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Activated H-*ras* Rescues E1A-Induced Apoptosis and Cooperates with E1A To Overcome p53-Dependent Growth Arrest

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The adenovirus E1A oncogene products stimulate DNA synthesis and cell proliferation but fail to transform primary baby rat kidney (BRK) cells because of the induction of p53-mediated programmed cell death (apoptosis). Overexpression of dominant mutant p53 (to abrogate wild-type p53 function) or introduction of apoptosis inhibitors, such as adenovirus E1B 19K or Bcl-2 oncoproteins, prevents E1A-induced apoptosis and permits transformation of BRK cells. The ability of activated Harvey-*ras* (H-*ras*) to cooperate with E1A to transform BRK cells suggests that H-*ras* is capable of overcoming the E1A-induced, p53-dependent apoptosis. We demonstrate here that activated H-*ras* was capable of suppressing apoptosis induced by E1A and wild-type p53. However, unlike Bcl-2 and the E1B 19K proteins, which completely block apoptosis but not p53-dependent growth arrest, H-*ras* expression permitted DNA synthesis and cell proliferation in the presence of high levels of wild-type p53. The mechanism by which H-*ras* regulates apoptosis and cell cycle progression is thereby strikingly different from that of the E1B 19K and Bcl-2 proteins. BRK cells transformed with H-*ras* and the temperature sensitive murine mutant p53(val 135), which lack E1A, underwent growth arrest at the permissive temperature for wild-type p53. p53-dependent growth arrest, however, could be relieved by E1A expression. Thus, H-*ras* alone was insufficient and cooperation of H-*ras* and E1A was required to override growth suppression by p53. Our data further suggest that two complementary growth signals from E1A plus H-*ras* can rescue cell death and thus permit transformation.

Apoptosis, or programmed cell death, is a fundamental biological process that eliminates cells during differentiation and development (31), defends hosts against emerging malignant cells (48), and modulates viral latency and replication (16, 23, 43). Apoptosis is predominantly characterized by the loss of cell viability, cellular chromatin condensation, and internucleosomal DNA fragmentation (52). These processes are tightly controlled by positive and negative modulators. Human adenovirus has developed regulators to modulate apoptosis (reviewed in references 38, 42, and 44). Although the mechanisms controlling apoptosis are still poorly understood, studies of adenovirus regulators may provide some insights.

The adenovirus E1A gene products promote both viral and host DNA replication (18, 36) in part by binding to host cell proteins Rb (46), p300 (37), cyclin A-cdk2 and cyclin E-cdk2 (12), p107 (9, 47), and p130 (15, 47). This stimulation of cellular DNA synthesis genetically cosegregates with the induction of apoptosis (41) through a wild-type p53-dependent pathway (8, 27). To combat the induction of apoptosis by E1A and p53, adenovirus also encodes a Bcl-2-related antideath gene product, the E1B 19,000-molecular-weight protein (19K protein) (5, 8, 32, 41, 45), which prolongs host cell viability, maximizes virus production, and counteracts host immune surveillance (reviewed in references 38, 42, and 44).

Regulation of apoptosis is also an integral part of the oncogenic transformation process. Positive growth-stimulatory signals such as E1A and the deregulation of c-*myc* permit uncontrolled cell proliferation but are commonly accompanied by apoptosis and abortive transformation (1, 4, 8, 11, 32, 45, 52). Thus, in order to achieve transformation, stimulation of cell proliferation must be coordinated with inhibition of cell death. In one model for cell transformation, the oncoproteins that cooperate with E1A or with c-*myc* to transform cells are potential apoptosis inhibitors. The E1B 19K protein and human proto-oncoprotein Bcl-2 were shown to be the inhibitors for E1A-induced cell death and thereby cooperate with E1A to transform baby rat kidney (BRK) cells (4, 8, 32).

Induction of apoptosis by E1A is largely p53 dependent. E1A expression induces p53 accumulation (5, 24), which mimics the cellular response to DNA damage upon UV or ionizing radiation treatments (19, 21, 28). Furthermore, dominant interfering mutant p53, cellular oncoprotein Mdm-2, and the E1B 55K protein, which directly bind to p53 and modulate p53 function (3, 35, 50), can cooperate with E1A to transform BRK cells (8, 40, 44), demonstrating that abrogation of wild-type p53 functions prevents cell death. Finally, E1A alone can transform p53-deficient ($p53^{-/-}$), but not normal p53-expressing ($p53^{+/+}$), primary mouse embryonic fibroblasts because of the absence of apoptosis (25), again consistent with the p53 dependence of E1A-induced apoptosis.

In order to understand the mechanisms of E1A-induced apoptosis, we have used a temperature-sensitive (*ts*) murine mutant p53 to suppress E1A-induced apoptosis (8). The mutant, p53(val 135), is predominantly in the wild-type conformation at the permissive temperature (32°C) and in the mutant conformation at the restrictive temperature (38.5°C) (29, 30). Therefore, E1A-plus-*ts* mutant p53-transformed BRK cells, such as the p53A cell line, grow normally at 38.5°C and undergo apoptosis at 32°C due to the presence of E1A and wild-type p53 (8). This system was utilized to examine the transforming gene products potentially involved in regulating

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apoptosis because the p53-dependent death switch can be turned on and off, depending on the temperature at which the cells were cultured.

Activated Harvey-*ras* (H-*ras*), harboring a mutation at codon 12, is highly oncogenic and commonly found in many human cancers (reviewed in reference 2). In vitro studies have shown that H-*ras* cooperates with E1A or with c-*myc* to transform cells (22, 33), leading to the possibility that H-*ras* is an apoptosis inhibitor. In this report, we have introduced the activated H-*ras* gene into the p53A cell line (8) and assayed for its inhibitory affects on E1A-induced apoptosis. The cell lines which expressed a high level of H-*ras* retained viability, whereas those which expressed low levels or no activated H-*ras* were completely eliminated through cell death by apoptosis. Surprisingly, unlike the E1B 19K and Bcl-2 proteins, which specifically inhibit E1A-induced apoptosis but fail to relieve wild-type p53-mediated growth suppression (4, 8, 34), H-*ras* expression permitted DNA synthesis and cell proliferation in the presence of high levels of wild-type p53. Furthermore, H-*ras* alone was insufficient to overcome p53-dependent growth suppression which, however, could be relieved by the cooperation between H-*ras* and E1A. The mechanism by which H-*ras* regulates apoptosis and cell cycle progression is thereby strikingly different from that of the E1B 19K and Bcl-2 proteins.

