# Wild-type p53 mediates apoptosis by E1A, which is inhibited by E1B

**Michael Debbas[1] and Eileen White[1–3]**

[1]Center for Advanced Biotechnology and Medicine and [2]Department of Biological Sciences, Rutgers University, Piscataway, New Jersey 08854 USA

**Transformation of primary rodent cells by the adenovirus E1A and E1B oncogenes is a two-step process, where E1A-dependent induction of proliferation is coupled to E1B-dependent suppression of programmed cell death (apoptosis). The E1B gene encodes two distinct transforming proteins, the 19K and 55K proteins, both of which independently cooperate with E1A. E1B 19K or 55K protein, or the human Bcl-2 protein, functions to suppress apoptosis and thereby permits transformation with E1A. The E1B 55K protein blocks p53 tumor suppressor protein function, indicating that p53 may mediate apoptosis by E1A. In the mutant conformation, p53 blocked induction of apoptosis by E1A and efficiently cooperated with E1A to transform primary cells. When p53 was returned to the wild-type conformation, E1A+p53 transformants underwent cell death by apoptosis. This induction of apoptosis by conformational shift of p53 from the mutant to the wild-type form was inhibited by expression of the E1B 19K protein. Thus, the p53 protein may function as a tumor suppressor by initiating a cell suicide response to deregulation of growth control by E1A. E1B 19K and 55K proteins provide separate mechanisms that disable the cell suicide pathway of p53.**

[*Key Words:* Apoptosis; E1A; E1B; p53, oncogenes; tumor suppressor genes]

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The E1A gene products interact with and perturb the function of key regulators of cell growth, such as the retinoblastoma protein (Whyte et al. 1988) and cyclin A (Pines and Hunter 1990). The sum of these interactions is induction of proliferation (Stabel et al. 1985; Kaczmarek et al. 1986) but also viability loss, intranucleosomal DNA fragmentation, and chromatin condensation (White et al. 1991,1992; Rao et al. 1992), all hallmarks of apoptosis (Wyllie 1980). Thus, induction of apoptosis by E1A may impede transformation despite the ability of E1A to recruit cells into a proliferative state. Evidence to support this scenario has come from the observations that inhibitors of apoptosis, the E1B 19K or the Bcl-2 proteins, will cooperate with E1A to transform primary cells (Rao et al. 1992; White et al. 1992).

The E1B 19K protein was originally identified as an inhibitor of DNA fragmentation and cell death in human cells productively infected with adenovirus. Adenovirus mutants that fail to express a functional E1B 19K gene product induce pronounced fragmentation of both viral and cellular DNA (Pilder et al. 1984; Takemori et al. 1984; White et al. 1984b), causing premature death of the host cell, which severely compromises virus yield (Pilder et al. 1984; Subramanian et al. 1984; White et al. 1984b,1991; White and Stillman 1987). These findings established a role for the E1B 19K protein as a cell survival maintenance factor required to prevent cell death triggered by adenovirus infection of human cells.

[3]Corresponding author.

The viral gene product responsible for eliciting the induction of DNA fragmentation in E1B 19K mutant virus-infected cells mapped to the E1A gene (White and Stillman 1987; White et al. 1991). Therefore, the E1B 19K protein acts to compensate for the cytotoxic consequences of E1A function. Because DNA fragmentation is an indicator of cell death by apoptosis, E1A was thought to induce apoptosis and the E1B 19K protein was believed to function as an inhibitor of apoptosis (White et al. 1991). These conclusions were substantiated by the findings that expression of the E1B 19K protein could block cell death induced by such heterologous agents as tumor necrosis factor-α (TNF-α) and Fas antigen (Gooding et al. 1991; Hashimoto et al. 1991; White et al. 1992), both of which are potent inducers of apoptosis (Laster et al. 1988; Itoh et al. 1991). Genetic analysis of a series of E1B 19K mutant proteins has shown that inhibition of TNF-α cytolysis and transforming activity cosegregate, consistent with both activities resulting from the same function, inhibition of apoptosis (White et al. 1992). By blocking apoptosis the E1B 19K protein may not only directly promote survival of infected cells but also may provide the additional advantage of permitting escape antiviral immune surveillance directed by TNF-α and Fas. Thus, the E1A and E1B gene products provide a model system for examining both positive and negative regulation of apoptosis.

Regulation of apoptosis may be of general importance in transformation and may not be limited to our observations with the transforming genes of adenovirus. The

human *bcl-2* proto-oncogene that becomes overexpressed as a result of translocation in human B-cell lymphomas has been shown to be an inhibitor of apoptosis. Bcl-2 is a mitochondrial protein that, when overexpressed, will block apoptosis in response to growth factor withdrawal in dependent lines (Vaux et al. 1988; Hockenbery et al. 1990; Nuñez et al. 1990), glucocorticoid treatment, radiation, and anti-CD3 antibodies (Sentman et al. 1991; Alnemri et al. 1992). In transgenic mouse models Bcl-2 overexpression will extend B-cell memory (Nuñez et al. 1991) and cause lymphomas (McDonnell and Korsmeyer 1991; Strasser et al. 1991). Finally, expression of the human *bcl-2* proto-oncogene will substitute for E1B in a transformation assay with E1A (Rao et al. 1992). Therefore, apoptosis is a significant obstacle to transformation by E1A, which can be overcome in multiple ways by the 19K, 55K E1B, or Bcl-2 protein.

