

Stabilization of the p53 tumor suppressor is induced by adenovirus 5 E1A and accompanies apoptosis

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Oncogenic transformation by human adenoviruses requires early regions 1A and 1B (E1A and E1B) and provides a model of multistep carcinogenesis. This study shows that the metabolic stabilization of p53 observed in adenovirus 5 (Ad5)-transformed cells can occur in untransformed cells expressing E1A alone. Stabilized p53 was localized to the nucleus and was indistinguishable from wild-type p53 with respect to its interactions with hsc70, PAb420, Ad5 p55^{E1B}, and SV40 large T antigen. Moreover, binding of Ad5 p55^{E1B} or SV40 large T antigen had no additional effect on p53 levels or turnover. Higher levels of p53 were also induced in a variety of cell types within 40 hr after transferring E1A genes. E1A also caused cells to lose viability by a process resembling apoptosis. The apoptosis appeared to involve p53, because p53 levels reverted to normal in surviving cells that had lost E1A, and E1B protected cells from the toxic effects of E1A. These results suggest that (1) the involvement of p53 in tumor suppression and/or apoptosis can be regulated at the level of protein turnover, and (2) a major oncogenic role for E1B is to counter cellular responses to E1A (i.e., stabilization of p53 and associated apoptosis) that preclude transformation by E1A alone. This represents the first physiological setting in which high levels of endogenous p53 are induced in response to an oncogenic challenge, with the apparent consequence of suppressing transformation.

[Key Words: p53 tumor suppressor; adenovirus 5; E1A; apoptosis]

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p53 was identified as a cellular protein associated with simian virus 40 (SV40) large T antigen (Lane and Crawford 1979; Linzer and Levine 1979) and later with the adenovirus 5 early region 1B (Ad5 p55^{E1B}) and human papilloma virus (HPV) types 16 and 18 E6 proteins (Sarnow et al. 1982; Werness et al. 1990). Although the gene was originally classified as a dominant-acting oncogene, current evidence indicates that p53 functions primarily as a tumor suppressor (Malkin et al. 1990; Srivastava et al. 1990; Levine et al. 1991; Donehower et al. 1992).

Mechanisms whereby p53 protects against neoplastic growth are unknown. Forced overexpression of wild-type p53 can suppress cell growth (Eliyahu et al. 1989; Finlay et al. 1989) and promote cell death by apoptosis (Yonish-Rouach et al. 1991; Shaw et al. 1992). However, the relevance of these experiments to the natural circumstances in which p53 participates in tumor suppression and/or apoptosis is unknown, because physiological changes in p53 levels or activity that might mediate cellular resistance to oncogenic transformation have not been identified.

Interactions between viral early region proteins and

p53 also contribute to oncogenic transformation by human adenoviruses SV40 and HPV (Levine 1990). Given the role of p53 as a tumor suppressor, the viral tumor antigens are thought to interfere with p53 functions that preclude transformation. This is illustrated by the HPV E6 protein that promotes the proteolytic degradation of p53 (Scheffner et al. 1990). Similarly, stable complexes between p53 and p55^{E1B} may inactivate p53 function by sequestering the protein outside the nucleus (Zantema et al. 1985) or by blocking interactions between p53 and other cellular targets (Yew and Berk 1992).

Because oncogenic transformation frequently involves the loss of p53 function, it seems paradoxical that the stability and levels of p53 are greatly increased in adenovirus- and SV40-transformed cells. Although the stabilization of p53 associated with viral tumor antigens may simply be coincidental, the phenomenon may have significance regarding p53 function or regulation. For example, p53 may have positive as well as negative effects on cell growth (Mercer et al. 1984; Shohat et al. 1987; Shaulsky et al. 1990; van den Heuvel et al. 1990). Thus, the tumor antigens could promote cell transformation, in part, by increasing the levels of p53. Alternatively, the stabilization of p53 may result from factors other than

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tumor antigen binding, possibly as part of a mechanism whereby cells attempt to resist viral transformation. Binding of the tumor antigen could allow transformation by countering the effects of increased p53 expression.

Although evidence supporting this last model is limited, several studies suggest that tumor antigen binding may not stabilize p53. First, p53 turnover decreases in adenovirus type 12-transformed cells, even though the protein does not form a stable, immunoprecipitable complex with Ad12 p55^{E1B} (Zantema et al. 1985). Second, the half-lives of free and T antigen-associated p53 are similar in SV40-transformed cells (Deppert and Haug 1986). In both cases, the effect of the tumor antigen on p53 turnover was attributed to metabolic changes associated with cell transformation, although neither study excluded the possibility of physical interactions between the tumor antigen and p53.

The present study analyzed p53 expression in normal and transformed REF52 cells. Although established as a permanent line, REF52 cells resemble primary cells in that transformation can require two or more oncogenes acting in concert (Ruley 1990). For example, REF52 cells are not transformed by either *ras* or adenovirus early region 1A (E1A) individually but are oncogenically transformed by combinations of the two oncogenes (Franza et al. 1986). SV40 large T antigen and dominant transforming forms of p53 also transform in collaboration with *ras* (Hirakawa and Ruley 1988; Hicks et al. 1991). Because p53 is the only known cellular gene product with this activity in REF52 cells [Myc is inefficient (Kohl and Ruley 1987)], we analyzed the effects of various oncogenes on cellular p53 expression. Quite unexpectedly, the half-

life of endogenous p53 was extended in all cells expressing Ad5 E1A. Moreover, the stabilization of p53 was associated with the selective loss of E1A-expressing cells by a process resembling apoptosis. Although it had no additional effect on p53 turnover, E1B protected cells against the toxic effects of E1A.

Results

p53 levels are elevated in cell lines expressing adenovirus E1A

p53 expression was analyzed in REF52 clones following immunoprecipitation using a broadly reactive anti-p53 antibody (PAb421). Cells expressing adenovirus E1A (clone 1A1) and cells transformed by T24 H-*ras* and E1A (e.g. RNA7) expressed significantly more p53 than the parental cell line (Fig. 1A). On average, the p53 levels in E1A-expressing clones were 10-fold higher than in REF52 cells and were similar to the levels expressed in 293 cells, a human cell line expressing adenovirus E1A and E1B. In 293 cells, a protein of ~55 kD coimmunoprecipitated with p53, indicating that p53 was complexed to Ad5 p55^{E1B} (Sarnow et al. 1982).