MATERIALS AND METHODS

Plasmids and cell lines. Cytomegalovirus expression vectors were utilized to express the adenovirus E1A (pCMVE1A) (41) or E1B 19K (pCMV19K) (39) protein as previously described. Plasmid pLTRcGval135 was utilized to express *ts* murine mutant p53(val 135) (30). Plasmids pneoCMVH-*ras* and pneoCMVH-*ras*-FD were generated by inserting the cDNA of H-*ras* into pcDNAI/neo (Invitrogen Corp.), which contains a neomycin resistance marker. The cDNA of H-*ras* encodes the human H-*ras* which harbors a point mutation at codon 12 (Gly to Val) and thus causes oncogenic activation. *ras*-FD contains an additional Cys-to-Ser substitution at codon 186 of the CAAX motif which abolishes farnesylation of the protein and anchoring to the cell membrane. Because of its inability to properly localize to membranes, the *ras*-FD mutant protein fails to transform (20) and thus serves as a nonfunctional control H-*ras*.

The p53A line was one of many cell lines established by transfecting primary BRK cells by electroporation with plasmids which express E1A and *ts* mutant p53 (8). To study the effect of H-*ras* on E1A-induced p53-mediated apoptosis, H-*ras*-expressing p53A derivatives were generated by electroporation of p53A with H-*ras*- or *ras*-FD-encoding plasmid DNAs at 38.5°C as previously described (4, 8). The transformants were selected for neomycin resistance and were individually cloned and maintained at 38.5°C.

The transformation assays of primary BRK cells were carried out as previously described (41). Briefly, BRK cells were prepared from 6-day-old baby Fisher rats and were electroporated with test DNA plus carrier DNA. Primary rat cells were cultured in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum for 3 to 4 weeks at 38.5°C, and plates were then stained with Giemsa stain. Foci with diameters of 5 mm or larger were scored as positive for transformation. Independent foci were also cloned and propagated as individual cell lines.

Viruses and viral infection. The E1B-lacking adenovirus (12SE1B$^-$) that expresses the 12S E1A gene product with the E1B gene deleted between nucleotides 1720 and 3322 has been previously described (5) and was utilized to express E1A. The 12SE1B$^-$ virus is unable to express either the E1B 19K or 55K protein. The control virus (PAC3), which expresses neither E1A nor E1B products but was otherwise identical to 12SE1B$^-$, was obtained from Yasha Gluzman (Cold Spring Harbor Laboratory). Cells were infected with adenovirus at a multiplicity of infection of 100 PFU per cell. The infected cells were further subjected to 5′-bromo-2′-deoxyuridine (BrdU) incorporation and cell cycle analysis (see below).

Antibodies and Western blot analysis. Monoclonal antibody (PAb248) directed against murine (wild-type and mutant) p53 was provided by Arnold J. Levine (Princeton University, Princeton, N.J.). The E1A-specific monoclonal antibody M73 was a gift from Ed Harlow (Massachusetts General Hospital, Charlestown). The anti-H-*ras* monoclonal antibody, Pan-*ras* Val-12 (Ab-1), was purchased from Oncogene Science. The cell extracts for Western blot (immunoblot) analysis were prepared from subconfluent cultures, and 20 μg of total protein from each cell line was subjected to sodium dodecyl sulfate-polyacrylamide gel analysis followed by blotting onto nitrocellulose membranes. After incubation with antibody, the immune complexes were detected by enhanced chemiluminescence as described by the manufacturer (Amersham).

Viability measurements and DNA fragmentation analysis. BRK transformants were plated at a density of 3×10^5 cells per 6-cm-diameter plate at 38.5°C. At 36 to 48 h postplating, when the cells stably attached to plates and were subconfluent, one plate of cells was trypsinized and total viable cell number per plate was quantitated by trypan blue exclusion. The remaining plates were continued in culture at 38.5°C or were shifted to 32°C, and the total viable cell number per plate was determined following incubation for the periods indicated in the figures. In DNA fragmentation assays, cells were harvested and the low-molecular-weight DNA was isolated by a modified Hirt procedure (17, 43). Fragmented DNA was normalized with respect to the initial viable cell number from the time of the shift to 32°C and was analyzed on 1% agarose gels and visualized by ethidium bromide staining as previously described (8).

BrdU incorporation and flow cytometry. Cells (1×10^6 to 5×10^6) in a 10-cm-diameter plate were labeled with 20 μM BrdU (Boehringer Mannheim Biochemicals) for 4 h. Cells were then trypsinized, washed with phosphate-buffered saline (PBS), and fixed with 70% ethanol. Nuclei were treated with 2 N HCl to remove histones and to denature double-stranded DNA. Following neutralization with 0.1 M $Na_2B_4O_7$ (pH 8.5), cells were stained with fluorescein-conjugated anti-BrdU antibody (Becton Dickinson Immunocytometry Systems), washed with PBS, and resuspended in PBS containing 10 μg of propidium iodide per ml to measure the DNA content per nucleus. Propidium iodide- and BrdU-labeled cells were subjected to analysis on an EPICS profile analyzer (Coulter). Unlabeled cells were stained in parallel with the same fluorescein isothiocyanate-conjugated antibody to serve as a negative control. Compartments were established such that cell fractions in G_0/G_1, early S, late S, and G_2/M were gated in compartments 1, 2, 3, and 4, respectively. Cells that fell into compartments 2 and 3 were scored as positive for BrdU incorporation, and less than 1% of the negative control cell population fell into these compartments.

RESULTS

High expression of activated H-*ras* rescues E1A-induced p53-dependent apoptosis. BRK cell lines transformed by E1A and *ts* mutant p53, such as the p53A cell line (8), grow normally at the restrictive temperature (38.5°C), at which p53 is predominantly in the mutant conformation (29, 30). However, at the permissive temperature (32°C), when p53 is predominantly in the wild-type conformation (29, 30), this and other similarly generated cell lines undergo apoptosis (8). To determine if H-*ras* can rescue the cell death, an H-*ras*-carrying plasmid (pneoCMVH-*ras*) was transfected into the p53A cell line and the transformants were selected for neomycin resistance. Sixteen independent clones, all of which expressed nearly equivalent amounts of the p53 and E1A proteins as in the parental line p53A (Fig. 1), were individually isolated. Among these, the five with moderate to high levels of H-*ras* expression (Fig. 1) were further investigated. Similarly, a negative control line, Ras-FD, which expressed a transformation-defective mutant H-*ras*, was obtained by transfection with pneoCMVH-*ras*-FD (Fig. 1).

To examine if H-*ras* expression prevented E1A-induced cell death, the cells were tested for cell viability at both restrictive (38.5°C) and permissive (32°C) temperatures. All cells grew normally at 38.5°C, as demonstrated by an increasing viable cell number (Fig. 2). One property of E1A-plus-H-*ras*-transformed BRK cell lines was that they lost viability upon reaching high cell density or growth factor deprivation (Fig. 2). This phenomenon is not observed in other cell lines transformed by E1A plus E1B or E1A plus Bcl-2 (Fig. 2) (4, 8). By day 4, all of the H-*ras*-expressing lines showed some degree of viability loss due to this p53-independent cell death at 38.5°C (Fig. 2).

