JOURNAL OF VIROLOGY, Oct. 1996, p. 7116–7124 Vol. 70, No. 10
0022-538X/96/$04.00+0

Induction of Apoptosis by Baculovirus Transactivator IE1

ELENA A. PRIKHOD'KO AND LOIS K. MILLER*

Departments of Entomology and Genetics, The University of Georgia, Athens, Georgia 30602-2603

Received 19 April 1996/Accepted 11 July 1996

Apoptosis is induced upon infection of SF-21 cells by mutants of the baculovirus *Autographa californica* nuclear polyhedrosis virus (Ac*M*NPV) lacking a functional *p35* gene which encodes a stoichiometric inhibitor of members of the interleukin-1β converting enzyme family of cysteine proteases (N. J. Bump et al., Science 269:1885–1888, 1995; R. J. Clem, M. Fechheimer, and L. K. Miller, Science 254:1388–1390, 1991). We found that transfection of SF-21 cells with the Ac*M*NPV *ie-1* gene was sufficient to induce apoptosis, which was characterized by fragmentation of cellular DNA into oligonucleosomal fragments and apoptotic body formation. No signs of apoptosis were observed in *Trichoplusia ni* TN-368 cells transfected with *ie-1*, a result which is consistent with the observation that *p35* mutants of Ac*M*NPV do not induce apoptosis in this cell line. Cotransfection of SF-21 cells with *p35* blocked *ie-1*-induced apoptosis, indicating that expression of *ie-1* activates apoptosis through a *p35*-inhibitable cysteine protease pathway. Cotransfection with Cp-*iap*, an active member of another family of antiapoptotic inhibitors of apoptosis (*iap*s), also inhibited IE1-induced apoptosis. Thus, *ie-1* may participate in inducing apoptosis in Ac*M*NPV-infected cells, although the dependence of induction on DNA replication suggests that *ie-1* is not the direct apoptotic signal during infection. The *ie-1* gene product, IE1, is known to be a potent transactivator of baculovirus gene expression that interacts with specific palindromic sequences which can act as transcriptional enhancers and as origins of DNA replication in transient assays.

Mutants of *Autographa californica* nuclear polyhedrosis virus (Ac*M*NPV) defective in the *p35* gene induce apoptosis during infection of SF-21 cells (10). The characteristic phenotype begins during the late phase of infection and includes extensive blebbing of the cell surface, nuclear condensation and fragmentation, degradation of cellular DNA into oligonucleosomal-size fragments, apoptotic body formation, and premature cell death, which limits virus replication and precludes occlusion body formation (10, 12, 24). The product of the *p35* gene, P35, is a stoichiometric inhibitor of the interleukin-1β converting enzyme (ICE) family of cysteine proteases which have a central role in triggering apoptosis in vertebrates as well as invertebrates (4, 46). P35 can function in a phylogenetically broad range of organisms to block apoptosis induced by a variety of signals (2, 23, 30, 37, 43). Some members of a second family of anti-apoptotic genes, the *iap* (inhibitor of apoptosis) family, can fully substitute for *p35* during Ac*M*NPV infection (3, 15). Although the molecular mechanism by which *iap*s block apoptosis is not known, the recent discovery of cellular homologs of *iap*s with anti-apoptotic activity suggests that the gene products, IAPs, are normal components of the cellular apoptotic pathway that have been acquired by baculoviruses to block cellular apoptosis during infection (11, 17, 22, 28, 42).

Induction of apoptosis by *p35* mutants exhibits cell line, tissue, and species specificity (12). Infection of TN-368 cells with *p35* mutants appears to be normal. Similarly, *p35* mutant infection of larvae of *Trichoplusia ni*, the species from which TN-368 cells were originally derived, is normal with regard to infectivity (intragastric [i.g.] 50% lethal dose, LD_{50}) and virulence (i.g. 50% lethal time, LT_{50}). In contrast, *p35* mutants have markedly reduced infectivity in larvae of *Spodoptera frugiperda*, from which SF-21 cells were derived. Some tissue specificity appears to be overlaid upon this species-specific response; *T. ni* larvae infected with *p35* mutant virus produce fewer occluded viruses, and larvae of both species present an abnormal pathology at death. It is not known why *p35* mutants exhibit these cell-, tissue- and species-specific effects nor is it known what feature(s) of virus infection triggers apoptosis.