Steady-state levels of p53 are frequently elevated in naturally occurring tumor cells and in oncogenically transformed cell lines. In most cases, the increase results from point mutations that stabilize the protein (Eliyahu et al. 1988; Finlay et al. 1988; Hinds et al. 1989). Many, but not all, of the mutant proteins share similar conformational alterations: They express an epitope recognized by PAb240 (Gannon et al. 1990) and bind hsc70, a con-

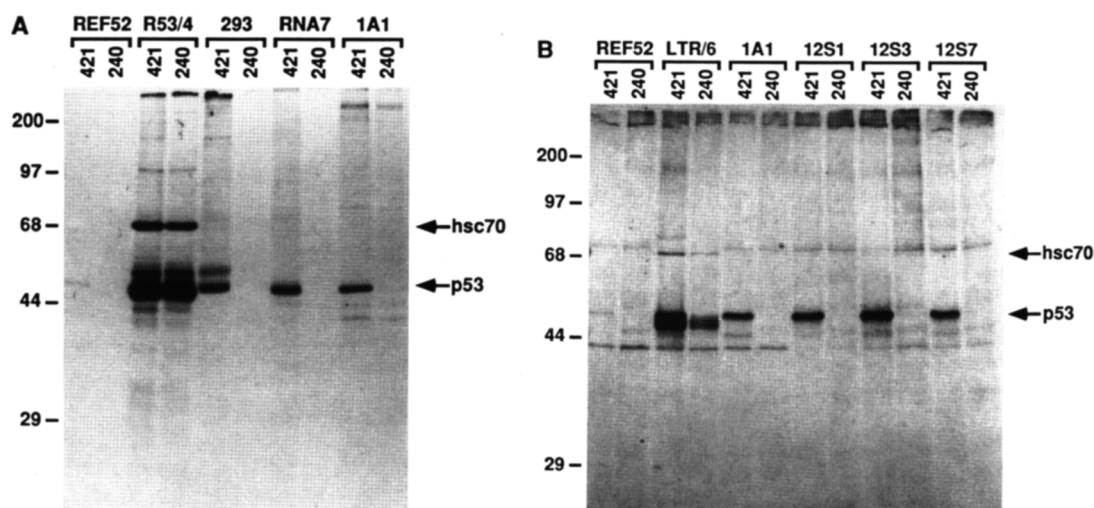


Figure 1. p53 levels in REF52 cells expressing adenovirus E1A. Cells were labeled for 4 hr with ³⁵S-labeled amino acids, and p53 was immunoprecipitated using either PAb421 (421) or PAb240 (240). The immunoprecipitated proteins were separated on 10% SDS-polyacrylamide gels and visualized by fluorography. (A) p53 levels in cell lines expressing the entire Ad5 E1A gene. (B) p53 levels in cell lines infected with a retrovirus expressing the 243R E1A protein, analyzed after minimal selection in culture (<20 population doublings). (REF52) A rat embryo fibroblast line; (R53/4) REF52 clone transformed with T24 H-*ras* and p53^{Pro-193}; (293) a human adenovirus-transformed cell line; (RNA7) REF52 cells transformed by T24 H-*ras* and E1A; (1A1) REF52 clone expressing Ad5 E1A; (LTR/6) REF52 clone expressing p53^{Val-135}; (12S1, 12S3, 12S7) REF52 clones expressing the 243R E1A protein. p53 and hsc70 are marked with arrows. The mobilities of molecular mass standards (in kilodaltons) are shown at left of each gel.

stitutively expressed member of the heat shock family (Pinhasi-Kimhi et al. 1986). For example, p53 from R53/4 cells (which express T24 H-ras and a mutant p53 gene, p53^{Pro-193}) complexed to a 70-kD protein and was immunoprecipitated with PAb240 (Fig. 1). In contrast, the p53 expressed in REF52, RNA7, and 1A1 cells neither associated with hsc70 nor reacted with PAb240 (Fig. 1A), suggesting that the p53 was wild type in conformation. Similar results were obtained with other REF52-derived cell lines expressing E1A (not shown).

Increased p53 expression requires only the 12S E1A product

Differential splicing generates two E1A transcripts, designated 12S and 13S, which encode proteins of 243 and 289 amino acids, respectively. Sequences required for oncogenic transformation are contained within the amino-terminal domain shared by the 289R and 243R proteins (Ruley 1990). Clones 1A1 and RNA7 express both the 243R and 289R proteins (Franza et al. 1986). Therefore, to determine whether the 243R protein was sufficient to increase p53 levels, a 12S cDNA was introduced into REF52 cells by retrovirus-mediated gene transfer, and p53 expression was assessed immediately after a sufficient number of cells were obtained for metabolic labeling (1×10^6 cells, obtained after 20 population doublings). p53 levels in 12S E1A-expressing cells (clones 12S1, 12S3, and 12S7) were 10 times higher than the parental cell line (Fig. 1B), indicating that the 243R protein was sufficient for p53 induction. Unlike p53^{Val-135}, none of the cellular p53s formed complexes with hsc70 or reacted with PAb240.

Nuclear localization of p53 in E1A-expressing cells

The intracellular localization of p53 was examined by immunofluorescent staining (Fig. 2). The staining pattern in REF52 cells was relatively weak and predominantly nuclear. Moreover, most cells in the population expressed similar levels of p53. Likewise p53 was predominantly nuclear in cells expressing the entire E1A gene, only 12S E1A, T24 H-ras and E1A, or T24 H-ras and 12S E1A. However, anti-p53 fluorescence was considerably more intense and heterogeneous than in normal REF52 cells, suggesting that individual cells expressed variable levels of p53. Finally, cells transformed by E1A and E1B also expressed high levels of p53, but the protein localized within distinct perinuclear regions (Zantema et al. 1985). Each cell type displayed only minimal fluorescence when the primary antibody was omitted (data not shown).