At 32°C, cell lines Ras1, Ras4, and Ras5, which expressed high levels of H-*ras* (Fig. 1), maintained a constant number of viable cells for the duration of the experiment (5 days) (Fig. 2). Ras2, however, proliferated at a rate similar to that of the positive control 4P line (a nearly 500% increase in viable cell number in 5 days) (Fig. 2). In contrast, Ras3 and 11 other clones which expressed low levels of H-*ras* dramatically lost viability by apoptosis with efficiency similar to that of the control cell line, Ras-FD, in which nearly the entire (>97%) population underwent apoptosis by day 2 (Fig. 2 and 3 and data not shown). These data suggested that high levels of expression

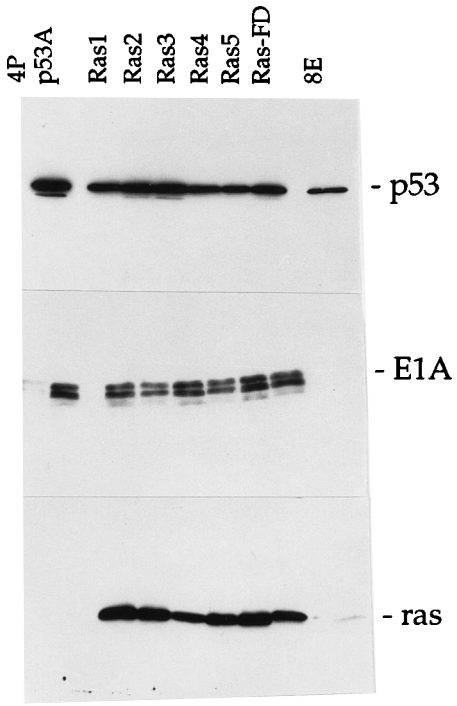

FIG. 1. Expression of activated H-*ras* in E1A-plus-*ts* p53(val 135)-transformed BRK cell lines. The E1A-plus-*ts* p53-transformed BRK line, p53A, was transfected with pneoCMVH-*ras* (cell lines Ras1 to Ras5) or pneoCMVH-*ras*-FD (cell line Ras-FD) to overexpress H-*ras* or nonfunctional *ras*-FD protein, respectively. 4P is an E1A-plus-E1B-transformed BRK cell line (40) and thus serves as a positive control for E1A expression and a negative control for H-*ras* and murine p53 expression. 8E (see Fig. 7) is an H-*ras*-plus-*ts* p53-transformed BRK cell line which serves as a positive control for H-*ras* and p53 expression and a negative control for E1A expression. Equal quantities of proteins from each cell line were analyzed for E1A, p53, and H-*ras* levels with monoclonal antibodies M73, PAb248, and Pan-*ras* Val-12 (Ab-1), respectively, by Western blotting.

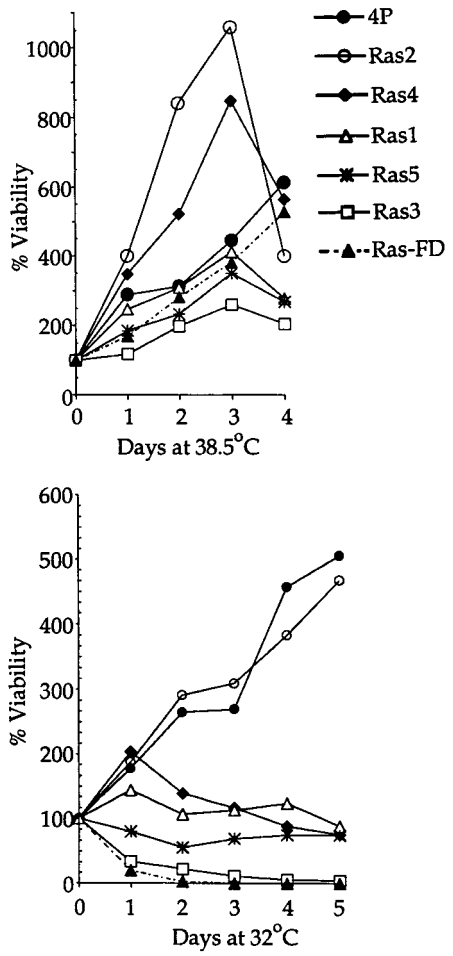

FIG. 2. High expression of H-*ras* rescues cell death induced by E1A and wild-type p53. The p53A-derived cell lines which express H-*ras* (Ras1 to Ras5) or *ras*-FD (Ras-FD) were assayed for viability at the permissive (32°C) and restrictive (38.5°C) temperatures. 4P does not express *ts* p53 and thus serves as a control for continued proliferation at 32°C. Each cell line was plated at an equal density, and 42 h postplating, the number of viable cells was quantitated by trypan blue exclusion (day 0). The remaining plates were either maintained at 38.5°C or shifted to 32°C, and then the number of viable cells per plate was determined for 4 consecutive days. Viability is expressed as a percentage of the number of total viable cells per plate relative to that at day 0.

of activated H-*ras* could prevent the loss of viability due to E1A-induced p53-mediated apoptosis. The dramatic difference in the viability of Ras1, Ras2, Ras3, Ras4, and Ras5 cells was visually distinguishable (Fig. 3 and data not shown), although they were morphologically round and clumpy (Fig. 3) compared with the parental p53A cell line or the E1B 19K protein- or Bcl-2-expressing derivatives (4, 8). Furthermore, H-*ras* expression did not affect p53 protein levels at 32 and 38.5°C (data not shown), excluding the possibility that H-*ras* acted by reducing wild-type p53 levels.

Maintenance of the viable cell number at nearly 100% at 32°C in most H-*ras*-expressing cell lines could be due to one of two possibilities: inhibition of apoptosis and induction of growth arrest (as the E1B 19K protein or Bcl-2 acts) (4, 8, 34) or equal rates of cell proliferation and cell death. To distinguish between these two possibilities, low-molecular-weight DNA was isolated to determine if DNA fragmentation, an indicator of apoptotic cell death, was occurring. As shown in Fig. 4, internucleosomally cleaved DNA fragments in multiples of approximately 200 bp were detected in all H-*ras*-expressing lines at 32°C, with an exception of the Ras2 line, which showed minimal DNA fragmentation. Apoptotic cells were not apparent in the Ras2 line for up to 4 days at 32°C. The Ras3 line, which expressed less H-*ras* protein (Fig. 1), showed significant amounts of apoptotic DNA fragmentation (Fig. 4) and lower cell viability (Fig. 2) than the cell lines (Ras1, Ras2, Ras4, and Ras5) which expressed more H-*ras*. In the Ras-FD cell line, however, degraded DNA was observed on day 1 at 32°C but not beyond that point, because more than 97% of the cells were dead and lysed prior to analysis. In conclusion, the Ras1, Ras4, and Ras5 lines remained partially susceptible to apoptosis, which indicated that surviving cells may continue to proliferate in order to compensate for cell loss through apoptosis.