Several lines of evidence suggest that the virus signal which induces apoptosis in SF-21 cells is related to viral DNA replication. Plasmid DNA transient-replication assays in SF-21 cells have identified nine viral genes that are necessary and sufficient for the replication and/or stability of plasmids carrying putative origins of virus DNA replication known as homologous region (hr) sequences (26, 29). One of these nine genes is *p35*. The fact that antiapoptotic *iap* genes such as Op-*iap* or Cp-*iap* are able to substitute functionally for *p35* in these transient assays strongly suggests that apoptosis is induced by one or a combination of the eight other replicative genes in these assays. An independent line of evidence suggests that viral DNA replication per se is required for the induction of apoptosis; the presence of aphidicolin, a known inhibitor of cellular and viral DNA polymerases, blocks apoptosis during *p35* mutant infections (13). Thus, DNA replication itself or some subsequent event such as late gene expression, which is dependent on viral DNA replication, is required to trigger apoptosis during *p35* mutant infections.

The product of *ie-1*, IE1, is a multifunctional protein which acts as a strong transcriptional activator in transient-expression assays (20, 32, 34) and is one of the nine genes required in the hr-dependent plasmid replication assays (26, 29). IE1 binds directly to hr sequences (7, 41) which serve both as enhancers (5, 19, 21, 32) and as putative replication origins in these assays (35, 36). Although the role of IE1 as a transactivator of early viral gene expression is well established, its roles in viral DNA replication and late gene expression, if any, require further definition. In this study, we report that transient IE1 expression alone induced apoptosis in SF-21 but not TN-368 cells and

* Corresponding author. Mailing address: Department of Entomology, The University of Georgia, 413 Biological Sciences Bldg., Athens, GA 30602-2603. Phone: (706) 542-2294. Fax: (706) 542-2279. Electronic mail address: Miller@bscr.uga.edu.

that coexpression of *p35* or Cp-*iap* genes blocked IE1-induced apoptosis.

MATERIALS AND METHODS

Cells. *S. frugiperda* IPLB-SF-21 (SF-21) cells (45) and *T. ni* TN-368 cells (25) were maintained at 27°C in TC-100 growth medium (GIBCO BRL, Gaithersburg, Md.) supplemented with 10% fetal bovine serum and 0.26% tryptose broth as described previously (33).

Reporter plasmid and other plasmid constructs. The plasmid pETCAThr5 was used as a reporter plasmid in transient-expression assays. This plasmid (34) contains the gene encoding chloramphenicol acetyltransferase (CAT) under the transcriptional control of the *etl* promoter (14) and a portion of hr 5 (38) of Ac*M*NPV.

The plasmids pH_3G and pH_3F contain the Ac*M*NPV genomic fragments *Hin*dIII-G and *Hin*dIII-F, respectively, and the plasmid pIE1/HC contains the Ac*M*NPV *ie-1* gene in a *Cla*I-*Hin*dIII fragment (94.7 to 96.9 map units [m.u.]); all are cloned in pBluescript II KS+ as described earlier (34). Plasmid pHEV was constructed by inserting the 5,511-bp Ac*M*NPV *Hin*dIII-*Eco*RV fragment (91.0 to 95.1 m.u.) from pH_3G into the *Eco*RV site of pBluescript II KS+. PIE1/HCfs has a frameshift mutation in the *ie-1* gene of pIE1/HC. To construct pIE1/HCfs, pIE1/HC was digested with *Nde*I, blunt-ended with T4 DNA polymerase, and then religated. The frameshift was confirmed by DNA sequencing.

Plasmid pHSP70PLVI$^+$CAT has been described previously and contains the CAT gene under the control of the *Drosophila melanogaster* hsp70 promoter (13). To construct pHSP70PLVI$^+$IE1, the CAT gene from pHSP70PLVI$^+$CAT was replaced with the PCR-amplified open reading frame (ORF) of the Ac*M*NPV *ie-1* gene (20). Primers used to amplify the 1,746-bp *ie-1* gene were a 5′ primer based on the 5′ end of the *ie-1* coding sequence in the sense orientation (5′-GCGAGATCTAATATGACGCAAATTAATA-3′) and a 3′ primer based on the 3′ end of *ie-1* in the antisense orientation (5′-TCCCCCGGGTTAAAGTTCGAATTTTTT-3′).

Transfections, transient-expression assays, and CAT assays. SF-21 or TN-368 cells (2.0×10^6 cells per 60-mm-diameter dish) were transfected by using Lipofectin reagent (GIBCO BRL). Cells were transfected with 2.0 μg of the reporter plasmid, pETCAThr5, and 1.0 μg of each of the other plasmids unless otherwise noted. Transfected cells were incubated at 27°C for 24 h before harvesting. CAT assays (18, 38) were performed by using 1/10 of the cell lysates or dilutions thereof for quantitation purposes.

Apoptosis assays and internucleosomal DNA fragmentation. SF-21 or TN-368 cells (0.5×10^6 cells per 35-mm-diameter dish) were transfected with 0.5 μg of the indicated plasmid by using Lipofectin. At various times after transfection, cells were examined by light microscopy. At 24 h posttransfection, the medium was removed and the cells were harvested and resuspended in 1 ml of TC-100 medium (without supplements) containing 0.04% trypan blue. Cells that excluded the dye were considered viable. Viable cells were counted in 5 grids of a hemocytometer. Relative cell viability was determined by dividing the number of viable test cells by the number of viable cells transfected with a control plasmid. The mean and standard error were calculated from three replicate samples.