Elevated p53 levels in cells transiently expressing E1A

p53 expression was monitored in primary mouse embryo fibroblasts by immunofluorescent staining 36–40 hr after transferring E1A genes by DNA-mediated gene transfer (Fig. 3). A β -galactosidase gene (pCH110) was introduced together with either E1A or control plasmids (e.g.,

pBluescript), to identify cells that had acquired exogenous DNA. The cells were stained with rabbit anti- β -galactosidase antibody and with murine antibodies specific for either E1A or p53, together with a mixture of anti-rabbit IgG (rhodamine) and anti-mouse IgG (fluorescein) secondary antibodies. Approximately 10% of the transfected cells expressed both E1A and β -galactosidase (Fig. 3A,B), and of these, 25–50% expressed discernibly higher levels of p53 (Fig. 3C,D). In contrast, higher levels of p53 were not induced in cells transfected with control plasmids (e.g., Bluescript) and pCH110 (Fig. 3E,F). Occasional cells that displayed anti-p53 fluorescence were present in both transfected and untransfected cultures and did not stain with anti- β -galactosidase. Similar results were also obtained following the transfer of E1A genes into REF52 and NIH-3T3 cells (data not shown), and high p53 levels were induced in HeLa cells infected with Ad5 mutants lacking E1B, as judged by Western blot analysis (E. White, pers. comm.). Thus, the induction of p53 by E1A is rapid and occurs in a variety of cell types.

Binding of SV40 large T antigen to p53 in cells expressing E1A

Oncogenic p53 variants lose the ability to bind SV40 large T antigen (Braithwaite et al. 1987; Wang et al. 1989). Consequently, various clones were infected by retroviruses expressing SV40 large T antigen and then analyzed for complexes containing both p53 and large T antigen (Fig. 4). As expected, p53 levels in REF52 cells were elevated following the transfer of large T antigen, and p53 was quantitatively precipitated by using antibodies against large T antigen (PAb419). In contrast, introduction of SV40 large T antigen into E1A-expressing cells (e.g., clone 1A1) had little additional effect on p53 levels, even though all of the p53 coprecipitated with large T antigen. Thus, although SV40 large T antigen and E1A increase p53 levels independently, the effects are not additive. Moreover, the p53 stabilized by E1A appears to maintain a wild-type conformation, capable of binding T antigen.

Binding of p55^{E1B} to p53 in cells expressing E1A

These observations suggest that the elevated levels of p53 in adenovirus-transformed cells might result largely, if not entirely, from E1A expression. To examine whether E1B had any additional effect on p53 levels or protein stability, cell lines expressing both E1A and E1B were constructed. Plasmids encoding Ad5 E1A and Ad5 E1B were introduced simultaneously into REF52 cells by cotransfection. Alternatively, E1B was introduced into cells already expressing E1A (clone 1A1). Clones were isolated in hygromycin, expanded, and analyzed after a limited number of passages.

p53 and p55^{E1B} were immunoprecipitated independently from [³⁵S]methionine-labeled cell lysates. All clones expressing E1A and E1B contained p53/p55^{E1B} complexes, yet p53 levels were no higher than in cells

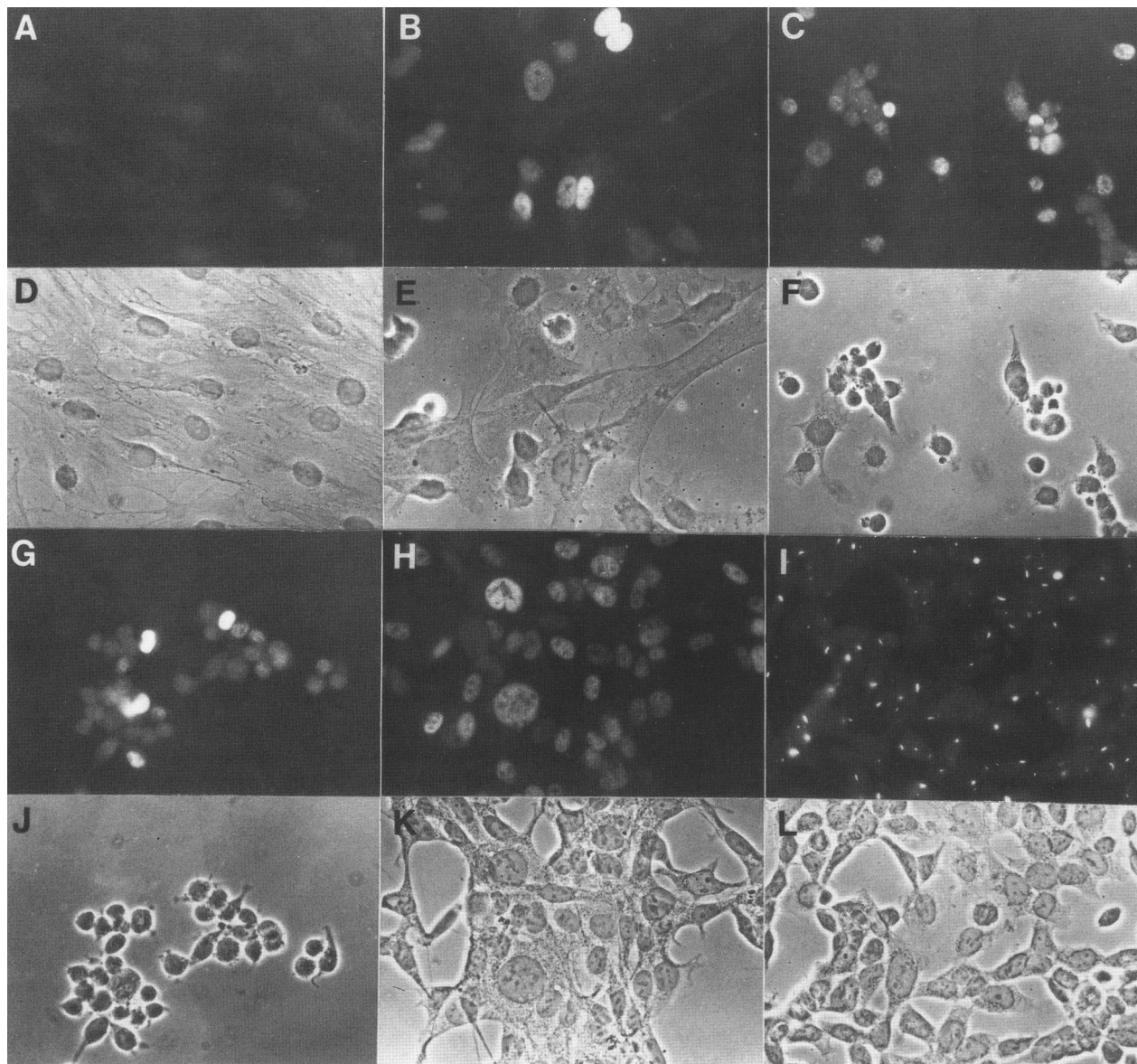


Figure 2. Localization of p53 in E1A-expressing cells. Cells were stained with PAb421 and FITC-labeled goat anti-mouse IgG. (A,D) REF52 cells; (B,E) 1A1, a clone expressing Ad5 E1A; (C,F) 12S1, a clone expressing a 243R E1A cDNA; (G,I) RNA9, a clone transformed by E1A and T24 H-ras; (H,K) r12S-2, a clone transformed by 12S E1A and T24 H-ras; (J,L) 1A1XX8, a cell line derived following transfer of Ad5 E1B into 1A1 cells. Fluorescent images are shown above phase-contrast photomicrographs of the same field.

