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Human Cytomegalovirus IE1 and IE2 Proteins Block Apoptosis

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Human cytomegalovirus-infected fibroblasts are resistant to the induction of apoptosis by superinfection with a mutant adenovirus unable to produce the viral E1B 19-kDa protein that normally causes an E1A protein-mediated apoptotic response. Two cytomegalovirus gene products that block apoptosis were identified. The IE1 and IE2 proteins each inhibit the induction of apoptosis by tumor necrosis factor α or by the E1B 19-kDa-protein-deficient adenovirus but not by irradiation with UV light. Our results suggest a new physiological role for the IE1 and IE2 proteins in the human cytomegalovirus replication cycle.

Human cytomegalovirus (HCMV) is a widespread human pathogen (reviewed in references 2 and 19). HCMV infections are generally asymptomatic in healthy children and adults, although they are responsible for some cases of mononucleosis and for transfusion disease in some people who receive HCMV-contaminated blood products. HCMV can cause serious disease in unborn children and in immunocompromised individuals. It is the leading viral cause of birth defects, a major life-threatening opportunistic infection in AIDS, and a common posttransplantation complication in allograft recipients.

The first set of viral genes to become active within HCMV-infected cells are the immediate-early genes (reviewed in references 31 and 32). The most abundantly expressed products of this first set of genes are termed the immediate-early 1 and 2 proteins (IE1 and IE2). These proteins are expressed from a single transcription unit, and they share 85 amino acids at their amino termini. Transient transfection assays indicate that the IE1 and IE2 proteins regulate transcription. IE2 (579 amino acids) is a promiscuous *trans*-activator. It is thought to activate, at least in part, through its ability to bind to the TATA-binding protein, transcription factor IIB, and Sp1 (3, 14, 26). IE2 negatively regulates its own promoter (40) by binding directly to a *cis*-acting repression site located between the initiation site and the TATA box (24, 27). The IE1 protein (491 amino acids) activates transcription through some but not all NF-κB sites (44), and it cooperates with the IE2 protein to synergistically activate many target promoters (23, 28, 50). The ability of the IE1 and IE2 proteins to regulate expression in short-term assays has led to the view that these proteins regulate transcription of both viral and cellular genes within HCMV-infected cells.

The IE2 protein is reported to interact with the p53 tumor suppressor protein and block its ability to activate transcription (49), and it also can bind to the retinoblastoma susceptibility protein, modulating its activity (13, 48). These associations with tumor suppressor proteins are reminiscent of those mediated by the oncoproteins of DNA tumor viruses. The IE1 and IE2 proteins might induce cell cycle progression through these interactions, and this perturbation could be responsible for the ability of HCMV to induce DNA synthesis in infected cells (19, 22).

Here we report a new biological function for the IE1 and IE2 proteins. Both of these proteins can inhibit the induction of apoptosis by tumor necrosis factor α (TNF-α) or the adenovirus E1A proteins. The ability to block apoptosis is presumably a key element of the mechanism by which the virus replicates and persists, influencing the course of HCMV pathogenesis in its infected host.

MATERIALS AND METHODS

Plasmids, cell lines, and viruses. Mammalian expression plasmids for the IE1 and IE2 proteins, pCGN-IE1 and pCGN-IE2, respectively, were derived from pCGN (56), a vector designed to fuse a 9-amino-acid epitope of the influenza virus hemagglutinin (flu tag) to expressed proteins. An *Eco*RI to *Hin*dIII fragment from pGEX72 (23) containing a full-length IE1 cDNA was purified and blunted by treatment with the Klenow polymerase. A *Kpn*I 8-mer linker was added to its ends, and the fragment was ligated with pCGN, which had been cleaved with *Kpn*I. pCGN-IE2 was constructed in the same way as pCGN-IE1 except that IE2 cDNA which was purified from pGEX86 (23) was used. pCGN-IE1 and pCGN-IE2 express the IE1 and IE2 proteins, respectively, with a flu tag fused to their amino termini under the control of the HCMV immediate-early promoter. pPuro-IE1 and pPuro-IE2 were generated by inserting the IE1- and IE2-coding sequences extending from the *Eco*RI to *Sal*I sites of pGEX72 and pGEX-86, respectively, into the *Eco*RI and *Sal*I sites of pBabe-puro (33). These plasmids express the IE1 and IE2 proteins under the control of the Moloney murine leukemia virus long terminal repeat and contain a puromycin-resistance gene.

To ensure that translation of deleted IE1 and IE2 derivatives is terminated at the correct position and that the protein fragments are transported into the nucleus, two pCGN-based expression vectors were made. pCFN contains the simian virus 40 T antigen nuclear localization signal and was constructed by inserting the sequence 5′-CTAGGCCAAAAAAGAAGAGAAAGGTAT-3′ at the *Xba*I site of pCGN. $pCFN_2$ was derived from pCFN by inserting an *Nhe*I linker DNA containing an amber codon at the *Bam*HI site. $pCFN_2$-$IE1^{1–136}$, pCFN-$IE1^{137–491}$, and pCFN-$IE1^{389–491}$ (superscript numbers indicate the first and last amino acid position of the fragment) were constructed by inserting the *Eco*RI to *Bsp*HI, *Bsp*HI to *Sal*I, and *Msc*I to *Sal*I fragments, respectively, of the IE1 coding region from pGEX72 into the *Kpn*I site of $pCFN_2$ or pCFN. $pCFN_2$-$IE2^{1–135}$ and $pCFN_2$-$IE2^{136–579}$ were constructed by inserting the *Xba*I to *Sma*I and *Sma*I to *Sal*I fragments, respectively, of the IE2 coding region from pGEX86 into $pCFN_2$. pUHG19K was made by inserting a 0.6-kb *Eco*RI-*Hin*dIII fragment containing the adenovirus E1B 19-kDa coding sequence from pCMV-19K (63) into the *Bam*HI site of pUHG10-3 (11). The prokaryotic expression plasmid pGEX72-13 encodes a glutathione-*S*-transferase (GST)-$IE1^{346–419}$ fusion protein and was constructed by deleting an *Eco*RI to *Bgl*II fragment from pGEX72. pGEX72 was first cleaved with *Bgl*II, the resulting single-stranded ends were blunted by treatment with Klenow polymerase, and an *Eco*RI 8-mer linker was added. The plasmid was then digested again by using *Eco*RI and religated. pHBcl-2 was made by cloning the *Sac*I to *Bam*HI fragment (158 bp) of pSFFV-Bcl2 (18) into the *Bam*HI site of pBluescript KS. The human *bax* sequence from nucleotides 331 to 501 was cloned by reverse transcription-PCR and inserted into pGEM-T (Promega) to generate pHBax-1. pCMV-E1A, an E1A expression plasmid, has been described previously (36).

HeLa cell lines constitutively expressing HCMV IE1 (HIE1-1 and HIE1-2), or IE2 (HIE2-1 and HIE2-2) were generated by transfecting (1) pPuro-IE1 or pPuro-IE2 into HeLa cells and selecting individual clones that are resistant to 5 μg of puromycin per ml. Two cell lines, HMrk-1 and HMrk-2, which are resistant to puromycin but do not express IE1 or IE2 at detectable levels, were saved for use as control lines. An $IE1^{389–491}$-expressing cell line was generated by cotrans-

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fecting pCGN-IE1$^{389–491}$ and pGK-Hyg into HeLa cells and selecting for growth in 0.1 mg of hygromycin per ml. One hygromycin-resistant cell line, HMrk-3, which does not express IE1$^{389–491}$, was used as a control line. The adenovirus E1B 19-kDa-protein-expressing cell line, HE1B-19K, was generated by cotransfection of pUHG15-1neo (11) and pUHG19K into HeLa cells. Neomycin-resistant colonies were selected in medium containing 0.2 mg of G418 per ml. In all cases, drug-resistant clones were screened by Western blot (immunoblot) for expression of the exogenous proteins.

HeLa cells and derivatives of HeLa cells expressing viral proteins were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum and appropriate drugs for the selection of transfected plasmids. Human foreskin fibroblasts were propagated in DMEM supplemented with 10% fetal calf serum. All cells were maintained as monolayers at 37°C in a 5% CO_2 atmosphere.

HCMV (Towne strain) was used to infect cells at a multiplicity of infection of 5 PFU per cell. Phenotypically wild-type Ad5 (*dl*309) and Ad5 lacking the gene encoding the E1B 19-kDa protein (*dl*337 [39]) were used to infect cells at a multiplicity of infection of 30 PFU per cell.

