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Modulation of p53-Mediated Transcriptional Repression and Apoptosis by the Adenovirus E1B 19K Protein

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BRK cell lines that stably express adenovirus E1A and a murine temperature-sensitive p53 undergo apoptosis when p53 assumes the wild-type conformation. Expression of the E1B 19,000-molecular-weight (19K) protein rescues cells from this p53-mediated apoptosis and diverts cells to a growth-arrested state. As p53 likely functions as a tumor suppressor by regulating transcription, the ability of the E1B 19K protein to regulate p53-mediated transactivation and transcriptional repression was investigated. In promoter-reporter assays the E1B 19K did not block p53-mediated transactivation but did alleviate p53-mediated transcriptional repression. E1B 19K expression permitted efficient transcriptional activation of the p21/WAF-1/cip-1 mRNA by p53, consistent with maintenance of the growth arrest function of p53. The E1B 19K protein is thereby unique among DNA virus-transforming proteins that target p53 for inactivation in that it selectively modulates the transcriptional properties of p53. The E1B 19K protein also rescued cells from apoptosis induced by inhibitors of transcription and protein synthesis. This suggests that cell death may result from the inhibition of expression of survival factors which function to maintain cell viability. p53 may induce apoptosis through generalized transcriptional repression. In turn, the E1B 19K protein may prevent p53-mediated apoptosis by alleviating p53-mediated transcriptional repression.

Expression of the adenovirus E1A proteins induces DNA synthesis in infected cells, and the cells respond by undergoing apoptosis (39, 49, 53, 55, 56), manifested by degradation of host cell DNA into nucleosomal-size fragments (*deg* phenotype) and enhanced cytopathic effect (*cyt* phenotype) (35, 46, 50, 53, 57). Apoptosis induced by E1A is p53 dependent and is blocked by events that specifically interfere with p53 function (6, 8, 29, 39). Expression of the adenovirus E1B 19,000-molecular-weight (19K) protein will specifically block E1A-triggered, p53-dependent apoptosis, which most likely contributes to the role the E1B 19K protein plays in oncogenesis (8).

The p53 protein is a nuclear phosphoprotein and a transcription factor capable of both repressing and transactivating transcription (12, 16, 45, 62). p53 has also been shown to induce apoptosis or growth arrest depending on the physiological circumstances (9, 13, 17, 22, 61). Both the transcriptional and the functional properties of the p53 tumor suppressor protein are thwarted by a number of different cellular and viral proteins which elicit neoplastic growth. The adenovirus E1B 55K protein complexes with p53 and inhibits its activity as a transcriptional activator (59), the papillomavirus E6 protein binds to p53 and initiates its degradation via the ubiquitin pathway (43), and the cellular protein MDM2 binds specifically to the transactivation domain of p53, thereby directly subverting its transactivation properties (34). In each of these instances the transcriptional and functional properties of the p53 protein are compromised. Therefore, a great deal of effort has been focused towards identifying the cellular genes that are either positively or negatively regulated by p53, as this may form the mechanistic basis for the ability of p53 to control growth arrest and apoptosis and to prevent the progression of neoplastic transformation.

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The E1B 19K protein will block p53-dependent apoptosis (8), as well as apoptosis induced by tumor necrosis factor alpha and by anti-Fas antibody (7, 56). In an effort to understand how the E1B 19K protein is able to overcome p53-mediated apoptosis, we analyzed the ability of the E1B 19K protein to modulate the transcriptional activity of p53. We report here that the E1B 19K protein did not inhibit p53-mediated transactivation but did alleviate p53-mediated transcriptional repression in promoter-reporter assays and that these effects cosegregate with the transforming ability of the E1B 19K protein. Northern (RNA) blot analysis of cell lines rescued from p53-mediated apoptosis by the E1B 19K protein displayed as efficient an induction of the p53-inducible gene *p21/WAF-1/cip-1* as that in control cell lines that do undergo apoptosis, consistent with preservation of the growth arrest function of p53. Interestingly, the E1B 19K protein was able to overcome apoptosis induced by treatment of cells with inhibitors of transcription or protein synthesis. These results suggest that p53 may induce apoptosis not through the activation of transcription but rather through transcriptional repression and that the E1B 19K protein may overcome p53-mediated apoptosis by alleviating p53-mediated transcriptional repression.

MATERIALS AND METHODS

Cell lines. The HeLa and Saos-2 cell lines were grown in monolayer culture in Dulbecco modified Eagle medium with 10% fetal bovine serum. Construction of the p53An1, p5319K1, p5319K2, and p53ABcl-2 4B baby rat kidney (BRK) cell lines and their use in viability assays have been previously described (6, 8). Briefly, all three cell lines were derived from primary BRK cells that were transfected with genes encoding adenovirus E1A and a murine temperature-sensitive p53 (*ts*p53 val135). The p53An1, p5319K1, and p5319K2 cell lines express the E1A proteins and the *ts*p53 protein that is predominantly in the mutant configuration at the restrictive temperature of 38.5°C and predominantly in the wild-type configuration at the permissive temperature of 32°C (33). The p53An1 cell line is transformed and proliferates when p53 is in the mutant configuration yet undergoes apoptosis rapidly upon incubation at the permissive temperature for wild-type p53 (wtp53) (8). The p5319K1 and p5319K2 cell lines also stably express the adenovirus E1B 19K protein and are protected from p53-mediated apoptosis at the permissive temperature (8). The KH215 cell line