expressing E1A alone (Fig. 5). A protein of ~155 kD associated with the p53/p55^{E1B} complex in cells expressing both E1A and E1B (Fig. 5). This protein coimmunoprecipitated with antibodies to either p53 or p55^{E1B}; therefore, recovery of the 155-kD protein did not result from antibody cross-reaction. The 155-kD protein was not associated with p53 in REF52 cells or in cells expressing E1A or T24 H-ras and E1A, suggesting that the protein bound specifically to p53/p55^{E1B} complexes.

Stabilization of p53 in cells expressing E1A

Levels of p53 gene transcripts and rates of protein turn-

over were measured to determine the mechanism whereby p53 levels were increased. As judged by Northern blot analysis, E1A had no obvious effect on p53 gene transcription. The levels of p53 transcripts in cells transformed with T24 H-ras and E1A and cells expressing E1A or 12S E1A were comparable to those present in normal REF52 cells (Fig. 6).

To examine p53 turnover, cells were labeled with [³⁵S]methionine for 1 hr and chased with excess unlabeled methionine for various times, and levels of labeled p53 were monitored by immunoprecipitation. Autoradiographs illustrating p53 turnover in representative clones are shown in Figure 7. The half-life of p53 in the

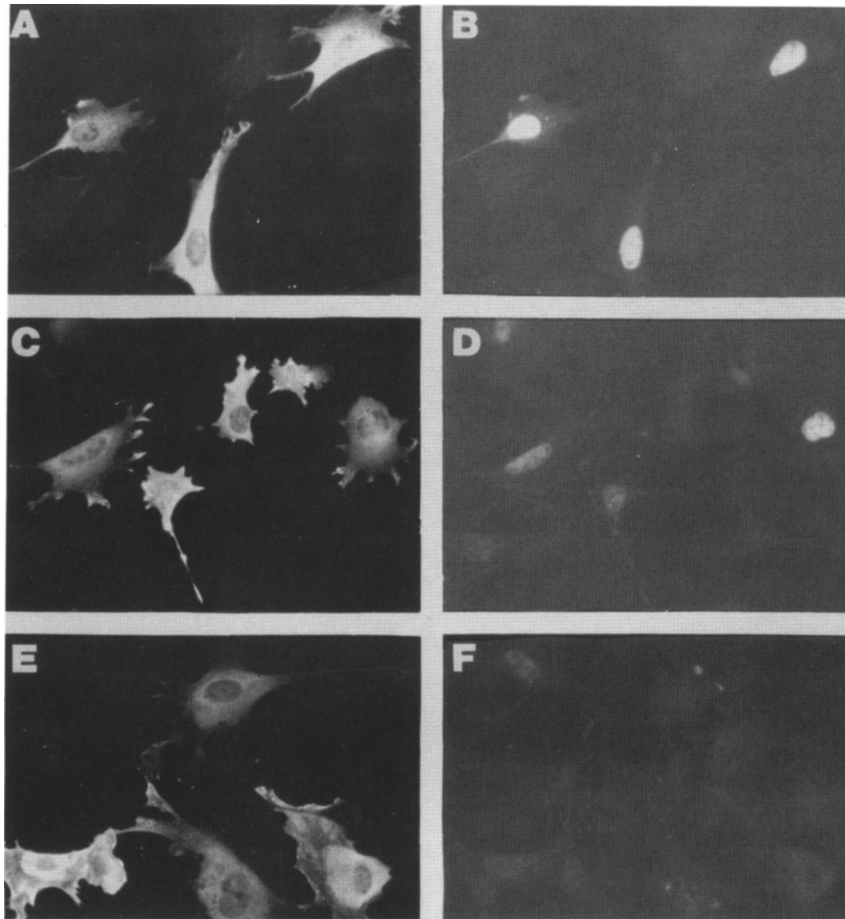


Figure 3. Induction of p53 following transient expression of E1A. Primary mouse embryo fibroblasts were cotransfected with pCH110 and either p1A (A-D) or pBluescript (E,F). After 36–40 hr, the cells were incubated with a rabbit anti- β -galactosidase antibody and with murine antibodies specific for either E1A or p53 and stained with a mixture of anti-rabbit IgG (rhodamine) and anti-mouse IgG (fluorescein) secondary antibodies. Cells in the same field were photographed to show anti- β -galactosidase fluorescence (A,C,E) and either anti-E1A (B) or anti-p53 fluorescence (D,F).

parental line was 20–30 min, consistent with previously reported values for wild-type protein (Gronostajski et al. 1984; Reich and Levine 1984). In contrast, p53 in clones expressing E1A were 5–10 times more stable, with half-lives exceeding 2 hr. Moreover, p53 half-lives in cells expressing E1A and those expressing both E1A and E1B were not significantly different. p53 half-lives were similarly extended in clones expressing the 12S E1A gene or transformed by E1A and T24 H-ras (not shown). This indicates that E1A is responsible for the increased stability of p53 in at least some cells transformed by human adenoviruses. However, we cannot exclude the possibility that E1B has an independent, but nonadditive, effect on p53 levels.

Elevated p53 levels require continuous E1A expression

E1A is selected against, such that clones transfected with E1A expression plasmids frequently lose the gene during serial passaging unless selection is maintained for a linked drug resistance gene or unless the cells are transformed by a collaborating oncogene (e.g., *ras* or E1B). In this study cells were allowed to lose E1A to study the association between E1A expression and high p53 levels.