Assays for apoptosis and immunofluorescence. Three different treatments were employed to induce apoptosis. First, TNF-α (1,000 U/ml) in combination with 25 μg of cycloheximide per ml was administered to cultures of HeLa cells, and apoptotic effects were examined 4 or 8 h later. Second, the E1B 19-kDa-protein-deficient Ad5 mutant, *dl*337, was used to infect cells and apoptosis was assayed 36 or 72 h later. Third, UV irradiation was performed at 40 J/m^2, and cells were examined 24 h later.

Apoptosis was monitored by terminal deoxynucleotidyltransferase (TdT) assays (10). Cells were grown either in tissue-culture dishes or directly on glass coverslips. Cells on coverslips were washed with phosphate-buffered saline (PBS) and fixed with freshly made 1% formaldehyde in PBS for 15 min at room temperature. They were rinsed with PBS and further fixed with 70% ethanol at −20°C for ≥1 h. When cells were grown in culture dishes, all cells (adherent and floating) were collected by trypsinization, fixed as described above, and allowed to dry on a coverslip. The coverslip with fixed cells was placed (upside down) in a dish containing 30 μl of TdT labeling mix (1× TdT reaction buffer, 2.5 mM $CoCl_2$, 0.1 mM dithiothreitol, 0.25 U of TdT per ml, 10 mM biotin-16-dUTP) (Boehringer Mannheim). The reaction was carried out at 37°C in a humidified incubator for 45 min. The cells were then rinsed briefly with PBST (PBS with 1% Triton X-100 and 0.5% Tween 20) and reacted with appropriate monoclonal antibodies (adenovirus E1A proteins [15], E2A protein [43], or the IE1 and IE2 proteins [Chemicon]) in PBST with 0.1% bovine serum albumin (BSA) at room temperature for 1 h. The samples were then washed three times with PBST and stained with a solution containing anti-mouse immunoglobulin G-rhodamine conjugate (Sigma) and avidin-fluorescein conjugate (Boehringer Mannheim) in PBST with 0.1% BSA at room temperature for 1 h. Samples were visualized by using a confocal microscope (Bio-Rad model MRC600).

Assays for gene expression. RNase protection assay (1) was employed to monitor mRNA levels. The antisense RNA probes for *bcl-2*, *bax*, or β-actin mRNA were prepared by in vitro transcription. pHBcl-2 template DNA linearized by cleavage with *Hin*dIII or pHBax-1 template linearized with *Not*I was transcribed with T7 polymerase, and *Bst*EII-cut pGEM3β-actin DNA was transcribed with SP6 polymerase. Total cellular RNA was purified from HeLa cells and cell lines constitutively expressing the different viral proteins. RNA (40 μg) was hybridized with the Bcl-2 or Bax probe, and 3 μg of RNA was hybridized with the β-actin probe.

Western blot analysis was used to confirm the expression of the IE1, IE2, and the E1B 19-kDa protein and to examine the steady-state levels of the Bcl-2 protein and Bax protein in various cell lines. MAB810 (Chemicon) recognizes both the IE1 and IE2 proteins. A monoclonal antibody, 1B12, was raised against the C terminus of IE1 as follows. The GST-IE1$^{346–419}$ fusion protein encoded by pGEX72-13 was purified from *Escherichia coli* through glutathione-Sepharose (47). The protein was eluted from the beads by using glutathione and used for immunizing mice. The hybridoma cell lines producing anti-IE1 monoclonal antibody were screened by using HCMV-infected cell extracts. Monoclonal antibodies to the E1B 19-kDa protein, Bax, and Bcl-2 were obtained from Oncogene Sciences, Santa Cruz Biotechnology, and Dako, respectively.

RESULTS

HCMV-infected human fibroblasts are protected from apoptosis induced by an adenovirus mutant lacking the gene encoding E1B 19-kDa protein. To explore the possibility that the HCMV genome encodes a gene product that antagonizes apoptosis, we asked whether primary human foreskin fibroblasts, the host cell generally used for HCMV propagation in the laboratory, became resistant to the induction of apoptosis after infection with HCMV. This experiment employed a mutant adenovirus, *dl*337 (39), which lacks the gene encoding the E1B 19-kDa protein. Mutations in this gene cause premature cellular destruction with extensive DNA fragmentation (39, 55, 64), and this phenotype results from the induction of apoptosis by the viral E1A proteins (25, 41). Apoptosis was monitored in our experiment by assaying DNA fragmentation, a diagnostic feature of apoptosis. Cells were fixed and chromatin was treated in situ with TdT to label the 3′-OH ends of DNA breaks with biotin-dUTP. The biotinylated product was then reacted with fluorescein-labeled avidin, and the reaction was visualized by immunofluorescence.

Human fibroblasts were infected with the adenovirus mutant, and 72 h later, all of the cells were confirmed to be infected by an immunofluorescence assay using a rhodamine-coupled antibody as the reporter for expression of the adenovirus E2A protein (Fig. 1A; red signal). About 40% of the infected cells exhibited DNA breakage characteristic of apoptosis in the TdT assay using fluorescein-conjugated avidin as the reporter (Fig. 1A; green and yellow signals [yellow results from the overlap of red and green signals]). These cells also exhibited morphological changes characteristic of apoptosis, such as condensed chromatin and shrunken cell bodies. When cells were first infected with HCMV and then superinfected with the adenovirus mutant 10 h later, no TdT labeling was observed at 72 h after infection with the adenovirus mutant, when all of the cells contained the adenovirus E2A protein (Fig. 1B). Control immunofluorescence assays confirmed that the HCMV IE1 protein and adenovirus E1A protein were expressed in the cells infected with both viruses (data not shown). Thus, HCMV apparently encodes a protein that can block the induction of apoptosis in primary human cells by an adenovirus variant lacking the gene encoding the E1B 19-kDa protein.

HCMV IE1 and IE2 proteins each block apoptosis induced by TNF-α. A computer search failed to identify an open reading frame in the HCMV genome encoding a protein with recognizable homology to a cellular or viral protein known to antagonize apoptosis. Without a homolog to test, we narrowed our search by reasoning that the HCMV protein responsible for protection probably functions very early after infection, instituting a block before the onset of cell death. Two sets of proteins could perform this function soon after infection: virion constituents or immediate-early proteins. Defective virions lacking viral DNA (20) failed to protect against apoptosis in the assay employed in Fig. 1 (data not shown). This experiment suggested that protection was not mediated by one of the approximately 30 virion proteins that are delivered to the cell by the defective particles. Therefore, we assayed the major immediate-early proteins, IE1 and IE2, for their ability to inhibit apoptosis.

HeLa cells, which undergo apoptosis when exposed to TNF-α (61, 65), were transfected with a plasmid expressing either the IE1 or IE2 protein, and 48 h later the cells were treated with TNF-α (1,000 U/ml) in the presence of cycloheximide (25 μg/ml), which enhances the cellular response to TNF-α treatment. A trypan blue exclusion assay indicated that the cells started dying about 4 h after treatment with TNF-α, and more than 80% of them were dead after 8 h (data not shown). The cultures were assayed by double immunofluorescence for expression of the HCMV IE1 or IE2 protein and reactivity in the TdT assay for apoptosis. Cells expressing the IE1 protein (red signal) survived exposure to TNF-α for either 4 h (Fig. 1C) or 8 h (Fig. 1D) and did not exhibit DNA breakage, while cells not expressing IE1 were labeled by the TdT treatment (green signal). In addition to DNA breakage, the dying cells exhibited nuclear shrinkage and chromatin condensation characteristic of apoptosis. The same result was obtained when HeLa cells were transfected with a plasmid expressing the IE2 protein. Cells expressing the viral protein (red