Cells were heat shocked in a 42°C water bath for 30 min beginning at 18 h posttransfection. The cells were then returned to 27°C and analyzed 6 to 12 h after heat shock.

For the analysis of internucleosomal DNA fragmentation, SF-21 cells were harvested 24 h posttransfection and incubated in lysis buffer (20 mM Tris-HCl [pH 7.6], 10 mM EDTA, 0.2% [vol/vol] Triton X-100, and 200-μg/ml protease K) at room temperature overnight. Lysates were extracted with 1:1 (vol/vol) phenol-chloroform. DNA was precipitated with ethanol, dissolved in 10 mM Tris-HCl–1 mM EDTA, and treated with 0.3 mg of RNase A per ml prior to electrophoresis with 1.5% agarose gels in a Tris-acetate-EDTA buffer. DNA was visualized by ethidium bromide staining.

RESULTS

The IE15 clone, containing the 89.2 to 5.7 m.u. region of the Ac*M*NPV genome, can induce apoptosis in SF-21 cells. In order to identify the viral gene(s) which might be involved in the induction of apoptosis in Ac*M*NPV-infected cells, we analyzed the ability of different clones from the Ac*M*NPV genomic library to induce apoptosis. The Ac*M*NPV genomic library is a set of overlapping 10- to 15-kbp cloned fragments which collectively represent the entire Ac*M*NPV genome (34). One clone of this genomic library, the HK5 clone, contains the anti-apoptotic *p35* gene (10). We investigated how the removal of this clone from the genomic library would affect SF-21 cells following transfection.

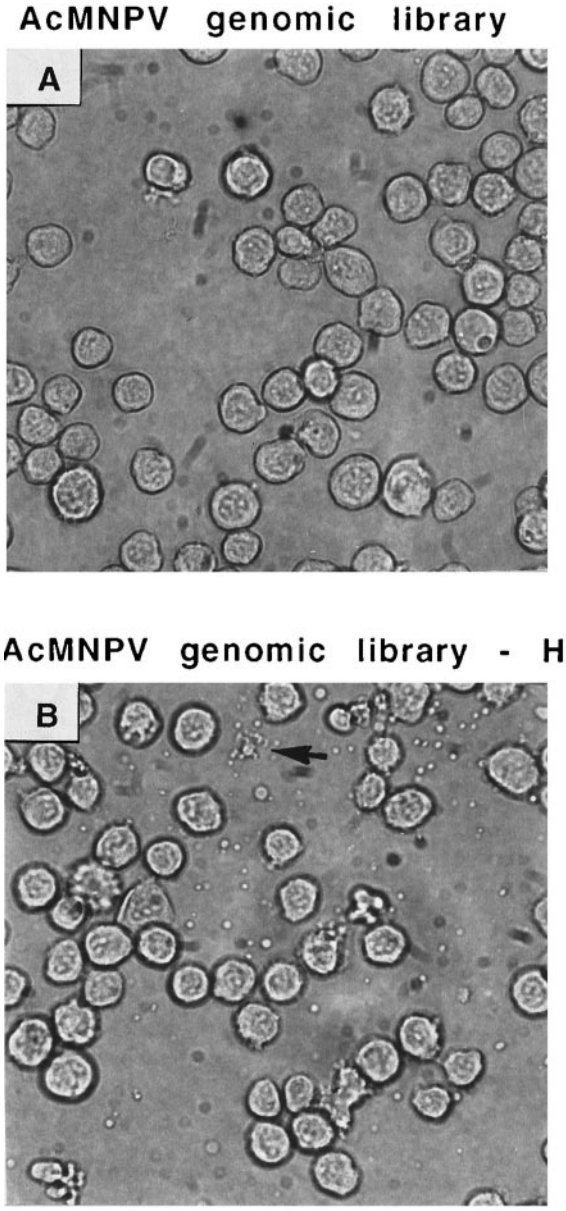
SF-21 cells transfected with either the entire Ac*M*NPV genomic library or this library lacking the HK5 clone were examined by light microscopy (Fig. 1A and B). Cells transfected with the Ac*M*NPV genomic library showed no signs of apoptosis at 48 h (Fig. 1A) and at more than 72 h after transfection (data not shown), but omission of the HK5 clone induced apoptotic activity (Fig. 1B). Cells exhibited signs of apoptosis as early as 18 to 24 h following transfection with the library lacking HK5; intensive membrane blebbing was accompanied by apoptotic body formation by 48 h posttransfection (Fig. 1B). To quantify the level of apoptotic activity, SF-21 cells transfected with either the entire Ac*M*NPV genomic library or this library lacking the HK5 clone were stained with 0.04% trypan blue. The percentages of viable cells in cultures transfected with the entire genomic library or with the library lacking the HK5 clone were 90 and 65%, respectively (Fig. 1C). Thus, in the absence of HK5, the Ac*M*NPV genomic library induced apoptosis in a substantial proportion of cells (ca. 25%) upon transfection of SF-21 cells.