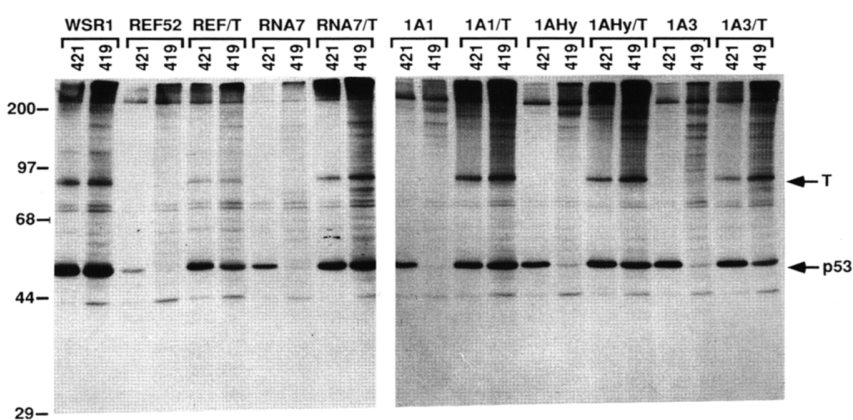
Clone 1A1, derived from a *neo*-resistant colony following transfer of p1Aneo (Franza et al. 1986), was passaged twice weekly in the absence of G418. The cells quickly (3 weeks, ~20 population doublings) lost the epithelial morphology characteristically associated with E1A and became indistinguishable from the parental cells (not shown). Loss of E1A expression in the resulting cell population (designated 1A1_{rev}) was demonstrated by immunoprecipitation using an E1A-specific monoclonal antibody (Fig. 8, right). Similarly, p53 levels in 1A1_{rev} cells were 10-fold lower than the original 1A1 clone, declining to the levels observed in normal REF52 cells (Fig. 8, left). These results indicate that the stabilization of p53 requires continuous E1A expression.

Cells expressing E1A undergo apoptosis

Recently, both E1A and p53 have been shown to induce apoptosis [Yonish-Rouach et al. 1991; Rao et al. 1992; Shaw et al. 1992; White et al. 1992]. We therefore examined E1A-expressing REF52 cells for signs of apoptosis, namely, reduced viability of cells grown at high densities or in media containing low serum and degradation of genomic DNA. Cultures of E1A-expressing cells contained significant numbers of dead cells when main-

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Figure 4. Interactions between p53 and SV40 large T antigen in cells expressing E1A. A temperature-conditional SV40 large T antigen (*tsA58*) was introduced into REF52 cells and clones expressing E1A. Cells were labeled for 4 hr with 100 μ Ci/ml of 35 S-labeled amino acids at 33°C, and p53/T antigen complexes were precipitated using monoclonal antibodies against p53 (PAb421) and large T antigen (PAb419). (WSR1) REF52 clone transformed by wild-type large T antigen and T24 H-*ras*; RNA7, a clone transformed by T24 H-*ras* and E1A; (1A1, 1AHy, 1A3) clones expressing Ad5 E1A. REF/T, RNA7/T, 1A1/T, 1AHy/T, and 1A3/T are cell populations derived from the same clones following infection with a retrovirus vector expressing *tsA58*. Bands corresponding to SV40 large T antigen (T) and p53 are indicated with arrows. The mobility of molecular size markers are shown at left.



tained in 10% FBS, as judged by trypan blue dye exclusion (Fig. 9A). In contrast, the viability of either REF52 cells or cells transformed by E1A and E1B was nearly 100%. The viability of cells transformed by T24 H-*ras* and E1A was higher than that of cells expressing E1A alone, except at higher cell densities where the cultures contained similar proportions of dead cells (not shown). Death of E1A-expressing cells was particularly pronounced in media containing 0.5% calf serum. Although E1B protected against the loss of cell viability associated with E1A, T24 H-*ras* did not.

Genomic DNA was isolated from various clones 3 days after seeding in media containing 10% FBS or 0.5% calf serum and analyzed by agarose gel electrophoresis. DNA from E1A-expressing clones generated a "ladder" characteristic of intranucleosomal cleavage (Fig. 9B). DNA degradation was particularly pronounced when cells were maintained in low serum. When cultured in low serum, cells transformed by T24 H-*ras* and E1A also contained degraded DNA, whereas no DNA degradation was observed in normal REF52 cells or cells transformed by E1A and E1B.

Discussion

The stabilization of p53 in cells transformed by Ad5 has been thought to result from physical interactions with the p55^{E1B} tumor antigen or from changes associated with oncogenic transformation. However, this study

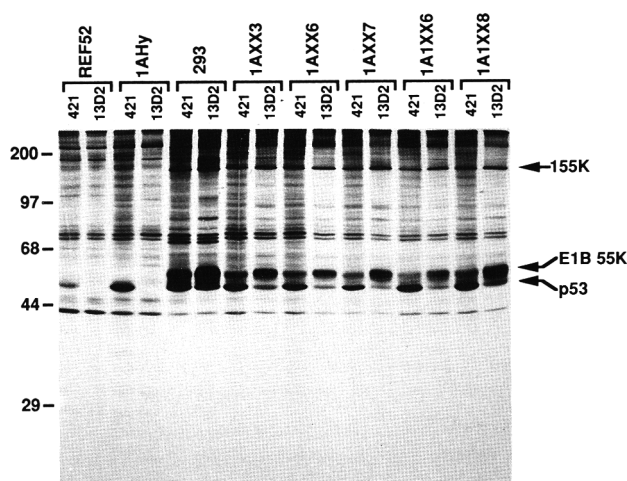


Figure 5. Interactions between p53 and p55^{E1B} in cells expressing E1A. Cells were labeled for 18 hr with 100 μ Ci/ml of 35 S-labeled amino acids; p53/p55^{E1B} complexes were precipitated using PAb421 (421) and 13D2, an antibody to p55^{E1B}. (1AHy) A clone expressing Ad5 E1A; (293) human cells expressing Ad5 E1A and E1B; (1AXX3, 1AXX6, 1AXX7) clones derived following cotransfection of E1A and E1B into REF52 cells; (1A1XX6, 1A1XX8) cell lines derived from clone 1A1 following introduction of E1B. The mobilities of p53, p55^{E1B} and a coprecipitating 155-kD protein are marked with arrows, and the mobilities of molecular size markers are indicated at left.

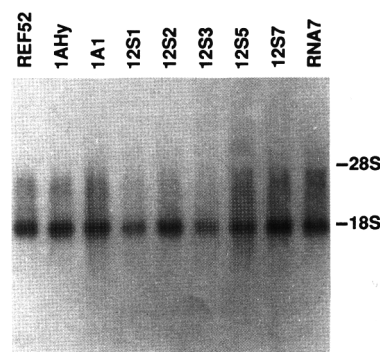


Figure 6. p53 gene expression. p53 transcripts were detected by northern blot analysis of total RNA following separation on a 1% agarose gel. A 32 P-labeled rat p53 cDNA fragment was used as a probe. (1AHy, 1A1) REF52 cells containing Ad5 E1A; (12S1, 12S2, 12S3, 12S5, and 12S7) clones expressing 12S E1A cDNA; (RNA7) cells transformed by T24 H-*ras* and Ad5 E1A. The locations of the 28S and 18S RNAs are shown at right.