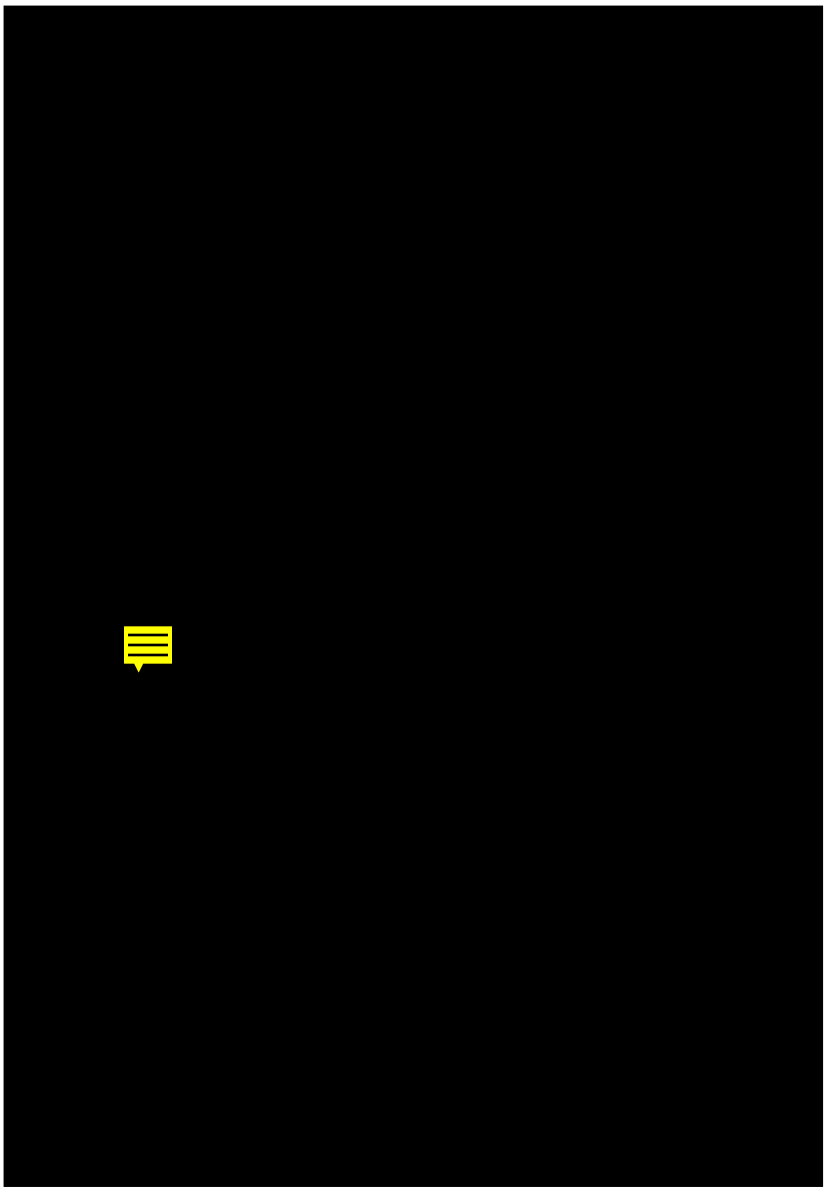

FIG. 1. Confocal immunofluorescence assays demonstrating that HCMV proteins block apoptosis. (A and B) Infection with HCMV blocks the induction of apoptosis by the adenovirus E1A protein, and transient expression of the IE1 or IE2 protein can inhibit the induction of apoptosis by TNF-α. Adenovirus mutant *dl*337, lacking the gene encoding the E1B 19-kDa protein, was used to infect either normal primary human fibroblasts (A) or human fibroblasts that were infected 10 h earlier with HCMV (B). Cells were fixed at 72 h after infection with adenovirus and assayed by double immunofluorescence for expression of the adenovirus E2A protein (red signal) and for DNA breakage (TdT assay, green and yellow signal). (C to H) HeLa cells were transfected with plasmids expressing the IE1 protein (C and D), the IE2 protein (E and F), the carboxy-terminal 103 amino acids of IE1 (G), or the adenovirus 13S E1A protein (H), and 48 h later they were treated with TNF-α (1,000 U/ml) and cycloheximide (25 μg/ml). After an additional 4 h (C and E) or 8 h (D, F, G, and H), cells were fixed and assayed by double immunofluorescence for expression of the relevant viral protein (red signal) and for DNA breakage (TdT assay, green and yellow signal).

nuclei) were protected from apoptosis (green signal) after exposure to TNF-α for either 4 h (Fig. 1E) or 8 h (Fig. 1F). The adenovirus E1A proteins, which can induce apoptosis within transfected cells and enhance apoptosis on treatment of HeLa cells with TNF-α (4, 6, 65), were used as a control for this experiment (Fig. 1H). Cells undergoing apoptosis in the absence of E1A proteins exhibited TdT reactivity only (green signal), while cells undergoing apoptosis in the presence of E1A proteins (red signal) exhibited reactivity to both the E1A antibody and in the TdT assay (the overlapping responses produce a yellow signal). This control demonstrated that it would have been possible to detect cells expressing the IE1 or IE2 protein and undergoing apoptosis; they would produce a yellow signal in the double immunofluorescence assay. No such cells were detected, indicating that transient expression of the IE1 or IE2 protein protects HeLa cells from TNF-α-induced apoptosis.

To further explore the protective roles of the HCMV immediate-early proteins, a series of HeLa cell lines were generated by coselection of an IE1 or IE2 expression construct with a drug resistance marker. Figure 2A displays an immunoblot analysis of seven clonal cell lines probed with a mixture of antibodies to the IE1 and IE2 proteins. Two cell lines express the 72-kDa IE1 protein (HIE1-1 and HIE1-2), two express the

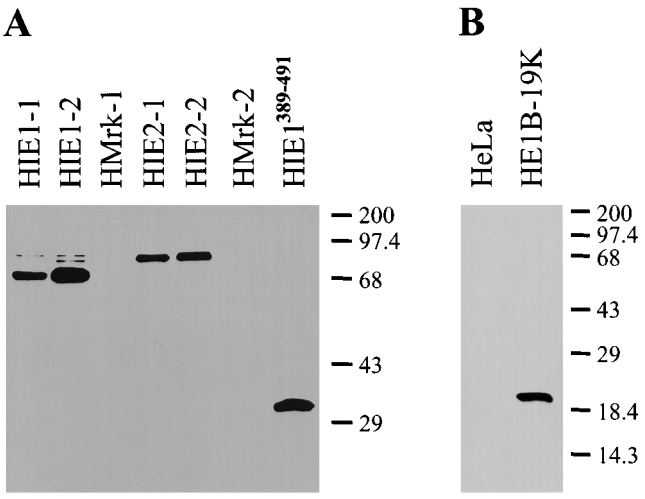

FIG. 2. Constitutive expression of viral proteins in HeLa cell lines. Whole-cell extracts were prepared from HeLa cell lines expressing the HCMV IE1 protein (HIE1-1 and HIE1-2), IE2 protein (HIE2-1 and HIE2-2), a carboxy-terminal fragment of IE1 ($HIE1^{389–491}$), and the adenovirus E1B 19-kDa protein (HE1B-19K) and assayed for expression of viral proteins by using antibodies specific for the IE1 or IE2 proteins (A) or an E1B 19-kDa protein-specific antibody (B). Lanes designated HMkr-1 and HMkr-2 received extracts of control cell lines that were coselected for the expression of IE1 or IE2 protein plus the puromycin resistance marker but failed to express a viral protein. The lane labeled HeLa received an extract of normal HeLa cells. The positions of molecular weight standards (in thousands) are indicated on the left.

86-kDa IE2 protein (HIE2-1 and HIE2-2), one expresses a carboxy-terminal 389- to 491-amino-acid segment of IE1 ($HIE1^{389–491}$), and two failed to express detectable amounts of a viral protein (HMkr-1 and HMkr-2) and were retained for use as negative controls. An additional control cell line, HE1B-19K, that expresses the adenovirus E1B 19-kDa protein was produced (Fig. 2B).

Each of the HeLa cell clones expressing a viral protein was substantially resistant to treatment with TNF-α and cycloheximide. A representative experiment is displayed in Fig. 3 in which nuclei were marked by staining cellular DNA with propidium iodide (red signal) and apoptosis was assayed at 8 h after TNF-α treatment by using the TdT assay. Greater than 90% of normal HeLa cells or the drug-resistant HeLa cell clones lacking a viral protein (HMrk-1 and HMrk-2) exhibited DNA breakage (green and yellow signals) and nuclear fragmentation indicative of apoptosis (Fig. 3A, C, and E). In contrast, only about 2% of the cells expressing the E1B 19-kDa protein were positive (Fig. 3B), and ~5% of the cells in the IE1- or IE2-expressing cell populations (HIE1-1 or HIE2-1) were positive in the TdT assay (Fig. 3D and F). The HIE1-2 and HIE2-2 cell lines also were tested in this assay, and the same results were obtained (data not shown).

