JOURNAL OF VIROLOGY, Oct. 1994, p. 6553–6566
0022-538X/94/$04.00+0

Vol. 68, No. 10

Functional Complementation of the Adenovirus E1B 19-Kilodalton Protein with Bcl-2 in the Inhibition of Apoptosis in Infected Cells

SHIUN-KWEI CHIOU,[1] CHING-CHUNG TSENG,[1] LAKSHMI RAO,[1] AND EILEEN WHITE[1,2]*

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

Received 12 May 1994/Accepted 18 July 1994

Expression of the adenovirus E1A oncogene induces apoptosis which impedes both the transformation of primary rodent cells and productive adenovirus infection of human cells. Coexpression of E1A with the E1B 19,000-molecular-weight protein (19K protein) or the Bcl-2 protein, both of which have antiapoptotic activity, is necessary for efficient transformation. Induction of apoptosis by E1A in rodent cells is mediated by the p53 tumor suppressor gene, and both the E1B 19K protein and the Bcl-2 protein can overcome this p53-dependent apoptosis. The functional similarity between Bcl-2 and the E1B 19K protein suggested that they may act by similar mechanisms and that Bcl-2 may complement the requirement for E1B 19K expression during productive infection. Infection of human HeLa cells with E1B 19K loss-of-function mutant adenovirus produces apoptosis characterized by enhanced cytopathic effects (*cyt* phenotype) and degradation of host cell chromosomal DNA and viral DNA (*deg* phenotype). Failure to inhibit apoptosis results in premature host cell death, which impairs virus yield. HeLa cells express extremely low levels of p53 because of expression of human papillomavirus E6 protein. Levels of p53 were substantially increased by E1A expression during adenovirus infection. Therefore, E1A may induce apoptosis by overriding the E6-induced degradation of p53 and promoting p53 accumulation. Stable Bcl-2 overexpression in HeLa cells infected with the E1B 19K$^-$ mutant adenovirus blocked the induction of the *cyt* and *deg* phenotypes. Expression of Bcl-2 in HeLa cells also conferred resistance to apoptosis mediated by tumor necrosis factor alpha and Fas antigen, which is also an established function of the E1B 19K protein. A comparison of the amino acid sequences of Bcl-2 family members and that of the E1B 19K protein indicated that there was limited amino acid sequence homology between the central conserved domains of E1B 19K and Bcl-2. This domain of the E1B 19K protein is important in transformation and regulation of apoptosis, as determined by mutational analysis. The limited sequence homology and functional equivalency provided further evidence that the Bcl-2 and E1B 19K proteins may possess related mechanisms of action and that the E1B 19K protein may be the adenovirus equivalent of the cellular Bcl-2 protein.

The recent growing interest in apoptosis stems from the recognition that it is a fundamentally important part of normal development and that its abnormal regulation may lead to neoplastic transformation. Apoptosis is observed under a large number of circumstances during mammalian development. One view states that most mammalian cells possess an innate program for cell death and that apoptosis is a default mechanism in the cell which requires the continuous presence of signals from other cells to prevent its activation (53). One signal from within the cell that blocks apoptosis is expression of the *bcl-2* proto-oncogene (26, 49, 60, 63, 69). Bcl-2 overexpression has been shown to suppress apoptosis upon growth factor withdrawal in various cell lines (2, 12, 49, 69). In transgenic animals, Bcl-2 overexpression results in extended B-cell memory (48) and the occurrence of follicular lymphomas (39, 40). Thus, abnormal regulation of Bcl-2 expression may contribute to the development of cancer. Indeed, the *bcl-2* gene is the most common translocation found in B-cell follicular lymphomas (66).

Another process during which apoptosis occurs is viral infection (reviewed in references 72 and 77). Viral infection and subsequent gene expression can trigger apoptosis, and some viruses encode apoptosis inhibitors as a means for escaping this host immune defense response. Adenoviruses represent a category of viruses that encode genes which both stimulate and suppress apoptosis (reviewed in references 72 and 77).

The adenovirus E1A gene encodes the capacity to stimulate host cell DNA synthesis (reviewed in reference 45), which creates an environment favorable for viral genome propagation. However, this required function of E1A induces the host cell to undergo apoptosis (76, 80, 81), which is likely responsible for impairing virus production (52, 64, 78). For this reason, adenoviruses encode a second function in the E1B gene that prolongs host cell viability by inhibiting apoptosis (54, 76, 80).

E1B encodes two different gene products, the E1B 55,000-molecular-weight protein (55K protein) and the E1B 19K protein, both of which can independently inhibit E1A-induced apoptosis, although the E1B 19K protein does so much more effectively (54). The E1B 19K protein has the additional ability to inhibit apoptosis mediated by tumor necrosis factor alpha (TNF-α) and Fas antigen (13, 14, 18, 80). In vitro studies have shown that TNF-α possesses antiviral activities (31, 42, 82) and that it is induced in response to viral infections (1, 67, 82). Fas antigen is a member of the TNF receptor family which is involved in regulating apoptosis in the immune system (71), and it has been shown to be selectively cytocidal to human immunodeficiency virus-infected cells (30). Thus, TNF-α and Fas antigen may both be involved in host cell immune surveillance systems against viral infection. The adenovirus E1B gene

* Corresponding author. Mailing address: Center for Advanced Biotechnology and Medicine, 679 Hoes La., Piscataway, NJ 08854. Phone: (908) 235-5329. Fax: (908) 235-5318. Electronic mail address: ewhite@mbcl.rutgers.edu.

encodes redundant functions for escaping not only the host cell apoptosis response-induced E1A but also those induced by TNF-α and Fas antigen, suggesting that complete blockage of apoptosis is a significant aspect of successful virus production.

The apoptosis-inhibitory activity of either the E1B 19K protein or the Bcl-2 protein is required for transformation of primary rodent cells in cooperation with E1A (54). In rodent cells, which are semipermissive for adenovirus infection, E1A expression alone results in abortive transformation because of the induction of apoptosis (54, 80). Coexpression of either the E1B 19K protein or the Bcl-2 protein with E1A inhibits apoptosis and results in efficient transformation of these cells (6, 10, 54, 80). Thus, regulation of apoptosis is an integral part of the multiple aspects of the adenovirus life cycle.

One way to approach the question of how apoptosis is regulated is to elucidate the molecular mechanisms by which apoptosis is induced by E1A and inhibited by E1B. E1A-induced apoptosis requires functional wild-type p53 and is completely blocked in rodent cells by dominant mutant forms of p53 (10, 79). Thus, p53 acts as a molecular switch to control cell death in the presence of E1A. Upon E1A expression, p53 protein accumulates to high levels because of the prolonged half-life of the protein (36). In baby rat kidney (BRK) cell lines transformed by E1A in cooperation with a temperature-sensitive dominant mutant of p53, cell viability is maintained at the restrictive temperature when p53 is in the mutant form, whereas cell death by apoptosis occurs when p53 is returned to the wild-type conformation at the permissive temperature (10). The E1B 19K protein blocks this wild-type p53-dependent apoptosis, suggesting a role for the E1B 19K protein in regulation of p53 function (10). Interestingly, the Bcl-2 protein also blocks this p53-dependent apoptosis, suggesting that the E1B 19K protein and the Bcl-2 protein may function by a related mechanism (6). The E1B 19K protein could be a functional homolog of Bcl-2, or the activity of the E1B 19K protein may be mediated by Bcl-2. In the latter case, the E1B 19K protein may upregulate expression of cellular apoptosis inhibitors (e.g., Bcl-2) in order to block apoptosis. This mechanism had been reported for the inhibition of apoptosis by another DNA virus, the Epstein-Barr virus (EBV), in the case of which the EBV latent membrane protein 1 (LMP-1) induces Bcl-2 overexpression and increases cell survival (20).

We demonstrate here that neither E1B 19K nor Bcl-2 blocked induction of p53 levels by E1A, suggesting that they both act downstream of p53 upregulation. This result supports earlier findings that both E1B 19K and Bcl-2 can block apoptosis but not growth arrest by p53 (6, 55). E1B 19K and Bcl-2 apparently resolve the ability of p53 to induce growth arrest from its ability to induce apoptosis, suggesting that they act upstream at a point in the p53 pathway where apoptosis and growth arrest are separable. Furthermore, E1B 19K expression completely inhibited apoptosis in cells that express extremely low levels of Bcl-2, and Bcl-2 levels were not upregulated. This suggests that the E1B 19K protein does not act through Bcl-2 but rather that the E1B 19K protein and the Bcl-2 protein may be functional counterparts.

To further delineate the functional relationship between E1B 19K and Bcl-2, the ability of Bcl-2 to complement E1B 19K function was tested by using assays which measure the ability of Bcl-2 to inhibit apoptosis induced during productive adenovirus infection, by TNF-α, and by Fas antigen. Bcl-2 functioned similarly to E1B 19K in all these assays, although it appeared quantitatively to possess less activity than the E1B 19K protein. Comparison of the amino acid sequences of E1B 19K and Bcl-2 family members demonstrated a modest degree of homology. Mutational analysis showed that the central region of the E1B 19K protein between amino acids 44 and 113, which had limited homology with Bcl-2 and related proteins, is also important for E1B 19K function. Therefore, the E1B 19K protein may be a viral homolog of the cellular Bcl-2 protein.

MATERIALS AND METHODS

Cell lines and plasmids. HeLa cells were grown in monolayer culture in Dulbecco modified Eagle medium with 10% fetal bovine serum. Primary Fisher BRK cells were prepared as previously described (75). The E1B 19K protein-expressing stable HeLa cell line (24) was generously provided by M. Mathews (Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.). Bcl-2-expressing cell lines were generated by introducing the plasmid expression vector pSFFVBcl-2 (26) into HeLa cells by the standard calcium phosphate-DNA precipitation method and selecting for growth in G418 (1 mg/ml). Eight of the independent clones were isolated and propagated in Dulbecco modified Eagle medium with 10% fetal bovine serum containing G418 and then checked for Bcl-2 expression by Western blot (immunoblot) analysis. Three of these clones were characterized further; two of them, Bcl-2 4C and Bcl-2 3C, expressed very high levels of Bcl-2, whereas Bcl-2 1 expressed low levels of Bcl-2. Neomycin-resistant control lines were generated in parallel with the Bcl-2-expressing lines by transfection of plasmid pSV2Neo into HeLa cells, and one of these lines was used in functional assays with the Bcl-2-expressing lines. The Saos-2 cell line was obtained from the American Type Culture Collection.

The plasmid expression vectors pCMV19K and pCMVE1A, which produce the E1B 19K protein and the E1A protein, respectively, have been described previously (74, 76). Mutant 19K proteins (see below) were expressed from the cytomegalovirus promoter on a pUC19 vector.

Viruses. Adenovirus type 5 (Ad5) virus Ad5*dl*337 (52) contains a deletion in the E1B 19K gene and was generously provided by T. Shenk (Princeton University, Princeton, N.J.). Ad5*dl*337 was derived from Ad5*dl*309 (27), which was used as the wild-type strain. The 12S virus expresses only the 12S E1A mRNA of the E1A gene. The E1A gene encodes two different mRNAs that are expressed early in infection, the 13S and 12S transcripts (4, 8, 29, 51, 81). The 12S product differs from the 13S product only by the absence of 46 internal amino acids due to alternative splicing. As a consequence, the 12S gene product is deficient in transcriptional activation and viruses which express only this gene product are defective for productive infection in HeLa cells (44, 46). However, the 12S protein retains transforming activity (17, 44, 46) and the ability to stimulate DNA synthesis when introduced into quiescent cells (28, 62), and it induces apoptosis (76, 81).