Although the mechanism by which the E1B 19K or Bcl-2 protein suppresses apoptosis is not known, the E1B 55K protein, which inhibits the function of the p53 tumor suppressor gene product (Sarnow et al. 1982; Yew and Berk 1992), also was able to suppress apoptosis and cooperate with E1A (Rao et al. 1992). This suggested that p53 may participate in the apoptosis pathway and that elimination of p53 function may block apoptosis.

p53 mutations producing loss of p53 function are among the most common genetic alterations found in human cancer (Vogelstein 1990; Levine et al. 1991). The mechanism by which p53 functions as a tumor suppressor is therefore central to preventing oncogenic transformation. When reintroduced into stable, transformed cell lines that have lost p53 function, wild-type p53 most often produces growth arrest at the $G_1$/S boundary, suggesting a role for p53 in regulating cell cycle progression (Finlay et al. 1989; Diller et al. 1990; Mercer et al. 1990; Michalovitz et al. 1990; Ginsberg et al. 1991b; Martinez et al. 1991). In some situations, however, reintroduction of wild-type p53 produces apoptosis, suggesting a role for p53 in induction of cell suicide (Yonish-Rouach et al. 1991; Shaw et al. 1992). To test the involvement of p53 in E1A-dependent apoptosis, the murine p53(val135) temperature-sensitive mutant (pLTRcGval135) (Michalovitz et al. 1990) was utilized in transformation assays with primary baby rat kidney (BRK) cells. Expression of mutant p53 was sufficient to block apoptosis by E1A and permit transformation with high frequency. When p53 was returned to the wild-type conformation, E1A+p53 transformants underwent apoptosis. Expression of the E1B 19K protein completely prevented induction of apoptosis by wild-type p53. Therefore, p53 function is required for induction of apoptosis by E1A, and the E1B 19K protein has been identified as an inhibitor of p53 function.

## Results

### *E1A and mutant p53 cooperate to transform primary rodent cells*

In primary BRK cells E1A expression is sufficient to initiate the formation of foci, most of which subsequently degenerate and die. Transformants emerge with low frequency under these circumstances. When E1A is cotransfected with either E1B 19K or 55K expression vectors, transformation occurs with high frequency (Fig. 1; White and Cipriani 1990; Rao et al. 1992; White et al. 1992). Although the mechanism by which the E1B 19K protein functions to suppress apoptosis was not known, the ability of the E1B 55K protein to do so suggested the involvement of p53. To test the role of p53 in induction of apoptosis following E1A expression, the p53(val135) temperature-sensitive mutant was used.

The p53(val135) protein behaves as a dominant-interfering mutant at the restrictive temperature (37.5°C) and reverts to the wild-type conformation at the permissive temperature (32°C). Primary BRK cells were transfected with E1A and mutant p53(val135) to test whether the blocking of p53 function would suppress E1A-dependent apoptosis. The frequency-of-focus formation by E1A was 10-fold less in the absence of the E1B 19K gene owing to the induction of cell death and focus regression as reported previously (Fig. 1; Rao et al. 1992; White et al. 1992). Mutant p53(val135) enhanced the transformation frequency at least 10-fold over E1A alone at the restrictive temperature (Fig. 1). Cooperation between mutant p53 and E1A was so efficient that it was the most potent transforming combination we have observed in this assay. No focus formation was detectable when mutant p53 was transfected alone or in combination with the E1B 19K expression vector (Fig. 1).

Transformed cell lines were generated from E1A+p53(val135)-transformed foci at the restrictive temperature. All E1A+p53(val135) transformants ex-

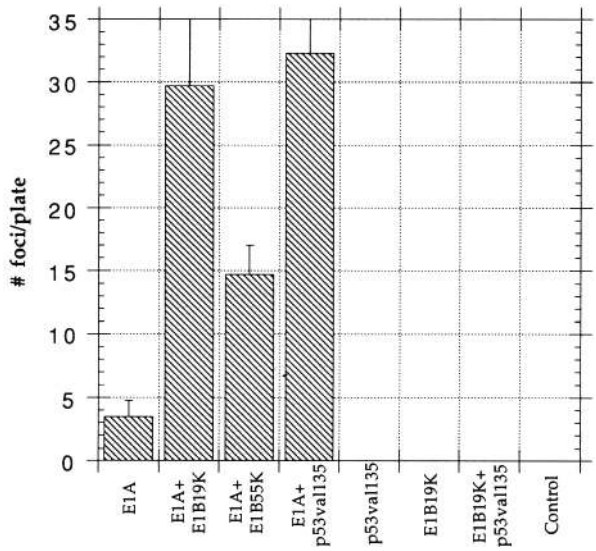


**Figure 1.** Cooperation between E1A and murine mutant p53 in primary BRK cells. Primary BRK cells were transfected by standard procedure with the following DNAs—E1A, pCMVE1A (White et al. 1991); E1B19K, pCMV19K; E1B55K, pCMV55K (White and Cipriani 1990); p53val135, pLTRcGval135 (Michalovitz et al. 1990)—and were maintained at 37.5°C. Focus formation was determined at 30 days post-transfection.