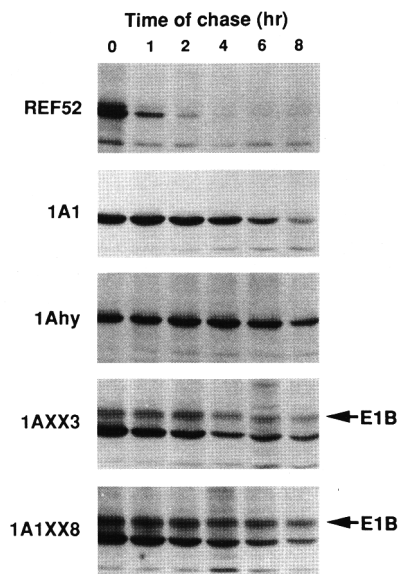


Figure 7. Stabilization of p53 in cells expressing E1A. Cells were incubated for 2 hr with medium lacking methionine, labeled for 1 hr with 100 μ Ci/ml of 35 S-labeled amino acids, washed twice with PBS, and chased with excess unlabeled methionine. Cells were lysed at various times, and p53 was immunoprecipitated using PAb421. (1A1, 1Ahy) Clones expressing Ad5 E1A; (1AXX3) REF52 clones derived by cotransfecting E1A and E1B; (1A1XX8) cell line derived from 1A1 following transfer of E1B.

demonstrates that the metabolic stabilization of p53 can occur in untransformed cells expressing E1A alone. Neither transformation by *ras* nor binding of p55^{E1B} or SV40 large T antigen extended the half-life of p53 beyond that observed in cells expressing E1A alone.

The mechanism whereby E1A increases the half-life of p53 is unknown. The effect is presumably indirect, as the two proteins are not known to interact physically (Egan et al. 1988; Whyte et al. 1989). Moreover, the effects of SV40 large T antigen and E1A on p53 turnover were not additive. Although the stabilized p53 was unaltered with regard to electrophoretic mobility, subcellular localization, or interactions with monoclonal antibodies and viral tumor antigens, we cannot exclude the possibility that p53 is modified in a manner that makes the protein less susceptible to proteolytic degradation. Alternatively, E1A may affect pathways involved in p53 turnover.

Previous studies have resulted in different conclusions concerning the roles of E1A and E1B (or transformation) on p53 levels and stability. The levels of p53 have been measured in cells expressing different segments of adenovirus early region 1 and have been correlated with the expression of E1B (Jochemsen et al. 1987; Mak et al. 1988; van den Heuvel et al. 1990) or E1A (Zantema et al. 1985). In each case, p53 stabilization was attributed to interactions with p55^{E1B} and/or changes associated with transformation. However, the correlation between p53 stability and E1B is probably indirect, because E1A ex-

pression is typically much higher in cells cotransfected with E1A and E1B than in cells transfected with E1A alone (van den Elsen et al. 1983b; Senear and Lewis 1986; Jochemsen et al. 1987; Yoshida et al. 1987). Both *cis*- and *trans*-acting effects of E1B on E1A transcription have been reported (Natarajan 1986; Herrman et al. 1987; Jochemsen et al. 1987; Yoshida et al. 1987). The phenomenon may also reflect selection against cells expressing higher levels of E1A in the absence of E1B (Fig. 8).

Although mutations may stabilize p53, several observations indicate that the p53 induced by E1A is structurally wild type and therefore is biochemically competent to function as a tumor suppressor. First, p53 levels were elevated in all clones expressing E1A, including those analyzed <48 hr after transfer of E1A. These included cells (primary mouse embryo fibroblasts, mouse 3T3 cells, and HeLa cells) known to contain only wild-type p53 genes. Second, in all 20 independent E1A-expressing clones analyzed, the stabilized p53 was localized to the nucleus and did not associate with hsc70 or react with PAb240. Third, all of the p53 was capable of binding SV40 large T antigen. Fourth, p53 levels reverted to normal as cells lost E1A, whereas it would be highly unlikely that the cells could concomitantly lose mutant p53 genes. Finally, mutant p53 genes enable *ras* to transform REF52 cells (Hicks et al. 1991), an unlikely interaction if the cells already contain mutant p53.

Recently, E1A has been found to induce a cytotoxic phenotype resembling apoptosis (White et al. 1991, 1992; Rao et al. 1992). The phenomenon accounts for the DNA degradation (*deg*) and cytotoxic (*cyt*) phenotypes associated with adenovirus strains containing mutations in the 19-kD E1B protein and probably contributes to the en-

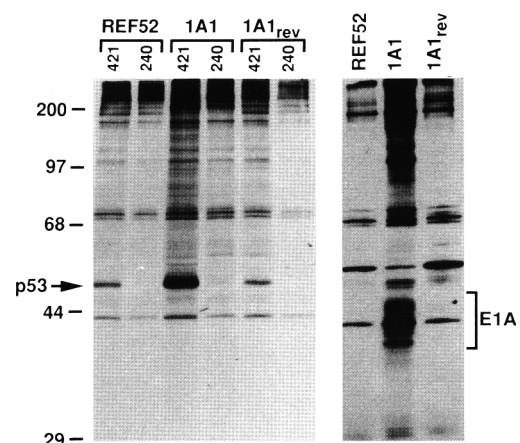


Figure 8. Elevated p53 levels require continuous E1A expression. Cells were labeled for 18 hr with 35 S-labeled amino acids. p53 was precipitated using either PAb421 (421) or PAb240 (240), and E1A polypeptides were precipitated using monoclonal antibody M73. (REF52) The parental rat embryo fibroblast line; (1A1) REF52 cells expressing E1A and 1A1_{rev}, derived by passaging 1A1 cells for ~20 population doublings (six passages) in the absence of G418 selection. Autoradiographs illustrating levels of p53 (left) and E1A (right) are shown. The mobilities of p53, E1A, and molecular size markers are indicated.