The E1B$^-$ virus, which contains a deletion in the E1B gene, was constructed by overlapping recombination (81) between plasmid pE1A (74), encoding early region 1 (map units 0 to 15.5 of the adenovirus genome) containing a deletion in the E1B gene (between nucleotides 1769 and 3322), and the large *Xba*I fragment from Ad5*dl*309. The 12SE1B$^-$ virus expresses only the 12S E1A mRNA, and it lacks the E1B gene. For construction of this virus, a *Cla*I-*Hpa*I fragment (nucleotides 916 to 1569) containing an E1A gene capable of expressing only the 12S product was substituted for the *Cla*I-*Hpa*I wild-type E1A fragment (nucleotides 916 to 1569) in plasmid pE1A (74). The resulting construct, plasmid p12SE1B$^-$, contained an insert representing 15.5 map units of the left end of the viral genome with the E1A 12S gene and a deletion in the E1B gene. The viral DNA insert from p12SE1B$^-$ was rebuilt into an

intact Ad5*dl*309 chromosome by overlapping recombination as previously described (81). Viruses were plaque purified twice on 293 cells and verified by restriction analysis.

Construction of E1B 19K mutations. A *Bam*HI-*Cla*I fragment from plasmid pCMV19K (74) encompassing a cytomegalovirus promoter region, the E1B 19K coding region (nucleotides 1711 to 2256), and a simian virus 40 polyadenylation signal region was subcloned into M13 vector for site-directed mutagenesis by modifying the *Cla*I site into an *Xma*I site. Expression from the E1B 55K open reading frame was prevented by an engineered stop codon at nucleotide 2022 (cytosine-to-thymidine conversion) of the adenovirus genome, corresponding to the second codon of the E1B 55K reading frame (3). Site-directed mutagenesis was performed by using a method for oligonucleotide-mediated mutagenesis as previously described (33).

Mutagenic sites were selected at amino acids that are conserved (≥75%) across 12 different serotypes of adenoviruses. The mutants generated were as follows: *pm*44R, AA-to-CG transition at nucleotides 1841 and 1842, lysine-to-arginine conversion at codon 44; *pm*51, T-to-C transition at nucleotide 1863, phenylalanine-to-serine conversion at codon 51; *pm*55, T-to-C transition at nucleotide 1875, leucine-to-serine conversion at codon 55; *pm*73, TT-to-GC transition at nucleotides 1928 and 1929, phenylalanine-to-alanine conversion at codon 73; *pm*81, T-to-C transition at nucleotide 1953, leucine-to-serine conversion at codon 81; *pm*113, TA-to-GC transition at nucleotides 2048 and 2049, tyrosine-to-alanine conversion at codon 113; *pm*123, GG-to-CT transition at nucleotides 2079 and 2080, tryptophan-to-serine conversion at codon 123 (see Fig. 7). The complete E1B 19K coding region from each of the mutant constructs was sequenced, and the entire fragment, with the cytomegalovirus promoter and the simian virus 40 polyadenylation signal, was subcloned into the pUC19 expression vector. Mutations were confirmed by restriction analysis and double-stranded DNA sequencing.

Transfections. HeLa cells were transfected by the standard calcium phosphate-DNA precipitation method, by using 10 μg of test plasmid DNA plus 10 μg of carrier salmon sperm DNA in each transfection. Electroporation was utilized for transfection of primary BRK cells as previously described (76). Briefly, 20 μg of linearized plasmid DNA plus 100 μg of carrier salmon sperm DNA was used for each sample (76).

Viral infections. All lines were infected with wild-type adenovirus (Ad5*dl*309) or E1B 19K$^-$ mutant virus (Ad5*dl*337) at a multiplicity of infection of 100. At 48 h postinfection, the morphological changes were observed by light microscopy and recorded by photography with a Nikon Diaphot microscope at a magnification of ×25, and low-molecular-weight DNA was isolated by a modified Hirt DNA isolation procedure (25, 78). Cell extracts were prepared in parallel for protein quantitations. Equivalent numbers of cells from each sample were analyzed for the presence of degraded DNA by agarose gel electrophoresis and ethidium bromide staining.

Western blot analysis. Cell extracts were prepared after transfection or infection, and the protein levels were examined by Western blot analysis. Immune complexes were detected by enhanced chemiluminescence (Amersham). E1B 19K mutant proteins were also visualized by indirect immunofluorescence as previously described (78). A monoclonal mouse anti-E1B 19K antibody was used to detect the E1B 19K protein (81). A monoclonal hamster antibody, 6C8 (26), generously provided by S. Korsmeyer (Washington University, St. Louis, Mo.), was used to specifically detect human Bcl-2 protein. The p421 monoclonal antibody against p53 (Oncogene Science) was used to detect human p53. A polyclonal rabbit antibody against E1B 19K was used for indirect immunofluorescence as previously described (78).

Transformation assays. Plasmids containing E1B 19K mutant cDNAs were cotransfected with pCMVE1A into primary BRK cells as previously described (76). Foci were observed and counted at 21 days posttransfection. Transformation efficiency was measured as the number of transformed foci observed for transfection of each E1B 19K mutant construct compared with that observed for transfection of the wild-type E1B 19K construct. At least eight independent transfections were analyzed for each DNA combination.

TNF-α cytolysis assays. E1B 19K proteins were transiently expressed in HeLa cells by transfection of the plasmid expression vectors carrying wild-type or mutant E1B 19K cDNAs. At 48 h posttransfection, when maximal transient expression occurs, cells were treated with TNF-α (2,000 U/ml) in the presence of cycloheximide (30 μg/ml) as previously described (80). Stable Bcl-2- and E1B 19K-expressing cell lines, with their Neo^r control cell lines, were treated directly with TNF-α and cycloheximide. The duration of treatment was 16 h, after which time cytolysis of susceptible cells was completed. The TNF-α-containing medium was then replaced with normal growth medium, and resistant cells were fixed and stained with Giemsa 5 days later. Resistance was measured by eluting the bound dye from viable cells and measuring the optical density at 590 nm of the light absorption of each eluate and then comparing the optical density reading from each test plate with that from the control for each experiment. The levels of resistance of the mutant E1B 19K protein-expressing cell lines were compared with that of the wild-type 19K protein-expressing cell line. The viabilities of the Bcl-2-expressing cells and the 19K protein-expressing cells were represented relative to that of the Neo^r control cell line.

Anti-Fas antibody cytolysis assay. E1B 19K mutant proteins were transiently expressed in HeLa cells as described above for the TNF-α cytolysis assay. Transfected cells were then treated with anti-Fas antibody (5 μg/ml), generously provided by P. H. Krammer (Deutsches Krebsforschungszentrum, Heidelberg, Germany), in the presence of protein A (5 μg/ml). Cell lines stably expressing Bcl-2 or E1B 19K proteins, and the Neo^r control cell lines, were treated directly with anti-Fas antibody (5 or 10 μg/ml) and protein A (5 μg/ml). The duration of the incubation with anti-Fas antibody was 6 days, at which time the antibody-containing medium was replaced by normal growth medium and resistance to anti-Fas antibody was measured as described for the TNF-α cytolysis assay.

RESULTS

E1A expression induces p53 accumulation in human HeLa cells. E1A expression in REF 52 cells has been shown to induce accumulation of the tumor suppressor gene product p53, which is accompanied by apoptosis (36). Adenovirus infection as well as E1A expression also induces apoptosis in HeLa cells (76, 78). HeLa cells normally contain very low levels of wild-type p53 protein because of the presence of the human papillomavirus E6 protein, which forms a complex with and promotes degradation of p53 protein in vitro (58). Since E1A-induced apoptosis has been shown to be p53 dependent (10), the possibility arises that E1A expression in HeLa cells could override the mechanism by which E6 protein degrades p53, thereby allowing p53 protein accumulation and apoptosis.

To examine this possibility, HeLa cells were mock infected or infected with adenovirus, and p53 levels were examined by Western blot analysis. The 12S and 12SE1B$^-$ viruses express only the 12S E1A product that is sufficient for induction of

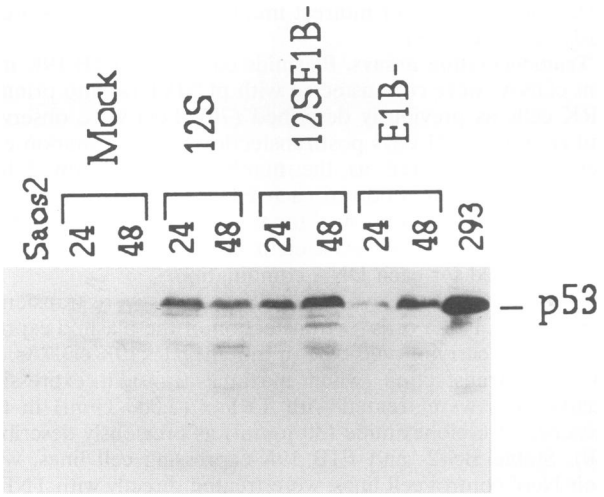

FIG. 1. Induction of p53 in HeLa cells by E1A expression. HeLa cells were mock infected or infected with 12S, 12SE1B$^-$, or E1B$^-$ virus, and p53 levels were examined by Western blot analysis at the times indicated by using a monoclonal human p53 antibody, pAb421. Each lane represents 20 μg of protein from a whole-cell extract. The position of the p53 protein is indicated. Infections with specific viruses are indicated above the lanes. Saos2, human osteosarcoma cell line null for the p53 gene; 293, human epithelial cell line transformed with E1A plus E1B used as a positive control for p53 expression (15). The numbers 24 and 48 indicate hours postinfection.

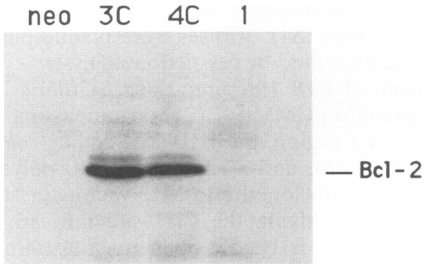

FIG. 2. Expression of Bcl-2 protein in HeLa cell lines. Bcl-2-expressing HeLa cell lines, along with a drug-resistant control line that expresses only low levels of endogenous Bcl-2, were established as described in Materials and Methods. Bcl-2 expression in these cell lines was determined by Western blot analysis with a hamster anti-human Bcl-2 monoclonal antibody, 6C8 (26), by using 20 μg of whole-cell extract from each sample. The position of the 26-kDa Bcl-2 protein is indicated. Neo, the neomycin-resistant control cell line; 4C, 3C, and 1, the three Bcl-2-overexpressing HeLa cell lines Bcl-2 4C, Bcl-2 3C, and Bcl-2 1, respectively.

apoptosis (76, 81). The E1B$^-$ virus encodes both the 12S and 13S E1A products (see Materials and Methods). Both the 12SE1B$^-$ virus and the E1B$^-$ virus possess a deletion of the entire E1B gene, in order to avoid any potential contribution of either the E1B 55K protein or the E1B 19K protein to the regulation of p53 levels. This was crucial, since the E1B 55K protein has been shown to enter into a stable protein complex with the p53 protein (57) and therefore could potentially increase the half-life of the p53 protein independently of E1A action.

The p53 protein is absent in the cell line Saos-2 derived from a human osteosarcoma (American Type Culture Collection), which is null for both alleles of the p53 gene locus and serves as a negative control for p53 levels (Fig. 1). In contrast, an E1A- plus E1B-transformed human embryo kidney cell line, 293 (15), constitutively expressed very high levels of wild-type p53 (Fig. 1). p53 levels in mock-infected HeLa cells are extremely low, as expected on the basis of the presence of human papillomavirus E6 protein (Fig. 1). Infection of HeLa cells with 12S, 12SE1B$^-$, and E1B$^-$ viruses resulted in the induction of high levels of p53 protein by 48 h postinfection (Fig. 1). The p53 protein induced by 12SE1A localized to the nucleus, as determined by indirect immunofluorescence (data not shown). Induction of p53 levels is exclusively E1A dependent, since it does not occur in HeLa cells infected by E1A 9S loss-of-function mutant adenoviruses (7). Furthermore, transient expression of E1A in HeLa cells induces both apoptosis (76) and p53 accumulation (7, 36). These observations demonstrate that E1A expression is necessary and sufficient to induce p53 levels in HeLa cells. The mechanism by which E1A increases p53 levels in HeLa cells is not yet known, but it could be via stabilization of the p53 protein as described for REF 52 cells (36).