pressed murine mutant p53 and E1A at comparable levels as determined by Western blotting of cell extracts with E1A and murine p53-specific monoclonal antibodies (Fig. 2,top). E1A protein levels in E1A + p53(val135) transformants were similar to those found in E1A + E1B-transformed cell lines (Fig. 2,top).

### *Wild-type p53 induces apoptosis in transformed cells*

The viability of E1A + p53(val135) transformants was examined when p53 was returned to the wild-type conformation. All lines grew efficiently (Fig. 2, bottom) and morphologically resembled E1A + E1B-transformed lines

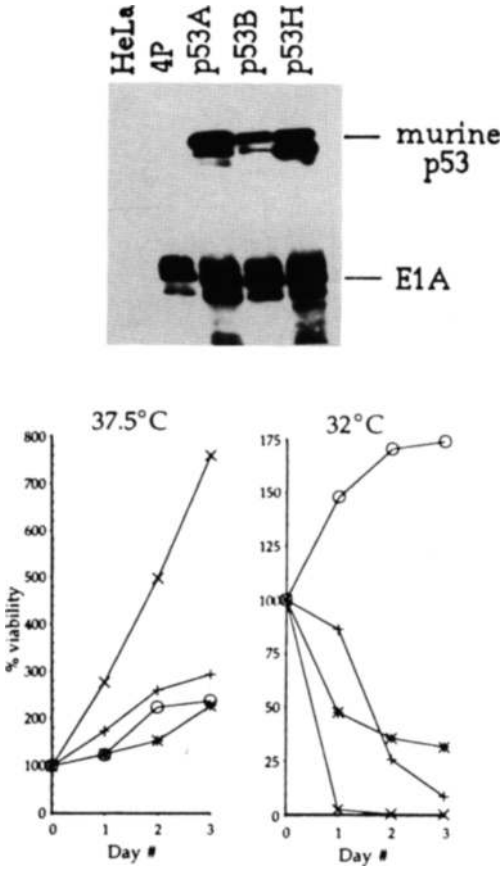


**Figure 2.** Induction of cell death in transformed BRK lines when p53 is in the wild-type conformation. (*Top*) Levels of murine p53 and E1A in transformed BRK lines. Three independent E1A + p53(val135) lines, p53A, p53B, p53H, were derived from foci of transfections described in Fig. 1. Equal amounts of cell extract for each line were analyzed for E1A and murine p53 levels, with monoclonal antibodies M73 and pAb248, respectively, by Western blotting. The 4P (White and Cipriani 1990) line is a previously characterized E1A + E1B-transformed BRK line as a reference for E1A levels. (*Bottom*) Viability of E1A + p53(val135) transformants at permissive temperature (32°C), where p53 is predominantly in the wild-type conformation, and at the restrictive temperature (37.5°C) where p53 is predominantly mutant. Viability as determined by Trypan blue exclusion is expressed as the percentage of the original viability at the time of shift to 32°C. (×) p53A; (*) p53B; (+) p53H; (○) 4P.

at the restrictive temperature (data not shown). Pronounced loss of viability occurred within 24 hr in all three independent E1A + p53(val135) lines after a shift from restrictive to permissive temperatures (Fig. 2, bottom). Viability loss continued for 72 hr (Fig. 2, bottom) and progressed nearly to completion if the lines were maintained at the permissive temperature for 2 weeks. Rare survivors arose as colonies at 32°C with a frequency of ~ 1 in $10^6$cells (data not shown). For comparison, an E1A + E1B-transformed BRK line 4P, which does not contain a temperature-sensitive mutant p53 protein (White and Cipriani 1990), not only maintained viability but continued to grow at 32°C (Fig. 2,bottom). Massive cytopathic effect was coincident with viability loss in the E1A + p53(val135) transformants but not in the E1A + E1B control cell line 4P (see below).

E1A + p53(val135) transformants were examined for indications of apoptosis, DNA fragmentation, and chromatin condensation, accompanying viability loss at the permissive temperature. When maintained at the restrictive temperature where viability was high, none of the lines demonstrated DNA fragmentation. By 24 hr after shift to the permissive temperature, and coincident with viability loss, chromosomal DNA was degraded into nucleosome-size fragments in all three E1A + p53(val135) lines (Fig. 3). DNA fragmentation persisted for 72 hr (Fig. 3). No DNA fragmentation was observed in the control cell line 4P at any time of incubation at 32°C (Fig. 3). Condensation of chromatin and DNA into large aggregates, another indicator of cell death by apoptosis, was also observed in E1A + p53(val135) transformants at 32°C (data not shown). The fastest growing E1A + p53(val135)-transformed line, p53A, displayed the most extreme induction of cell death and was utilized to test the ability of the E1B 19K protein to inhibit apoptosis by wild-type p53.