H-*ras* expression permits DNA synthesis in the presence of E1A and wild-type p53. The ability of H-*ras*-expressing cell lines to undergo apoptosis without a decrease in the viable cell number suggested that expression of activated H-*ras* allowed cells to proliferate in the presence of high levels of wild-type p53. To address this possibility, the rate of DNA synthesis in these cell lines was measured by their ability to incorporate the thymidine analog BrdU (Fig. 5 and Table 1). To measure the DNA content per nucleus, each sample was additionally stained with propidium iodide before being subjected to cell cycle analysis.

The H-*ras*-expressing lines Ras1, Ras2, and Ras5 were able to incorporate BrdU at 32°C at a rate similar to that of the 4P positive control cell line, indicating that DNA synthesis was

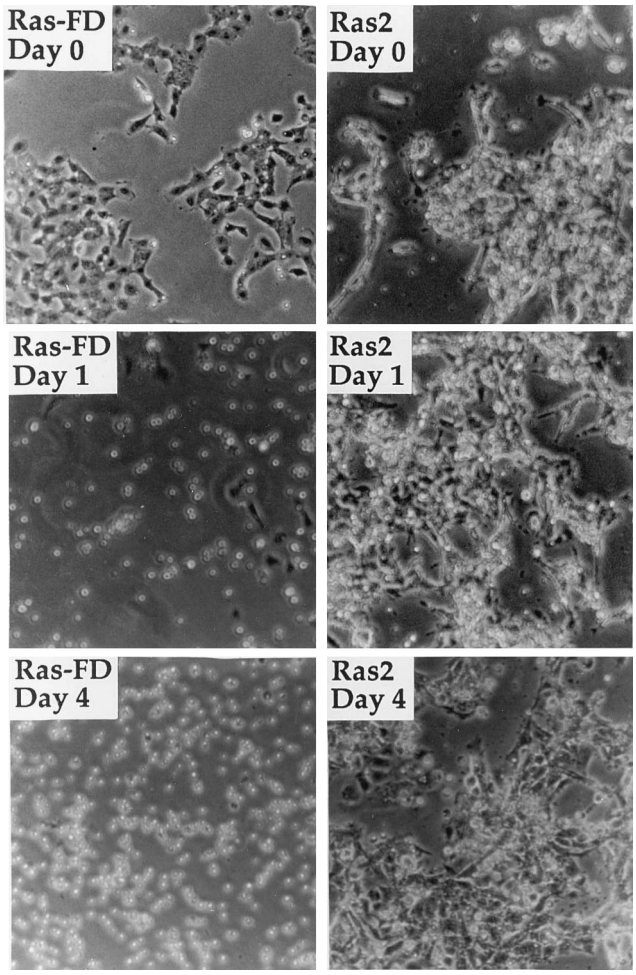

FIG. 3. Expression of activated H-*ras* prevents the morphological aspects of apoptosis induced by E1A and wild-type p53. In parallel to the experiment shown in Fig. 2, an H-*ras*-expressing cell line (Ras2) or the nonfunctional *ras*-FD expressing cell line (Ras-FD) was photographed at the restrictive temperature (38.5°C) (day 0) or after incubation at the permissive temperature (32°C) for 1 or 4 days.

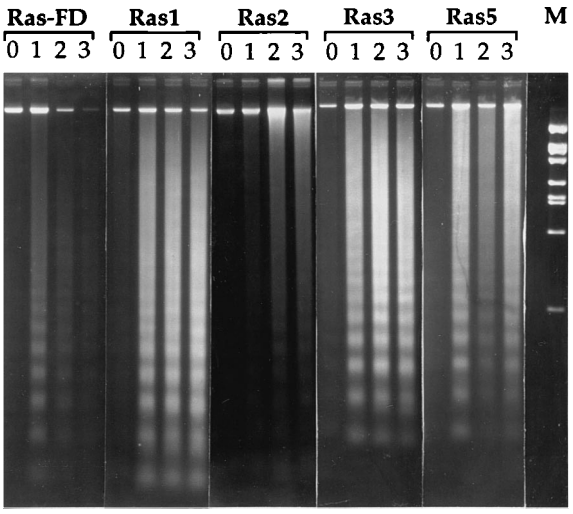

FIG. 4. Analysis of DNA integrity in H-*ras*-expressing cell lines. Low-molecular-weight degraded DNAs were isolated by a modified Hirt DNA method in parallel with the assay for the number of viable cells shown in Fig. 2. DNA was isolated from cells at the time of the shift to 32°C (lanes 0) or after incubation at 32°C for 1, 2, and 3 days (lanes 1 to 3, respectively) and analyzed by agarose gel electrophoresis. Lane M, *Hin*dIII-digested Ad5*dl*309 viral DNA was used as molecular weight markers.

taking place at the temperature permissive for wild-type p53 (Fig. 5 and Table 1). In contrast, the Ras-FD negative control line completely underwent apoptosis by day 2 (data not shown). Our data suggested that in the presence of E1A, H-*ras* expression relieved wild-type p53-dependent growth suppression and permitted DNA synthesis.

The 19K1 and Bcl-2 4B cell lines, which were also derived from the same parental p53A line and additionally express either the E1B 19K or the Bcl-2 protein, maintain nearly 100% viability at 32°C without any indications of apoptosis (4, 8, 34). In comparison to the BrdU incorporation profiles of H-*ras*-expressing cells, those of the 19K1 and Bcl-2 4B lines gradually lost BrdU incorporation ability at 32°C (Fig. 5 and Table 1). By day 4 at 32°C, there was an approximately fivefold reduction in BrdU incorporation in both cell lines compared with the level at 38.5°C. In contrast, the 4P and the H-*ras*-expressing cell lines retained about 50% BrdU incorporation relative to that at 38.5°C, suggesting that the twofold reduction in BrdU incorporation in the 4P and H-*ras*-expressing cell lines was due to the incubation at the lower temperature rather than the presence of ectopically expressed wild-type p53 (Table 1). These observations demonstrate that both the 19K1 and Bcl-2 4B cells lines undergo growth arrest at 32°C. Thus, E1B 19K and Bcl-2 protein expression blocks only apoptosis and fails to relieve growth suppression by wild-type p53. Interestingly, the population of cells in the G_2/M phase increased from about 9% (at 38.5°C) to approximately 54% after incubation at 32°C for 4 days (Table 1 and data not shown). The accumulation of cells with a 4 N DNA content indicated that the p53-dependent growth arrest took place predominantly in the G_2/M phase in the E1B 19K and Bcl-2 protein-expressing p53A derivatives (Fig. 5).