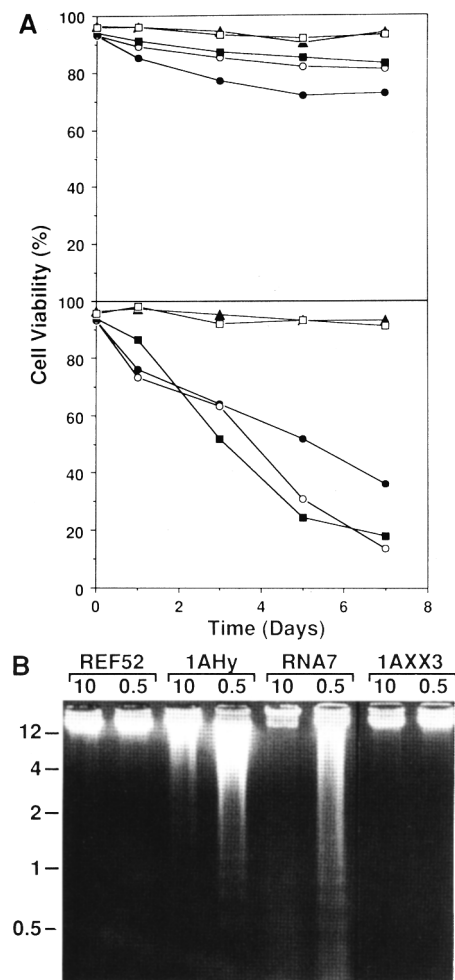


Figure 9. E1A-induced apoptosis. Cells (3×10^4 cells/60-mm dish) were seeded in 10% FCS (top) or 0.5% calf serum (bottom). At various times, the viability of floating and adherent cells was assessed by trypan blue exclusion. Fresh medium was added 3.5 days after seeding. (▲) REF52; (●) Ad5 E1A; (○) 12S E1A; (■) T24 H-ras + E1A; (□) Ad5 E1A + Ad5 E1B. Each point represents the average from three independent cell clones. At least 200 cells were counted for each determination. [B] Cells (2×10^5 cells/150-mm dish) were seeded in 10% FCS or 0.5% calf serum. After 3 days, genomic DNA was isolated from both floating and adherent cells, fractionated by agarose gel electrophoresis, and stained with ethidium bromide. (REF52) Parental line; (1AHy) a clone expressing Ad5 E1A; (RNA7) a clone transformed by T24 H-ras and E1A; (1AXX3) a clone transformed by E1A and E1B.

hanced sensitivity of E1A-expressing cells to killing by tumor necrosis factor- α (TNF- α). Thus, the 19-kD E1B and Bcl-2 proteins each protect cells from E1A-induced apoptosis, and p19^{E1B} protects against killing by TNF- α . Similarly, a significant proportion of REF52 cells expressing E1A are nonviable, such that the E1A gene is quickly lost during serial passaging. Cell death is particularly pronounced in low serum and is accompanied by DNA degradation, as is characteristic of cells undergoing apoptosis.

Several observations suggest that the stabilization of p53 mediates E1A-induced apoptosis. First, both E1A and p53 induce apoptosis (White et al. 1991, 1992; Rao et al. 1992; Shaw et al. 1992; Yonish-Rouach et al. 1991). Second, p53 stabilized by E1A is present in the nucleus and is structurally indistinguishable from the wild-type tumor suppressor protein. Third, p53 levels revert to normal as cells lose E1A. Fourth, E1B allows cells to tolerate E1A without affecting p53 levels. This also suggests that the stabilization of p53 is not simply a consequence of DNA degradation (Maltzman and Czyzyk 1984; Kastan et al. 1991) or apoptosis. Finally, in the report by Debbas and White (this issue), p19^{E1B} was found to protect cells from p53-induced apoptosis while a dominant-negative form of p53 (p53^{val135}) allowed E1A to transform in the absence of E1B.

Myc, like E1A and dominant transforming forms of p53, can promote the establishment of primary cells into permanent lines and transform primary cells in collaboration with *ras* oncogenes (Ruley 1990). Whereas Myc can also induce apoptosis (Evan et al. 1992), E1A activities involved in both transformation and apoptosis in REF52 cells appear to be independent of *c-myc*. In particular, *c-myc* transcription is unaffected by E1A (Kohl and Ruley 1987), and the levels of *c-myc* transcripts decline when cells expressing E1A are exposed to conditions (low serum) that promote apoptosis (M. Ragozzino and H.E. Ruley, unpubl.). It will be important to test whether the half-life of p53 changes during Myc-induced apoptosis.

It is perhaps significant that T24 H-ras did not completely protect against E1A-induced apoptosis, even though E1A is not selectively lost from cells cotransformed with T24 H-ras. Presumably, a selective advantage provided by *ras* and/or transformation compensates for cell losses owing to E1A. Because cells transformed by *ras* and E1A are highly tumorigenic, escape from apoptosis appears to be neither a prerequisite for, nor a consequence of, oncogenic transformation in vitro.

Two E1B proteins, p55^{E1B} and p19^{E1B}, collaborate separately with E1A to transform cultured cells and contribute to the oncogenicity of human adenoviruses (Bernards et al. 1986; Barker and Berk 1987; White and Cipriani 1990). Both E1B proteins protect against the consequences of p53 stabilization. Binding of p55^{E1B} blocks p53 functions (Yew and Berk 1992) and sequesters the protein from the nucleus (Zantema et al. 1985). p19^{E1B} and, to a lesser extent, p55^{E1B}, protect against E1A-induced apoptosis (Rao et al. 1992; White et al. 1992), and p19^{E1B} protects against p53-induced apoptosis (Debbas and White, this issue). Thus, an important, if not the primary, oncogenic role of both E1B proteins is to counter cellular responses to E1A (i.e., stabilization of p53 and associated apoptosis) that preclude transformation by E1A alone. This would also explain why no transforming activities have been attributed to E1B in the absence of E1A (van den Elsen et al. 1983a).

In summary, p53 turnover can be regulated in response to E1A. This represents the first example of a physiological setting in which high levels of endogenous p53 are

induced in response to an oncogenic challenge, with the apparent consequence of suppressing transformation. The stabilization of p53 appears to guard cells against unscheduled proliferation induced by E1A. Similarly, the stabilization of native p53 and associated apoptosis may provide a natural defense against tumor progression *in vivo*.