Since both of the immediate-early proteins were able to block the induction of apoptosis in HeLa cells by TNF-α, we initially suspected that the protective function resided in the 85 amino-terminal residues shared by the two proteins whose mRNAs are derived from a single transcription unit and share three exons at their 5′ ends. However, an analysis of several deleted variants of the proteins (Fig. 4A) indicated that this is not the case. For this experiment, the deleted variants were each fused to an amino-terminal segment containing a flu epitope and the simian virus 40 T antigen nuclear localization signal. Duplicate HeLa cell cultures were transfected with plasmids expressing each of the fusion proteins. Beginning 48 h after transfection, one set of the duplicate cultures was treated for 8 h with TNF-α and cycloheximide, while the second set received only cycloheximide. An immunofluorescence assay with an antibody to the flu epitope demonstrated that each culture was transfected at high efficiency, expressing the fusion protein in the nuclei of over 40% of the cells (data not shown). After 8 h of drug treatment, a sufficient number of microscopic fields in cultures that received only cycloheximide were scanned to count 500 transfected cells expressing a flu epitope-tagged protein. The same number of microscopic fields were then scanned for the presence of transfected cells in cultures that were treated with TNF-α and cycloheximide, and the proportion of transfected cells that survived the TNF-α treatment was determined (Fig. 4B). A total of 93% of the cells expressing the wild-type IE1 protein survived the TNF-α treatment (Fig. 4B, IE1). This level of protection is consistent with the proportion of cells resistant to apoptosis observed in HeLa cell lines constitutively expressing the IE1 protein (Fig. 3D). Only 15% of the cells expressing an amino-terminal fragment of IE1 survived the treatment with TNF-α, while 81 to 85% of cells expressing carboxy-terminal segments of the IE1 protein were protected (Fig. 4B, compare $IE1^{1–136}$ with $IE1^{137–491}$ and $IE1^{389–491}$). Although the amino-terminal 136 amino acids of the IE1 protein appeared to provide a modest degree of protection, the carboxy-terminal segment protected much more effectively and nearly as well as wild-type protein. This result was confirmed in experiments in which apoptosis was assayed by the TdT procedure after treatment of $IE1^{389–491}$-expressing cells with TNF-α in a transient transfection assay (Fig. 1G) or in a stable cell line, $HIE1^{389–491}$ (Fig. 2A and data not shown). The wild-type IE2 protein protected 85% of the transfected cells in which it was expressed (Fig. 5B, IE2). The amino-terminal 135 amino acids of this protein clearly provided some protection, sparing 36% of transfected cells, while the remaining 444 amino acids of the IE2 protein protected 85% of transfected cells, blocking apoptosis as effectively as the full-length protein (Fig. 4B, $IE2^{1–135}$ and $IE2^{136–579}$). As expected, transfection with the adenovirus E1A protein, which induces apoptosis, left very few survivors after treatment with TNF-α (Fig. 4B, E1A).

We conclude that the HCMV IE1 and IE2 proteins both function to block apoptosis induced by TNF-α, protecting HeLa cells when they are either transiently or constitutively expressed. Their antiapoptotic activities reside principally within domains where the two proteins do not share amino acid sequences, raising the possibility that they interfere with apoptosis by different mechanisms.

HCMV IE1 and IE2 proteins block apoptosis induced by a mutant adenovirus unable to express the E1B 19-kDa protein. Next, we tested whether the IE1 and IE2 proteins also suppress the premature apoptotic cell death induced by the E1B 19-kDa protein-deficient adenovirus mutant. It seemed likely that they would be active in this assay since infection of primary human fibroblasts with HCMV blocked the induction of apoptosis by the mutant adenovirus (Fig. 1A and B).

Normal HeLa cells and HeLa cells constitutively expressing viral proteins (Fig. 2) were infected with either wild-type adenovirus or the E1B 19-kDa protein-deficient virus, *dl*337. At 36 h after infection, cells were fixed and analyzed by a double immunofluorescence assay for E2A expression as a marker for adenovirus infection (red signal) and for DNA breakage with the TdT assay (green and yellow signal). As predicted by earlier work, wild-type virus did not induce apoptosis (Fig. 5A, C, E, and G) while the mutant virus induced apoptosis in normal HeLa cells (Fig. 5B) but not in HeLa cells expressing the E1B 19-kDa protein (Fig. 5D). Both IE1 and IE2 proteins also

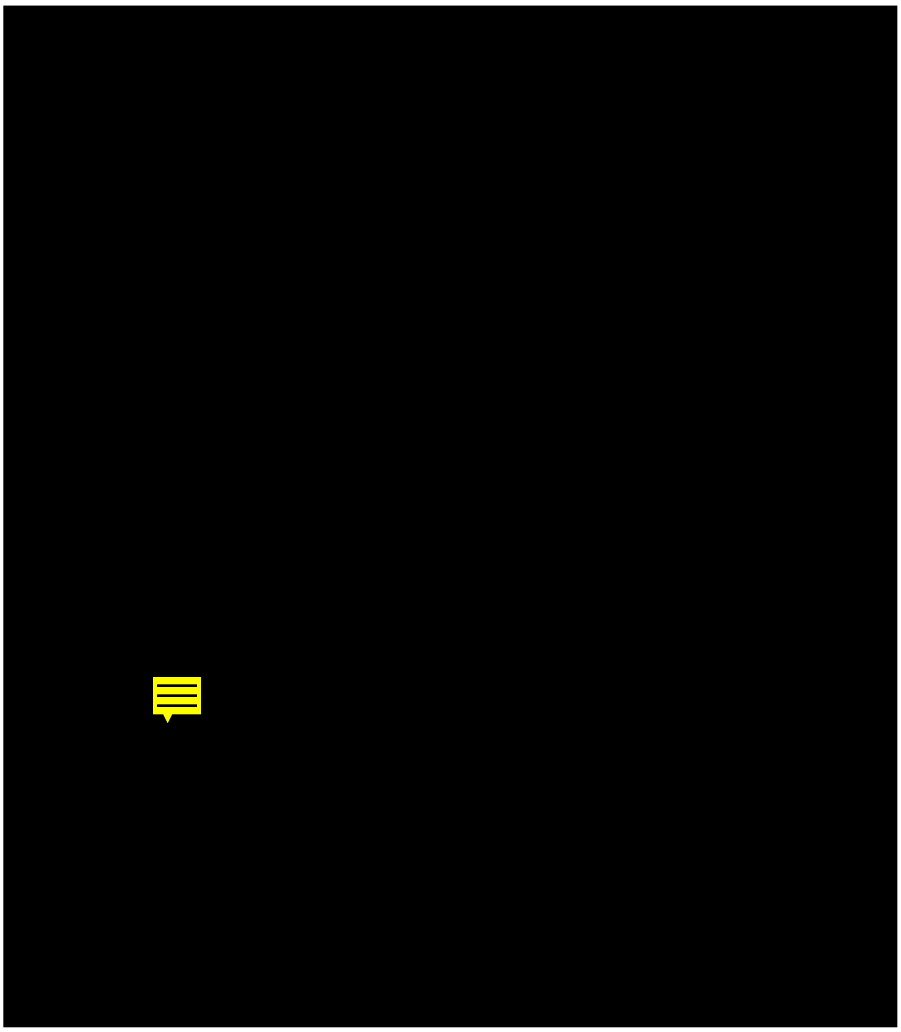

FIG. 3. Confocal immunofluorescence assay demonstrating that cells constitutively expressing the IE1 or IE2 protein are resistant to the induction of apoptosis by TNF-α. Cells were treated with TNF-α (1,000 U/ml) and cycloheximide (25 μg/ml) for 8 h, trypsinized, and fixed. A double immunofluorescence assay was performed; DNA was identified by staining with propidium iodide (red signal), and DNA breakage was monitored by the TdT assay (green and yellow staining). (A) HeLa cells; (B) HE1B-19K cells; (C) HMkr-1 cells; (D) HIE1-1 cells; (E) HMkr-2 cells; (F) HIE2-1 cells.

inhibited *dl*337-induced apoptosis, although the IE1 protein was more effective. Greater than 95% of HeLa cells expressing IE1 (HIE1-1 cells) were resistant to apoptosis induced by the mutant adenovirus (Fig. 5F), but IE2-expressing cells (HIE2-1 cells) were only partially resistant, with ~70% of the cells protected in repeated assays (Fig. 5H). Similar results were obtained when HIE1-2 and HIE2-2 cells (Fig. 2A) were assayed (data not shown).

The HCMV IE1 and IE2 proteins do not block the induction of apoptosis by UV irradiation. The HCMV IE1 and IE2 proteins behaved similarly to the adenovirus E1B 19-kDa protein, protecting against apoptosis induced by either TNF-α or expression of adenovirus E1A proteins. However, the HCMV and adenovirus proteins differed in their abilities to prevent cell killing by UV irradiation.

HeLa cell lines constitutively expressing the E1B 19-kDa, IE1, or IE2 protein were irradiated with UV light (40 J/m^2), and 24 h later, the cells expressing the E1B 19-kDa protein appeared normal (Fig. 6A, panel 1) while cultures expressing the IE1 or IE2 protein contained many rounded, dying cells (Fig. 6A, panels 2 and 3). When assayed for apoptosis by the TdT assay at 24 h, cells expressing the E1B 19-kDa protein scored negative (Fig. 6A, panel 4) whereas all cells in the cultures expressing the IE1 or IE2 proteins scored positive for DNA breakage (Fig. 6A, panels 5 and 6).