The role which H-*ras* plays in the regulation of apoptosis appears to be different from that of the E1B 19K and Bcl-2 proteins. While most of the H-*ras*-expressing cell lines (Ras1, Ras4, and Ras5) maintained viability at the permissive temperature, as do the E1B 19K or Bcl-2 protein-expressing counterparts, H-*ras* expression permitted DNA synthesis and cell proliferation whereas the E1B 19K protein or Bcl-2 did not (Fig. 5 and Table 1) (4, 34). The rate of cell proliferation as indicated by BrdU incorporation (Table 1) was somewhat equal for most of the H-*ras*-expressing cell lines and the 4P control line, which corresponded to a 150% increase in viable cell numbers per 24 h (Fig. 2). Similarly, the Ras-FD control cell line continued DNA synthesis at a rate not significantly different from that of the H-*ras*-expressing counterparts prior to undergoing massive apoptosis (data not shown). The dramatic loss of viability at the permissive temperature in Ras-FD (80% cell death per 24 h [Fig. 2]) was therefore primarily due to apoptosis rather than a lack of cell proliferation. Thus, the rate of cell death greatly exceeds the rate of cell proliferation in the absence of activated Ras. To maintain viability, reduction of the rate of apoptosis may be more significant than modulation of cell proliferation in these H-*ras*-expressing p53A derivatives. Although Ras2 showed negligible levels of apoptosis, the rate of cell death in Ras1, Ras4, and Ras5 was approximately equal to the rate of cell proliferation, which would be sufficient to maintain the viable cell number at 100%. In conclusion, the apoptosis induced by E1A and wild-type p53 appeared to be significantly rescued by the high expression of

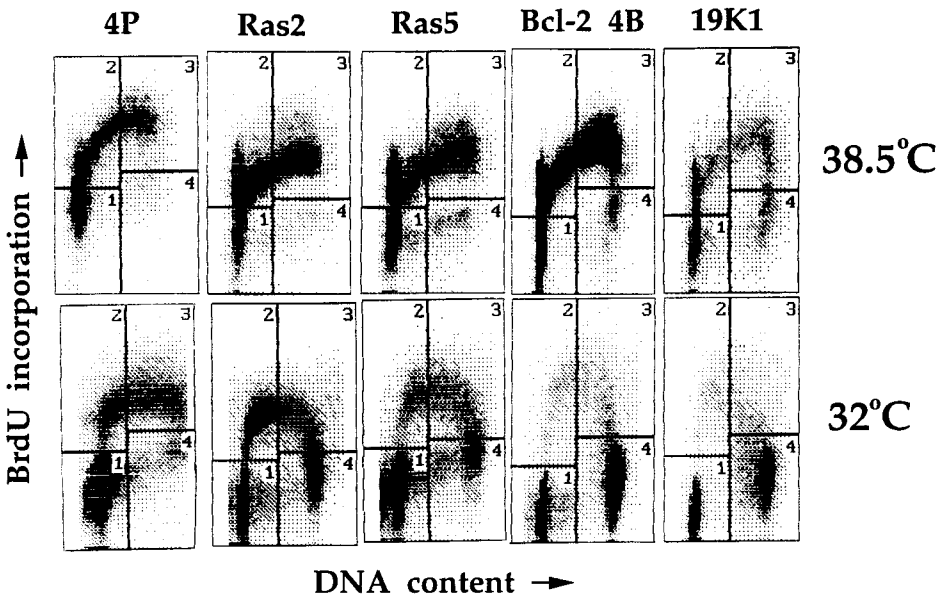

FIG. 5. BrdU incorporation and cell cycle analysis of p53A-derived cell lines. 4P is an E1A-plus-E1B-transformed BRK cell line which lacks the *ts* p53, whereas Ras1 and Ras5 were derived from p53A and express H-*ras*. 4B and 19K1 were also derived from the same p53A line but additionally express Bcl-2 (4) and the E1B 19K protein (8), respectively. Cells were propagated at 38.5°C (top panels) or were shifted to 32°C and cultured for 3 additional days (bottom panels). Cells were labeled with BrdU and stained with fluorescein isothiocyanate-conjugated anti-BrdU antibody to monitor BrdU incorporation. Propidium iodide was used to measure the DNA content per nucleus. Cells were then subjected to flow cytometry analysis, and the percentages of cells gated in compartments 2 (early S) and 3 (late S) were scored as positive for BrdU incorporation as summarized in the Table 1. Cells in compartments 1 and 4 possess 2 N (G_1-phase) and 4 N (G_2-phase) DNA contents, respectively.

H-*ras*, suggesting that H-*ras* had the ability to suppress cell death.

Activated H-*ras* and mutant p53(val 135) cooperate to transform primary BRK cells. To further understand the role that H-*ras* plays in regulation of p53 function, we transfected H-*ras* and the *ts* mutant p53 into BRK cells and selected for transformants at 38.5°C (Fig. 6). Transfection of E1A alone into BRK cells produced abortive transformation and few foci because of apoptosis (32, 45), but E1A efficiently cooperated with *ts* mutant p53 and with activated H-*ras* to transform BRK cells and gave rise to greater than 30 foci per plate (Fig. 6). H-*ras* cooperated with *ts* mutant p53 to transform BRK cells with lower efficiency than did E1A plus mutant p53 with an average of five foci per plate, whereas transfection of mutant p53 or H-*ras* DNA alone, carrier DNA, or control *ras*-FD did not give rise to any foci (Fig. 6). As H-*ras* was able to cooperate with mutant p53 to transform BRK cells, this suggested that H-*ras* possessed a growth-stimulatory function, which is consistent with the observation that activated H-*ras* induces proliferation of quiescent cells (13). Furthermore, endogenous wild-type p53 function is apparently an impediment for transformation by H-*ras* alone, since abrogation of p53 function permits trans-

TABLE 1. BrdU incorporation permitted by H-*ras* expression in the presence of wild-type p53 and E1A

Day[a]	% of cells from indicated cell line incorporating BrdU[b]					
	4P	Ras1	Ras2	Ras5	Bcl-2 4B	19K1
0	56.6	73.0	74.4	64.4	60.8	50.9
1	45.5	42.2	47.0	36.0	26.7	21.9
2	33.1	28.2	43.9	33.8	28.5	28.7
3	34.2	33.4	42.1	33.4	14.0	18.2
4	29.6	31.5	38.3	31.7	8.5	11.6

[a] Cells were propagated at 38.5°C and were shifted to 32°C at day 0, and BrdU incorporation was analyzed for four consecutive days. Cells were labeled with 20 μM BrdU for 4 h prior to harvest for the analysis.