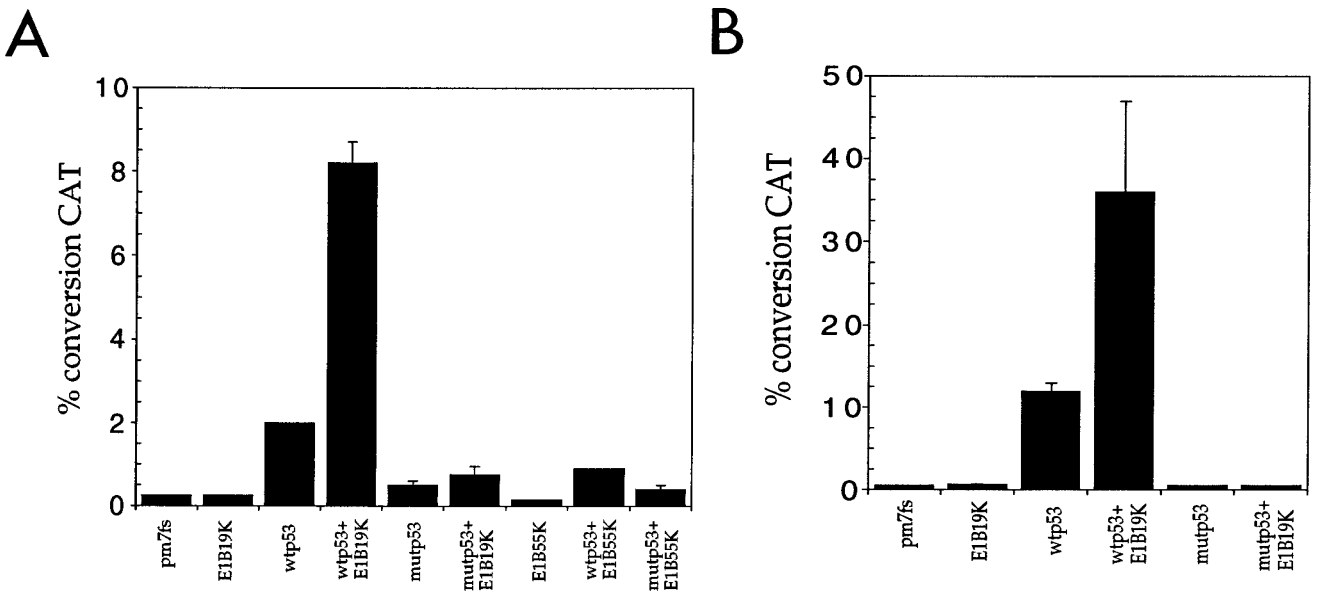

FIG. 1. The E1B 19K protein enhances p53-mediated transactivation. HeLa (A) or Saos-2 (B) cells growing in log phase were transfected with 5 μg of p50-2CAT, 5 μg of each experimental plasmid, and carrier DNA for a total of 20 μg of DNA per transfection. The p50-2CAT plasmid contains two copies of the p53-binding region from the muscle creatine kinase promoter joined in tandem and placed upstream of the CAT gene (62). The pm7fs plasmid is identical to the pCMV19K plasmid with the exception that it contains a frameshift mutation at amino acid 7 and does not express a functional E1B 19K protein (56). Cells were harvested 48 h posttransfection. CAT assays were performed as described in Materials and Methods. CAT activity was quantitated with the Image-Quant program on a PhosphorImager. The data represent the averaged results from transfections that were performed in duplicate.

was derived from primary BRK cells transformed with adenovirus E1A and a murine non-temperature-sensitive mutant p53, KH215 (47). This cell line is not susceptible to p53-mediated apoptosis at either the restrictive or the permissive temperature.

Cell viability assays. The BRK cell lines were plated at a density of 5×10^5 cells per 6-cm plate at 38.5°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 following incubation for increasing lengths of time. Where indicated, cells were plated at a density of 1.5×10^6/6-cm plate at 38.5°C. Twenty-four hours postplating, cycloheximide (30 μg/ml), anisomycin (100 μM), or actinomycin D (2.5 μg/ml) was added to the culture media, and cells were allowed to incubate for an additional 24 h at 38.5°C. The number of viable cells present was subsequently determined by trypan blue exclusion and represented as a percentage of the number of viable cells present at the time the drug was added.

Plasmids. Plasmid expression vectors directing expression of the E1B 19K protein (pCMV19K) and the E1B 55K protein (pCMV55K) from the constitutive cytomegalovirus (CMV) promoter have been previously described (51, 52, 56). The pm7fs plasmid is identical to the pCMV19K plasmid with the exception that it contains a frameshift mutation in the E1B 19K coding region at amino acid 7 and does not express a functional E1B 19K protein (56). Each of the pm7, pm28, pm44R, pm51, pm55, pm87, pm102, pm113, and pm123 expression constructs expresses the E1B 19K protein containing a specific missense mutation within the E1B 19K coding region, and these constructs have been described elsewhere (7, 56). Expression constructs pC53-SN_3 and pC53-C_x22AN_3 express the human wtp53 and mutant p53 (mutp53), respectively (1, 21). For promoter-reporter assays, the p50-2CAT plasmid contains two copies of the p53-binding region from the muscle creatine kinase promoter joined in tandem and placed upstream of the chloramphenicol acetyltransferase (CAT) gene (62). The pTATACAT and pInrCAT plasmids both contain the simian virus 40 21-bp repeats and either the adenovirus major late promoter TATA box or the terminal transferase gene initiator element, respectively, upstream of the CAT gene (30).

DNA transfections and CAT assays. Cells growing in log phase were plated at 50% confluency 24 h before transfection. DNA, prepared as a calcium phosphate precipitate (adjusted to 20 μg total with single-stranded salmon sperm DNA), was added to the cells which were glycerol shocked 4 h later as previously described (56).

Cells were harvested 48 h posttransfection, washed with phosphate-buffered saline, resuspended in 0.25 M Tris (pH 8.0), and processed for CAT assays as previously described (56). Protein estimations were performed with the Bio-Rad protein assay reagent. Equal amounts of protein extracts were used for the CAT reactions, which consisted of protein extract, 1.2 mM acetyl coenzyme A (Sigma), and 0.1 μCi of ^{14}C-chloramphenicol (Amersham). Reactions containing extracts from cells transfected with the p50-2CAT reporter construct were incubated at 37°C for 1.5 h, and reactions containing extracts from cells transfected with either the pInrCAT or the pTATACAT reporter construct were incubated at 37°C for 4 h. Reactions were terminated with the addition of 1 ml of ethyl acetate and processed for thin-layer chromatography as previously described (56). Thin-layer chromatography plates were quantitated by using the Image-Quant program on a PhosphorImager (Molecular Dynamics). The histograms with accompanying error bars in Fig. 1 represent the averaged results from transfections that were performed in duplicate. Each set of histograms in Fig. 2 through 5 represents the averaged results of two identical experiments.

Northern blot analysis. Cytoplasmic RNA was isolated as previously described (54). Ten micrograms of cytoplasmic RNA was denatured and size fractionated on a 1% agarose gel containing formaldehyde. The gel was blotted onto nitrocellulose, and the blot was subsequently prehybridized, hybridized, and washed as previously described (54). The probes used for Northern blot analysis were a cDNA corresponding to mouse *p21/WAF-1/cip-1* (generously provided by G. Hannon, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.) and a cDNA corresponding to mouse β-actin (generously provided by B. Antoni, University of Medicine and Dentistry of New Jersey, Piscataway). Northern blots were quantitated by using the Image-Quant program on a PhosphorImager (Molecular Dynamics).

RESULTS

The E1B 19K protein enhances wild-type p53-mediated transactivation in promoter-reporter assays. wtp53 has been shown to transactivate genes that harbor a p53-binding consensus sequence (11, 12, 44, 62). Since the E1B 19K protein overcomes p53-mediated apoptosis, it was of interest to determine the ability of the E1B 19K protein to modulate the transactivation potential of wtp53. The p50-2CAT plasmid was used for this purpose, as it contains two copies of a p53-responsive element upstream of the CAT gene and is transactivated effectively by wtp53 and not by mutp53 (62). HeLa cells were used in transfection assays because they lack detectable levels of endogenous p53 (3, 40).

Transfection of p50-2CAT alone or with the E1B 19K non-expressing control plasmid pm7fs (56) produced a basal level of CAT expression that was not altered by coexpression of the E1B 19K protein (Fig. 1A). Expression of wtp53 with p50-

2CAT dramatically stimulated CAT expression above basal p50-2CAT expression (Fig. 1A), as previously reported (62). Surprisingly, coexpression of the E1B 19K protein with wtp53 did not inhibit but rather augmented the transactivation potential of wtp53 four- to fivefold over and above the transactivation observed with wtp53 alone (Fig. 1A). Expression of mutp53, which lacks the ability to activate transcription from constructs containing strong p53-binding sites (12, 14, 22, 23, 62), had no significant effect on the background levels of CAT activity (Fig. 1A). Coexpression of the E1B 19K protein with mutp53 did not significantly affect the levels of CAT activity observed with expression of mutp53 alone (Fig. 1A). This indicates that the synergistic transactivation of E1B 19K and wtp53 is specific for wtp53 and is not due to generalized nonspecific increased levels of CAT expression.