### *E1B 19K protein blocks induction of apoptosis by wild-type p53*

p53A was transfected with a neomycin-resistance marker alone or, additionally, with the E1B 19K plasmid expression vector pCMV19K. Drug-resistant clones were screened for 19K expression by Western blotting with a monoclonal antibody directed against the E1B 19K protein. Two independent p53A clones were identified that expressed the 19K protein (p53A19K1, p53A19K2) in parallel with two independent control p53A derivatives that were only neomycin resistant (p53An1, p53An2) (Fig. 4,top). All four lines displayed similar growth rates (Fig. 4,bottom) and morphology (data not shown) when maintained at the restrictive temperature. Therefore, E1B 19K expression did not enhance the transformed cell phenotype or growth properties in a conspicuous way. The ability of the E1B 19K protein to inhibit apoptosis upon conformational shift of p53 from the mutant to the wild-type form was examined.

The p53A derivatives that were merely drug resistant rapidly lost viability when shifted to 32°C as did the original parental line p53A (Fig. 4, bottom). The p53A

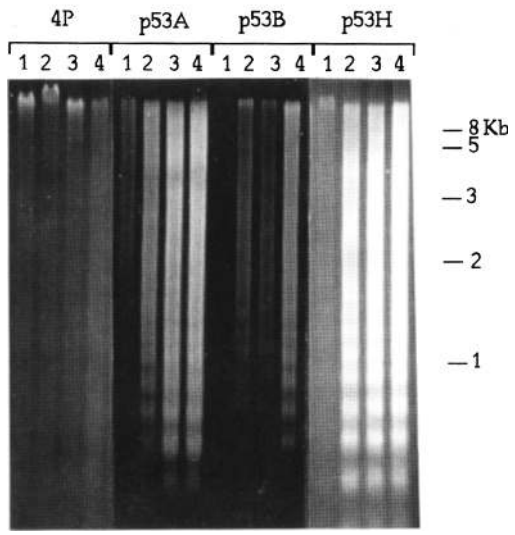


**Figure 3.** Induction of DNA fragmentation by wild-type p53. DNA integrity was determined in the three independent E1A + p53(val135)- transformed BRK lines, p53A, p53B, p53H, and the E1A + E1B control line, 4P, at the restrictive temperature (37.5°C, lane *1*) and following shift to the permissive temperature (32°C) for 24 (lane *2*), 48 (lane *3*), and 72 (lane *4*) hr. Low-molecular-weight DNA was isolated by a modified Hirt assay (Hirt 1967) from nonadherant (apoptotic) cells. Cell lines were equalized with respect to the viable cell number at the time of the shift to 32°C. The presence of degraded DNA was determined by electrophoresis of Hirt DNA samples on 1% agarose gels, and the DNA was visualized with ethidium bromide. Presence of low-molecular weight DNA in a nucleosome ladder pattern is indicative of apoptosis.

derivatives that expressed the E1B 19K protein, however, maintained viability after shift to 32°C (Fig. 4, bottom). Expression of the E1B 19K protein also prevented the induction of pronounced cytopathic effect that accompanied viability loss in E1A + p53(val135) transformants (Fig. 5). Interestingly, the 19K-expressing lines did not grow efficiently at 32°C, suggesting that 19K expression was sufficient to block apoptosis but insufficient to completely overcome induction of growth arrest by wild-type p53 in these lines. After 3 days the 19K-expressing lines did appear to grow at a slow rate. Any potential effects on the cell cycle in these lines is currently under investigation. The growth-suppressive effect of p53 in these 19K-expressing transformants was reversible, with cell proliferation being restored upon return to the restrictive temperature (data not shown).

The integrity of chromosomal DNA was monitored as an indicator of death by apoptosis in the p53A derivatives at 37.5°C and after shift to 32°C. Low-molecular-weight (degraded) DNA was selectively extracted from all four lines with increasing time at the permissive temperature. Although no DNA fragmentation was apparent at the high temperature, pronounced DNA fragmentation into a nucleosome "ladder" pattern was induced in the 19K-minus lines at 32°C (Fig. 6). Although low-molecular-weight DNA was extracted from the equivalent numbers of cells in all four lines, little DNA was present in the Hirt extracts of the 19K-expressing lines because