To determine whether any one of the library clones could induce apoptosis, SF-21 cells were transfected with each genomic clone individually. With the exception of IE15, none of the library clones individually induced signs of apoptosis upon transfection in SF-21 cells (data not shown). However, a high level of apoptosis (only 45% viable cells remaining) was obtained when cells were transfected with the IE15 clone which contains the Ac*M*NPV region from 89.2 to 5.7 m.u. (Fig. 2) alone. At 18 h posttransfection, the initial phases of apoptosis, including active blebbing at the cell surfaces, were observed in IE15-transfected SF-21 cells. The blebbing intensified, and by 24 h posttransfection, apoptotic bodies were visible (Fig. 3A, top left). Apoptosis in IE15-transfected cells was confirmed by observation of cellular DNA degradation into oligonucleosomal fragments (Fig. 3B, lane 2). Apoptotic morphology and chromatin degradation did not occur in control SF-21 cells transfected with the vector plasmid pBluescript KS+ (Fig. 3A, bottom right; Fig. 3B, lane 1). No signs of apoptosis could be detected in *T. ni* TN-368 cells transfected with the IE15 clone (data not shown).

IE1 induces apoptosis in SF-21 cells. In order to further define the gene or genes that were responsible for apoptotic activity of the IE15 library clone, we tested subclones from this region (Fig. 2). The addition of the pH_3G and pH_3F plasmids, which together represent most of the IE15 lambda clone, induced apoptosis to an extent comparable to that of the intact IE15 clone (Fig. 3A, compare top micrographs; Fig. 3B, compare lanes 2 and 3). Transfection of plasmid pH_3G alone caused apoptotic morphology and chromatin degradation in SF-21 cells (Fig. 3B, lane 4), while pH_3F had little or no apoptotic activity at 24 h posttransfection (data not shown).

The region of pH_3G responsible for this apoptotic activity was defined by using two subclones of pH_3G, pHEV and pIE1/HC (Fig. 2). SF-21 cells transfected with pHEV, the subfragment of pH_3G from the *Hin*dIII site at 91 m.u. to the *Eco*RV site at 95.1 m.u., did not undergo apoptosis (Fig. 3B, lane 5). Plasmid pIE1/HC induced apoptotic activity resulting in the formation of apoptotic bodies and internucleosomal degradation of chromosomal DNA (Fig. 3A, lower left; Fig. 3B, lane 6).

The relative level of apoptotic activity in SF-21 cells transfected with IE15 or with the different subclones of this region was also quantitated by staining with trypan blue and determining the number of viable cells (Fig. 3C). Approximately 45% of pIE1/HC-transfected cells were dead by 24 h posttransfection. Plasmid pIE1/HC contains only one complete ORF, the gene *ie-1* which encodes IE1. The level of apoptosis was relatively uniform for transfections with 0.3 to 1.0 μg of pIE1/HC (data not shown). A frameshift mutation introduced



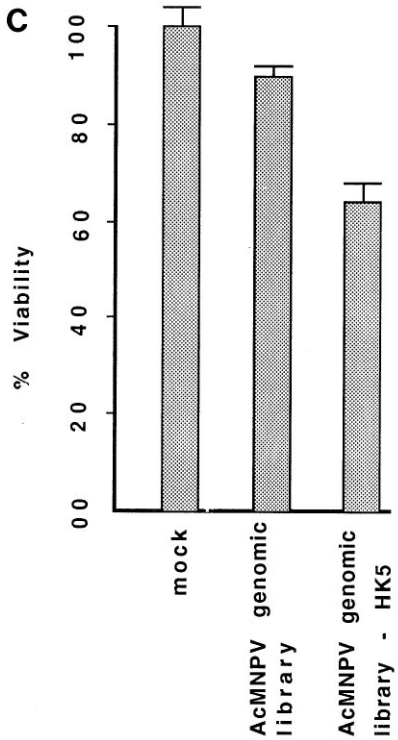


FIG. 1. Apoptosis in SF-21 cells transfected with an Ac*M*NPV genomic library with or without the HK5 lambda clone which contains *p35*. Monolayers of SF-21 cells (0.5×10^6 cells per 35-mm-diameter dish) were transfected with the entire Ac*M*NPV genomic library (0.5 μg of each genomic clone) (A) or with this library lacking the HK5 clone (B). After transfection, cells were maintained at 27°C in complete TC-100 medium. Cells were examined by light microscopy and photographed at 24 h posttransfection. The arrow in panel B indicates a cluster of apoptotic bodies. The percentage of viable cells in SF-21 cultures transfected with the entire Ac*M*NPV clone library or with this library lacking the HK5 lambda clone was compared with mock-transfected cells (C). Cells were stained with trypan blue 24 h posttransfection, and viable cells, indicated by exclusion of the dye, were counted as described in Materials and Methods.