The E1B 55K protein, which enters into a complex with p53 and inhibits both transactivation and repression by p53 (59), inhibited wtp53-mediated transactivation without affecting the level of CAT activity from expression of mutp53 (Fig. 1A). Thus, the E1B 19K protein significantly augmented p53-mediated transactivation in contrast to the E1B 55K protein, which abrogated p53-mediated transactivation.

There is the inherent possibility that the augmentation of p53-mediated transactivation by the E1B 19K protein that we observed is merely a reflection of increased cell viability from inhibition of p53-mediated apoptosis. To address this possibility we measured the capacity of the E1B 19K protein to enhance p53-mediated transactivation in the p53-null cell line, Saos-2, which is not vulnerable to p53-mediated apoptosis but rather arrests in the G_1 phase of the cell cycle in response to expression of exogenously introduced wtp53 (9, 18). E1B 19K protein expression did not alter the basal level of CAT activity from p50-2CAT in Saos-2 cells (Fig. 1B). Expression of wtp53 generated a level of CAT activity that was approximately 12-fold higher than the background level of CAT activity (Fig. 1B). Coexpression of the E1B 19K protein with wtp53 augmented the transactivation potential of wtp53 three- to fourfold over that for wtp53 alone (Fig. 1B). The CAT activity from expression of mutp53 was comparable to background levels, and this was unaltered by coexpression of the E1B 19K protein (Fig. 1B). This indicates that the synergistic effect that the E1B 19K protein has on p53-mediated transactivation in Saos-2 cells is specific for wtp53 and that the ability of the E1B 19K protein to augment p53-mediated transactivation is not simply a reflection of the ability of the E1B 19K protein to overcome p53-mediated apoptosis.

The E1B 19K protein alleviates p53-mediated transcriptional repression in promoter-reporter assays. The p53 protein has been shown to repress the expression of genes that do not harbor a p53-binding consensus sequence both in vivo and in vitro (16, 30, 38, 42, 44, 45). Therefore, it was of interest to determine the ability of the E1B 19K protein to modulate the transcriptional repression potential of p53. For this series of assays the pInrCAT and pTATACAT reporter plasmids were used; both of these plasmids contain the simian virus 40 21-bp repeats and either the terminal transferase gene initiator element or the adenovirus major late promoter TATA box, respectively, upstream of the CAT gene (30). The use of CAT reporter plasmids containing minimal promoter elements without any upstream enhancer elements simplifies the analysis of potential modulation of p53-mediated transcriptional repression by the E1B 19K protein.

The basal level of CAT expression observed from pInrCAT was not affected by expression of the E1B 19K protein but was repressed approximately eightfold by wtp53 in HeLa cells (Fig. 2A). Others have reported that the pInrCAT reporter is not repressed by wtp53, although these experiments were performed in cell types other than HeLa (30). The E1B 19K protein completely derepressed p53-mediated repression of pInrCAT to basal levels even at the lowest concentration of the E1B 19K plasmid transfected (Fig. 2A). The level of CAT activity observed upon expression of mutp53 was approximately twofold higher than background levels (Fig. 2A). Nevertheless, the E1B 19K protein did not affect this level of CAT activity, indicating that the ability of the E1B 19K protein to alleviate p53-mediated transcriptional repression is specific to wtp53 (Fig. 2A).

An identical experiment was then performed with the pTATACAT reporter plasmid. Expression of the E1B 19K protein appeared to have a slight inhibitory effect on the background levels of CAT activity observed (Fig. 2B). This result, however, was not generally reproducible. wtp53 repressed the background levels of CAT activity approximately sixfold even at the lowest concentration of wtp53 plasmid DNA transfected (Fig. 2B), as has been previously reported (30). Coexpression of the E1B 19K protein completely alleviated p53-mediated transcriptional repression of pTATACAT even at the lowest concentration of E1B 19K plasmid DNA transfected (Fig. 2B). mutp53 appeared to increase CAT activity from pTATACAT approximately twofold over basal levels; however, this effect was unaltered by coexpression of the E1B 19K protein (Fig. 2B). This indicates that the ability of the E1B 19K protein to alleviate p53-mediated repression of pTATACAT in HeLa cells is specific for wtp53. The fact that mutp53 reproducibly transactivated both pInrCAT and pTATA and that the E1B 19K protein did not upregulate this effect argues against the possibility that the E1B 19K protein augments wtp53-mediated transactivation of p50-2CAT by simply increasing plasmid stability.

To address the possibility that the alleviation of p53-mediated transcriptional repression by the E1B 19K protein is a secondary effect of inhibition of p53-mediated apoptosis, we assayed the ability of the E1B 19K protein to alleviate p53-mediated transcriptional repression of pInrCAT in Saos-2 cells. Expression of the E1B 19K protein caused a slight increase in CAT activity over background levels, but expression of wtp53 repressed the background levels of CAT activity approximately sixfold (Fig. 3). Coexpression of the E1B 19K protein with wtp53 completely alleviated p53-mediated transcriptional repression (Fig. 3). Similar results were obtained when we used the pTATACAT reporter plasmid (data not shown). Thus, the E1B 19K protein can alleviate p53-mediated transcriptional repression of pInrCAT and pTATACAT in Saos-2 cells as well as in HeLa cells. This suggests that p53-mediated transcriptional repression of pInrCAT and pTATA CAT is not merely a result of p53-induced apoptosis and that the ability of the E1B 19K protein to alleviate p53-mediated transcriptional repression is not simply a reflection of the E1B 19K protein overcoming p53-mediated apoptosis.

At this point we can conclude that the E1B 19K protein both enhances p53-mediated transactivation and alleviates p53-mediated transcriptional repression in promoter-reporter assays. In turn, these results do not reflect generalized increased cell viability due to inhibition of apoptosis by the E1B 19K protein, nor can they be attributed to the ability of the E1B 19K protein to increase plasmid stability.

Modulation of the transcriptional activity of p53 by the E1B 19K protein cosegregates with transformation and inhibition of apoptosis. Comparison of the amino acid sequence of the E1B 19K protein from 12 different serotypes of adenovirus has indicated that the central region of the protein (amino acids 81 to 113) is highly conserved, the amino terminus (amino acids 1