Survival after irradiation with UV light (40 J/m^2) was monitored by trypan blue exclusion (Fig. 6B). At 6 days after irradiation, only 0 to 8% of the cells expressing either no viral protein, the IE1 protein, or the IE2 protein remained viable, while 52% of cells expressing the E1B 19-kDa protein survived. This result is consistent with an earlier report that ectopic expression of the adenovirus protein can protect cells from death induced by UV light (57).

The HCMV IE1 and IE2 proteins do not alter the expression of *bcl-2* or *bax*. Since the HCMV IE1 and IE2 proteins are known primarily for their ability to modulate transcription of viral and cellular genes, we asked if they might alter the expression of genes which have been shown to be involved in apoptosis, specifically members of the *bcl-2* gene family and interleukin-1β-converting enzyme (ICE) gene family. Members of the Bcl-2-related family of proteins share two short sequence motifs, known as the BH1 and BH2 domains, that are

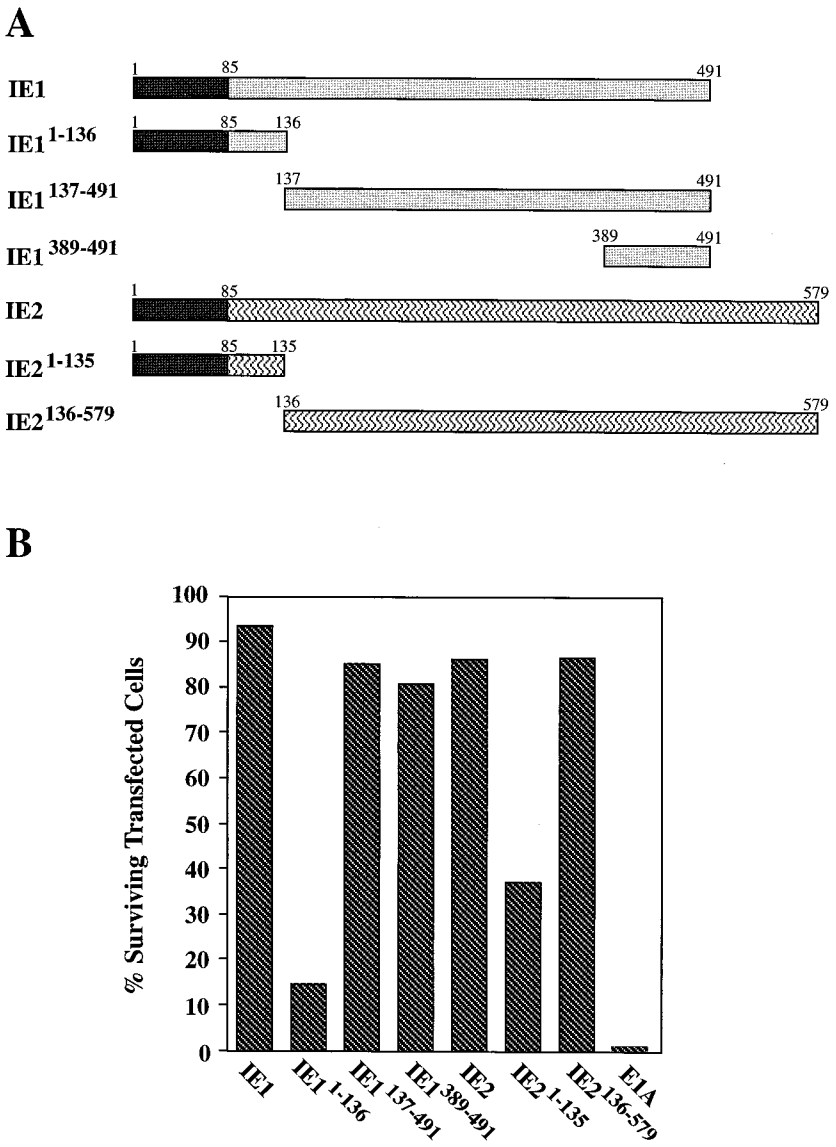

FIG. 4. Sequences downstream of the shared amino-terminal domain in the IE1 and IE2 proteins protect cells from TNF-α-induced apoptosis. (A) Schematic diagram of mutant proteins. HCMV polypeptides were fused to an amino-terminal segment containing the flu epitope tag and a nuclear localization signal. IE1 or IE2 sequences present in the fusion proteins are represented by bars, and the first and last amino acids of the IE protein segments are indicated by superscripts. The darker regions at the amino terminus of some fusion proteins indicate the 85 residues that the immediate-early proteins share. (B) Quantitative assay of cells expressing portions of IE1 and IE2 proteins that survive exposure to TNF-α. Duplicate HeLa cell cultures were transfected with plasmids expressing the IE1 or IE2 fusion proteins or the 13S E1A protein; one of each duplicate culture was treated with TNF-α (1,000 U/ml) and cycloheximide (25 μg/ml) at 48 h after transfection, and the other set was treated with cycloheximide only. The cells were fixed and assayed by immunofluorescence to identify the transfected cells by using a monoclonal antibody to the flu epitope. Each bar represents the percentage of 500 cells expressing the indicated fusion protein that survived treatment with TNF-α. Cells were scored as survivors when they remained attached to the plate and their nuclei did not display the shrinkage and condensation of chromatin characteristic of apoptosis.

essential for their function (66, 68). Some family members, such as the Bcl-2 protein, inhibit apoptosis (9, 18, 51, 60), while other members, such as the Bax protein, promote cell death (37). The IE1 and IE2 proteins could function by enhancing the expression of a protective protein or inhibiting the expression of a death-promoting protein.

We assayed the steady-state levels of Bcl-2 and Bax gene products in HeLa cell lines constitutively expressing the E1B 19-kDa, IE1, or IE2 protein. RNase protection assays indicated that the levels of Bcl-2 and Bax mRNAs were not affected by expression of the viral proteins (Fig. 7A), and protein blot assays failed to detect significant differences in the levels of Bcl-2 or Bax proteins (Fig. 7B). We also found no differences in the levels of Bad (67), Bag-1 (54), or ICE (30) mRNA in this set of cell lines (data not shown).

DISCUSSION

Three different assays were employed to test the abilities of the HCMV IE1 and IE2 proteins to block apoptosis. The IE1 or IE2 protein blocked the induction of apoptosis by TNF-α, when tested in HeLa cells transiently expressing the HCMV proteins (Fig. 1C-G) or in HeLa cells constitutively expressing them (Fig. 3 and 4B). The IE1 or IE2 protein also inhibited apoptosis induced by the adenovirus E1A proteins within HeLa cells infected with an E1B 19-kDa protein-deficient ad-