Induction of p53 in HeLa cells by the 12S and 12SE1B$^-$ viruses suggested that the 12S product of the E1A gene is sufficient for induction of p53. Furthermore, p53 levels induced by infection of HeLa cells with the 12S virus did not differ significantly from those found in HeLa cells infected with the 12SE1B$^-$ virus. This supports the findings of Lowe and Ruley (36) and suggests that E1B expression does not alter the induction of p53 by E1A. E1A is primarily responsible for high levels of p53 in cells expressing both E1A and E1B, as the E1B 19K protein inhibits p53-dependent apoptosis without affecting the levels of p53 in cells. Furthermore, E1B 19K expression in HeLa cells does not affect the nuclear localization of p53 (data not shown), suggesting that the E1B 19K protein does not directly interact with p53 but that it acts indirectly to modify p53 function in the induction of apoptosis. Thus, p53 levels are induced by E1A expression during adenovirus infection of HeLa cells, which is consistent with a role for the p53 protein in E1A-associated apoptosis in HeLa cells as well as in primary rodent cells (10).

E1B 19K and Bcl-2 act downstream of p53 induction. Since Bcl-2 can inhibit p53-dependent apoptosis (6), one possible mechanism by which the Bcl-2 protein functions may be to prevent p53 accumulation in response to E1A expression. HeLa cells express extremely low levels of endogenous Bcl-2 (Fig. 2). When the Bcl-2 protein is expressed in HeLa cells during infection by mutant adenovirus deleted in the E1B 19K gene, any alterations in the induction of p53 levels by E1A may be attributed to the expression of Bcl-2. Thus, HeLa cells provide ideal conditions for testing the effect of Bcl-2 expression on p53 induction by E1A.

The human *bcl-2* gene was introduced into HeLa cells by using neomycin selection, and resistant colonies were cloned and screened for human Bcl-2 protein expression. Three independent cell lines, two that expressed high levels of Bcl-2 (the Bcl-2 3C and Bcl-2 4C lines) and one that expressed lower levels of Bcl-2 (Bcl-2 1), were evaluated for p53 induction by E1A (Fig. 2). A control cell line, Neo^r, which was selected in parallel by transfection of HeLa cells with the neomycin resistance marker alone, expressed only endogenous and extremely low levels of Bcl-2 (Fig. 2).

The Bcl-2 3C, Bcl-2 4C, and Bcl-2 1 cell lines, and the Neo^r control, were infected with the 12SE1B$^-$ virus, and levels of p53 protein expression were examined at various time points after infection by using Western blot analysis. Infection of the Neo^r control cell line with 12SE1B$^-$ virus resulted in a significant rise in p53 protein levels (Fig. 3). In the 12SE1B$^-$

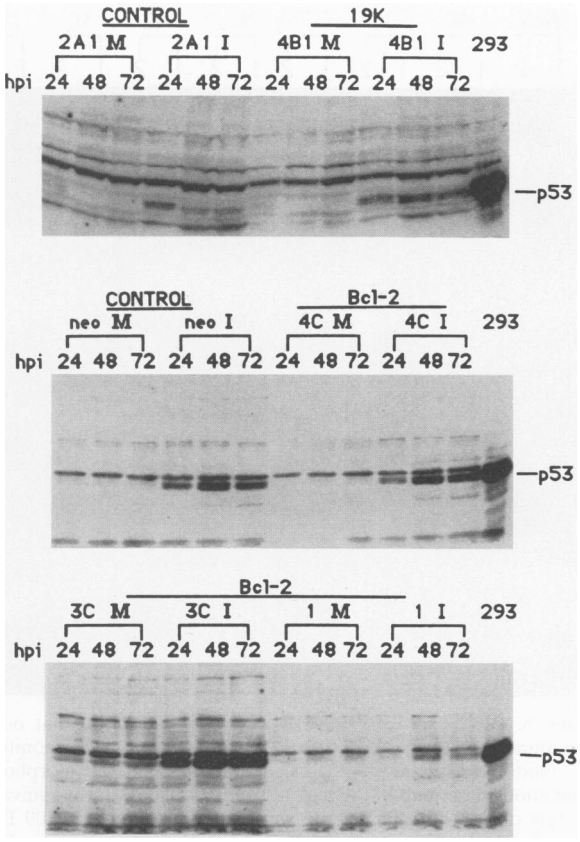

FIG. 3. E1B 19K expression and Bcl-2 expression do not block induction of p53 by E1A. p53 protein levels were examined by Western blotting of Bcl-2- and E1B 19K-expressing HeLa cell line extracts at 24, 48, and 72 h postinfection (hpi) with the 12SE1B$^-$ virus, which contains a deletion of the E1B gene and expresses only the 12S E1A gene product. An anti-human p53 monoclonal antibody, pAb421, was utilized to detect human p53. M, mock infection; I, infected cells. 4B1, stable 19K protein-expressing cell line; 2A1, control cell line for 4B1 which does not express the 19K protein (24); 293, E1A- plus E1B-transformed human epithelial cell line (15); neo, the neomycin-resistant control cell line; 4C and 3C, Bcl-2 4C and Bcl-2 3C lines, which expressed high levels of Bcl-2 protein; 1, Bcl-2 1 line, which had a very low level of Bcl-2 expression.

virus-infected Bcl-2-expressing cell lines, p53 induction by E1A was unaffected by Bcl-2 expression, since p53 levels were as high as that found in the Neo^r control cell line (Fig. 3). Thus, Bcl-2 expression does not alter p53 induction by E1A during adenovirus infection of HeLa cells.

The 19K protein-expressing stable cell line (4B 1) and the corresponding control cell line which does not express the E1B 19K protein (2A 1) (24) were tested for p53 accumulation in parallel with the Bcl-2-expressing lines. E1B 19K expression also did not affect induction of p53 accumulation by E1A, since p53 accumulation in the infected 4B 1 line was as high as that in the infected 2A 1 control line (Fig. 3). As in the case of E1B 19K expression during adenovirus infection, E1B 19K expression in stable HeLa cell lines also does not affect induction of p53 levels by E1A.

The inability of the Bcl-2 and E1B 19K proteins to prevent p53 accumulation in response to E1A expression suggests that inhibition of p53-dependent apoptosis by the E1B 19K and Bcl-2 proteins occurs downstream of p53 accumulation. This result is consistent with that observed with BRK cell lines transformed by E1A plus p53(val135), for which expression of E1B 19K or Bcl-2 did not affect p53 levels but did prevent apoptosis (6, 55).

Bcl-2 expression in human cells inhibits apoptosis during adenovirus infection. E1B 19K expression from the adenovirus genome (78) or in stable cell lines (23) prevents the occurrence of the *cyt* and *deg* phenotypes associated with the induction of apoptosis by E1A during productive adenovirus infection (76). The ability of Bcl-2 to functionally substitute for the E1B 19K protein in transformation suggested the possibility that Bcl-2 may complement E1B 19K function during productive adenovirus infection of HeLa cells.

The three Bcl-2-expressing cell lines, 4C, 3C, and 1, with the Neo^r control cell line, were infected with a wild-type adenovirus (Ad5*dl*309) or with a mutant adenovirus with the E1B 19K gene deleted (Ad5*dl*337) and were observed for the appearance of the *cyt* and *deg* phenotypes. At 48 h postinfection the Ad5*dl*337-infected neomycin-resistant control cell line presented extreme cytopathic effects (*cyt* phenotype), with an early onset and increased severity of cell rounding and detachment from the plate surface (Fig. 4). The Bcl-2 1 cell line, which expressed low levels of Bcl-2, also displayed a *cyt* phenotype comparable to that seen in the control cell line at 48 h postinfection (Fig. 4). The Bcl-2 3C and Bcl-2 4C lines, which expressed high levels of Bcl-2 protein, remained morphologically intact and resembled the Ad5*dl*309-infected controls (Fig. 4).

DNA fragmentation (*deg* phenotype) was examined concurrently with observation of the *cyt* phenotype, and it was apparent in the Ad5*dl*337-infected Neo^r control and in the Bcl-2 1 cell line but not in cells infected with Ad5*dl*309 wild-type adenovirus (Fig. 5). The Bcl-2 3C and Bcl-2 4C lines showed less DNA fragmentation upon Ad5*dl*337 infection than the Ad5*dl*337-infected Bcl-2 1 cell line and the Neo^r control cell line did (Fig. 5). The suppression of DNA fragmentation by Bcl-2 was more striking in *Hin*dIII-digested DNA samples (Fig. 5, right panel). However, the inhibition of DNA degradation by Bcl-2 was not as efficient as the inhibition of enhanced morphological changes, nor was it as effective as inhibition of the *deg* phenotype by the E1B 19K protein, as shown by the complete absence of degraded DNA in Ad5*dl*309-infected cells (Fig. 5). Rescue of the *deg* phenotype by Bcl-2 expression during adenovirus infection has also been observed in Bcl-2-expressing Rat-1 cell lines, with more dramatic differences in the degree of DNA degradation (data not shown). Thus, Bcl-2 can suppress cell death by apoptosis during adenovirus infection in a dose-dependent way and in a manner similar to that of the E1B 19K protein, although it does so less effectively.

Bcl-2 protein levels are not altered during adenovirus infection of HeLa cells. To examine the possibility that E1A may induce apoptosis by decreasing Bcl-2 expression, or that the E1B 19K protein functions by inducing Bcl-2 expression, HeLa cells infected with Ad5*dl*309 or with Ad5*dl*337 were examined for alterations in endogenous Bcl-2 protein levels. Mock-infected HeLa cells had extremely low levels of Bcl-2 expression, and infection of HeLa cells with either virus did not result in any increase or decrease of Bcl-2 protein levels (Fig. 6). Also, Bcl-2 cooperates with E1A to transform primary BRK cells (54), and Bcl-2 protein levels in transformed BRK cell lines [see E1A+Bcl-2(D) in Fig. 6] remain high despite overexpression of E1A (Fig. 6). Therefore, neither E1A nor the E1B 19K protein functions through alteration of Bcl-2 expression, suggesting that E1A does not induce apoptosis through direct disposal of Bcl-2 and that the E1B 19K protein

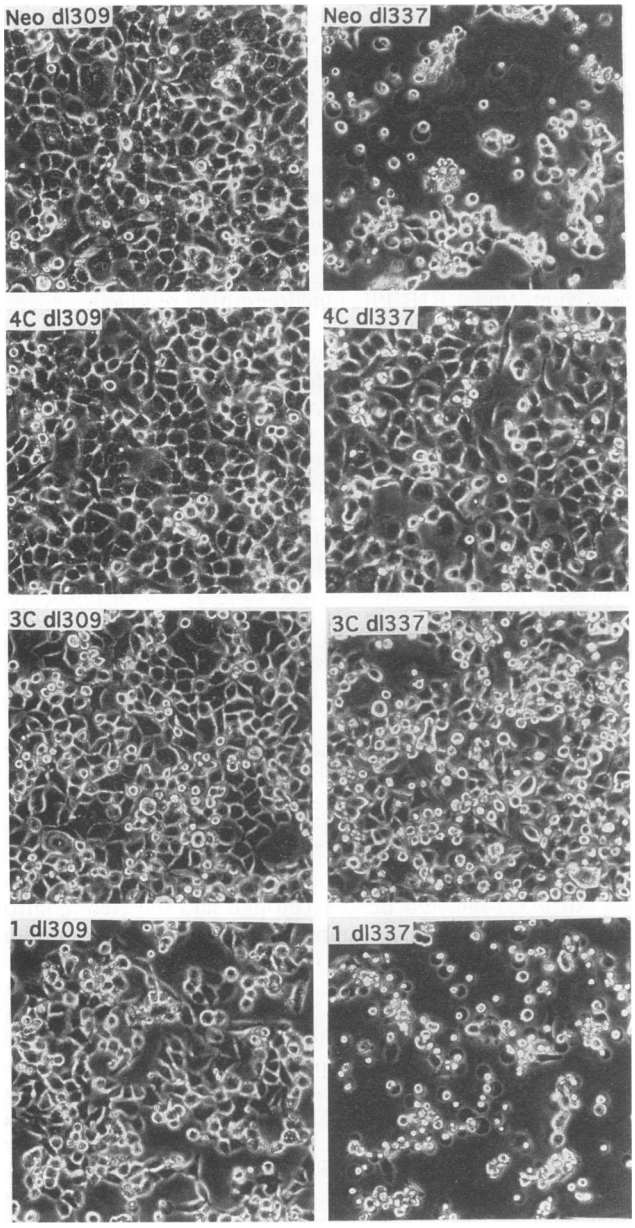

FIG. 4. Bcl-2 expression blocks the induction of the cytopathic effect that accompanies E1B 19K$^-$ mutant adenovirus infection. The Bcl-2 3C, Bcl-2 4C, and Bcl-2 1 cell lines and the neomycin-resistant control cell line, Neo, were infected with an E1B 19K$^-$ mutant adenovirus (Ad5*dl*337) and with wild-type adenovirus (Ad5*dl*309). Cells were photographed at 48 h postinfection, when severe morphological changes (*cyt* phenotype) become apparent in cells infected with an E1B 19K$^-$ mutant adenovirus. The high-level Bcl-2 protein-expressing cell lines 3C and 4C did not display the *cyt* phenotype at this time. Magnification, ×25.