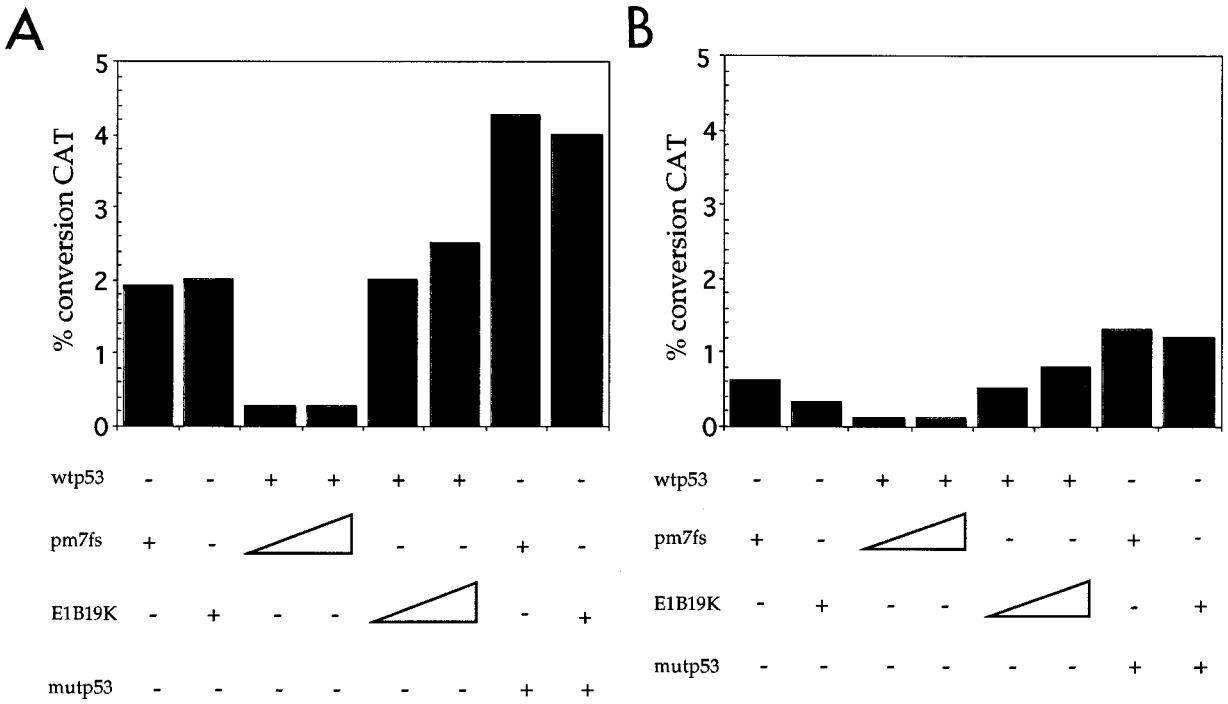

FIG. 2. The E1B 19K protein alleviates p53-mediated transcriptional repression of pInrCAT and pTATACAT in HeLa cells. HeLa cells were transfected with 10 μg of either the pInrCAT (A) or the pTATACAT (B) reporter plasmid and 1 μg of either the E1B 19K, the wtp53, or the mutp53 expression vector. The pInrCAT and pTATACAT plasmids both contain the simian virus 40 21-bp repeats and either the terminal transferase gene initiator element or the adenovirus major late promoter TATA box, respectively, upstream of the CAT gene (30). One and two micrograms of the pm7fs and CMV19K expression vectors were used for titration analysis. Cells were harvested 48 h posttransfection. CAT assays were performed and quantitated as described in Materials and Methods. The data represent the averaged results from two identical experiments.

to 80) is moderately conserved, and the carboxyl terminus (amino acids 117 to 175) is poorly conserved (7, 56). This suggested that the central region and perhaps the amino terminus of the E1B 19K protein are important for its function. Therefore, a series of E1B 19K mutants were constructed to generate missense mutations by single amino acid substitutions at specific conserved residues throughout the E1B 19K protein but predominantly within the highly conserved central portion of the protein. Only those E1B 19K mutants that were shown to express a stable protein will be discussed. The level of expression of each E1B 19K mutant protein was determined by transiently expressing the corresponding plasmid construct in HeLa cells and performing Western blot (immunoblot) analysis 48 h posttransfection (7). Therefore, the assay conditions for Western blot analysis were identical to those under which the promoter-reporter assays were performed.

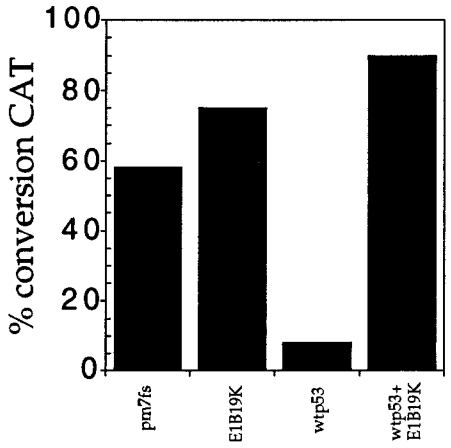

FIG. 3. The E1B 19K protein alleviates p53-mediated transcriptional repression of pInrCAT in Saos-2 cells. Saos-2 cells were transfected with 10 μg of pInrCAT and 1 μg of either the pm7fs, the E1B 19K, or the wtp53 expression vector. Cells were harvested 48 h posttransfection. CAT assays were performed and quantitated as described in Materials and Methods. The data represent the averaged results from two identical experiments.

Each E1B 19K mutant contains a single amino acid substitution either within the moderately conserved amino terminus (pm7, pm28, pm44R, pm51, and pm55), the highly conserved central portion of the protein (pm87, pm102, and pm113), or the poorly conserved carboxyl terminus of the protein (pm123). Characterization of these E1B 19K mutants for transforming activity, inhibition of apoptosis by tumor necrosis factor alpha and anti-Fas antibody, and level of protein expression has been published elsewhere (7, 56). The results from this characterization demonstrated that the transformation ability of the E1B 19K protein cosegregates with its ability to overcome apoptosis induced by tumor necrosis factor alpha and anti-Fas antibody.

The transforming ability of the E1B 19K protein may, in turn, be correlated with its ability to modulate p53-mediated transcription. The E1B 19K mutants described above, which differ in their individual transforming abilities (7, 56), were therefore assayed for their ability to enhance p53-mediated transactivation of p50-2CAT in HeLa cells. The percent conversion of CAT activity from expression of wtp53 alone was arbitrarily set at a value of 1.0. To determine the fold increase in transactivation, the percent conversion of CAT activity from coexpression of wtp53 with either the wild-type E1B 19K protein or each of the E1B 19K mutants was quantitated and

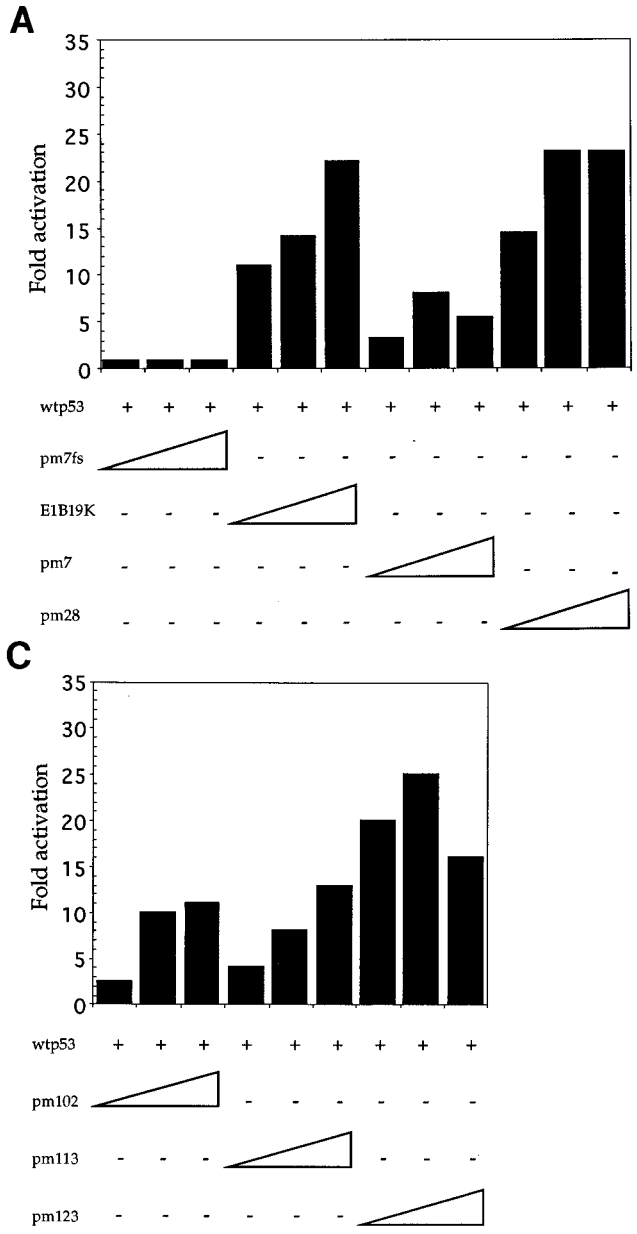

FIG. 4. Assay of the ability of different E1B 19K mutants to enhance p53-mediated transactivation of p50-2CAT in HeLa cells. HeLa cells were cotransfected with 5 μg of both p50-2CAT and the wtp53 expression vector. One, 2.5, and 5 μg of each of the E1B 19K mutant expression vectors were used for titration analysis. Cells were harvested 48 h posttransfection. CAT assays were performed and quantitated as described in Materials and Methods. The fold activation in CAT activity from expression of wtp53 alone was arbitrarily set at 1.0. The data represent the averaged results from two identical experiments.

