, p53 induction by E1A may require both pRb and p300 binding, but in the absence of functional pRb, p300 binding by E1A is sufficient for p53 induction.

The requirement of p300 binding for p53 accumulation in adenovirus-infected HeLa cells suggests possible mechanisms for p53 protein stabilization by E1A. Infections by wild-type and mutant E1A viruses that retained p300-binding activity were able to cause p53 accumulation to high levels by extending the half-life of the p53 protein, whereas E1A mutant viruses defective for p300 binding lost the ability to cause the accumulation of p53. One explanation for these observations is that the E1A-p300 complex may directly stabilize the p53 protein. Alternatively, p300 may regulate p53 turnover indirectly through its role as a transcriptional coactivator (2) which E1A perturbs.

The observation that expression of E1A containing CR3 during adenovirus infection inhibited high levels of p53 accumulation by 12S E1A supports the idea that p53 is stabilized when bound to another cellular factor. It has been reported that binding of 13S E1A to TATA-binding protein (TBP) disrupts the p53-TBP association, relieving p53-mediated transcriptional repression (14). Displacement of p53 from TBP by expression of 13S E1A may promote the degradation of p53 by the E6-associated proteolysis pathway in HeLa cells. Also, since the CR3 of E1A is involved in transcriptional activation of viral genes, it is possible that expression of downstream viral genes contributes to p53 degradation. Downregulation of p53 levels by viral gene products may be important for virus reproduction. Other than the apoptosis-promoting consequence of high p53 levels, p53 has been shown to repress the transcription of genes that promote cellular proliferation, such as c-*myc* (20) and the cyclin A gene (49). Since 13S E1A can disrupt the p53-TBP association, relieving the p53-mediated repression of transcription (14), cell cycle progression may be restored upon expression of 13S E1A, and viral gene expression may be maximized. p53 has also been shown to inhibit cell cycle progression through induction of p21/WAF-1/cip-1 expression (8, 11, 48). Downregulation of p53 levels by expression of 13S E1A may also restore cell cycle progression through inhibition of *p21/WAF-1/cip-1* induction by p53.

Our data also showed that the 12S E1A gene product can induce p53-independent apoptosis during productive adenovirus infection of human cells and that apoptosis does not require binding to pRb and/or p300. This is in conflict with earlier reports showing that induction of apoptosis maps to the amino-terminal region of E1A involved in interactions with pRb and p300 (25) and that E1A-induced apoptosis is p53 dependent (7). Since these earlier experiments were done with rodent cells and the results reported here were obtained with human cells, the most obvious hypothesis for the discrepancy in E1A function is that the specific cellular factors involved in the induction of apoptosis by E1A may be cell type dependent. E1A may induce apoptosis through a solely p53-dependent pathway in rodent cells but through multiple p53-dependent and -independent pathways in human cells. Alternatively, E1A-induced apoptosis in human cells may occur through only the p53-independent mechanism.

The mechanism of the p53-independent apoptosis in human cells is not known. It is unlikely that binding of E1A to pRb and/or p300 is involved in this p53-independent apoptosis, since pRb binding and p300 binding are not required for apoptosis in Saos-2 cells. pRb binding and p300 binding appar-

ently are associated only with p53-dependent apoptosis, as observed in rodent cells (25). It is possible that interaction of E1A with other cellular partners mediates the p53-independent apoptosis pathway in human cells. In addition, the involvement of cysteine proteases in the induction of apoptosis has been established (reviewed in reference 26). It is unclear whether these protease activities are regulated by p53 during productive adenovirus infection. It is possible that activation of cysteine proteases is also involved in the p53-independent pathway through which E1A can induce apoptosis in adenovirus-infected cells.

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