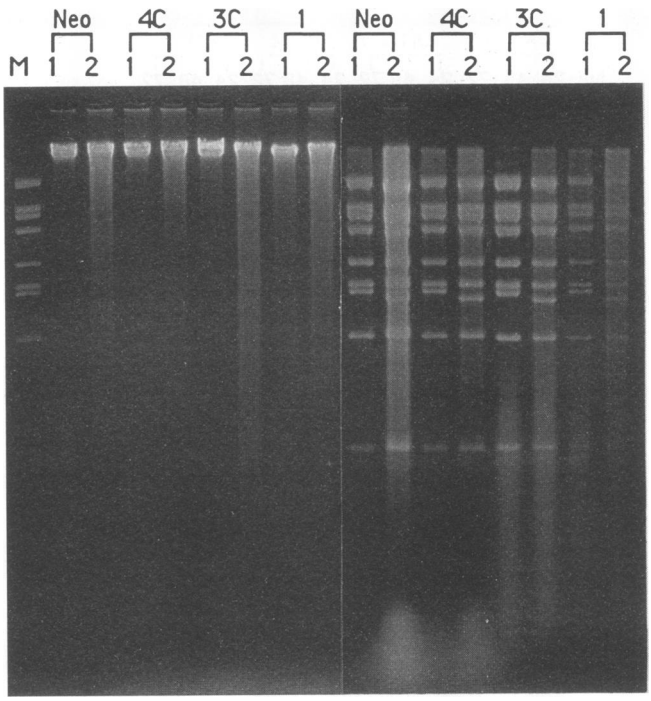

FIG. 5. Bcl-2 expression blocks DNA fragmentation that occurs upon mutant adenovirus infection. DNA fragmentation was monitored by a modified Hirt assay (25, 78) in parallel with the morphology observations described in the legend to Fig. 4. DNAs from equivalent numbers of cells in each sample were analyzed. M, Ad5*dl*309 DNA digested with *Hin*dIII as molecular weight markers. Lanes 1, Hirt DNA from cells infected by wild-type virus (Ad5*dl*309); lanes 2, Hirt DNA from cells infected by E1B 19K$^-$ mutant virus (Ad5*dl*337). Neo, the neomycin-resistant control cell line; 4C, 3C, and 1, the three Bcl-2-expressing cell lines. Left panel, undigested Hirt DNA. Right panel, Hirt DNA preparations digested with *Hin*dIII (bands are replicated viral DNA).

functions instead of, and not through, Bcl-2 to inhibit apoptosis. However, this result does not exclude the possibility that other Bcl-2-related proteins could be involved in the induction of apoptosis by E1A or the inhibition of apoptosis by the E1B 19K protein or that E1A or the E1B 19K protein could regulate Bcl-2 activity by other mechanisms.

Mutational analysis of the E1B 19K protein. Comparison of the E1B 19K amino acid sequence conservations from 12 different serotypes of adenoviruses indicated that the central region (amino acids 81 to 113) of the E1B 19K protein is highly conserved, the N terminus (amino acids 1 to 80) is moderately conserved, and the C terminus (amino acids 117 to 175) is poorly conserved (80). This suggests that the central region and perhaps the amino-terminal regions of the E1B 19K protein are important for function. Previously characterized site-directed mutations in the E1B 19K gene indicated that this is the case (80). Functional analysis of mutants *pm*7, *pm*28, *pm*44, *pm*87, *pm*91, and *pm*102, each containing a missense mutation in a conserved amino acid at the position indicated by the number in its designation, revealed that transforming activity and TNF-α resistance functions of the E1B 19K protein cosegregate (80). This observation supports the conclusion that so far all of the activity of the E1B 19K protein can be attributed to the inhibition of apoptosis (80). However, there may be activities associated with the E1B 19K protein that are not yet apparent. Furthermore, the central and amino-terminal regions of the protein between amino acid residues 44 and 102 are important regions involved in E1B 19K protein function (80). To better delineate boundaries or key residues of the functional region of the E1B 19K protein, mapping studies were continued to generate seven additional E1B 19K mutations (Fig. 7).

The mutants *pm*51, *pm*55, *pm*73, *pm*81, *pm*113, and *pm*123

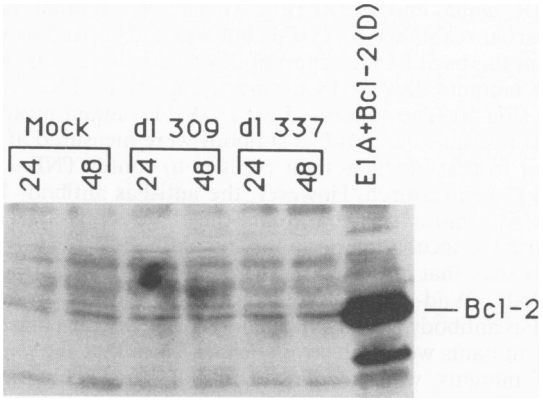

FIG. 6. Bcl-2 protein levels are not upregulated during adenovirus infection of HeLa cells. HeLa cells were infected with wild-type or E1B 19K$^-$ mutant adenoviruses. Cell extracts were made at 24 and 48 h postinfection, and Bcl-2 protein expression was monitored by Western blotting using the 6C8 (human Bcl-2) monoclonal antibody (26). Numbers indicate hours postinfection. Mock, mock infection; dl 309, lanes containing cells infected with the wild-type adenovirus Ad5*dl*309; dl 337, lanes containing cells infected with the E1B 19K mutant virus Ad5*dl*337; E1A+Bcl-2 (D), a control BRK cell line transformed with E1A and human Bcl-2 (54).

were constructed to generate missense mutations at conserved residues (at least 75% conserved across 12 different serotypes of adenoviruses) located at the positions indicated by the numbers in their designations. The *pm*44 mutation (80), in which the positively charged lysine residue was replaced by a glutamic acid, is identical to the mutation found in the *cyt*6 mutant adenovirus (65) and is completely defective for transforming activity and inhibition of apoptosis (65, 80). This mutation is located in a hydrophilic region of the protein, where a positive charge could have functional significance. The positive charge was restored to residue 44, by substituting an arginine residue for the original lysine residue, to address the importance of the positive charge at that position for E1B 19K protein function. These seven new mutants, plus the seven previously generated mutants, were characterized by assays which measure the ability of each mutant protein to function to inhibit apoptosis in transformation and that mediated by TNF-α or Fas antigen.

Transforming activity of the E1B 19K protein cosegregates with TNF-α and anti-Fas antibody resistance. The E1B 19K mutant proteins were analyzed for transforming efficiency in cooperation with E1A, TNF-α resistance, and anti-Fas antibody resistance, in comparison with the wild-type E1B 19K protein. In all three assays, mutants with >40% retention of wild-type activity were considered nondefective, whereas ≤40% retention of wild-type activity denoted a defective mutant.

Mutant 19K proteins were each coexpressed with E1A in BRK cells, and transforming ability was measured in a standard focus formation assay (76). Three of the mutants, *pm*28, *pm*44R, and *pm*123, were as efficient as the wild-type 19K protein in transformation (Fig. 8). The *pm*7 and *pm*55 mutant proteins had partial transforming activity but were defective according to our criterion, and all the other mutants were completely defective (Fig. 8).

To map the TNF-α and anti-Fas antibody resistance functions of the E1B 19K protein, mutant proteins were transiently expressed in HeLa cells, and at the time of maximum protein expression (48 h) the cells were treated with TNF-α or with an antibody directed against Fas antigen. After TNF-α treatment the degree of survival resulting from expression of each mutant protein was measured and compared with that resulting from wild-type 19K protein expression, as previously described (80).

Wild-type E1B 19K protein rescued cells from TNF-α treatment-induced apoptosis very efficiently, as did *pm*28,

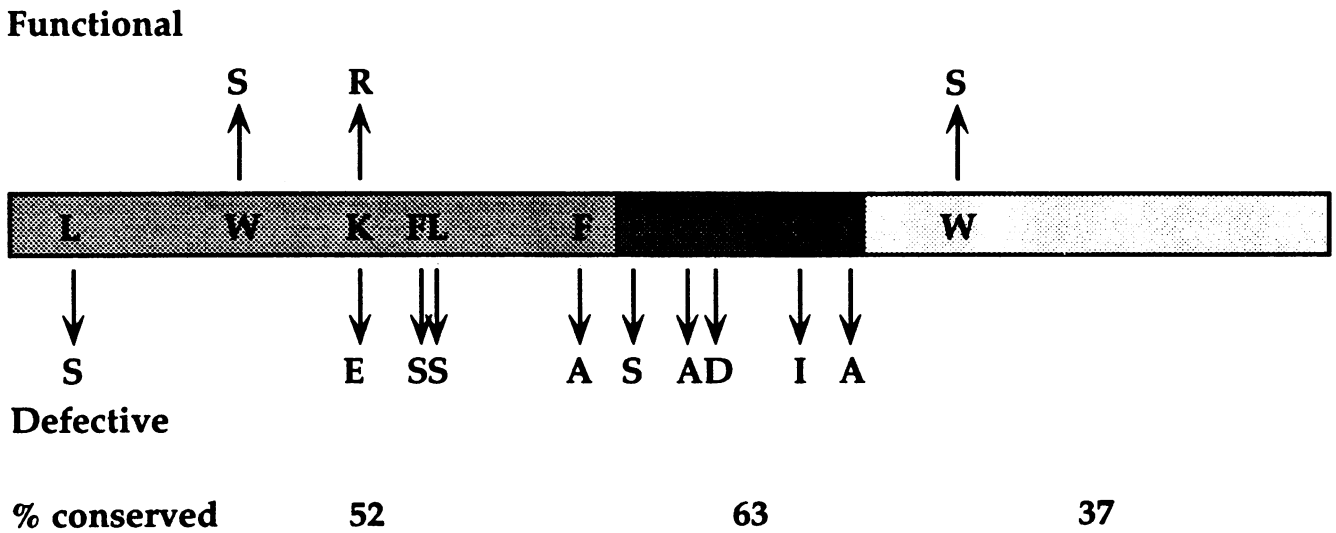

FIG. 7. Mutational analysis of the E1B 19K protein. Diagram of the E1B 19K protein showing locations of missense mutations. The arrows represent positions of amino acid substitutions, and the three shaded areas indicate three regions of differential conservation among the amino acid sequences from different adenovirus serotypes. The shaded region on the left corresponds to amino acid residues 1 to 81, the middle region corresponds to residues 82 to 116, and the region on the right corresponds to residues 117 to 175 of the E1B 19K protein. These residue numbers indicate positions of amino acids from the amino terminus (1) to the carboxy terminus (175) of the E1B 19K protein. The percent conservation in sequence across the 12 serotypes is indicated beneath each shaded region. Mutations resulting in functional proteins are shown at the top, and mutations that affect E1B 19K protein function or stability are shown below the shaded regions.