divided by the percent conversion of CAT activity from expression of wtp53 alone. Increasing amounts of DNA encoding the wild-type E1B 19K and each of the E1B 19K mutants were used to determine the maximum proportional increase in p53-mediated transactivation obtainable over the range of DNA concentrations.

The wild-type E1B 19K protein enhanced p53-mediated transactivation of p50-2CAT 23-fold, and the pm28, pm44R, pm55, and pm123 E1B 19K mutants were also able to enhance p53-mediated transactivation to this extent (Fig. 4). The remaining mutants possessed impaired activity in the p53-dependent transactivation assay. The pm7 E1B 19K mutant enhanced p53-mediated transactivation only eightfold, whereas the pm51 and pm87 E1B 19K mutants enhanced p53-mediated transactivation only twofold and fourfold, respectively (Fig. 4A and B). The pm102 E1B 19K mutant was also somewhat defective in that it enhanced p53-mediated transactivation a maximum of 10-fold (Fig. 4C). The pm113 E1B 19K mutant enhanced p53-mediated transactivation a maximum of 13-fold (Fig. 4C). However, it appears that this percent increase in transactivation would have been more extreme had more pm113 expression construct been transfected, as there was a proportional increase in the level of transactivation throughout the titrating range of pm113 (Fig. 4C). Thus, the wild-type E1B 19K protein and those E1B 19K mutants which retain full transformation function and the ability to overcome p53-mediated apoptosis enhance p53-mediated transactivation to the same extent. However, those E1B 19K mutants that have both an impaired transformation ability and an impaired ability to overcome p53-mediated apoptosis are defective in their ability to enhance p53-mediated transactivation.

The E1B 19K mutants were also assayed for their ability to alleviate p53-mediated transcriptional repression of pInrCAT in HeLa cells. One hundred percent derepression represented the ability to alleviate p53-mediated transcriptional repression of pInrCAT to background levels. The wild-type E1B 19K protein and the pm7, pm28, pm44R, pm55, and pm123 E1B 19K mutants completely alleviated p53-mediated transcriptional repression of pInrCAT (Fig. 5). The pm51, pm87, pm102, and pm113 E1B 19K mutants each alleviated p53-mediated repression of pInrCAT to different extents but poorly compared with the wild-type E1B 19K protein (Fig. 5).

A comparison of the ability of the E1B 19K mutants to modulate p53-mediated transcription suggests a linkage between the ability of the E1B 19K protein to modulate p53-mediated transcription and the transformation potential of the E1B 19K protein (Table 1). The numbers in Table 1 pertaining to the percent increase in transactivation over wtp53 alone are derived from the data in Fig. 4 and correspond to the maximum fold increase in transactivation that was obtained at saturating levels of CAT activity. The maximum fold increase in transactivation obtained by wild-type E1B 19K was assigned a value of 100%. The activity of mutant E1B 19K proteins is

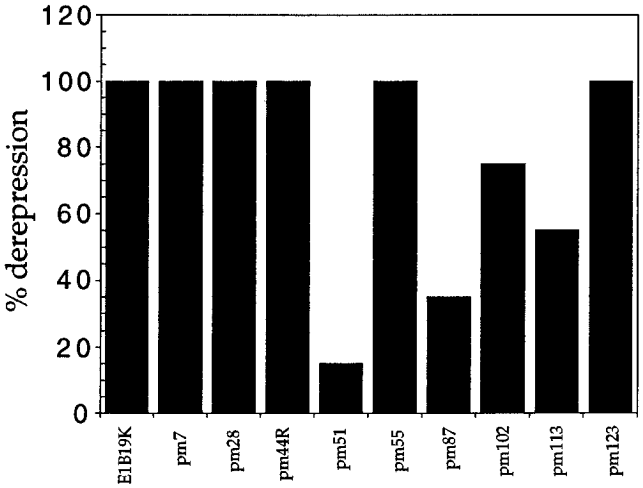

FIG. 5. Assay of the ability of different E1B 19K mutants to alleviate p53-mediated transcriptional repression of pInrCAT in HeLa cells. HeLa cells were cotransfected with 10 μg of pInrCAT and 1 μg of both the wtp53 and the E1B 19K mutant expression constructs. One hundred percent derepression represents the ability to alleviate p53-mediated repression of pInrCAT to background levels. The data represent the averaged results from two identical experiments.

expressed as a percentage of wild-type E1B 19K activity. Values for percent derepression are taken from the data in Fig. 5. Those E1B 19K mutants that retained at least 40% of wild-type activity with respect to transformation are designated nondefective for transformation (7, 56).

There was a positive correlation between the transformation potential of the E1B 19K mutants and their ability to enhance the transactivation potential of p53. The wild-type E1B 19K protein and those E1B 19K mutants (pm28, pm44R, pm55, and pm123) which retained at least 40% of wild-type activity with respect to transformation enhanced p53-mediated transactivation to the same extent (Table 1). The pm7 and pm102 E1B 19K mutants were partially able to enhance p53-mediated transactivation, and pm7 retained partial transformation potential whereas pm102 was completely defective for transformation (Table 1). The pm113 E1B 19K mutant, which was negative for transformation, was interesting in that it enhanced p53-mediated transactivation proportionally to the amount of the pm113 expression construct that was transfected but still did not enhance p53-mediated transactivation to the same extent as those E1B 19K mutants that were positive for transformation (55 versus 100%) (Table 1).

TABLE 1. Comparison of the transformation abilities of E1B 19K mutants with their effects on p53-mediated transactivation and repression

Mutant	% Transactivation[a]	% Derepression[b]	% Transformation ability[c]	Protein level[d]
pm7	35	100	30	++
pm28	100	100	70	++
pm44R	100	100	100	+++
pm51	10	15	10	+
pm55	100	100	40	++
pm87	20	35	10	+
pm102	45	75	5	++
pm113	55	55	10	+
pm123	100	100	100	+++

[a] From Fig. 4. For the wild-type E1B 19K protein the maximum fold increase in transactivation over wtp53 alone that was obtained at saturating levels of CAT activity was assigned a value of 100%. The maximum fold increase in p53-mediated transactivation by each of the E1B 19K mutants obtained at saturating CAT activity is represented as a percentage of wild-type E1B 19K activity.

[b] From Fig. 5.

[c] Relative to that of wild-type E1B 19K. The data are from reference 7.

[d] Determined by Western blot analysis of whole-cell extracts from transfected HeLa cells. Protein level for the wild type, ++. The data are from reference 7.