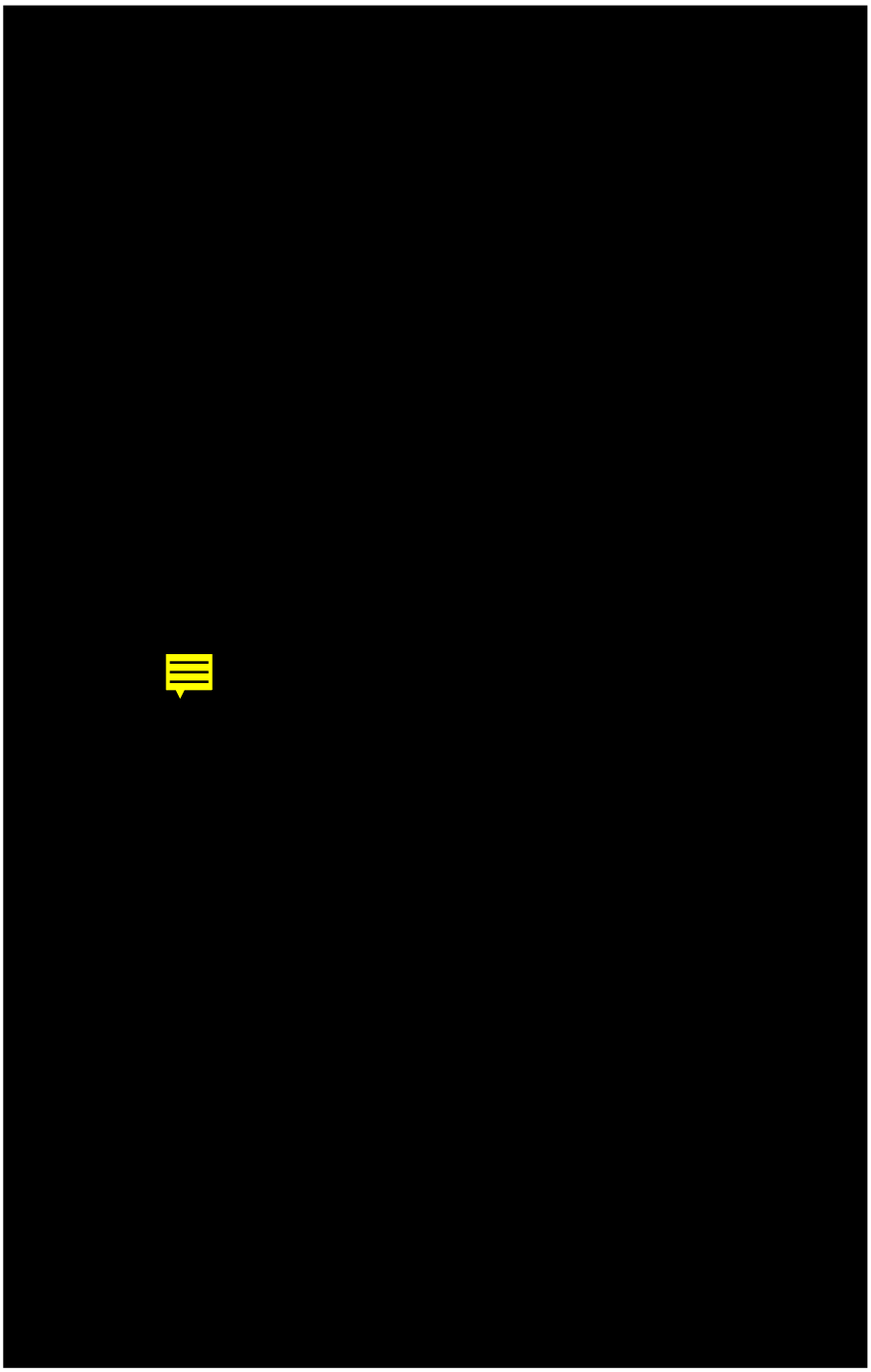

FIG. 5. Confocal immunofluorescence assay demonstrating that cells constitutively expressing the IE1 or IE2 protein are resistant to the induction of apoptosis by the adenovirus E1A protein. Cells were infected with either wild-type (A, C, E, and G) or mutant *dl*337 (B, D, F, and H) adenovirus and fixed 36 h later. A double immunofluorescence assay was performed; expression of the adenovirus E2A protein (red signal) and DNA breakage (TdT assay: green and yellow signal) were monitored. (A and B) HeLa cells; (C and D) HE1B-19K cells; (E and F) HIE1-1 cells; (G and H) HIE2-1 cells.

enovirus (Fig. 5), and presumably, the IE1 and IE2 proteins are substantially responsible for the resistance of HCMV-infected cells to the induction of apoptosis by superinfection with the adenovirus mutant (Fig. 1A and B). The IE1 and IE2 proteins, however, failed to protect HeLa cells from the induction of apoptosis by UV irradiation (Fig. 6). Thus, the HCMV immediate-early proteins can inhibit apoptosis mediated by some but not all inducers.

Programmed cell death is a key element of a host organism's defense against viral infections, inhibiting viral spread and persistence (reviewed in reference 45). The IE1 and IE2 proteins are the first HCMV gene products to be expressed after

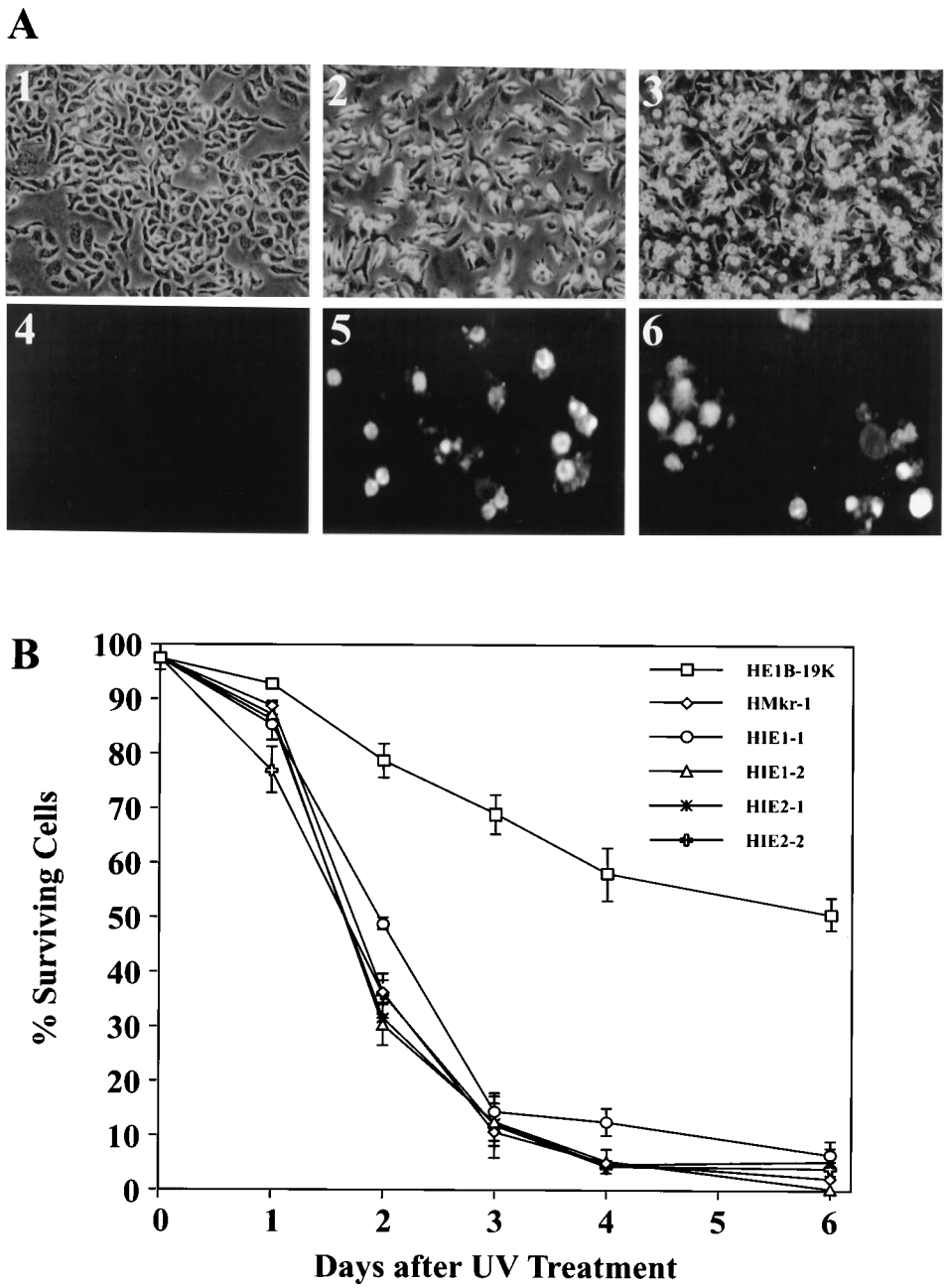

FIG. 6. Cells constitutively expressing IE1 and IE2 proteins are not resistant to killing by UV irradiation. (A) HE1B-19K cells (panels 1 and 4), HIE1-1 cells (panels 2 and 5), and HIE2-1 cells (panels 3 and 6) were irradiated (40 J/m^2), and 24 h later cultures were examined by phase microscopy (panels 1 to 3) and by TdT assay for DNA breakage (panels 4 to 6). (B) UV irradiation killing curves for IE1 and IE2 expressing cell lines. After irradiation (40 J/m^2), cultures were assayed in triplicate for viability at the indicated times by trypan blue exclusion.

infection, allowing them to antagonize apoptosis before the antiviral defense is instituted by the host cell. Such protection is probably extremely important to HCMV given that its replication within an infected cell appears to progress very slowly in comparison to many other viruses. A block to apoptosis could also be critical to the well-documented ability of HCMV to establish lifelong persistence in its human host, although it is not yet possible to assign persistence functions to specific viral genes because the principal mechanisms of HCMV persistence are poorly understood (reviewed in references 31 and 46).