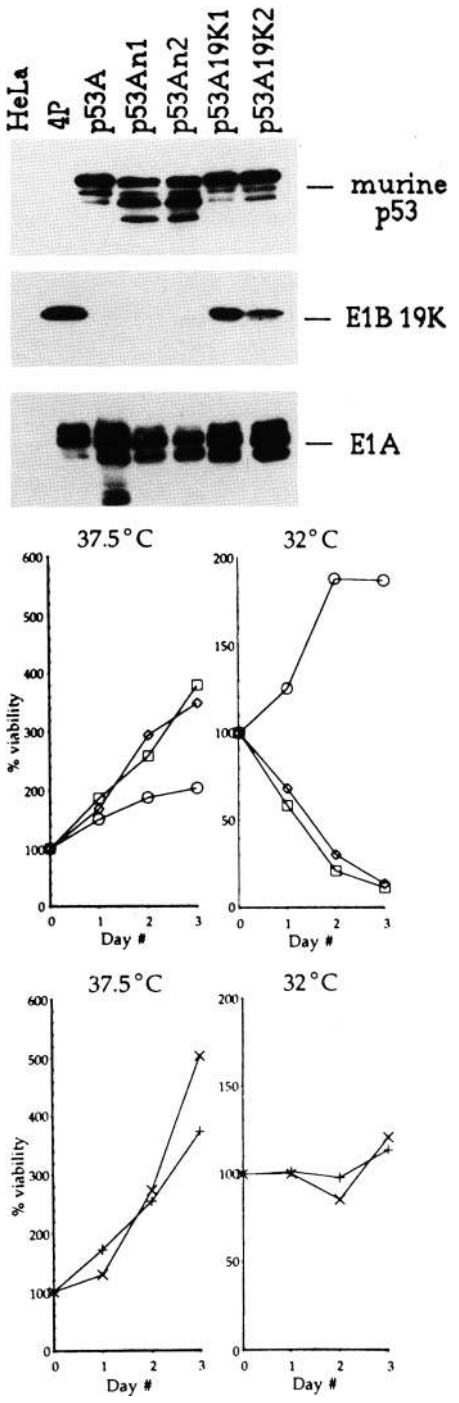


**Figure 4.** The E1B 19K protein blocks cell death induced by wild-type p53. (*Top*) Expression of E1A, murine p53, and the E1B 19K protein in the E1A + p53(val135) transformant p53A derivatives by Western blotting with murine p53, E1B 19K, and E1A-specific monoclonal antibodies. (*Bottom*) Viable cell number, as described in Fig. 2, was determined for four independent p53A derivatives that did (p53A19K1, p53A19K2) or did not (p53An1, p53An2) express the E1B 19K protein. (□) p53An1; (◇) p53An2; (○) 4P; (×) p53A19K1; (+) p53A19K2.

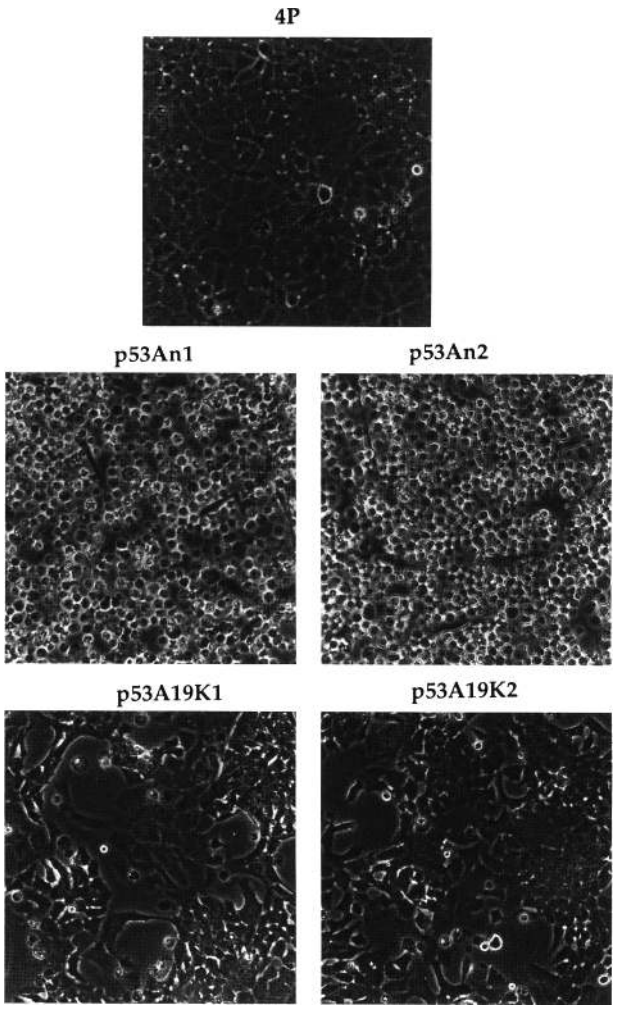


**Figure 5.** Induction of cytopathic effect by wild-type p53 is blocked by E1B 19K expression. The 19K-expressing and -non-expressing p53- derived cell lines described in Fig. 4 were photographed 48 hr after the shift from restrictive to permissive temperatures. Magnification, 25×.