Materials and methods

Cell culture

REF52 cells were maintained in Dulbecco's modified Eagles medium (DMEM) supplemented with 5% fetal bovine serum (FBS), 5% calf serum, penicillin (50 U/ml), and streptomycin (50 µg/ml). REF52 cells expressing Ad5 E1A (e.g., clone 1A1) or transformed by T24 H-ras and E1A (e.g., clone RNA7) have been described (Franza et al. 1986). Clones R53/4 and 52LTR/6 expressed mutant p53 genes [p53^{Pro-193} and p53^{Val-135}, respectively (Hicks et al. 1991)], and were obtained from Dr. Michael Mowat (Manitoba Institute of Cell Biology, Canada). ψ 2 cells producing the tsA58-3 and LJ-12S retroviruses (Jat and Sharp 1989) were maintained on DMEM containing 10% calf serum.

Gene transfer

Retroviruses expressing the Ad5 E1A 12S cDNA were isolated following transfection of pLJ-12S into ψ 2 cells. LJ-12S expresses a 12S E1A cDNA and a *neo* gene from the viral long terminal repeat (LTR) and an SV40 early promoter, respectively (M. Timmers, unpubl.). Cells expressing LJ-12S were isolated in media supplemented with 0.4 mg/ml of G418.

The tsA58-3 retrovirus expresses a temperature-sensitive large T antigen (tsA58) and *neo* (Jat and Sharp 1989). To introduce SV40 large T antigen into clones already expressing *neo*, 5×10^6 tsA58-3 producer cells were γ -irradiated for 27 min (3000 rads) and cocultivated with 2×10^3 target cells for 3 days at 37°C in the presence of 2 µg/ml of polybrene. Colonies of infected target cells arose by 10 days at 37°C while the irradiated producer cells detached from the plate. Colonies were pooled and transferred to the permissive temperature for large T expression (33°C) for at least 3 days before analysis. No cells remained in parallel cultures containing only irradiated producer cells.

Ad5 E1A and E1B genes were introduced into cells by calcium phosphate coprecipitation. p1AHygro (Ad5 E1A linked to a hygromycin B resistance gene) was cotransfected with p5XX (Ad5 *Xba*I-*Xho*I genomic E1B fragment), and stable transfectants were isolated in medium containing 100 µg/ml of hygromycin B. Alternatively, p5XX was introduced into 1A1 cells by cotransfecting pY3 (Blochinger and Diggelmann 1984) and selecting for hygromycin B resistance. Stable lines were isolated and expanded in 25 µg/ml of hygromycin B. For transient expression studies, cells were transfected with pCH110 (Hall et al. 1983) and either p1A (Franza et al. 1986) or pBluescript (Stratagene) plasmid DNAs (15 and 5 µg/ml, respectively).

Immunoprecipitation

Monoclonal antibodies PAb419, PAb421, and M73 react with SV40 large T antigen, p53, and adenovirus E1A, respectively (Harlow et al. 1981, 1985). PAb240 reacts with most dominant-transforming forms of p53 but does not recognize wild-type p53 (Gannon et al. 1990). 13D2 reacts with the p53^{E1B} product and was obtained from Dr. Eileen White (Rutgers University, NJ).

Cellular proteins were labeled with 100 µCi/ml of ³⁵S-Express protein labeling mix (New England Nuclear) for either 4 or 18 hr in methionine-free medium (Flow Labs) containing 5% dialyzed FBS. For 18-hour labelings, 5% normal DMEM was included. Cell lysates were prepared (Hinds et al. 1987), normalized to equivalent trichloroacetic acid-precipitable counts (usually 2×10^7 cpm), and cleared of nonspecific IgG-binding proteins (Harlow et al. 1986). p53, large T antigen, and E1B (Hinds et al. 1987) and E1A proteins (Harlow et al. 1986) were precipitated, fractionated on 10% SDS-polyacrylamide gels, and visualized by fluorography. Signal intensities were quantified using a Molecular Dynamics PhosphorImager and ImageQuant software.

For pulse-chase experiments, 5×10^5 cells were seeded into 100-mm dishes and allowed to adhere overnight. Dishes were washed twice with phosphate-buffered saline (PBS) and incubated in the presence of methionine-free medium for 2 hr. Cultures were pulse-labeled for 1 hr with 200 µCi of ³⁵S-labeled amino acids, washed twice with PBS, and chased for various times with normal growth medium supplemented with 10 mM unlabeled methionine. At various intervals, cells were lysed and the amount of labeled p53 was determined by immunoprecipitation as described above.

Immunofluorescence

For immunofluorescence experiments, cells were seeded on glass coverslips at subconfluent densities and allowed to adhere overnight. The cells were washed twice with PBS and fixed in freshly prepared formaldehyde solution [4% (wt/vol) paraformaldehyde and 0.4% (vol/vol) picric acid in PBS]. After 15 min at room temperature, the fixed cells were washed in PBS, permeabilized in methanol/acetone (1 : 1) for 2 min, and washed again in PBS. To minimize nonspecific staining, the coverslips were preincubated in PBS containing 5% goat serum for 1 hr at room temperature. The primary antibody (PAb421 hybridoma tissue culture supernatant diluted 1 : 10 in PBS/5% goat serum) was applied to the coverslips, and the samples were incubated for 2 hr at 37°C. Subsequently, the coverslips were washed in PBS and incubated with fluorescein isothiocyanate (FITC)-labeled goat anti-mouse IgG (1 : 200 dilution in PBS/5% goat serum; Calbiochem) for 20 min at 37°C. Finally, the samples were washed extensively in PBS/0.2% Tween 20 and mounted in PBS/glycerol (1 : 3) containing 0.1% (wt/vol) *p*-phenylenediamine (Sigma). Control cultures were treated identically, except the primary antibody was omitted. Transiently transfected cells were analyzed in a similar manner except anti- β -galactosidase (Promega) and mixtures of primary (PAb421, PAb248, and PAb246 for p53, or M58 and M73 for E1A) and secondary (rhodamine/anti-rabbit IgG and fluorescein-anti-mouse IgG) antibodies were used.

Cell viability

Cells were seeded at 3×10^4 cells/60-mm dish in 10% FCS or 0.5% calf serum. At various times thereafter, floating and adherent cells were pooled, and viability was assessed by trypan blue exclusion. Fresh medium was added 3.5 days after seeding. At least 200 cells were counted for each determination. Degradation of genomic DNA was taken as evidence of cell death by apoptosis. Cells were seeded at 2×10^5 cells/150-mm dish in 10% FCS or 0.5% calf serum. After 3 days, genomic DNA was isolated from pools of floating and adherent cells and analyzed by agarose gel (1%) electrophoresis.

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Stabilization of the p53 tumor suppressor is induced by adenovirus 5 E1A and accompanies apoptosis.

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