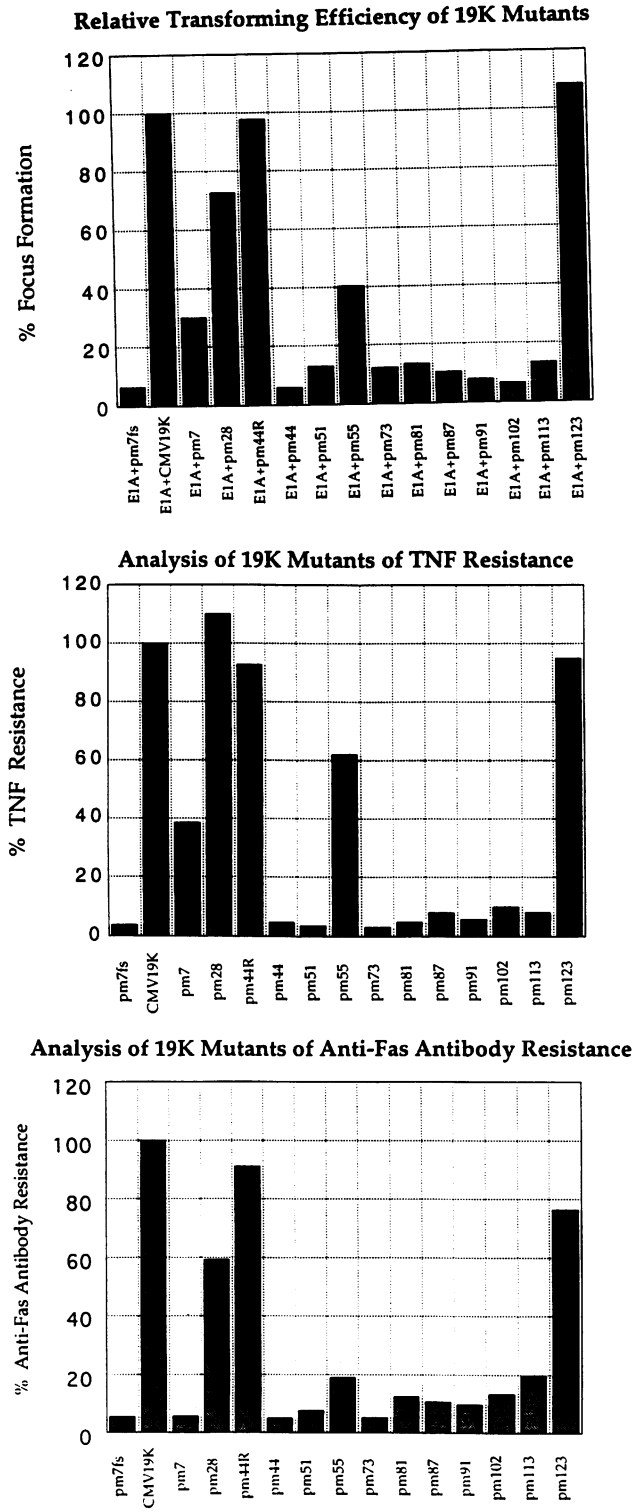

FIG. 8. Quantitation of transforming efficiency, resistance to TNF-α, and inhibition of anti-Fas antibody cytotoxicity. Transformation assays were performed by E1A cooperation assays with each mutant E1B 19K expression vector in primary BRK cells as described previously (80). Transformation efficiency was expressed as the percent focus formation of cells transfected with E1A plus mutant E1B 19K compared with the focus formation of cells transfected with E1A plus wild-type E1B 19K. The TNF-α cytolysis assays were performed by transient expression of wild-type E1B 19K protein and each mutant E1B 19K protein in HeLa cells as described in Materials and Methods. Anti-Fas antibody cytolysis assays were performed as described for the TNF-α cytolysis assays. TNF-α and anti-Fas antibody resistance was measured as the percent survival of mutant E1B 19K-expressing cells compared with the survival of the wild-type E1B 19K-expressing cells after TNF-α or anti-Fas antibody treatment.

*pm*44R, *pm*55, and *pm*123 (Fig. 8). The *pm*7 mutant allowed for partial resistance to TNF-α but was categorized as defective on the basis of the criterion described above (Fig. 8). All other mutants displayed extreme sensitivity to TNF-α treatment (Fig. 8). The abilities of the E1B 19K mutant proteins to confer resistance to anti-Fas antibody were measured in a way similar to that in which their abilities to confer TNF-α resistance were measured. However, the anti-Fas antibody killing assay was more stringent than the TNF-α cytolysis assay because the incubation period in the antibody assay was much longer than that in the TNF cytolysis assay (see Materials and Methods). Wild-type E1B 19K protein produced resistance to anti-Fas antibody, as did *pm*28, *pm*44R, and *pm*123 (Fig. 8). All other mutants were defective (Fig. 8). Note that the *pm*7 and *pm*55 mutants, which conferred partial transforming activity and resistance to TNF-α, were more defective in inhibiting anti-Fas antibody cytolysis than in the TNF-α assays. The increased stringency of the anti-Fas antibody cytolysis assay is likely responsible for ablating even the partial activity displayed by these two mutants. E1B 19K mutants which retained transforming activity also retained the ability to block apoptosis mediated by TNF-α and Fas antigen (*pm*28, *pm*44R, and *pm*123), whereas mutants that lost transforming capability became sensitive to apoptosis mediated by TNF-α and Fas antigen (*pm*7, *pm*44, *pm*51, *pm*55, *pm*73, *pm*81, *pm*87, *pm*91, *pm*102, and *pm*113). These results showed that the transforming ability and antiapoptotic activity of the E1B 19K protein cosegregate and that the region primarily responsible for this ability and activity is located between amino acid residues 44 and 113 in the central and amino-terminal domains. There may be involvement of the extreme amino terminus in E1B 19K function as well, since the *pm*7 mutant was defective in these functional assays. Restoration of a positive charge to the *pm*44 mutant, irrespective of whether the residue at amino acid 44 was arginine or lysine, resulted in reversion of the functional activity of the E1B 19K protein to wild-type levels. This indicates that conservation of charge at this location is important for E1B 19K function.

Expression of mutant E1B 19K proteins in HeLa cells. To examine the possibility that defective E1B 19K mutant proteins may be expressed at levels that are insufficient for E1B 19K protein activity, plasmid vectors carrying mutant or wild-type E1B 19K cDNA expressed under the control of the cytomegalovirus promoter were transiently expressed in HeLa cells, and mutant protein levels were monitored by Western blot analysis. Compared with the level of wild-type E1B 19K protein, mutant proteins *pm*123, *pm*44R, *pm*7, *pm*28, *pm*55, and *pm*102 were detected at high levels and *pm*51 and *pm*87 were detected at intermediate levels (Fig. 9). The mutants *pm*44, *pm*73, *pm*81, *pm*91, and *pm*113 were undetectable by this method (Fig. 9). However, all the mutant proteins were detected by indirect immunofluorescence (data not shown). The transient expression assay used is not a sensitive method for protein expression because of the possibility of low transfection efficiency, and this may be the cause of the low protein levels observed. On the other hand, the *pm*44, *pm*73, *pm*81,

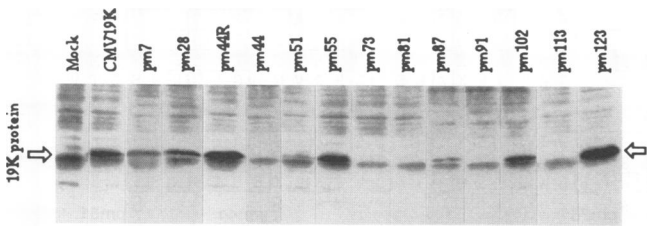

FIG. 9. Expression of E1B 19K mutant proteins in HeLa cells. Wild-type and mutant E1B 19K plasmid expression vectors were transfected into HeLa cells by the calcium phosphate-DNA precipitation method, and E1B 19K proteins were transiently expressed. The wild-type E1B 19K plasmid was used as a positive control for E1B 19K protein expression, whereas pm7fs, which has a frameshift mutation at codon 7 of the E1B 19K coding region and does not express the E1B 19K protein, was used as a negative control. A 20-μg sample of whole-cell extract for each transfection was analyzed for E1B 19K expression by Western blotting. The position of the E1B 19K protein is indicated.

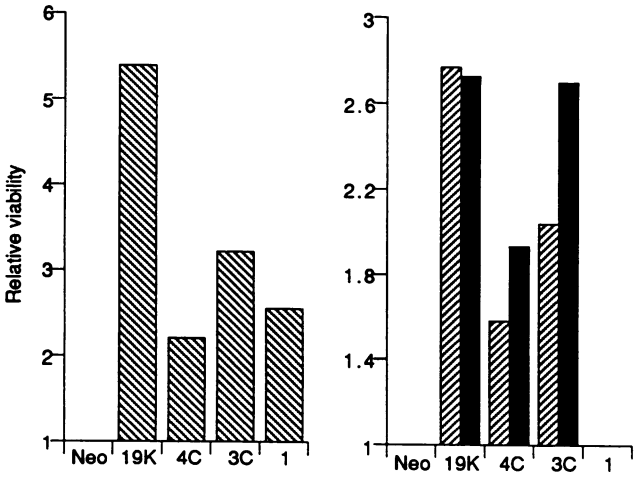

FIG. 10. Bcl-2 confers resistance to apoptosis mediated by TNF-α and Fas antigen. Stable HeLa cell lines expressing Bcl-2, i.e., Bcl-2 3C, Bcl-2 4C, and Bcl-2 1, or the E1B 19K protein, i.e., 4B (indicated by 19K), and the neomycin-resistant control cell line that expresses only endogenous Bcl-2 (Neo) were treated with TNF-α and anti-Fas antibody as described in Materials and Methods. Resistance to TNF-α or anti-Fas antibody treatment is expressed as relative viability compared with the viability of the neomycin-resistant control cell line. ▧, cells treated with TNF-α; ▨, cells treated with 5 μg of anti-Fas antibody per ml; ■, cells treated with 10 μg of anti-Fas antibody per ml.

*pm*91, and *pm*113 mutants may substantially affect the stability of the E1B 19K protein, thereby causing the mutant proteins to fail to accumulate. Therefore, the biological activity of these particular mutants likely reflects alterations in protein structure and/or stability, producing low E1B 19K protein levels rather than a loss of the functional region.

Bcl-2 expression inhibits apoptosis induced by TNF-α and Fas antigen. Bcl-2 has been shown to inhibit TNF-α and anti-Fas antibody cytotoxicity in some situations (22, 38) but not in others (68). The capacity of Bcl-2 to inhibit apoptosis may depend on the presence of cellular factors and/or on the cell type. In HeLa cells, the E1B 19K protein is sufficient to inhibit apoptosis induced by TNF-α and Fas antigen, which demonstrates that the capacity to inhibit apoptosis induced by TNF-α and Fas antigen exists in these cells. Thus, HeLa cells could be used as an appropriate model for comparison of Bcl-2 and E1B 19K protein activity.

The viabilities of Bcl-2-expressing HeLa cell lines and the Neo^r control line in TNF-α and anti-Fas antibody cytotoxicity assays were examined. The levels of TNF-α and anti-Fas antibody resistance of these cell lines were then compared with those of an E1B 19K protein-expressing HeLa cell line. The neomycin-resistant control cell line was susceptible to TNF-α killing, while the cell line expressing the E1B 19K protein maintained viability at a level greater than five times that of the Neo^r negative control cell line (Fig. 10). The Bcl-2-expressing cell lines 3C, 4C, and 1 had survival rates in response to TNF-α treatment that were increased at least twofold over that of the Neo^r control (Fig. 10). Resistance in all three lines was lower than that of the E1B 19K protein-expressing HeLa cell line (Fig. 10). Similarly, Bcl-2 expression confers resistance to Fas antigen-mediated apoptosis. Bcl-2 1 (low-level Bcl-2 expression) and the Neo^r control line were susceptible to anti-Fas antibody killing, whereas the Bcl-2 3C and Bcl-2 4C cell lines (high-level Bcl-2 expression) showed a degree of resistance to Fas antigen-mediated apoptosis similar to that of the E1B 19K protein-expressing cell line (Fig. 10). Thus, Bcl-2 functions similarly to the E1B 19K protein in the cellular context in which the E1B 19K protein has been demonstrated to inhibit apoptosis induced by TNF-α and anti-Fas antibody.