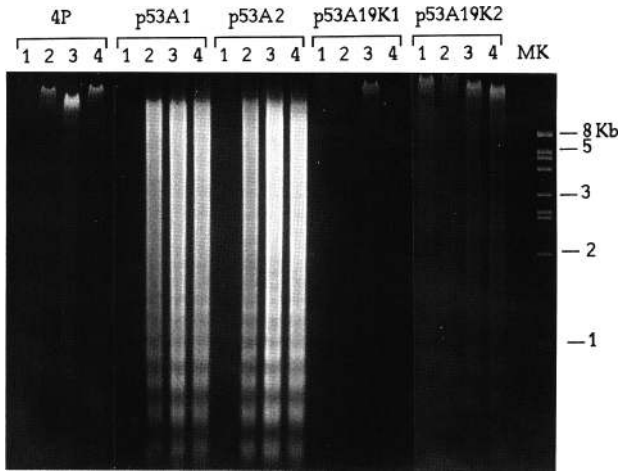


**Figure 6.** The E1B 19K protein blocks induction of DNA fragmentation by wild-type p53. DNA fragmentation was monitored with a modified Hirt assay (Hirt 1967) in parallel with the viability assay in Fig. 4. (Lane *1*) Cells at 37.5°C at the time of the shift to 32°C; (lanes *2–4*) incubation for 24 (lane *2*), 48 (lane *3*), and 72 (lane *4*) hr at 32°C. (MK) Ad5*dl*309 DNA digested with *Hin*dIII as molecular weight markers.

the DNA was intact and partitioned with the high-molecular-weight DNA fraction (Fig. 6). Expression of the E1B 19K protein completely suppressed the induction of DNA fragmentation that occurred with conformational shift of p53 from the mutant to the wild-type form. Thus, the E1B 19K protein was capable of completely inhibiting induction of apoptosis resulting from the presence of wild-type p53 in transformed BRK cells.

## Discussion

### *Apoptosis requires E1A expression in the presence of wild-type p53*

In previous studies it became apparent that expression of E1A was detrimental to cell viability unless the protective function of E1B was provided (White and Stillman 1987; White et al. 1991). The p53 tumor suppressor gene product can also overcome apoptosis in primary cells when in the mutant conformation and must be maintained, as mutant or E1A transformants will undergo apoptosis. Furthermore, the E1B 55K protein that sequesters wild-type p53 into an inactive complex creating a p53 null state will rescue apoptosis in E1A-expressing cells (Rao et al. 1992). Thus, E1A does not induce apoptosis in the absence of wild-type p53. This provides evidence that p53 is directing apoptosis in response to E1A expression during transformation of primary rodent cells (Fig. 7, top). Because p53 is ubiquitously expressed at low levels in normal cells that are not undergoing apoptosis

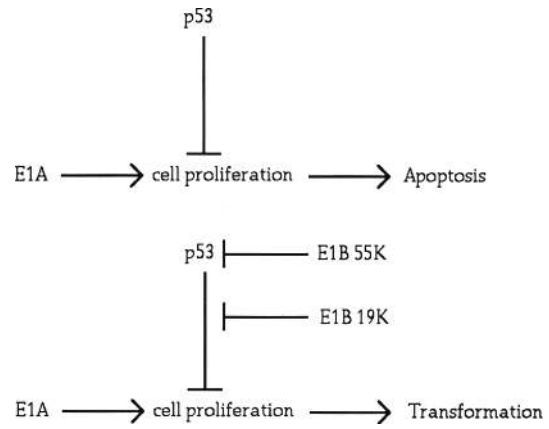


**Figure 7.** Model for regulation of cell proliferation and apoptosis by E1A and E1B gene products (for explanation, see discussion).

(Levine et al. 1991), induction of apoptosis must reflect a change in status of the cell by E1A and modification of p53 activity.

### *Induction of proliferation by E1A may be incompatible with induction of growth arrest by p53*

Mapping studies indicate that induction of apoptosis is a function of the E1A amino terminus that encompasses conserved region 1 (White et al. 1991). Specifically, deletion of amino acids 22–107 (PS*dl* mutation) in the amino-terminal region of E1A will prevent induction of apoptosis in adenovirus E1B 19K mutant virus-infected cells (White et al. 1991). Removal of sequences elsewhere in E1A, such as the *trans*-activation domain in the 13S unique region (conserved region 3), or the retinoblastoma protein- binding region in conserved region 2, does not alleviate apoptosis (White et al. 1991). The amino terminus of E1A is required for induction of cellular DNA synthesis, enhancer repression, and transformation (for review, see Shenk and Flint 1991), but which of these activities is related to apoptosis remains to be determined. The PD*dl* mutation in E1A, however, can be distinguished from the other E1A mutants examined because it produces a mutant protein that is completely defective for induction of cellular DNA synthesis (Moran and Zerler 1988; Stein et al. 1991). The ability of E1A to release restrictions on growth control and induce S-phase, therefore, correlates with induction of apoptosis.

These genetic studies have raised the possibility that induction of cell proliferation by E1A is incompatible with growth suppression by p53 (Fig. 7, top). The consequence of E1A overcoming p53 cytostasis is apoptosis. In that way the cell may regulate inappropriate, abnormal, or untimely DNA synthesis by eliminating cells through p53-dependent apoptosis. The process could be instigated by viral infection or by the accumulation of mutations that lead to the progressive loss of growth control in emerging transformed cells.