at the *Nde*I site (95.4 m.u.) within the IE1 coding sequence eliminated the apoptotic activity of *ie-1* (Fig. 3C), indicating that IE1 caused apoptosis in SF-21 cells in the absence of other viral gene products in transient assays.

The IE1 ORF under transcriptional control of an insect heat shock promoter enhances transcription and induces apoptosis in SF-21 cells. To ensure that the *ie-1* coding sequence alone had apoptotic activity and that *ie-1* was optimally expressed in both SF-21 and TN-368 cells, we constructed a plasmid, pHSP70PLVI$^+$IE1, in which PCR-amplified coding sequences of IE1 were placed under the transcriptional control of the *D. melanogaster* hsp70 promoter. The hsp70 promoter is known to direct relatively high levels of expression in SF-21 cells in the absence of heat shock (31), but the level of expression can also be increased with heat shock (13). This promoter provides approximately 30% more expression in TN-368 cells than in SF-21 cells as determined by using the reporter plasmid pHSP70PLVI$^+$CAT (data not shown). The hsp70 promoter was supplied within the context of pHSP70PLVI$^+$, which also provides a poly(A) signal and 3 kb of Ac*M*NPV viral sequence flanking the polyhedrin locus (13).

To determine if the PCR-amplified *ie-1* gene under the control of the hsp70 promoter was functional, we tested the ability of pHSP70PLVI$^+$IE1 to transactivate an early viral promoter in transient-expression assays. Previous studies have shown that *ie-1* is necessary and sufficient for the transactivation of the Ac*M*NPV *etl* promoter (34). Therefore, we analyzed the ability of pHSP70PLVI$^+$IE1 to transactivate the CAT gene driven by the *etl* promoter in the reporter plasmid pETCA-Thr5. Negligible CAT activity was observed upon transfection of pETCAThr5 alone in SF-21 cells (Fig. 4A, left bar graph). When SF-21 cells were cotransfected with pHSP70PLVI$^+$IE1 and the reporter plasmid pETCAThr5, a significant increase of CAT gene expression was observed even without heat shock. Slightly higher levels of CAT activity (approximately 1.5-fold higher) were obtained if the transfected cells were heat shocked at 18 h posttransfection (Fig. 4, left bar graph, compare lanes 3 and 4). The plasmid pIE1/HC stimulated slightly higher levels of CAT activity from the *etl* promoter than that determined for the plasmid pHSP70PLVI$^+$IE1 with heat shock.