Sequence similarities between the E1B 19K protein and the Bcl-2 family members. Since E1B 19K and Bcl-2 are qualitatively indistinguishable with regard to function, a comparison of their amino acid sequences was performed to assess any potential sequence homology. It is now clear that Bcl-2 is a member of a growing family of related proteins identified in eukaryotic cells, DNA viruses, and the nematode *Caenorhabditis elegans*. Some have been demonstrated to function in regulating apoptosis, while the roles of others have not yet been established. These proteins include the mammalian Bax (50), Bcl-x_L (5), Bcl-x_S (5), Mcl-1 (32), and A1 (35) proteins, the LMW-5HL (47) and BHRF1 (19) proteins from African swine fever virus and EBV, respectively, and the Ced-9 protein from *C. elegans* (21).

In multiple sequence alignments, the degree of homology between the E1B 19K protein and Bcl-2 family members was limited, but the region of greatest homology, which corresponds to the region between amino acids 39 and 123 of the E1B 19K protein, was also the region most homologous among the other Bcl-2 family members (Fig. 11). There is a 40% conservation (identity) in amino acid sequence between the E1B 19K protein and one or more of the other Bcl-2 family members (Fig. 11). From sequence conservation data and mutational analysis, we know that this region of homology overlaps the region of the E1B 19K protein that is important for functional and/or structural integrity, i.e., residues 44 to 113 (see above). Note that many conserved residues in the E1B 19K protein that are important for E1B 19K protein function and/or structure as determined by mutagenesis are also conserved in the Bcl-2 family members. For example, the glycine 87 residue is invariant in all 12 adenovirus serotypes and results in complete loss of E1B 19K protein function when replaced by an alanine residue in the *pm*87 mutation (Fig. 8) (80). This glycine is also conserved in all the Bcl-2 family members. However, not all conserved residues in the E1B 19K protein and the Bcl-2 family of proteins are important. Tryptophan 123, which occurs outside the central conserved region of the E1B 19K protein, is also highly conserved in the E1B 19K protein and the Bcl-2 family members, but when it is mutated

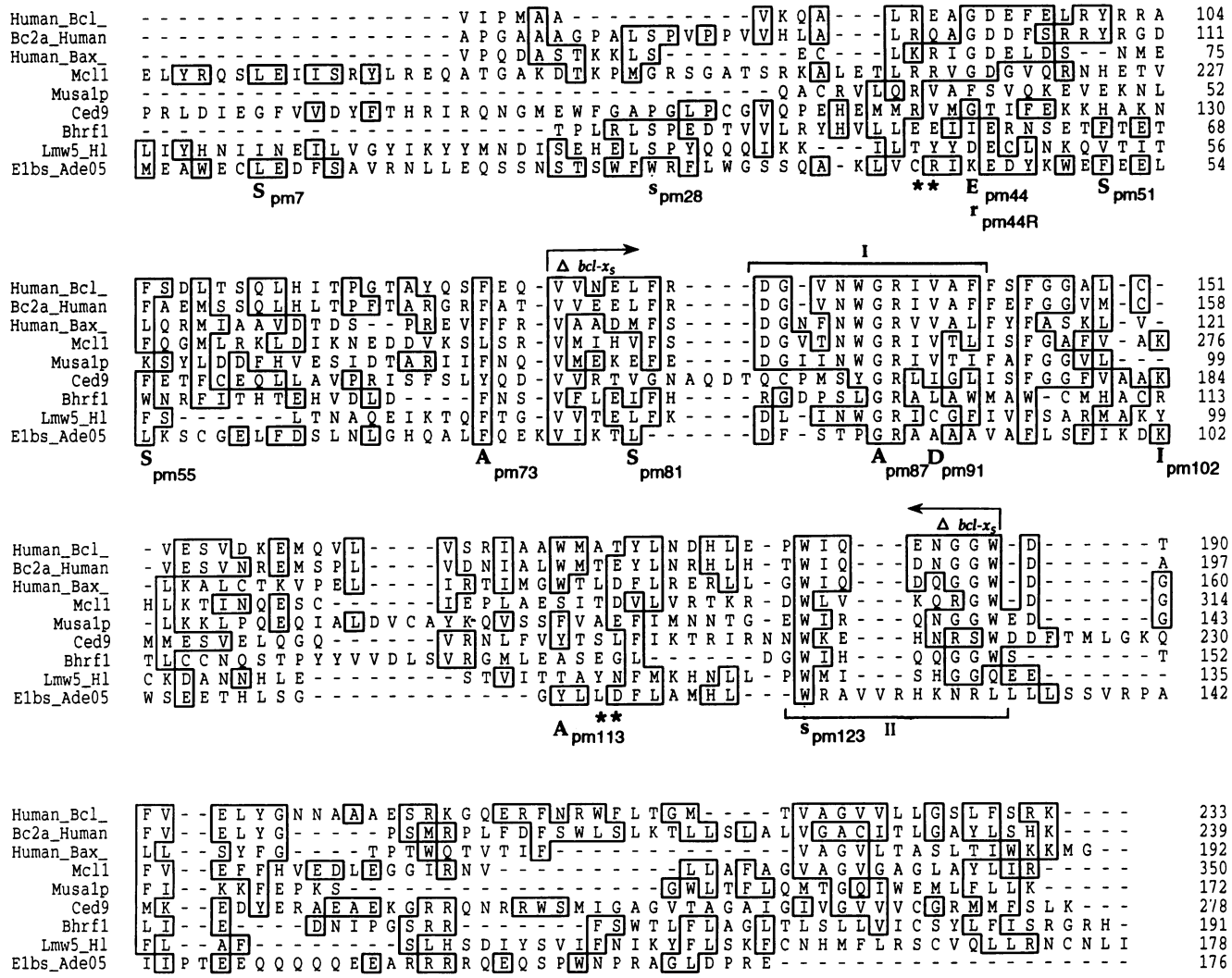

FIG. 11. Amino acid sequence homology between Bcl-2 family members. The E1B 19K amino acid sequence was compared with those of the Bcl-2 family members. All amino acid sequences were retrieved from the GenBank and EMBL data banks. Sequence alignments were performed by using Pileup software (Genetics Computer Group) and were represented by using Prettyplot (European Molecular Biological Laboratory software). Human Bcl, human Bcl-x_L protein; Bc2a Human, human Bcl-2 protein; Human Bax, human Bax protein; Mcl1, murine Mcl-1 protein; Musalp, murine A1 protein; Ced9, Ced-9 protein from *C. elegans*; Bhrf1, BHRF1 protein from EBV; Lmw5 H1, African swine fever virus LMW5-HL protein; E1bs Ade05, Ad5 E1B 19K protein. Boxed amino acids represent conserved residues (59). Positions of E1B 19K missense mutations, and the names of the mutations, are indicated below the E1B 19K protein sequence: capital letters indicate defective mutants, and lowercase letters indicate nondefective mutants. Asterisks indicate the approximate boundaries of the 19K protein conserved functional region. The conserved region absent from *bcl-*x_s is indicated by arrows. Conserved regions I and II in Bcl-2 family members are indicated.

to serine, it does not measurably affect E1B 19K protein function (*pm*123 mutation; Fig. 8). There are two conserved regions observed among Bcl-2 family members, indicated by I and II in Fig. 11. Of these, region I displays a high level of homology with the E1B 19K sequence (Fig. 11). Because the highest degree of conservation among the E1B 19K proteins occurs in this region, and because this is the region which displays the highest level of homology among the Bcl-2 family members, important functional and/or structural information may be provided by these sequences. In particular, glycine 87 may be important for E1B 19K protein function and/or structure. In contrast, homology in conserved region II of the Bcl-2 family members is not conserved in the E1B 19K sequence (Fig. 11), and mutation of the conserved tryptophan (*pm*123) does not affect E1B 19K function (Fig. 8). Determination of the functional significance of conserved regions I and II of Bcl-2 will require mutational analysis of these regions.

DISCUSSION

The adenovirus E1B 19K protein has the capacity to inhibit apoptosis in a variety of situations, and it possesses transforming ability. As previously described, the E1B 19K protein inhibits apoptosis induced by p53 (10, 54), TNF-α (13, 80), and Fas antigen (18). The E1B 19K protein also cooperates with E1A to transform BRK cells (41, 54, 75, 80). Functional analysis of the E1B 19K protein revealed that it may possess a single antiapoptosis function which can also account for its

transforming ability, since the ability of the E1B 19K protein to inhibit TNF-α cytotoxicity cosegregates with its ability to cooperate with E1A in transformation (80). Further mutagenesis studies showed that the ability of E1B 19K to inhibit apoptosis mediated by both TNF-α and Fas antigen cosegregates with its transforming ability (Fig. 8). Thus, the primary biological function of the E1B 19K protein may indeed be inhibition of apoptosis.

Apoptosis may be an important defense mechanism at the cellular level. Many mammalian cells undergo apoptosis when exposed to viruses (reviewed in references 72 and 77). Premature death of infected cells may limit virus propagation to surrounding uninfected cells by minimizing virus production. Because of this, viruses encode gene products which function to inhibit apoptosis to defeat this host cell suicide defense mechanism. The adenovirus E1B 19K protein provides such an antiapoptotic function for maximizing adenovirus production.

Adenoviruses represent only one example of viruses that possess antiapoptotic activity. Another virus, EBV, is capable of establishing persistent infection in human B lymphocytes by expression of latent proteins that protect these B cells from apoptosis (16). The mechanism for this EBV activity involves expression of the EBV LMP-1, which induces Bcl-2 protein expression (20). Expression of Bcl-2 has also been shown to allow persistent Sindbis virus infection of mammalian cells, whereas infection with this virus would otherwise be cytotoxic to these cells (34). Thus, Bcl-2 may play a role in the inhibition of apoptosis during viral infection.

In addition to modulation of endogenous Bcl-2, viral homologs of the Bcl-2 protein have also been found. EBV encodes a gene product, the BHRF1 protein, which has sequence homology to Bcl-2 (Fig. 11), shows a localization pattern similar to that of Bcl-2, and inhibits apoptosis in a fashion analogous to that of Bcl-2 (19). Since the Bcl-2 protein appears to be an important regulator in the mammalian antiapoptotic pathway, it is not surprising that a virus which developed the capacity to inhibit apoptosis encodes a gene product that mimics Bcl-2 in function. Another viral protein, the LMW5-HL protein from African swine fever virus, also contains limited sequence homology to the Bcl-2 protein, although it is not yet known whether it has the ability to regulate apoptosis (47). Bcl-2 may thus be a common target for viral attempts to mimic a host antiapoptotic mechanism. This raises the possibility that Bcl-2 homologs may exist in other viruses capable of evading the host apoptotic response to viral infection. The E1B 19K protein is certainly the adenoviral functional equivalent, and perhaps the structural homolog, of Bcl-2.

The E1B 19K and Bcl-2 proteins are functional counterparts on the basis of a number of observations. The E1B 19K protein can functionally replace Bcl-2 in a wide range of circumstances. This was demonstrated by the ability of both proteins to inhibit apoptosis induced by diverse factors like p53, TNF-α, and anti-Fas antibody (6, 10, 54). Bcl-2 had also been reported to have the ability to confer resistance to TNF-α cytolysis in other cellular settings (22), and its expression had been shown to be downregulated in B cells from chronic lymphocytic leukemias upon induction of anti-APO-1 (anti-Fas) antibody-mediated apoptosis, suggesting that downregulation of the Bcl-2 protein levels allowed the B cells from chronic lymphocytic leukemias to undergo APO-1-mediated apoptosis (38). There are still other situations in which Bcl-2 has been shown to inhibit apoptosis (26, 49, 60, 63, 69). It is not yet known whether the E1B 19K protein also functions to inhibit apoptosis under those circumstances. E1B 19K and Bcl-2 are also functionally interchangeable in the same cellular settings. In HeLa cells, where Bcl-2 levels are low and E1B 19K expression is sufficient to rescue apoptosis induced by adenovirus infection, TNF-α, or Fas antigen, artificial overexpression of Bcl-2 can inhibit apoptosis caused by these factors. Furthermore, E1B 19K and Bcl-2 are functionally complementary in inhibiting p53-mediated apoptosis in the BRK system, as described earlier (6, 10, 54). On the basis of the functional resemblance it is not surprising that the E1B 19K and Bcl-2 proteins also share similar localizations to cytoplasmic and nuclear membranes (43, 73) and that they share a modest degree of sequence homology.