The ability of the E1B 19K mutants to alleviate p53-mediated transcriptional repression also correlated for the most part with their respective transformation abilities. The pm7 E1B 19K mutant, however, which retained only 30% of wild-type activity with respect to transformation, was able to completely alleviate p53-mediated transcriptional repression (Table 1). Thus, E1B 19K mutants with an impaired ability to modulate either p53-mediated transactivation or transcriptional repression were also defective in transformation and as inhibitors of apoptosis.

The E1B 19K protein overcomes p53-mediated apoptosis but not p53-mediated growth arrest. In light of the fact that the E1B 19K protein modulates the transcriptional properties of wtp53, it was of interest to determine how the E1B 19K protein affects the growth arrest and apoptotic properties of p53 as well. These experiments are especially relevant since E1A triggers p53-mediated apoptosis and the E1B 19K protein must overcome this effect in order to cooperate with E1A to fully transform primary rodent cells (8).

The p53An1, p5319K1, and p5319K2 cell lines were derived from the same parental line generated from primary BRK cells transformed by E1A and the murine *ts*p53 val135 (8). The p5319K1 and p5319K2 cell lines are independent clones that also stably express the E1B 19K protein, whereas p53An1 does not. All three cell lines are transformed and proliferate when p53 is in the mutant configuration at 38.5°C, whereas only p53An1 undergoes apoptosis when p53 is returned to the wild-type configuration at 32°C (8). The E1B 19K-expressing cell lines p5319K1 and p5319K2 were analyzed for the induction of apoptosis or growth arrest at the permissive temperature of 32°C. 4P, an E1A- plus E1B-transformed BRK cell line, which does not contain a temperature-sensitive p53 mutation, was used as a positive control for viability at the permissive temperature. The E1A- plus p53 val135-transformed line p53An1, which undergoes apoptosis at the permissive temperature (8), served as a positive control for induction of apoptosis.

The viable number of cells in both the p5319K1 and the p5319K2 cell lines remained constant with no signs of apoptosis up to 10 days following incubation at the permissive temperature (Fig. 6). By 24 hours at the permissive temperature, DNA synthesis, as measured by bromodeoxyuridine incorporation, was substantially inhibited and complete by 48 h (27). After 10 days at the permissive temperature both cell lines were shifted back up to the restrictive temperature, at which point growth resumed (Fig. 6), indicating that the growth arrest inflicted by wtp53 was reversible. The p5319K1 line continued to divide for at least two additional passages at the restrictive temperature (data not shown). The 4P control cell line continued to grow subsequent to its incubation at the permissive temperature as expected, whereas the p53AN1 cell line underwent complete p53-mediated apoptosis within 4 days subsequent to its incubation at the permissive temperature (Fig. 6). Thus, the E1B 19K protein overcomes p53-mediated apoptosis while preserving p53-mediated growth arrest.

The E1B 19K protein does not enhance the p53-mediated transactivation of *p21/WAF-1/cip-1*. *p21/WAF-1/cip-1* is a p53-inducible gene, the protein product of which effectively inhibits G_1-specific cyclin-dependent kinases (11, 19, 58). This, in turn, can lead to the inhibition of DNA synthesis and the suppres-

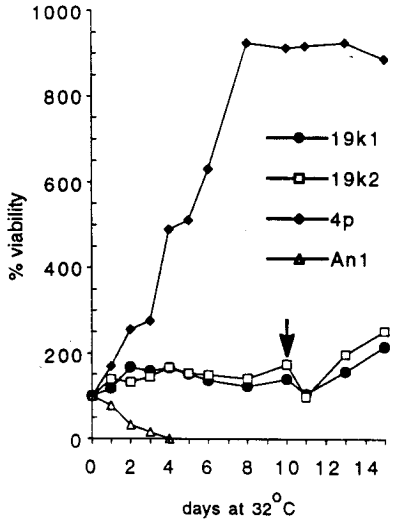

FIG. 6. Induction of long-term growth arrest in apoptosis-resistant cell lines by E1B 19K expression. Viability of E1B 19K-expressing E1A-plus-p53 val135 transformants at the permissive temperature for wtp53 was determined over an extended period of 14 days. Arrow, point at which the E1B 19K-producing lines p5319K1 and p5319K2 were returned to the restrictive temperature of 38.5°C. 4P, an E1A-plus-E1B transformant which does not contain a temperature-sensitive mutation, was used as a positive control for viability at the permissive temperature. The E1A-plus-p53 val135 neomycin-resistant transformed line p53An1, which undergoes apoptosis at the permissive temperature, served as a control for induction of apoptosis.

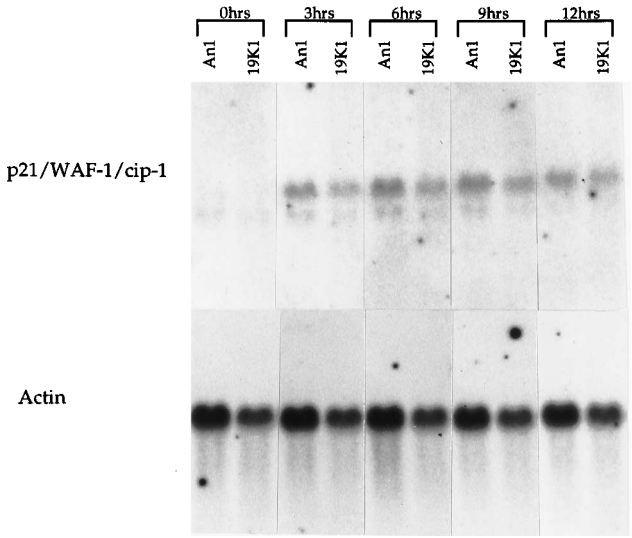

FIG. 7. The E1B 19K protein does not enhance the p53-mediated transactivation of p21/WAF-1/cip-1. The p53An1 and p5319K1 cell lines were incubated at the permissive temperature for 3, 6, 9, and 12 h. The 0-h time point represents cells growing in log phase at the restrictive temperature just prior to incubation at the permissive temperature. Cytoplasmic RNA was isolated at the indicated time points, fractionated on a formaldehyde-agarose gel, and blotted onto nitrocellulose. The blot was hybridized with a probe corresponding to mouse p21/WAF-1/cip-1 or mouse β-actin.

sion of tumor cell growth in culture (11, 19). In addition, the growth suppression induced by p21/WAF-1/cip-1 is reversible and is therefore believed to represent growth arrest and not apoptosis (19). Since the E1B 19K protein enhances p53-mediated transactivation in promoter-reporter assays, it seemed possible that the E1B 19K protein was implementing p53-mediated growth arrest in the BRK cell lines by enhancing the p53-mediated transactivation of p21/WAF-1/cip-1. To address this issue, RNA was extracted from the p53An1 and p5319K1 cell lines at various time points after the cells were shifted to the permissive temperature and Northern blot analysis for expression of p21/WAF-1/cip-1 was subsequently performed. The level of expression of p21/WAF-1/cip-1 was normalized against the level of expression of β-actin as a means of standardizing any sample variations in the levels of RNA.