[b] During a 4-h labeling period. The numbers were derived from the percentage of cells in compartments 2 and 3 in Fig. 5, which represents predominantly the fraction of cells in the S phase of the cell cycle. Cell lines are described in the legend to Fig. 5.

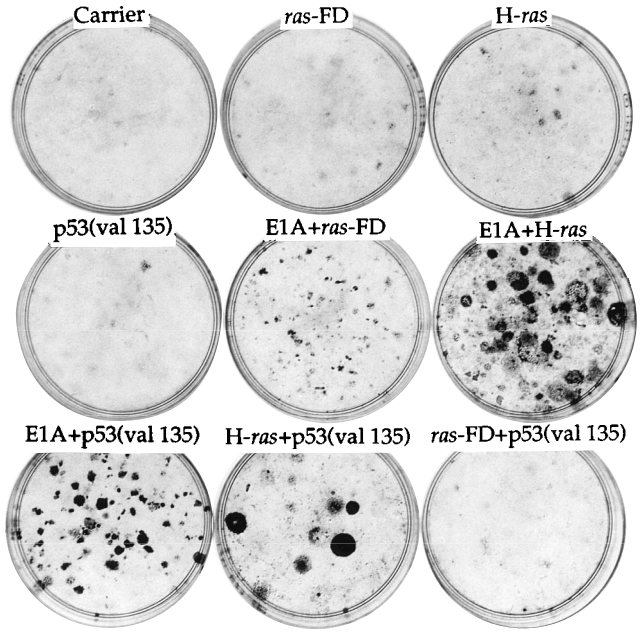

FIG. 6. Activated H-*ras* cooperates with *ts* mutant p53(val 135) or with E1A to transform BRK cells. Ten micrograms of the indicated plasmid DNA plus carrier DNA was electroporated into primary BRK cells. Cells were cultured at 38.5°C, and foci were stained with Giemsa stain at 3 to 4 weeks posttransfection and then photographed.

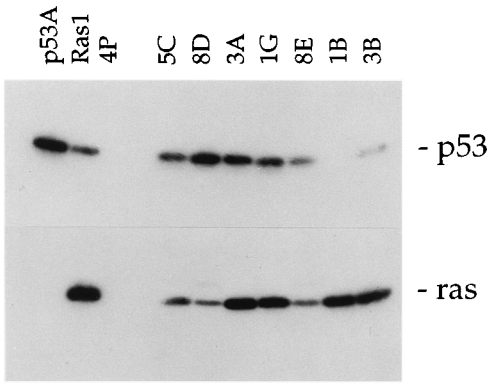

FIG. 7. Levels of p53 and H-*ras* expression in the H-*ras*-plus-*ts* p53(val 135)-transformed BRK cell lines. The H-*ras*-plus-*ts* p53 transformants (lines 5C, 8D, 3A, 1G, 8E, 1B, and 3B) and other control cell lines (p53A, Ras1, and 4P) were propagated at 38.5°C. Equal quantities of protein from each cell line were subjected to Western blot analysis, and the levels of p53 and H-*ras* were determined with PAb248 and Pan-*ras* Val-12 (Ab-1) antibodies, respectively.

formation by H-*ras* and exogenously introduced wild-type p53 suppresses transformation by E1A and H-*ras* (14). However, unlike E1A, which provides both growth-stimulatory and apoptotic signals, H-*ras* apparently induces growth stimulation without apoptosis in this setting.

H-*ras* is insufficient to overcome p53-mediated growth suppression which can be relieved by E1A expression. Seven independent H-*ras*-plus-*ts* mutant p53-transformed BRK cell lines which were established from foci represented in Fig. 6 expressed various levels of H-*ras* and mutant p53 (Fig. 7). Among these, clones 3B, 8D, and 1G, which expressed different ratios of H-*ras* and mutant p53 proteins, were further characterized for their viability at 32°C, at which p53 is predominantly in the wild-type conformation. As shown in Fig. 8, the H-*ras*-plus-*ts* mutant p53 transformants grew rapidly at 38.5°C. However, cell proliferation appeared to be significantly suppressed at 32°C when p53 reverted to the wild-type conformation (Fig. 8). In the same assay, an E1A-plus-H-*ras* transformant which was established from foci shown in Fig. 6 and did not express *ts* mutant p53 continued to proliferate at 32°C (Fig. 8).

To further substantiate whether the H-*ras*-plus-*ts* p53 transformants underwent growth arrest, BrdU incorporation and cell cycle analyses were performed. As shown in Table 2 and Fig. 9, these cell lines (3B and 8D) underwent growth arrest, as indicated by a substantial reduction in the incorporation of BrdU and complete depletion of cells in S phase by 24 h at 32°C. Furthermore, the cells remained healthy, with no indications of apoptosis (data not shown). Interestingly, both cell lines accumulated populations nearly equally distributed with a 2 and 4 N DNA content (Fig. 9), suggesting that growth arrest took place in both G_1 and G_2/M.

Cooperation between H-*ras* and E1A in focus formation suggests that together they are capable of overcoming growth suppression by wild-type p53. To test this hypothesis, E1A was introduced into H-*ras*-plus-*ts* p53-transformed BRK cell lines (3B and 8D) by infection with an E1B-lacking adenovirus which contained E1A (12SE1B$^-$ virus) or control virus which lacked both E1A and E1B (PAC3 virus). After the 3B and 8D cell lines had achieved growth arrest at 32°C (Fig. 9 and Table 2), the cells were infected and were analyzed for BrdU incorporation at 24 and 48 h postinfection. By 48 h postinfection, cells infected by the E1A-expressing virus (12SE1B$^-$ virus) had reentered S phase and the ability to incorporate BrdU was