Although the sequence homology between the 19K protein and Bcl-2 family members is limited, the specific region that is conserved, conserved region I in Bcl-2 and amino acids 44 to 113 of E1B 19K (Fig. 11), may be significant for regulation of apoptosis, although proof of this significance will require the identification of the biochemical mechanisms of inhibition of apoptosis in both cases. The E1B 19K and Bcl-2 proteins may interact with similar, if not the same, cellular proteins. Cellular Bcl-2-binding proteins, which include Bcl-2 itself, Bax (50), and R-*ras* (11), have begun to be identified. The identification of cellular E1B 19K-binding proteins is also in progress, and it should provide insight into how apoptosis is regulated.

Although Bcl-2 and the E1B 19K protein appear to be functionally and structurally homologous, Bcl-2 appears to be less potent than the E1B 19K protein in inhibiting apoptosis induced during productive infection and apoptosis induced by TNF-α and Fas antigen. The E1B 19K protein may possess either additional or stronger activity than Bcl-2 that enables the E1B 19K protein to more efficiently inhibit apoptosis. This characteristic of the E1B 19K protein may be essential during adenovirus infection and may account for differences in amino acid sequence between the E1B 19K protein and Bcl-2 family members. Therefore, it would not be unexpected that a viral version of a cellular protein would have greater ability to inhibit apoptosis. Furthermore, it has been shown that Bcl-2 itself is regulated by other members of the Bcl-2 family of proteins. Given that there is an entire family of Bcl-2-related proteins with high levels of sequence homology to Bcl-2, the possibility that E1B 19K may be even more similar to another member of the Bcl-2 protein family yet to be found cannot be eliminated.

The mechanism of apoptosis inhibition by E1B 19K is currently being investigated. Unlike EBV LMP-1 expression, E1B 19K expression does not cause deregulation of Bcl-2 expression. E1B 19K activity in mammalian cells would likely be similar to that of BHRF1, since both of these proteins are Bcl-2 homologs. We have also observed that both E1B 19K and Bcl-2 modify p53 function (6, 10, 54). However, E1B 19K and Bcl-2 do not block induction of p53 levels by E1A, nor do they affect localization of p53 (see above; also see reference 6). The E1B 19K protein does not bind p53 (70), suggesting that inhibition of p53-dependent apoptosis by E1B 19K occurs downstream of p53. Since both E1B 19K and Bcl-2 affect p53 function, it is possible that other viral Bcl-2 homologs, like BHRF1, would possess similar activities.

p53 function may be induced in response to viral infections, since it has been shown to be an inducer of apoptosis in many circumstances (9, 10, 37, 61, 84). Induction of p53 levels and apoptosis by E1A (36) (Fig. 1) created the neccessity for other adenoviral proteins to inhibit p53 function. The E1B 55K protein has been shown to complex with the p53 protein (57) and inhibit the activity of p53 as a transcription factor (83). In infected human cells, the E1B 55K protein is complexed with a 25-kDa protein from the adenovirus E4 region and is apparently not bound to p53 (56). The E1B 19K protein may

have evolved as an alternate Bcl-2-like activity to circumvent p53 function. Other viral proteins, the simian virus 40 large T antigen (57) and the human papillomavirus E6 protein (58), have also been shown to complex with the p53 protein to inhibit its functions. Thus, p53 appears to be a common target for inactivation by viral proteins. This suggests that apoptosis may be a common cellular pathway for defense against viral infections and that p53 is a key component of this pathway.

ACKNOWLEDGMENTS

We thank S. Korsmeyer, A. Levine, and P. Krammer for generously providing the antibodies and M. Mathews for cell lines.

This work was supported by grants from the NIH (CA53370 and CA60088), the American Cancer Society (MV71975), and the New Jersey Commission on Science and Technology to E.W. S.-K.C. was supported by an NIH biotechnology predoctoral fellowship. L.R. was supported by a C.A.B.M. predoctoral fellowship.

REFERENCES

1. **Aderka, D., H. Holtman, L. Toker, T. Hahn, and D. Wallach.** 1986. Tumor necrosis factor induction by Sendai virus. J. Immunol. **136**:2938–2942.
2. **Allsopp, T. E., S. Wyatt, H. F. Paterson, and A. M. Davies.** 1993. The proto-oncogene *bcl-2* can selectively rescue neurotrophic factor-dependent neurons from apoptosis. Cell **73**:295–307.
3. **Barker, D. D., and A. J. Berk.** 1987. Adenovirus proteins from both E1B reading frames are required for transformation of rodent cells by viral infection and DNA transfection. Virology **156**:107–121.
4. **Berk, A. J., and P. A. Sharp.** 1978. Structure of adenovirus 2 early mRNAs. Cell **14**:695–711.
5. **Boise, L. H., M. Gonzalez-Garcia, C. E. Postema, L. Ding, T. Lindsten, L. A. Turka, X. Mao, G. Nuñez, and C. Thompson.** 1993. *bcl-x*, a *bcl-2*-related gene that functions as a dominant regulator of apoptotic death. Cell **74**:597–608.
6. **Chiou, S.-K., L. Rao, and E. White.** 1994. Bcl-2 blocks p53-dependent apoptosis. Mol. Cell. Biol. **14**:2556–2563.
7. **Chiou, S.-K., and E. White.** Unpublished data.
8. **Chow, L. T., T. R. Broker, and J. B. Lewis.** 1979. Complex splicing patterns of RNAs from the early regions of adenovirus 2. J. Mol. Biol. **134**:265–303.
9. **Clarke, A. R., C. A. Purdie, D. J. Harrison, R. G. Morris, C. C. Bird, M. L. Hooper, and A. H. Wyllie.** 1993. Thymocyte apoptosis induced by p53-dependent and independent pathways. Nature (London) **362**:849–852.
10. **Debbas, M., and E. White.** 1993. Wild-type p53 mediates apoptosis by E1A which is inhibited by E1B. Genes Dev. **7**:546–554.
11. **Fernandez-Sarabia, M., and James R. Bischoff.** 1993. Bcl-2 associates with the *ras*-related protein R-*ras* p23. Nature (London) **366**:274–275.
12. **Garcia, I., I. Martinou, Y. Tsujimoto, and J.-C. Martinou.** 1992. Prevention of programmed cell death of sympathetic neurons by the *bcl-2* proto-oncogene. Science **258**:302–304.
13. **Gooding, L. R., L. Aquino, P. J. Duerksen-Hughes, D. Day, T. M. Horton, S. Yei, and W. S. M. Wold.** 1991. The E1B 19,000-molecular-weight protein of group C adenoviruses prevents tumor necrosis factor cytolysis of human cells but not of mouse cells. J. Virol. **65**:3083–3094.
14. **Gooding, L. R., R. Ranheim, A. E. Tollefson, L. Aquino, P. Duerksen-Hughes, T. M. Horton, and W. S. M. Wold.** 1991. The 10,400- and 14,500-dalton proteins encoded by region E3 of adenovirus function together to protect many but not all mouse cell lines against lysis by tumor necrosis factor. J. Virol. **65**:4114–4123.
15. **Graham, F. L., J. Smiley, W. C. Russell, and R. Nairu.** 1977. Characteristics of a human cell line transformed by DNA from adenovirus type 5. J. Gen. Virol. **36**:59–77.
16. **Gregory, C. D., C. Dive, S. Henderson, C. A. Smith, G. T. Williams, J. Gordon, and A. B. Richardson.** 1991. Activation of Epstein-Barr virus latent genes protects human B cells from death by apoptosis. Nature (London) **349**:612–614.
17. **Haley, K. P., J. Overhauser, L. E. Babiss, H. S. Ginsberg, and N. C. Jones.** 1984. Transformation properties of type 5 adenovirus mutants that differentially express the E1A gene products. Proc. Natl. Acad. Sci. USA **81**:5734–5738.
18. **Hashimoto, S., A. Ishii, and S. Yonehara.** 1991. The E1B oncogene of adenovirus confers cellular resistance to cytotoxicity of tumor necrosis factor and monoclonal anti-Fas antibody. Int. Immunol **3**:343–351.
19. **Henderson, S., D. Huen, M. Rowe, C. Dawson, G. Johnson, and A. Rickinson.** 1993. Epstein-Barr virus-coded BHRF1 protein, a viral homologue of Bcl-2, protects human B cells from programmed cell death. Proc. Natl. Acad. Sci. USA **90**:8479–8483.
20. **Henderson, S., M. Rowe, C. Gregory, D. Croom-Crater, F. Wang, R. Longnecker, E. Keiff, and A. Rickinson.** 1991. Induction of Bcl-2 expression by Epstein-Barr virus latent membrane protein 1 protects infected B cells from programmed cell death. Cell **65**: 1107–1115.
21. **Hengartner, M., and H. R. Horvitz.** 1994. C. elegans cell survival gene *ced*-9 encodes a functional homolog of the mammalian proto-oncogene *bcl-2*. Cell **76**:665–676.
22. **Hennet, T., G. Bertoni, C. Richter, and E. Peterhans.** 1993. Expression of Bcl-2 protein enhances the survival of mouse fibrosarcoid cells in tumor necrosis factor-mediated cytotoxicity. Cancer Res. **53**:1456–1460.
23. **Herrmann, C. H., C. V. Dery, and M. B. Mathews.** 1987. Transactivation of host and viral genes by the adenovirus E1B 19K tumor antigen. Oncogene **2**:25–35.
24. **Herrmann, C. H., and M. B. Mathews.** 1989. The adenovirus E1B 19-kilodalton protein stimulates gene expression by increasing DNA levels. Mol. Cell. Biol. **9**:5412–5423.
25. **Hirt, B.** 1967. Selective extraction of polyoma DNA from infected mouse cultures. J. Mol. Biol. **26**:365–369.
26. **Hockenbery, D., G. Nuñez, C. Milliman, R. D. Schreiber, and S. Korsmeyer.** 1990. Bcl-2 is an inner mitochondrial membrane protein that blocks programmed cell death. Nature (London) **348**:334–336.
27. **Jones, N., and T. Shenk.** 1979. An adenovirus type 5 early gene function regulates expression of other viral genes. Proc. Natl. Acad. Sci. USA **76**:3665–3669.
28. **Kaczmarek, L., B. Ferguson, M. Rosenberg, and R. Baserga.** 1986. Induction of cellular DNA synthesis by purified adenovirus E1A proteins. Virology **152**:1–10.
29. **Kitchingman, G. R., and H. Westphal.** 1980. The structure of adenovirus 2 early nuclear and cytoplasmic RNAs. J. Mol. Biol. **137**:23–48.
30. **Kobayashi, N., Y. Hamamoto, N. Yamamoto, A. Ishii, M. Yonehara, and S. Yonehara.** 1990. Anti-fas monoclonal antibody is cytocidal to human immunodeficiency virus-infected cells without augmenting viral replication. Proc. Natl. Acad. Sci. USA **87**:9620–9624.
31. **Kohase, M., D. Henriksen-De Stefano, L. T. May, J. Vilcek, and P. B. Sehgal.** 1986. Induction of β_2-interferon by tumor necrosis factor: a hemoeostatic mechanism in the control of cell proliferation. Cell **45**:659–666.
32. **Kozopas, K. M., T. Yang, H. L. Buchan, P. Zhou, and R. W. Craig.** 1993. MCL1, a gene expressed in programmed myeloid cell differentiation, has sequence similarity to BCL-2. Proc. Natl. Acad. Sci. USA **90**:3516–3520.
33. **Kunkel, T. A., J. D. Roberts, and R. A. Zakour.** 1987. Rapid and efficient site-specific mutagenesis without phenotypic selection. Methods Enzymol. **154**:367–382.
34. **Levine, B., Q. Huang, J. T. Isaacs, J. C. Reed, D. E. Griffin, and J. M. Hardwick.** 1993. Conversion of lytic to persistent alphavirus infection by the *bcl-2* cellular oncogene. Nature (London) **361**: 739–742.
35. **Lin, E. Y., A. Orlofsky, M. S. Berger, and M. B. Prystowsky.** 1993. Characterization of A1, a novel hemopoietic-specific early response gene with sequence similarity to *bcl-2*. J. Immunol. **151**: 1979–1988.
36. **Lowe, S., and H. E. Ruley.** 1993. Stabilization of the p53 tumor suppressor is induced by adenovirus-5 E1A and accompanies apoptosis. Genes Dev. **7**:535–545.
37. **Lowe, S. W., E. M. Schmitt, S. W. Smith, B. A. Osborne, and T.**