Expression of p21/WAF-1/cip-1 was virtually undetectable in the p53An1 and p5319K1 cell lines grown at the restrictive temperature but was clearly inducible in both cell lines within 3 h subsequent to incubation at the permissive temperature (Fig. 7). The levels of expression of p21/WAF-1/cip-1 in the p53An1 and p5319K1 cell lines, however, did not differ significantly from each other at any time points (Fig. 7). We have also observed this to be the case with the other E1B 19K-expressing clone, p5319K2 (data not shown). These results suggest that enhanced p53-mediated transactivation by expression of the E1B 19K protein in promoter-reporter assays is not necessarily reflected in the transcriptional activation of cellular genes. Nonetheless, it appears that the E1B 19K protein permits p53-mediated transactivation of p21/WAF-1/cip-1 under conditions in which the E1B 19K protein implements p53-mediated growth arrest.

The E1B 19K protein overcomes apoptosis induced by mRNA and protein synthesis inhibitors. The ability of the E1B 19K protein to alleviate p53-mediated transcriptional repression but not transactivation suggests that p53-dependent apoptosis may be caused by transcriptional repression rather than transactivation. Evidence to support this possibility has been provided by Caelles et al. (5), who have shown that p53-dependent apoptosis can be induced in the presence of inhibitors of transcription and protein synthesis. The transcription inhibitor actinomycin D and the protein synthesis inhibitors cycloheximide and anisomycin have been shown to induce apoptosis, presumably by preventing the expression of cell survival factors (2, 31, 32). Therefore, we investigated whether the E1B 19K protein is capable of protecting cells from apoptosis induced by these reagents.

The p53An1 cell line undergoes apoptosis in the presence of inhibitors of transcription and protein synthesis at either the permissive or the restrictive temperature (as indicated by viability loss [Fig. 6]) and intranucleosomal DNA cleavage (data not shown). Therefore, these reagents probably induce apoptosis in the p53An1 cell line through a p53-independent mechanism. However, the *ts*p53 val135 protein translocates to the nucleus in the presence of cycloheximide (15), and whether the *ts*p53 val135 mutant is still in the mutant configuration and has the capacity to induce apoptosis once inside the nucleus is not known. Thus, it cannot be stated unequivocally that treatment of the p53An1 cell line with cycloheximide induces apoptosis in a p53-independent manner. The KH215 cell line was derived from BRK cells that had been transformed with E1A and a murine non-temperature-sensitive mutant p53 (KH215) (47) which is constitutively nuclear, remains in the mutant configuration when in the nucleus, and is thus not capable of inducing apoptosis (15). Therefore, any apoptosis induced in this cell line in the presence of cycloheximide cannot be mediated through p53. The KH215 cell line thus serves as a positive control for apoptosis induced exclusively by the presence of inhibitors of transcription or protein synthesis.

Both the p53An1 and the KH215 cell lines succumbed to apoptosis induced by cycloheximide, actinomycin D, or anisomycin, as the number of viable cells present after 24 h in

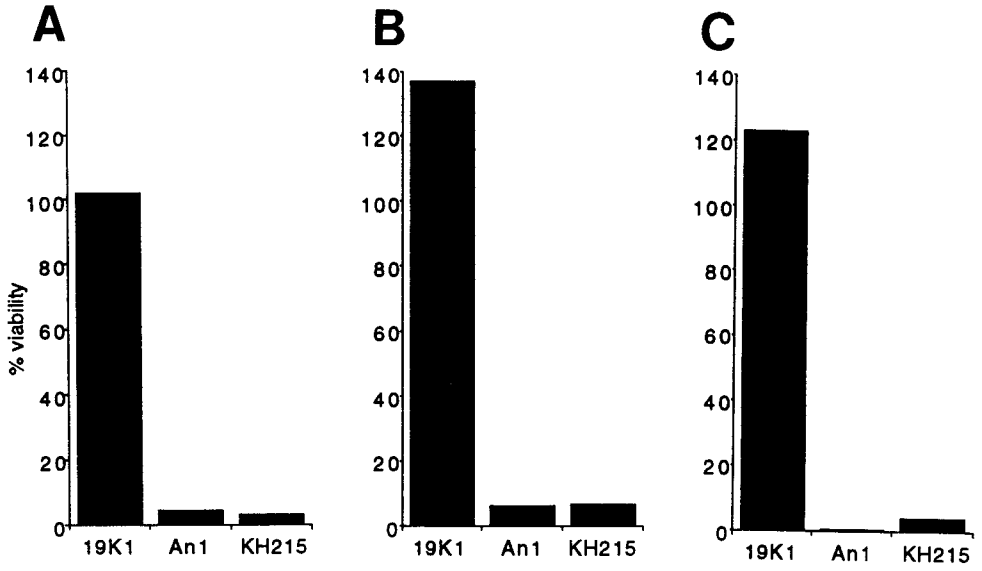

FIG. 8. The E1B 19K protein overcomes apoptosis induced by actinomycin D, anisomycin, or cycloheximide. The indicated cell lines were incubated with either cycloheximide (30 μg/ml) (A), anisomycin (100 μM) (B), or actinomycin D (2.5 μg/ml) (C) for 24 h at 38.5°C. The number of viable cells was subsequently determined by trypan blue exclusion and is represented as a percentage of the number of viable cells present at the time the drug was added. The KH215 cell line expresses E1A and a non-temperature-sensitive mutp53, KH215 (47).

drug-treated media was at most 5% of the number of viable cells present at the time the drug was added (Fig. 8). However, the p5319K1 cell line was completely rescued from apoptosis induced by either actinomycin D, anisomycin, or cycloheximide, as the number of viable cells present in the drug-treated plates was at least as large as the number of cells that had been present at the time the drug was added (Fig. 8). Low-molecular-weight DNA prepared from the p53An1 and KH215 cells that had been treated with cycloheximide, actinomycin D, or anisomycin for 24 h revealed the presence of nucleosomal-size DNA fragments, characteristic of apoptotic cells. No DNA fragmentation was observed in the 19K1 cell line treated under identical conditions (data not shown). Therefore, the E1B 19K protein overcomes p53-independent apoptosis induced by inhibitors of transcription and protein synthesis.

DISCUSSION

We have demonstrated in this report that the adenovirus E1B 19K protein enhances p53-mediated transactivation and alleviates p53-mediated transcriptional repression in promoter-reporter assays. We have also demonstrated that these activities cosegregate with the transformation ability of the E1B 19K protein. These activities are not indirect effects of the ability of the E1B 19K protein to prevent p53-mediated apoptosis, as the results were reproducible in the Saos-2 cell line, which, upon transient expression of wtp53, does not display any visible signs of apoptosis (9, 18, 41). We have also established Saos-2 cell lines that express extremely high levels of a transcriptionally functional human *ts*p53 and have observed no signs of cell death after up to 4 days of incubation at the permissive temperature (41). The E1B 19K protein has been shown to enhance the expression of reporter plasmids newly introduced into cells by increasing the stability of the transfected plasmid DNA (20a). This was not observed under the conditions of our experiments, however, as expression of the E1B 19K protein was not associated with an increase in the background level of CAT activity from any of the reporter plasmids tested. Moreover, in repeated experiments the E1B 19K protein was incapable of enhancing the ability of mutp53 to transactivate the pInrCAT and pTATACAT plasmid constructs. Therefore, the ability of the E1B 19K protein to modulate p53-mediated transcription is specific for wtp53 and, in turn, cannot be explained by general increases in plasmid stability.