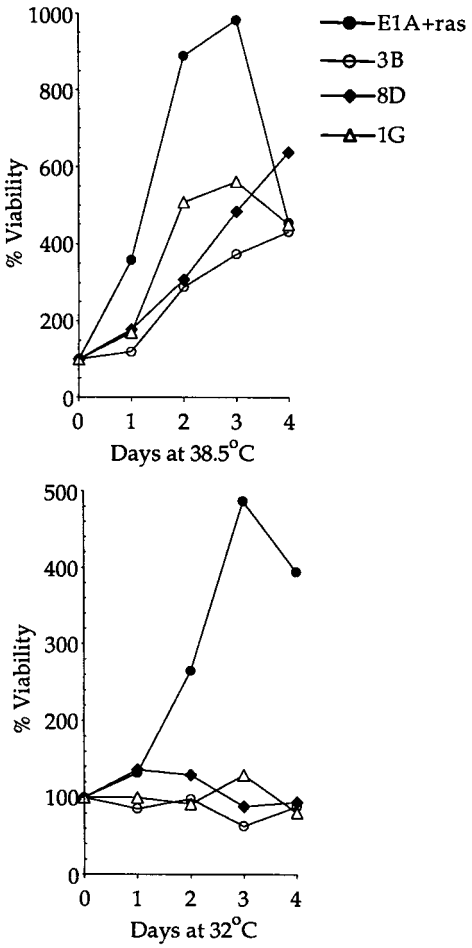

FIG. 8. H-*ras*-plus-*ts* p53(val 135) transformants undergo p53-dependent growth arrest at the permissive temperature (32°C). The H-*ras*-plus-*ts* p53-transformed BRK cell lines (3B, 8D, and 1G) were incubated at 38.5°C, and the viability was determined by trypan blue exclusion (day 0). The remaining plates were incubated at 38.5°C or shifted to 32°C, and the viable cell number was quantitated following incubation for the number of days indicated. Viability is expressed as a percentage of the number of total viable cells per plate relative to that at day 0. The E1A and H-*ras* transformant (E1A+ras) lacks *ts* p53 and served as control for continued proliferation.

significantly restored (Fig. 9 and Table 2). In contrast, cells infected by the control virus (PAC3) lacking both E1A and E1B remained growth arrested under the same conditions (Fig. 9 and Table 2). Therefore, E1A expression in the presence of H-*ras* was able to overcome p53-mediated growth arrest and permit continued DNA synthesis. This effect was more pronounced in the cell line which expressed less p53 and more H-*ras* (cell line 3B) than the cell line that expressed more p53 and less H-*ras* (cell line 8D) (Fig. 7 and 9 and Table 2), suggesting that the levels of wild-type p53 influence the targets which E1A subverted to overcome growth arrest.

DISCUSSION

This study suggests that oncogenically activated H-*ras* can rescue cells from p53-dependent apoptosis in response to adenovirus E1A expression. Derivatives of p53A, an E1A-plus-*ts* mutant p53(val 135) transformant, which express high levels of H-*ras* (Ras1, Ras2, Ras4, and Ras5) retain viability at the temperature permissive for wild-type p53, whereas those which

TABLE 2. E1A and activated H-*ras* cooperation to relieve p53-mediated suppression of DNA synthesis

Day[a]	% of cells from indicated cell line incorporating BrdU[b]		
	E1A+*ras*	3B	8D
0	61.9	37.3	49.4
1	42.8	12.2	8.8
2	37.5	8.9	9.7
3	33.7	7.9	5.7
4	32.7	12.4	7.4
6	32.2	26.9, 74.6[c]	23.7, 45.7[c]

[a] As described in Table 1, footnote *a*, cells were propagated at 38.5°C and then were shifted to 32°C to assay their ability to incorporate BrdU.

[b] During the 4-h labeling period. Numbers for days 0, 4, and 6 are presented graphically in Fig. 9. Cell lines are described in the legend to Fig. 9. The E1A+*ras* cell line was uninfected by adenovirus in this assay.

[c] Both the 3B and 8D cell lines were infected at day 4 at 32°C with E1A-lacking (Pac3) (first value) or E1A-expressing (12SE1B$^-$) (second value) adenoviruses. Infected cells were assayed for BrdU incorporation at 48 h postinfection (day 6 at 32°C).

express no or low levels of activated H-*ras* dramatically lose viability by apoptosis, indicating that suppression of cell death is H-*ras* specific.

Activated H-*ras* also possesses growth-stimulatory activity and can cooperate with E1A to override p53-dependent growth suppression, which thereby permits cell proliferation. Thus, H-*ras* may exert two activities to maintain viability, one to suppress apoptosis and another to permit cell proliferation in the presence of wild-type p53. The similar rates of DNA synthesis in Ras1, Ras2, and Ras5 cell lines as well as the 4P control line suggest that they proliferate equally well at the permissive temperature. The variation in the viability was most likely due to a difference in the rate of cell death. The significant reduction in the rate of cell death in the cell lines which expressed high levels of H-*ras* suggests that H-*ras* rescues E1A-induced apoptosis by modulating the rate of cell death more than the rate of cell proliferation. Coexpression of H-*ras* abolishes all signs of apoptosis and permits focus formation by E1A in BRK transformation assays. This is consistent with the finding of a reduced rate of cell death in the H-*ras*-expressing p53A derivatives. Furthermore, promoting cell proliferation frequently is associated with accelerated cell death (as E1A and E2F act) (38, 42, 44, 51) and not maintenance of cell viability. In this setting, suppression of cell death may play a more important role than does enhancing cell replication in countering cell loss through apoptosis.

The failure of H-*ras* to completely suppress apoptosis in most of the p53A derivatives at the permissive temperature may be due to high levels of ectopically expressed murine mutant p53(val 135). BRK cells transformed by E1A plus H-*ras* in which only endogenous rat p53 is expressed show no indication of apoptosis (data not shown). However, the Ras2 cell line, in which apoptosis appeared to be completely prevented at the permissive temperature, has either sustained a mutation or expressed sufficient levels of H-*ras* to abrogate apoptosis.

Activated H-*ras* behaves similarly to its downstream target, an activated form of signal transducing protein Raf-1, which is able to suppress apoptosis and promote growth of interleukin-3-dependent myeloid cells in a Bcl-2-independent manner (6, 7). These observations suggest that the signal transduction in the Ras pathway plays a role in modulating apoptosis and controlling cell cycle progression. Cell death induced by withdrawal of growth factors or mitogens (49; reviewed in refer-