Alternatively, the indirect consequences of loss of growth control may cause p53-dependent apoptosis. Abnormal or deregulated DNA synthesis produced by E1A could create DNA damage sufficient to provoke a growth arrest and cell suicide response by p53. p53 functions as a cell cycle control checkpoint in response to DNA damage. p53 protein accumulates in response to DNA damage (Maltzman and Czyzyk 1984), producing cell cycle arrest at $G_1$/S and repair (Kastan et al. 1991,1992; Kuerbitz et al. 1992) and, perhaps, apoptosis when DNA damage is so severe as to be irreparable. With loss of p53 function, the $G_1$/S checkpoint is bypassed, allowing replication of damaged DNA to take place (Kastan et al. 1991,1992; Kuerbitz et al. 1992) and transmission of genetic alterations including gene amplification (Livingstone et al. 1992; Yin et al. 1992). E1A expression has been linked to induction of DNA damage (Braithwaite et al. 1983; Caporossi and Bacchetti 1990), and p53 accumulation has been observed in response to E1A expression (Lowe and Ruley, this issue; E. White and L. Rao, in prep.). Thus, p53 responds to E1A expression in a way that mimics its response to DNA damage.

### *The role of the E1B 19K protein in preventing apoptosis by p53*

The experiments presented here demonstrate that the E1B gene encodes redundant functions, represented by the 55K and 19K proteins, for bypassing the activity of p53. The E1B 55K protein directly inhibits p53 function (Fig. 7, bottom) because physical association between 55K and p53 is usually required for blocking p53 activity in transformation and as a transcription factor (Yew and Berk 1992). However, in productively infected human cells the E1B 55K protein is complexed with the 25K protein from the E4 region of adenovirus, which may interfere with p53 association (Sarnow et al. 1984). This may necessitate the need for an auxiliary mechanism encoded by the E1B 19K protein for disabling the function of p53.

The E1A and E1B 19K proteins together will overcome growth arrest by p53 as their expression will transform cells (White and Cipriani 1990; Rao et al 1992; White et al. 1992). Presumably under these circumstances their expression is sufficient to overcome growth arrest by the endogenous wild-type p53 present in the primary BRK cells (Fig. 7, bottom). The E1A + p53(val135) transformants express much larger amounts of wild-type p53 at the permissive temperature than in the primary cells. The levels of E1B 19K expression in these lines may not be enough to completely block the growth inhibitory effects of wild-type p53, which may account for the reduced growth capacity of these cells at the permissive temperature. Ongoing experiments will distinguish whether E1B 19K protein function is restricted to inhibition of apoptosis by p53 or can be extended to other aspects of p53 function.

The E1B 19K protein may block p53 function by a direct or indirect mechanism (Fig. 7, bottom). Although no physical interaction between the E1B 19K and p53 proteins has been reported, the results presented here indicate that this possibility should be placed under closer scrutiny. Interference in p53 function, however, need not be direct. Changes in the localization and phosphorylation state of p53 are known to affect p53 function and may be modified by the E1B 19K protein. Examination of the p53 protein in cell lines that express the E1B 19K protein in comparison to those that do not, may help to address this issue. It is also possible that the E1B 19K protein acts far downstream of p53 in the apoptotic pathway. The E1B 19K protein resides in the nuclear envelope where it can potentially modify chromatin structure to prevent the endonucleolytic cleavage of DNA that occurs during apoptosis (White et al. 1984a). In that way p53-dependent apoptosis could be bypassed indirectly (Fig. 7, bottom).

How wild-type p53 causes growth arrest and apoptosis is not known. The wild-type p53 protein specifically binds to DNA at a defined consensus sequence (El-Deiry et al. 1992) and can act as a transcriptional activator in a

sequence-specific fashion (Farmer et al. 1992; Zambetti et al. 1992). p53 can also repress transcription, although no specific sequence requirement has been identified (Ginsberg et al. 1991a). The E1B 55K protein and the E6 protein of human papillomavirus can both alter the transcriptional activity of p53 (Lechner et al. 1992; Yew and Berk 1992). This suggests that modulation of cellular gene transcription by p53 may be important for its function as a tumor suppressor. Whether transcriptional activation or repression by p53 are affected by the E1B 19K protein is currently under investigation.

p53 may also play a direct role in cellular DNA replication. Translocation of p53 from the cytoplasm to the nucleus occurs at the $G_1$/S boundary, and expression of wild-type p53 can cause growth arrest at $G_1$/S (Baker et al. 1990; Diller et al. 1990; Mercer et al. 1990; Martinez et al. 1991). Wild-type but not mutant p53 has been shown to bind to sequences within the SV40 origin of replication, raising the possibility that p53 may prevent DNA synthesis by a direct mechanism (Bargonetti et al. 1991). It will be interesting to examine whether these activities of p53 are related to induction of apoptosis and are also affected by the E1B 19K protein.