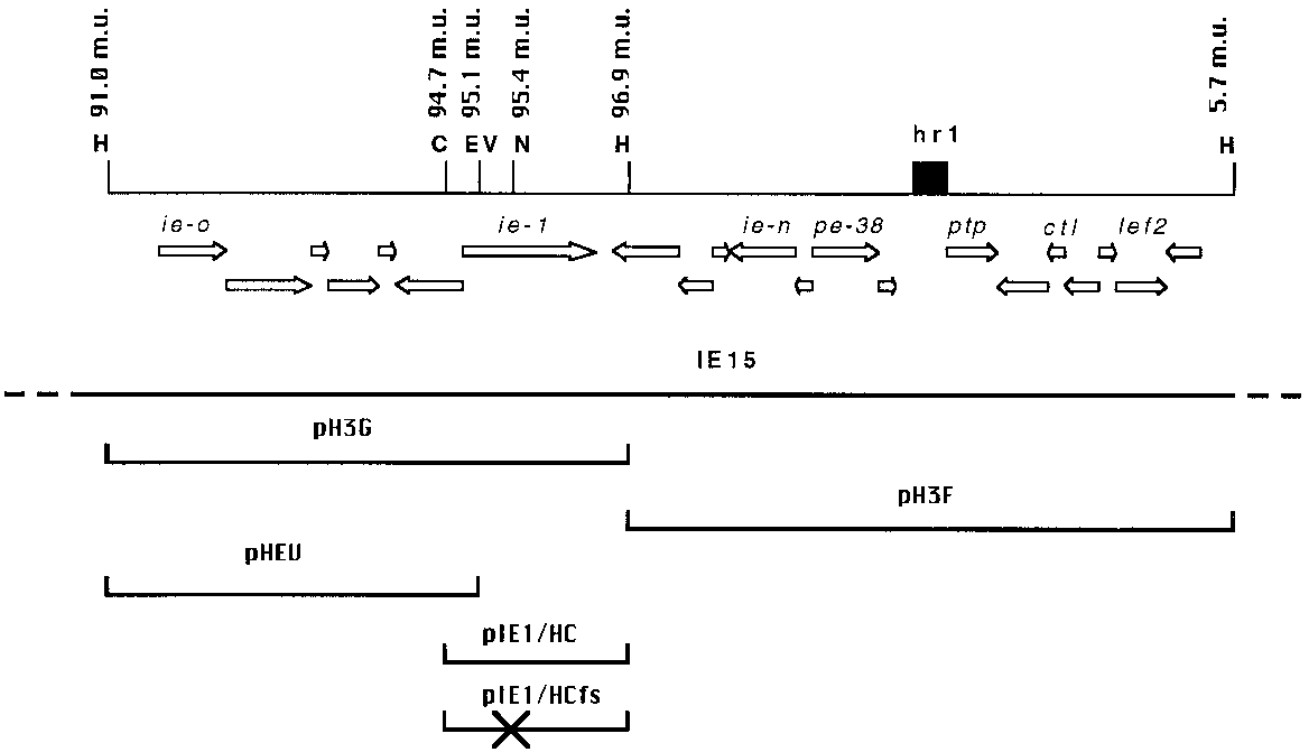


FIG. 2. Subclones in the region of the Ac*M*NPV genome encompassed by the IE15 clone. Plasmid subclones used in analysis of the apoptotic activity of the IE15 lambda clone (34) are shown below a physical map with key restriction sites and respective m.u. Positions of ORFs between 91.8 and 5.7 m.u. are derived from the sequence of Ayres et al. (1). Subclones are indicated by lines below the ORF arrows; the position of a frameshift mutation within the *ie-1* ORF of pIE1/HCfs is indicated by an X. Restriction sites are abbreviated as follows: H, *Hin*dIII; C, *Cla*I; EV, *Eco*RV; N, *Nde*I.

The ability of *ie-1* under the transcriptional control of the hsp70 promoter to induce apoptosis in SF-21 cells was also tested (Fig. 4B, left bar graph). Approximately 25% of SF-21 cells transfected with pHSP70PLVI$^+$IE1 underwent apoptosis. If pHSP70PLVI$^+$IE1-transfected cells were heat shocked, the level of apoptotic activity increased and was comparable to that observed with pIE1/HC. This apoptotic response was due to IE1, since control transfection with pHSP70PLVI$^+$CAT, which expresses the CAT gene under hsp70 promoter control, did not induce apoptosis.

IE1 does not induce apoptosis in TN-368 cells. We then tested whether IE1 can also induce apoptosis in TN-368 cells, which do not undergo apoptosis upon infection with *p35* mutant virus and which are generally more resistant to apoptosis (10, 13). Expression of the *ie-1* ORF transactivated CAT gene expression from the reporter plasmid pETCAThr5 whether *ie-1* was under hsp70 promoter control or the control of its own promoter (Fig. 4A, right bar graph). However, no apoptotic activity was observed in TN-368 cells transfected with either pIE1/HC or pHSP70PLVI$^+$IE1 even after heat shock (Fig. 4B, right bar graph). Thus, *ie-1* expression initiated a host cell-specific apoptotic response in SF-21 but not in TN-368 cells.