Jacks. 1993. p53 is required for radiation-induced apoptosis in mouse thymocytes. Nature (London) **362**:847–849.
38. **Mapara, M. Y., R. Bargou, C. Zugek, H. Dohner, F. Ustaoglu, R. Jonker, P. H. Krammer, and B. Dorken.** 1993. APO-1 mediated apoptosis or proliferation in human chronic B lymphocytic leukemia: correlation with *bcl-2* oncogene expression. Eur. J. Immunol. **23**:702–708.
39. **McDonnell, T. J., N. Deane, F. M. Platt, G. Nunez, U. Jaeger, J. P. McKearn, and S. J. Korsmeyer.** 1989. *bcl-2*-immunoglobulin transgenic mice demonstrate extended B cell survival and follicular lymphoproliferation. Cell **57**:79–88.
40. **McDonnell, T. J., and S. J. Korsmeyer.** 1991. Progression from lymphoid hyperplasia to high-grade malignant lymphoma in mice transgenic for the t(14:18). Nature (London) **349**:254–256.
41. **McLorie, W., C. J. McGlade, D. Takayesu, and P. E. Branton.** 1991. Individual adenovirus E1B proteins induce transformation independently but by additive pathways. J. Gen. Virol. **72**:1467–1471.
42. **Mestan, J., S. Mittnacht, H. Hillen, D. Blohm, A. Moller, H. Jacobsen, and H. Kirchner.** 1986. Antiviral effects of recombinant tumor necrosis factor *in vitro*. Nature (London) **323**:816–819.
43. **Monagan, P., D. Robertson, T. A. Amos, M. J. Dyer, D. Y. Mason, and M. F. Greaves.** 1992. Ultrastructural localization of Bcl-2 protein. J. Histochem. Cytochem. **40**:1819–1825.
44. **Montell, C., G. Courtois, C. Eng, and A. Berk.** 1984. Complete transformation by adenovirus 2 requires both E1A proteins. Cell **36**:951–961.
45. **Moran, E.** 1993. E1A/T antigen/E7 and the cell cycle. Curr. Opin. Gen. Dev. **3**:63–70.
46. **Moran, E., T. Grodzicker, R. J. Roberts, M. B. Mathews, and B. Zerler.** 1986. Lytic and transforming functions of individual products of the adenovirus E1A gene. J. Virol. **57**:765–775.
47. **Neilan, J. G., Z. Lu, C. L. Afonszo, G. F. Kutish, M. D. Sussman, and D. L. Rock.** 1993. An African swine fever virus gene with similarity to the proto-oncogene *bcl-2* and the Epstein-Barr virus gene *BHRF1*. J. Virol. **67**:4391–4394.
48. **Nuñez, G., D. Hockenbery, T. J. McDonnell, C. M. Sorensen, and S. J. Korsmeyer.** 1991. Bcl-2 maintains B cell memory. Nature (London) **353**:71–73.
49. **Nuñez, G., L. London, D. Hockenbery, J. P. McKearn, and S. J. Korsmeyer.** 1990. Deregulated *bcl-2* gene expression selectively prolongs survival of growth factor-deprived hematopoietic cell lines. J. Immunol. **144**:3602–3610.
50. **Oltvai, Z. N., C. L. Millman, and S. J. Korsmeyer.** 1993. Bcl-2 heterodimerizes in vivo with a conserved homolog, Bax, that accelerates programmed cell death. Cell **74**:609–619.
51. **Pericaudet, M., G. Akusjarvi, A. Virtanen, and U. Pettersson.** 1979. Structure of two spliced mRNAs from the transforming region of human subgroup C adenoviruses. Nature (London) **281**:694–696.
52. **Pilder, S., J. Logan, and T. Shenk.** 1984. Deletion of the gene encoding the adenovirus 5 early region 1B 21,000-molecular-weight polypeptide leads to degradation of viral and host cell DNA. J. Virol. **52**:664–671.
53. **Raff, M. C.** 1992. Social controls on cell survival and cell death. Nature (London) **356**:398–400.
54. **Rao, L., M. Debbas, P. Sabbatini, D. Hockenbery, S. Korsmeyer, and E. White.** 1992. The adenovirus E1A proteins induce apoptosis which is inhibited by the E1B 19K and Bcl-2 proteins. Proc. Natl. Acad. Sci. USA **89**:7742–7746.
55. **Sabbatini, P., L. Rao, and E. White.** Modulation of p53-mediated transcription, apoptosis, and growth arrest by the adenovirus E1B 19K protein. Submitted for publication.
56. **Sarnow, P., P. Hearing, C. W. Anderson, D. N. Halbert, T. Shenk, and A. J. Levine.** 1984. Adenovirus early region 1B 58,000-dalton tumor antigen is physically associated with an early region 4 25,000-dalton protein in productively infected cells. J. Virol. **49**:692–700.
57. **Sarnow, P., Y. S. Ho, J. Williams, and A. J. Levine.** 1982. Adenovirus E1b-58 kd tumor antigen and SV40 large tumor antigen are physically associated with the same 54 kd cellular protein in transformed cells. Cell **28**:387–394.
58. **Scheffner, M., B. A. Werness, J. M. Hulbregtse, A. J. Levine, and P. M. Howley.** 1990. The E6 oncoprotein encoded by human papillomavirus types 16 and 18 promotes the degradation of p53. Cell **63**:1129–1136.
59. **Schwartz, R. M., and M. O. Dayhoff.** 1979. Dayhoff table, p. 353–358. *In* M. O. E. Dayhoff (ed.), Atlas of protein sequence and structure. National Biomedical Research Foundation, Washington, D.C.
60. **Sentman, C. L., J. R. Shutter, D. Hockenbery, O. Kanagawa, and S. J. Korsmeyer.** 1991. *bcl-2* inhibits multiple forms of apoptosis but not negative selection in thymocytes. Cell **67**:879–888.
61. **Shaw, P., R. Bovey, S. Tardy, R. Sahli, B. Sordat, and J. Costa.** 1992. Induction of apoptosis by wild-type p53 in a human colon tumor derived cell line. Proc. Natl. Acad. Sci. USA **89**:4495–4499.
62. **Stabel, S., P. Argos, and L. Philipson.** 1985. The release of growth arrest by microinjection of adenovirus E1A DNA. EMBO J. **4**:2329–2336.
63. **Strasser, A., A. W. Harris, and S. Cory.** 1991. *bcl-2* transgene inhibits T cell death and perturbs thymic self-censorship. Cell **67**:889–899.
64. **Subramanian, T., M. Kuppuswamy, J. Gysbers, S. Mak, and G. Chinnadurai.** 1984. 19-kDa tumor antigen coded by early region E1b of adenovirus 2 is required for efficient synthesis and for protection of viral DNA. J. Biol. Chem. **259**:11777–11783.
65. **Takemori, N., C. Cladaras, B. Bhat, A. J. Conley, and W. S. M. Wold.** 1984. *cyt* gene of adenoviruses 2 and 5 is an oncogene for transforming function in early region E1B and encodes the E1B 19,000-molecular-weight polypeptide. J. Virol. **52**:793–805.
66. **Tsujimoto, Y., and C. M. Croce.** 1986. Analysis of the structure, transcripts, and protein products of *bcl-2*, the gene involved in human follicular lymphoma. Proc. Natl. Acad. Sci. USA **83**:5214–5218.
67. **Turtinen, L. W., A. Assimacopoulos, and A. T. Haase.** 1989. Increased monokines in cytomegalovirus infected myelomonocytic cell cultures. Microb. Pathog. **7**:135–145.
68. **Vanhaesebroeck, B., J. C. Reed, D. DeValck, J. Grooten, T. Miyashita, S. Tanaka, R. Beyaert, F. VanRoy, and W. Fiers.** 1993. Effect of *bcl-2* proto-oncogene expression on cellular sensitivity to tumor necrosis factor-mediated cytotoxicity. Oncogene **8**:1075–1081.
69. **Vaux, D. L., S. Cory, and T. M. Adams.** 1988. *bcl-2* promotes the survival of haemopoietic cells and cooperates with *c-myc* to immortalize pre-b cells. Nature (London) **335**:440–442.
70. **Verwaerde, P., and E. White.** Unpublished observations.
71. **Watanabe-Fukunaga, R., C. I. Brannan, N. G. Copeland, N. A. Jenkins, and S. Nagata.** 1992. Lymphoproliferation disorder in mice explained by defects in Fas antigen that mediates apoptosis. Nature (London) **356**:314–317.
72. **White, E.** 1993. Regulation of apoptosis by the transforming genes of the DNA tumor virus adenovirus. Proc. Soc. Exp. Biol. Med. **204**:30–39.
73. **White, E., S. H. Blose, and B. Stillman.** 1984. Nuclear envelope localization of an adenovirus tumor antigen maintains the integrity of cellular DNA. Mol. Cell. Biol. **4**:2865–2875.
74. **White, E., and R. Cipriani.** 1989. Specific disruption of intermediate filaments and the nuclear lamina by the 19-kDa product of the adenovirus E1B oncogene. Proc. Natl. Acad. Sci. USA **86**:9886–9890.
75. **White, E., and R. Cipriani.** 1990. Role of adenovirus E1B proteins in transformation: altered organization of intermediate filaments in transformed cells that express the 19-kilodalton protein. Mol. Cell. Biol. **10**:120–130.
76. **White, E., R. Cipriani, P. Sabbatini, and A. Denton.** 1991. The adenovirus E1B 19-kilodalton protein overcomes the cytotoxicity of E1A proteins. J. Virol. **65**:2968–2978.
77. **White, E., and L. R. Gooding.** 1994. Regulation of apoptosis by human adenoviruses, p. 111–141. *In* L. D. Tomei and F. O. Cope (ed.), Apoptosis II: the molecular basis for apoptosis in disease. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
78. **White, E., T. Grodzicker, and B. W. Stillman.** 1984. Mutations in the gene encoding the adenovirus early region 1B 19,000-molecular-weight tumor antigen cause degradation of chromosomal DNA. J. Virol. **52**:410–419.
79. **White, E., L. Rao, S.-K. Chiou, C.-C. Tseng, P. Sabbatini, M.**

Gonzalez, and P. Verwaerde. 1994. Regulation of apoptosis by the transforming gene products of adenovirus, p. 47–62. *In* E. Mihich and R. T. Shimke (ed.), Apoptosis. Plenum Press, New York.

80. **White, E., P. Sabbatini, M. Debbas, W. S. M. Wold, D. I. Kusher, and L. Gooding.** 1992. The 19-kilodalton adenovirus E1B transforming protein inhibits programmed cell death and prevents cytolysis by tumor necrosis factor α. Mol. Cell. Biol. **12:**2570–2580.
81. **White, E., and B. Stillman.** 1987. Expression of the adenovirus E1B mutant phenotypes is dependent on the host cell and on synthesis of E1A proteins. J. Virol. **61:**426–435.
82. **Wong, G. H., and D. V. Goeddel.** 1986. Tumor necrosis factors α and β inhibit virus replication and synergize with interferons. Nature (London) **323:**819–822.
83. **Yew, P. R., and A. J. Berk.** 1992. Inhibition of p53 transactivation required for transformation by adenovirus early 1B protein. Nature (London) **357:**82–85.
84. **Yonish-Rouach, E., D. Resnitzky, J. Lotem, L. Sachs, A. Kimchi, and M. Oren.** 1991. Wild-type p53 induces apoptosis of myeloid leukaemic cells that is inhibited by interleukin-6. Nature (London) **352:**345–347.