It remains unclear whether the IE1 and IE2 proteins are the only HCMV proteins that antagonize apoptosis or whether additional viral gene products contribute to the protection from cell death that we have observed within HCMV-infected human fibroblasts (Fig. 1B). It is conceivable that a variety of viral gene products employ different modes of action to inhibit cell death sponsored by inducers acting through multiple pathways in different cell types. In this regard, the IE1 and IE2 proteins probably differ in the mechanisms by which they inhibit cell death. The initial mapping studies that we performed indicate that the common amino-terminal sequence shared by

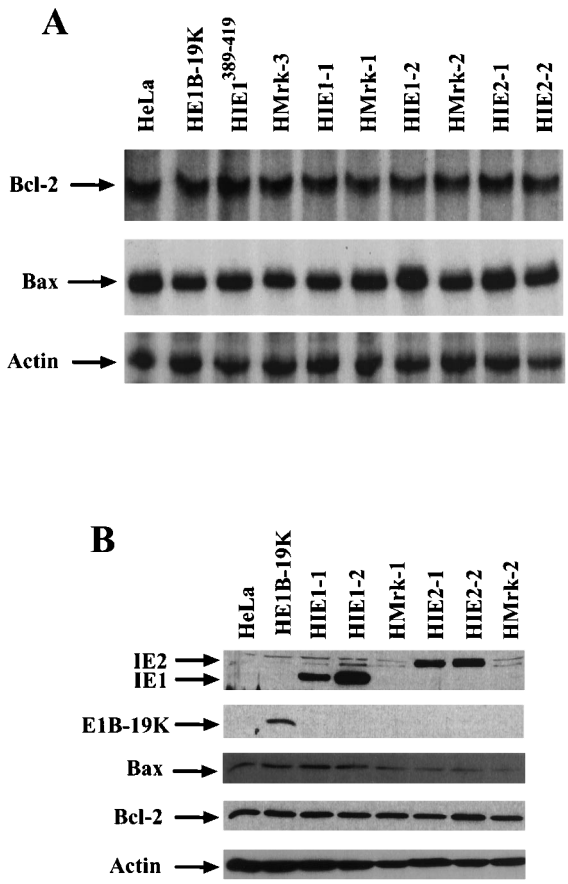

FIG. 7. Expression of the *bcl-2* and *bax* genes is not altered in cells constitutively expressing IE1 and IE2 proteins. (A) Ribonuclease protection assay. Total cell RNA was prepared from the indicated cell lines, and aliquots were hybridized with ^{32}P-labeled antisense probes specific for Bcl-2, Bax, or β-actin mRNA (β-actin as a normalization control). (B) Western blot assay. Whole-cell proteins were prepared from the indicated cell lines and analyzed (50 μg per sample) by using monoclonal antibodies specific for Bcl-2 protein, Bax protein, β-actin, E1B 19-kDa protein, or IE1 and IE2 proteins.

the two proteins is not responsible for their abilities to antagonize cell death. Rather, the antiapoptosis function resides primarily to the carboxy-terminal side of the shared domain in both proteins (Fig. 4). We have not been able to identify any sequence elements shared by the two proteins in the domains that mitigate the induction of apoptosis, and this leads us to suspect that the two proteins function by different mechanisms. Further, we have observed that the induction of apoptosis by adenovirus E1A protein is more effectively inhibited by the IE1 protein than by the IE2 protein (Fig. 5). The apparent efficacy of the two HCMV proteins in this assay could be influenced by differences in their steady-state levels. However, both proteins are abundantly expressed in these cells, so it is likely that the IE1 protein protects more efficiently because its mode of action differs from that of the IE2 protein.

Several viral genes that antagonize programmed cell death have been described. As mentioned above, the adenovirus E1B 19-kDa protein blocks the induction of apoptosis by the adenovirus E1A protein during both lytic growth and oncogenic transformation (41). The cellular Bcl-2 protein can substitute for the E1B 19-kDa protein (41), the E1B 19-kDa protein contains sequences homologous to the BH1 and BH2 conserved domains that are characteristic of Bcl-2 family members (66, 68), and the adenovirus protein can associate with a cellular Bcl-2 family member termed Bak (7). These observations indicate that the E1B 19-kDa protein is a homolog of the cellular Bcl-2 protein. The Epstein-Barr virus BHRF1 and African swine fever virus LMW5-HL proteins also block apoptosis and have homologies to BH1 and BH2 (16, 35, 58), while the Epstein-Barr virus LMP-1 protein induces expression of the cellular Bcl-2 protein (17). The cowpox virus CrmA protein blocks apoptosis by inhibiting ICE and ICH-1_L. These cysteine proteases, which are mammalian homologs of the *Caenorhabditis elegans ced-3* gene (70), induce apoptosis when overexpressed in mammalian cells (8, 30, 42, 62). Finally, the simian virus 40 T antigen and the human papillomavirus E6 protein modulate apoptosis by interfering with the function of the p53 tumor suppressor protein (29, 38, 53), which can induce apoptotic cell death under a variety of conditions (69; reviewed in reference 12). Thus, three general mechanisms for antagonizing apoptosis have been described for viral systems: mimicking or inducing the Bcl-2 protein, inhibiting the activity of ICE family members, or blocking p53 function.

How do the HCMV IE1 and IE2 proteins inhibit apoptosis? The two proteins are localized in the nucleus (Fig. 1), rather than in mitochondrial membranes, nuclear membranes, and the endoplasmic reticulum as is characteristic of Bcl-2 family members (5, 18, 21), and neither has recognizable homology to the BH1 or BH2 motif present in Bcl-2 family members. Therefore, the IE1 and IE2 proteins are not likely to be mimics of Bcl-2. Further, the nuclear localization of the two HCMV proteins predicts that they probably do not directly inhibit members of the ICE family of cysteine proteases, which reside in the cytoplasm (59).

It is possible that the IE1 and IE2 proteins inhibit apoptosis in part by modulating the activity of p53. p53 levels are elevated and can induce apoptosis in response to the adenovirus E1A protein, DNA damage, and other stimuli in some cell types (reviewed in reference 12). Transfection of human fibroblasts with a plasmid expressing both IE1 and IE2 proteins has been shown to induce elevated levels of p53 (34), and the IE2 protein has been reported to interact with p53 and to inhibit its ability to activate transcription of a reporter gene in transfected primary human smooth muscle cells (49). Perhaps, then, the IE2 protein interacts directly with p53 and inhibits its ability to induce apoptosis. However, since E1A can induce cell death in human SAOS2 cells (52), which do not express p53, IE1 and IE2 must have blocked p53-independent as well as p53-dependent cell death pathways to protect cells from E1A-induced apoptosis in our assays (Fig. 5). So, a possible effect of IE2 on p53 function cannot completely explain the ability of the IE2 protein to block E1A-induced apoptosis. Moreover, the IE1 protein, which is not known to influence p53 function, blocks apoptosis induced by E1A even more efficiently than the IE2 protein (Fig. 5), raising the possibility that this protein functions downstream of p53 in the pathway through which the tumor suppressor protein induces cell death.

Given the well-established ability of the IE1 and IE2 proteins to regulate transcription, one might speculate that they inhibit apoptosis by inducing the expression of a cellular gene that favors survival, such as *bcl-2*, or by repressing a cellular gene that favors death, such as *bax*. However, our initial experiments failed to detect a change in *bcl-2* or *bax* gene expression at either the mRNA or protein level (Fig. 7). We also did not observe a change in the level of Bad (67), Bag-1 (54), or ICE (30) mRNA (data not shown). Work is in progress to test for IE1- or IE2-mediated changes in the expression of these genes after exposure to apoptotic inducers and for al-

tered expression of additional cellular proteins that influence apoptosis.