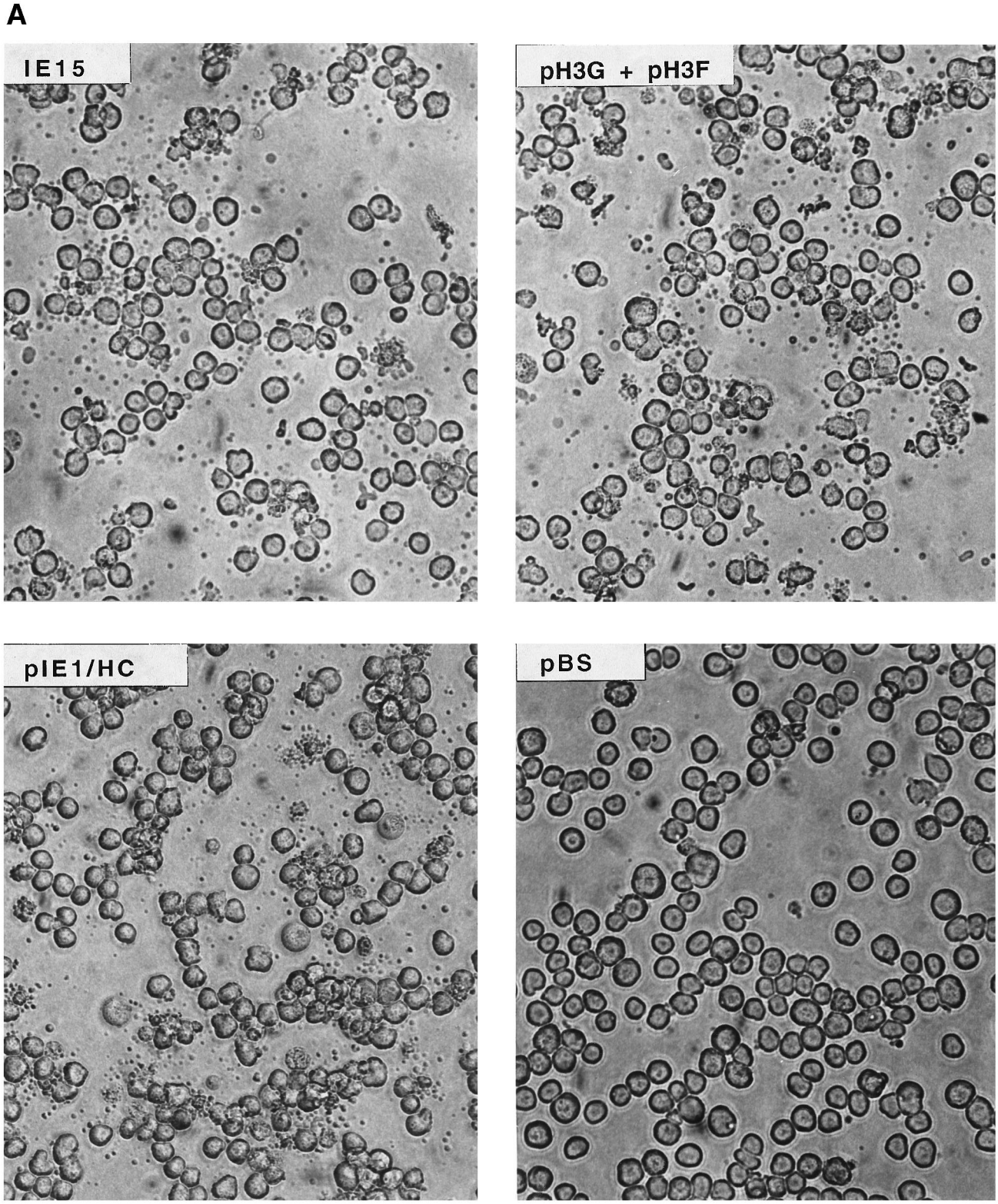
P35 and Cp-IAP inhibit IE1-induced apoptosis. Baculoviruses have at least two types of genes which are able to prevent cellular apoptosis in SF-21 cells: *p35* and *iap* (10, 11, 13, 15). Previously it was shown that *p35* can block apoptosis induced by the gene encoding ICE when both genes are placed under hsp70 promoter control and are cotransfected into SF-21 cells (4).

To determine whether genes with antiapoptotic activity can block IE1-induced apoptosis, the *p35*, Cp-*iap*, and Ac-*iap* genes were transiently expressed in SF-21 cells under the transcriptional control of the hsp70 promoter. SF-21 cells were first transfected with pHSP35VI$^+$, pHSCpIAPVI$^+$, pHSAcIAPVI$^+$, or pHSP70PLVI$^+$CAT, and 3 h later the cells were transfected with pHSP70PLVI$^+$IE1 and heat shocked. Apoptotic activity was examined 8 to 12 h after heat shock. The protective effect of P35 and Cp-IAP was evident by microscopic observation of cell morphology (data not shown), by analysis of cellular DNA degradation (Fig. 5A), and by measurement of cell viability (Fig. 5B). Complete blockage of IE1-induced membrane blebbing and DNA degradation was observed for plasmids expressing either P35 or Cp-IAP (Fig. 5A). Both P35 and Cp-IAP also blocked IE1-induced cell death (Fig. 5B), although P35 appeared to restore viability to only approximately half of the cells. However, at 36 h posttransfection, control transfections revealed that cells transfected with only the P35 gene exhibited reduced cell numbers compared with cells transfected with a plasmid expressing the CAT gene. Thus, P35 gene expression has a slightly negative effect on cell growth; no visual evidence of apoptosis was observed in cells transfected only with *p35*. No protection was observed with the plasmid pHSAcIAPVI$^+$ or with the control plasmid pHSP70PLVI$^+$CAT expressing the CAT gene from the hsp70 promoter.

Thus, both P35 and Cp-IAP but not Ac-IAP are able to block apoptosis initiated by IE1 in transient-expression assays.