The E1B 19K protein not only has the ability to block apoptosis induced by wild-type p53 but also by TNF-α and Fas antigen. If inhibition of p53 function is central to the ability of the E1B 19K protein to block apoptosis, TNF-α and Fas antigen may utilize the p53 pathway to induce apoptosis. This would explain the ability of the E1B 19K protein to inhibit apoptosis by such apparently diverse stimuli and would suggest a role for p53 in negative selection and antiviral immune surveillance.

The discovery that adenovirus has evolved mechanisms for both inducing proliferation and blocking apoptosis is generally significant and may represent the basic requirements for the development of cancer. Amplification of c-*myc* proto-oncogene expression, which has growth-deregulating properties analogous to E1A, similarly produces apoptosis (Bissonnette et al. 1992; Evan et al. 1992; Fanidi et al. 1992). Therefore, apoptosis may be a response to aberrant proliferation. p53 mutations are the most common genetic alterations found in human cancer (Levine et al. 1991). A p53-mediated cell suicide response may be an important cellular defense against cancer and may necessitate the evolution by DNA tumor viruses of multiple mechanisms for disabling the process.

## Materials and methods

### *Plasmids and primary cell transformation assays*

Cytomegalovirus (CMV) promoter constructs were utilized to express the E1A (pCMVE1A) (White et al. 1991), E1B 19K (pCMV19K), and 55K (pCMV55K) proteins (White and Cipriani 1990) in transformation assays. The plasmid pLTRcGval135 (Michalovitz et al. 1990) was utilized to express murine mutant p53. The p53(val135) protein is temperature sensitive and is predominantly in the mutant conformation at 37.5°C and predominantly in the wild-type conformation at 32°C (Michalovitz et al. 1990; Gannon and Lane 1991; Martinez et al. 1991). Transformation assays and continuous propagation of transformants containing the p53(val135) protein were carried out at 37.5°C.

Primary Fisher BRK cells were prepared as described previously (White et al. 1991). Plasmids were introduced by electroporation, and cells were maintained in Dulbecco's modified Eagle medium with 5% fetal bovine serum. At 30 days posttransfection, plates were stained with Giemsa and foci were tabulated. Focus diameter of 5 mm or greater was used as a criterion for focus formation. A minimum of eight plates from two independent experiments for each plasmid combination was analyzed. Independent transformed foci were cloned at ~6 weeks after transfection and maintained as continuous cell lines. Three E1A + p53(val135) transformants, p53A, p53B, and p53H, were evaluated further for E1A and murine p53 levels and induction of apoptosis. The transformed BRK cell line, p53A, that derived from transfection of pCMVE1A and pLTRcGval135 plasmids was transfected with the pCMV19K plasmid and a neomycin-resistance marker by electroporation. Independent cell lines were selected with G418 and characterized for 19K expression by Western blotting with 19K-specific antibodies. Two lines, p53A19K1 and p53A19K2, were evaluated further for induction of apoptosis. Control cell lines p53An1 and p53An2, derived from p53A containing only the neomycin-resistance marker, were constructed in parallel.

### *Antibodies and Western blotting*

Monoclonal antibodies directed against murine p53 (pAb248 and pAb2C2) were generously provided by Dr. Arnold J. Levine (Princeton University, NJ). The E1A-specific monoclonal antibody M73 was generously provided by Dr. Ed Harlow (Massachusetts General Hospital, Charlestown, MA). E1B 19K-specific antibodies have been described previously (White et al. 1984a). Cell extracts for Western analysis were prepared from subconfluent cultures, and 20 μg of protein from each cell line was analyzed by polyacrylamide gel electrophoresis and semidry blotting onto nitrocellulose membranes by standard procedures. Following antibody incubations, immune complexes were detected by enhanced chemiluminescence (ECL) according to the manufacturer's specifications (Amersham). The E1A + E1B-transformed BRK cell line 4P (White and Cipriani 1990) was utilized as a control for E1A and E1B expression.

### *Viability and DNA fragmentation analysis*

Transformed BRK cell lines were plated at a density of $1.5 \times 10^5$ cells per 6-cm plate at 37.5°C. Forty hours postplating, when the cells were completely attached to the substrate, the cells were trypsinized and the viable cell number per plate was determined by Trypan blue exclusion. The remaining plates were shifted to 32°C, and the viable cell number was determined after incubation for 24, 48, and 72 hr. For DNA fragmentation assays, apoptotic cells were harvested from the culture medium of transformed BRK cell lines by low-speed centrifugation. The apoptosis cells were then subjected to a modified Hirt procedure, which permits the selective isolation of low-molecular-weight degraded DNA from high-molecular-weight intact chromosomal DNA (Hirt 1967; White et al. 1984b). Hirt supernatant fractions from BRK lines were equalized with respect to the original cell number at the time of the shift to 32°C. Hirt DNA was analyzed by electrophoresis in a 1% agarose gel and visualized by ethidium bromide staining.

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# Wild-type p53 mediates apoptosis by E1A, which is inhibited by E1B.

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