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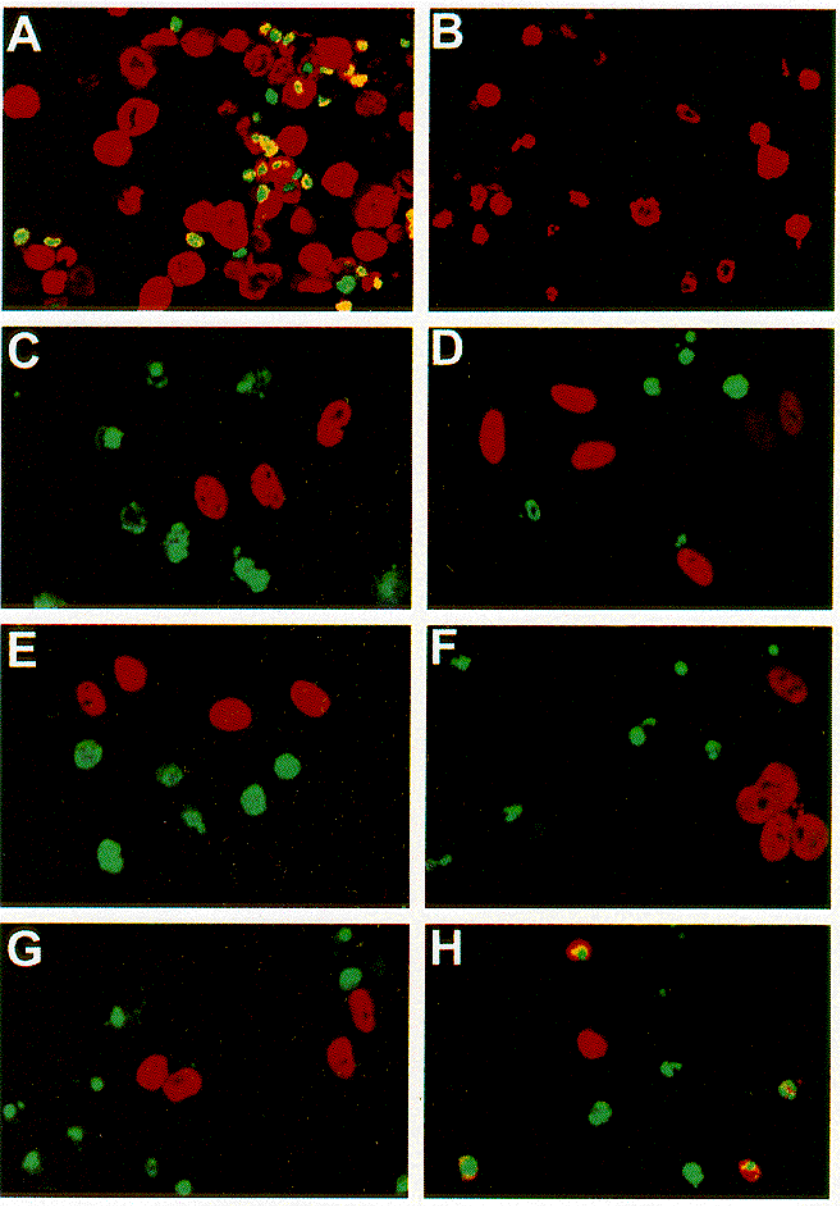
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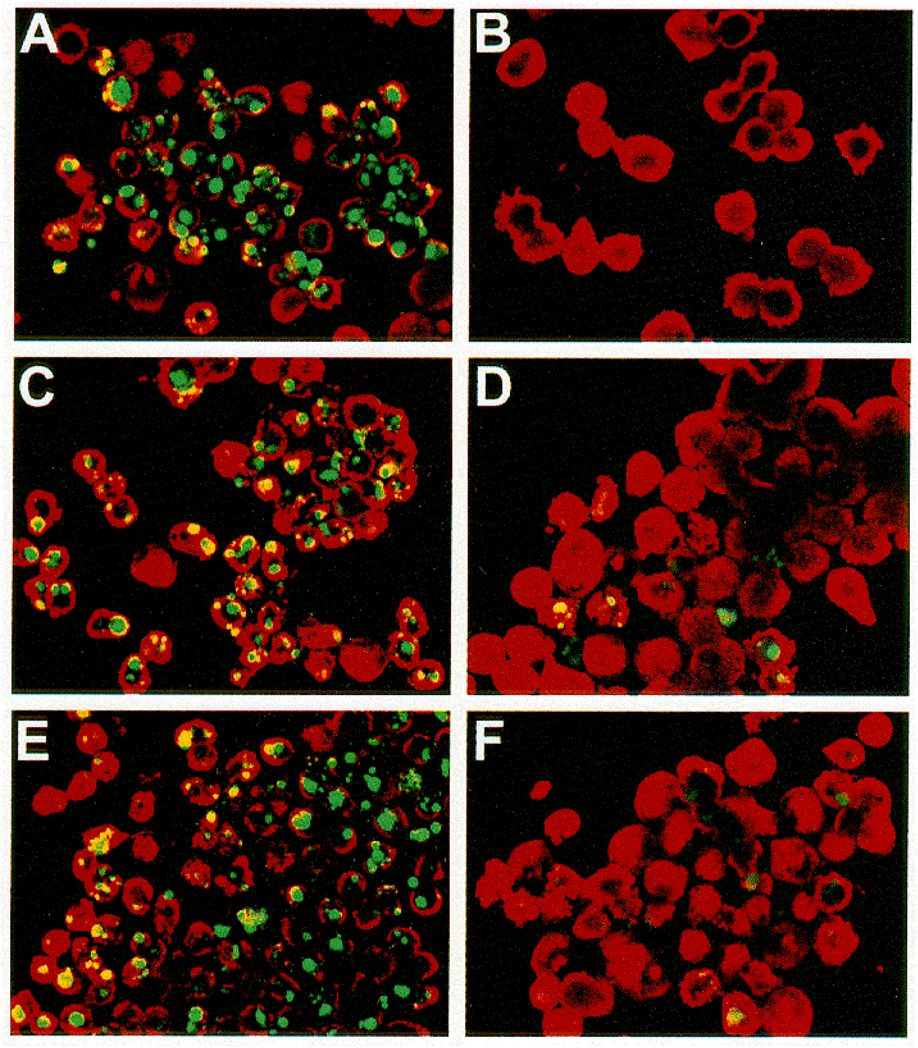
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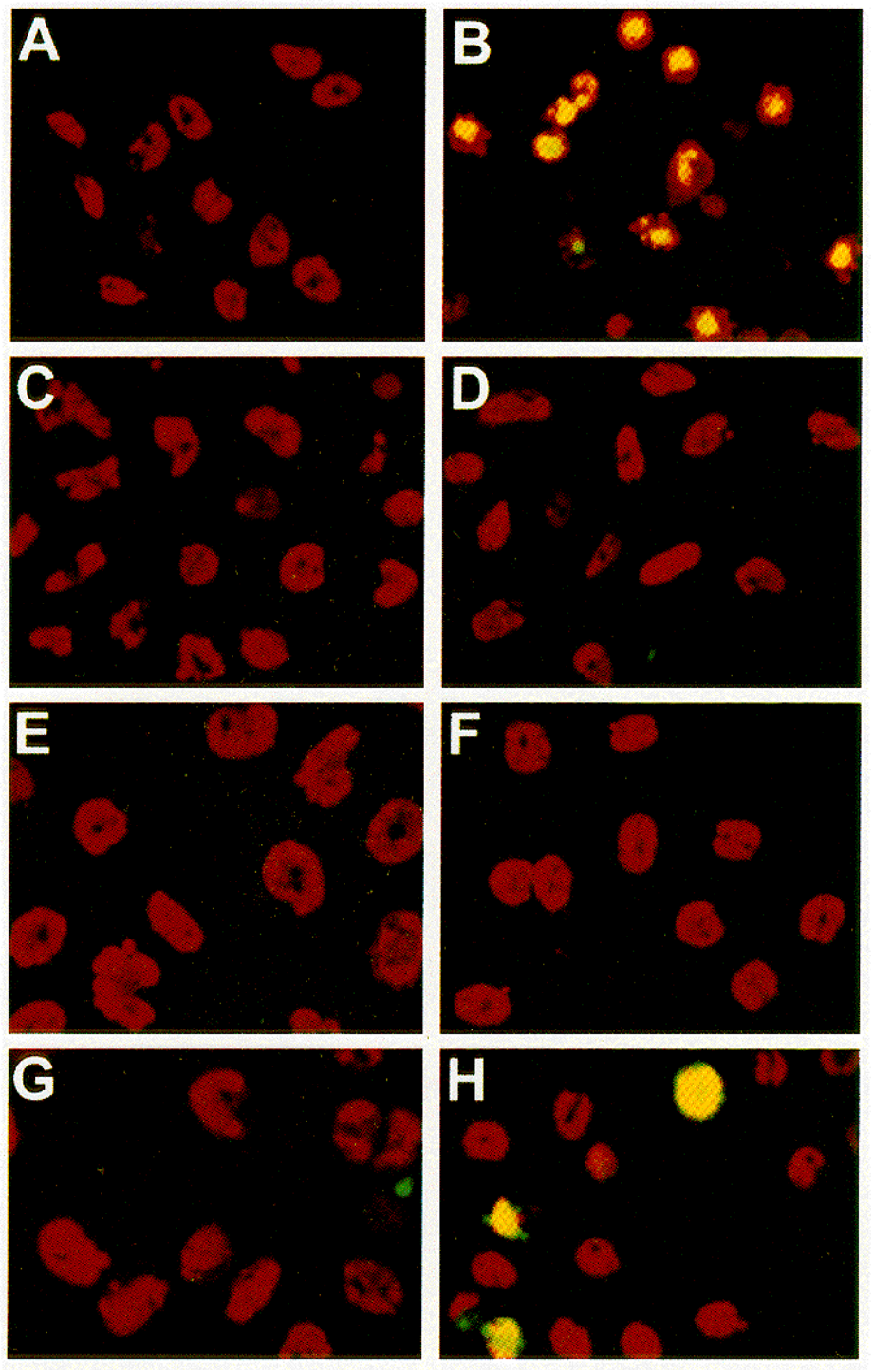
A
B
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