We have also demonstrated in this report that the E1B 19K protein preserves the growth arrest function of p53. In so doing, the E1B 19K protein may allow the cell to escape p53-mediated apoptosis. This concept is not without precedence. Indeed, the ectopic expression of the wt p53 in myeloid leukemic cells, which are devoid of endogenous p53, induces apoptosis in these cells (60, 61). However, in the presence of interleukin-6, to which these cells are highly responsive, p53-mediated apoptosis is overcome and, instead, the cells undergo G_1 arrest (60). It has been suggested that the commitment of these cells to p53-mediated apoptosis takes place preferentially in the G_1 phase of the cell cycle and that the continuation of cycling inevitably results in apoptosis (60). Therefore, the induction of growth arrest by interleukin-6 or the E1B 19K protein prior to the commitment point may offer the cell a means of escaping p53-mediated apoptosis.

The growth arrest function of p53 may be directly linked to its transactivational properties in view of the recent discovery and partial characterization of the p21/WAF-1/cip-1 protein. The expression of p21 is p53 inducible, and p21 binds preferentially to cyclin-dependent kinase 2 in vitro and inhibits the kinase activity of G_1-specific cyclin-dependent kinases (11, 19, 58). The overexpression of p21/WAF-1/cip-1 inhibits DNA synthesis in human diploid fibroblasts and in mouse NIH 3T3 cells (19) and suppresses the growth of tumor cells in culture (11). Moreover, the coexpression of the simian virus 40 large tumor antigen counteracts the ability of p21/WAF-1/cip-1 to inhibit DNA synthesis, suggesting that p21/WAF-1/cip-1 implements the inhibition of DNA synthesis by inducing growth arrest and not apoptosis (19). The E1B 19K protein not only preserves p53-mediated growth arrest but enhances p53-mediated transactivation in promoter-reporter assays. In light of

this information, it seems plausible that the E1B 19K protein might preserve p53-mediated growth arrest by enhancing p53-mediated transactivation of the p21/WAF-1/cip-1 gene. However, Northern blot analysis has revealed no significant differences in the levels of expression of the p21/WAF-1/cip-1 gene in the p53An1, p5319K1, and p5319K2 cell lines grown under conditions in which the E1B 19K protein diverts cells to a p53-mediated growth-arrested state. Therefore, apoptosis is not the result of a failure to induce p21/WAF-1/cip-1 expression, nor does the E1B 19K protein function by interfering with the transactivational function of p53. This also implies that enhancement of p53-mediated transactivation by the E1B 19K protein in promoter-reporter assays is not reflected in the p53-mediated transactivation of cellular genes. The p53 protein has been documented to transcriptionally repress the CMV promoter (45). The pC53-SN_3 construct used in our promoter-reporter assays expresses p53 from the CMV promoter. Therefore, after p53 is expressed, it would, to a certain extent, transcriptionally repress its own expression in a *cis*-mediated fashion. This would compromise the levels of p53 protein expressed from a plasmid driven by the otherwise constitutive CMV promoter. In turn, only a limited degree of p53-mediated transactivation would be possible. Since the E1B 19K protein alleviates p53-mediated transcriptional repression, coexpression of the E1B 19K protein in this scenario would predictably result in an increased expression of the p53 protein and, therefore, augmented p53-mediated transactivation. In fact, immunofluorescence analysis of Saos-2 cells transfected with the p53 and E1B19K expression constructs has demonstrated that, when p53 is coexpressed with the E1B 19K protein, there is a two- to three-fold increase in the number of cells expressing the p53 protein compared with cells transfected with the p53 expression construct alone (data not shown). This observation cannot be explained by the ability of the E1B 19K protein to overcome p53-mediated apoptosis, as Saos-2 cells that either transiently or stably express high levels of wtp53 do not display any visible signs of apoptosis (41). Therefore, the ability of the E1B 19K protein to enhance p53-mediated transactivation in promoter-reporter assays is likely a reflection of the ability of the E1B 19K protein to alleviate p53-mediated transcriptional repression. Obviously a demonstration of the ability of the E1B 19K protein to alleviate p53-mediated transcriptional repression of cellular genes rather than reporter genes would be preferable. The c-*myc* proto-oncogene has been shown to be transcriptionally repressed by p53 (25). However, we have been unable to detect c-*myc* mRNA in our rat cell system. Therefore, looking for transcriptional repression of c-*myc* by p53 and alleviation of this transcriptional repression by the E1B 19K protein will probably not be possible. Other cellular genes and approaches, however, are under investigation.

We have also demonstrated in this report that the E1B 19K protein alleviates p53-mediated apoptosis, as well as apoptosis induced by treatment of cells with the transcription inhibitor actinomycin D and the protein synthesis inhibitors cycloheximide and anisomycin. This observation strongly supports the contention that the ability of the E1B 19K protein to overcome p53-mediated apoptosis is derived from the ability of the E1B 19K protein to alleviate p53-mediated transcriptional repression. Indeed, the ability of the p53 protein to induce apoptosis may result from its ability to transcriptionally repress the expression of genes that are necessary for cell survival (5). The fact that cells depend upon the continuous expression of survival factors in order to maintain their viability has been well documented (10, 37). The E1B 19K protein may maintain cell viability by alleviating p53-mediated transcriptional repression of these survival factors. Members of the emerging Bcl-2 family of proteins could conceivably represent the survival factors in this scenario (4, 24, 26).

It has been purported that the p53 protein causes transcriptional repression by binding to and sequestering the TATA box-binding protein and interfering with transcriptional initiation (28, 38, 44). The E1B 19K protein could conceivably alleviate p53-mediated transcriptional repression by affecting the interaction of p53 with the TATA box-binding protein or with other basal transcription factors (20, 36, 63). When expressed in cells, the E1B 19K protein does not localize to the nucleus, as does wt p53. Instead, the E1B 19K protein localizes to the cytoplasmic membranes, to the nuclear membrane, and to the nuclear lamina (50, 51). Moreover, the E1B 19K protein does not appear to physically interact with the p53 protein (48). Therefore, any effects the E1B 19K protein has on the transcriptional properties of p53 and its interaction with the basal transcriptional complex are likely to be indirect.

In order to better understand how the transformation function of the E1B 19K protein may be related to its ability to modulate p53-mediated transcription, we analyzed the ability of nine E1B 19K mutants with different transformation potentials to both enhance p53-mediated transactivation and alleviate p53-mediated transcriptional repression. All E1B 19K mutants that were capable of completely alleviating p53-mediated transcriptional repression were positive for transformation. The pm7 E1B 19K mutant retained partial transforming activity despite its ability to completely alleviate p53-mediated transcriptional repression. In addition, the pm102 E1B 19K mutant alleviated p53-mediated transcriptional repression 75% as efficiently as the wild-type E1B 19K protein, yet it was defective in its transformation function. Perhaps, having the capacity to alleviate p53-mediated transcriptional repression is not sufficient for transformation, and other as yet unidentified functions of the E1B 19K protein are necessary to confer full transformation potential.

The results from this study suggest a model that could conceivably explain the ability of the E1B 19K protein to overcome p53-mediated apoptosis. The ability of p53 to simultaneously transactivate and transcriptionally repress may lead to apoptosis. However, if the ability of p53 to transcriptionally repress is abrogated but its transactivation potential is left intact, then growth arrest may ensue. This diverting of cells to a p53-mediated growth-arrested state may be a mechanism which the E1B 19K protein employs in order to rescue cells from p53-mediated apoptosis. In addition, by alleviating p53-mediated transcriptional repression, the E1B 19K protein may prevent the destruction of survival factors which function to promote cell viability. This ability to selectively modulate p53-mediated transcription may therefore confer upon the E1B 19K protein its full transformation potential.

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