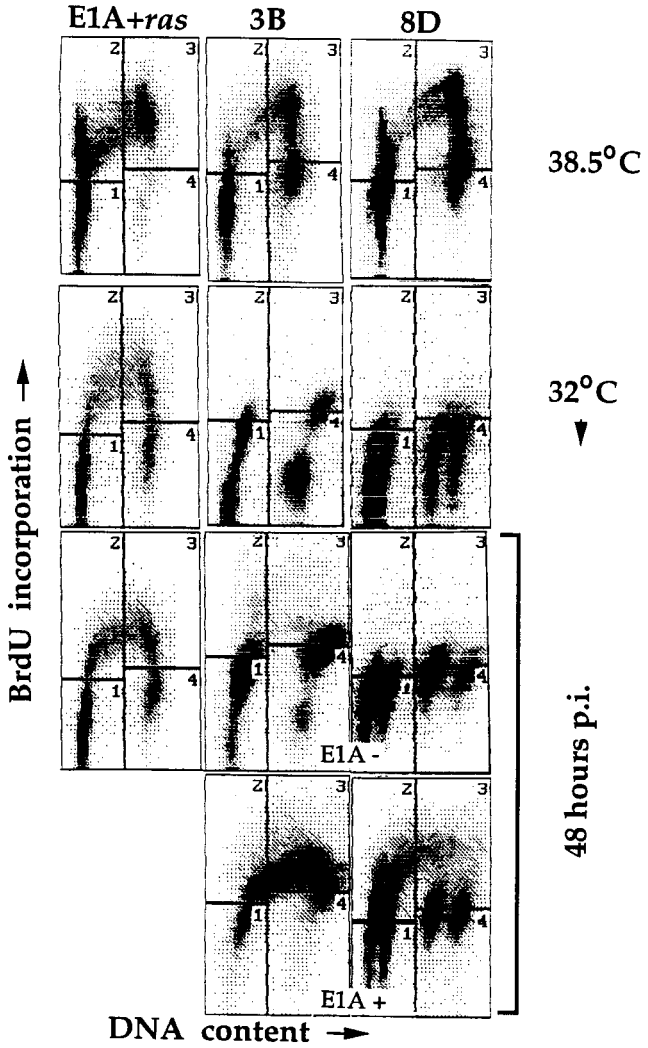

FIG. 9. E1A expression overcomes p53-dependent growth arrest. An E1A-plus-H-*ras*-transformed BRK cell line (E1A+*ras*) and the H-*ras*-plus-*ts* p53 BRK transformants (3B and 8D) were assayed for BrdU incorporation at 38.5°C or after incubation for 4 days at 32°C. Growth arrest was maintained for 4 days, at which time cells were infected by the E1A-expressing (E1A+) or E1A-lacking control (E1A−) adenoviruses. At 48 h postinfection (p.i.), cells were subjected to a BrdU incorporation assay and cell cycle analysis as described in the legend to Fig. 5. The percentage of cells incorporating BrdU is summarized in Table 2. The splitting of the G_1 and G_2/M populations, particularly in the growth-arrested cells, was due to an artifact of DNA denaturation and was not observed in untreated nuclei (data not shown). The E1A-plus-H-*ras* transformant (E1A+*ras*) does not express any *ts* p53 and thus serves as a control for continued proliferation at both temperatures.

ences 6 and 10) could also be mediated by the same signal transduction pathway.

Cooperation between H-*ras* and mutant p53 in transformation indicates that H-*ras* alone possesses a growth-stimulatory activity sufficient to transform cells if the wild-type p53 function is abrogated. H-*ras*-plus-*ts* p53 transformants underwent growth arrest but not apoptosis at the temperature permissive for wild-type p53, suggesting that the growth-stimulatory function of H-*ras* is insufficient to overcome cytostasis imposed by wild-type p53. However, addition of E1A overcame the G_1 arrest (and most likely G_2 arrest, although this was not examined directly) at which p53-mediated growth arrest normally takes place (29, 30). In contrast, E1B 19K or Bcl-2 protein-

expressing p53A derivatives underwent growth arrest predominantly in G_2/M (Fig. 5), suggesting that E1A expression was sufficient to override the G_1 but not the G_2/M checkpoint in these cells. Although either E1A or H-*ras* was capable of promoting cell proliferation, E1A was unable to overcome apoptosis mediated by p53 whereas H-*ras* was unable to relieve the growth suppression induced by p53. Cooperation between E1A and H-*ras*, however, permitted both apoptosis and growth arrest to be overcome. Thus, E1A and H-*ras* may modulate cell cycle progression in two distinct and complementary ways. In addition, E1A cooperates with mutant p53 to transform BRK cells more efficiently than H-*ras* and mutant p53 (Fig. 6), suggesting that E1A harbors more-potent growth-stimulatory activity than does H-*ras*. Thus, in the E1A-plus-mutant p53 transformants, wild-type p53 fails to suppress E1A-dependent growth stimulation and may thereby trigger cell death.

Unlike the E1B 19K and Bcl-2 protein-expressing counterparts which completely escaped from cell death but underwent growth arrest (4, 8, 34), the H-*ras*-expressing p53A derivatives were still partially susceptible to apoptosis, which might result from continued proliferation. In the presence of high levels of wild-type p53, continued cell cycle progression may be detrimental to cells and indirectly lead to cell death due to the presence of the mutually incompatible signals resulting from simultaneous growth stimulation and suppression. Other evidence suggests that cycling cells are more susceptible to cell death than growth-arrested cells (1, 7, 53). The E1B 19K or Bcl-2 protein-expressing p53A derivatives and the H-*ras*-plus-*ts* mutant p53-transformed BRK cells underwent growth arrest and were able to completely escape apoptosis at the temperature permissive for wild-type p53. This suggests that exit from the cell cycle could prevent cell death. Since induction of apoptosis by E1A cosegregates with induction of DNA synthesis (41), unscheduled DNA synthesis may lead to p53-dependent cell death. Furthermore, the Ras-FD cell line continued DNA synthesis and failed to growth arrest prior to undergoing apoptosis (data not shown), suggesting that growth arrest is not a prerequisite for apoptosis.

Finally, H-*ras* and the E1B 19K or Bcl-2 protein apparently modulate strikingly different pathways to overcome cell death and regulate cell cycle progression. In contrast to H-*ras*, the E1B 19K protein fails to cooperate with mutant p53 to transform BRK cells (8), suggesting that the E1B 19K protein does not possess growth-stimulatory activity whereas E1A and H-*ras* do. Both the E1B 19K and Bcl-2 proteins function similarly in the inhibition of cell death induced by not only E1A and p53 but also tumor necrosis factor alpha and Fas antigen (5), indicating that they may act on a common target(s) to inhibit apoptosis. Similarly, in the presence of E1A, E1B (55K and 19K proteins) expression prevents the p53-dependent apoptosis upon anticancer drug treatment or serum starvation whereas H-*ras* fails (25, 26). Furthermore, H-*ras* synergizes with the E1B 19K protein in cooperation with E1A to transform BRK cells (40), suggesting that H-*ras* and the E1B 19K protein act on different pathways to suppress cell death induced by E1A and thus exert an additive effect on transformation. Taken together, cooperation between oncogenes in transformation may be achieved by providing sufficient and complementary growth-stimulatory signals which result in rescuing cell death (E1A plus H-*ras*). Alternatively, providing a signal to stimulate cell proliferation in conjunction with inhibition of apoptosis (E1A plus the E1B 19K or Bcl-2 protein) or stimulating cell growth plus abrogation of wild-type p53 function (E1A plus mutant p53 or the E1B 55K protein) also permits transformation (4, 8, 32).

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