DISCUSSION

We have demonstrated that transient expression of the Ac-*M*NPV *ie-1* gene induced apoptosis in SF-21 cells but not in TN-368 cells. The placement of *ie-1* under the control of a



promoter which drives higher levels of expression in TN-368 cells than in SF-21 cells ensured that *ie-1* was being expressed in this cell line. The pattern of cell line-specific induction of apoptosis by IE-1 is similar to that of *p35* mutant-induced apoptosis. However, the cell line specificity of induction may be due to either a specific interaction of IE1 with factors in the apoptotic signal transduction pathway of SF-21 cells or the refractivity of TN-368 cells to apoptosis in general. TN-368

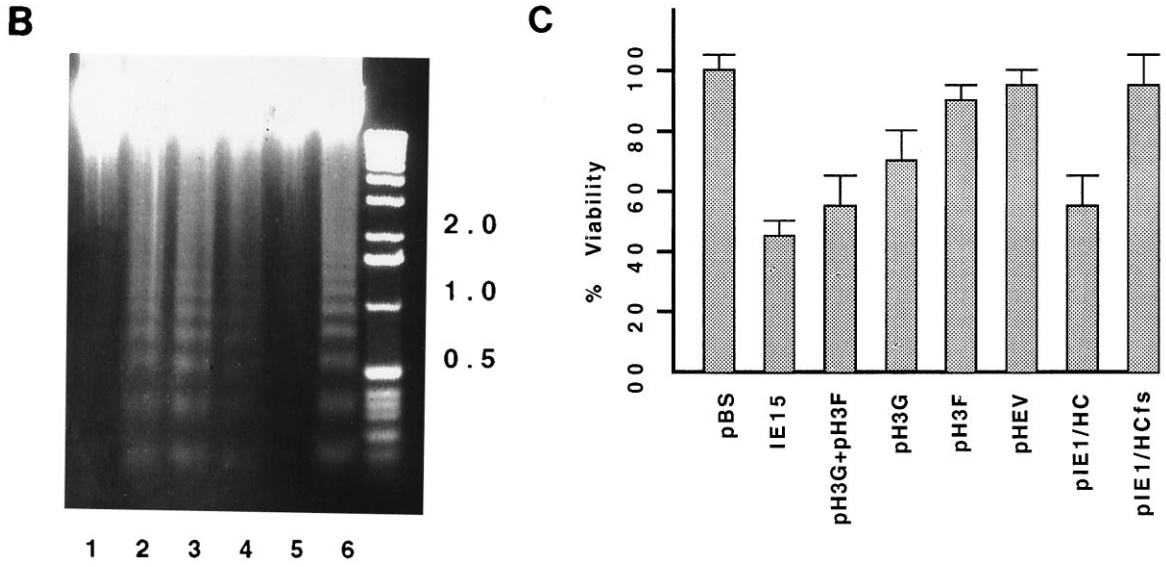


FIG. 3. Analysis of the apoptotic activity in SF-21 cells transfected with IE15 or different subclones of the IE15 region at 24 h posttransfection. (A) Light microscopy of SF-21 cells transfected with the IE15 lambda clone (upper left), a combination of the pH_3G and pH_3F plasmids (upper right), or plasmid pIE1/HC containing the *ie-1* gene (lower left). As a negative control, cells were transfected with pBluescript II KS+ (pBS) (lower right). (B) Internucleosomal DNA fragmentation. Total cellular DNA was extracted from SF-21 cells transfected with IE15 (lane 2), a combination of plasmids pH_3G and pH_3F (lane 3), plasmid pH_3G (lane 4), pHEV (lane 5), or pIE1/HC (lane 6). At 24 h posttransfection, cells and apoptotic bodies were collected, lysed, and subjected to agarose gel electrophoresis. DNA from SF-21 cells transfected with pBluescript II KS+ is shown as a control (lane 1). DNA molecular weight markers (size in kilobase pairs) are indicated at the right. (C) Percentage of viable SF-21 cells remaining at 24 h after transfection with different subclones of IE15. Cells transfected with pBluescript II KS+ (pBS) served as a control for 100% cell viability.

cells are not totally refractive to apoptosis since transfection with the gene encoding ICE protease induces apoptosis (4), but they are more refractive than SF-21 cells to actinomycin D, which induces widespread apoptosis in SF-21 cells (15). If TN-368 cells lack a component of the apoptotic signal transduction pathway upstream of ICE proteases, then any apoptotic inducer acting upstream of this component would be expected to exhibit this type of cell line specificity.

Although IE1 induces apoptosis in transfected SF-21 cells, IE1 is probably not the sole signal which induces apoptosis during *p35* mutant infections of SF-21 cells. Prior data show that aphidicolin blocks apoptosis in *p35* mutant-infected SF-21 cells (13). Aphidicolin, an inhibitor of DNA synthesis, blocks viral replication at the interface between early and late gene expression and, as an early gene, IE1 should be expressed in the presence of aphidicolin. There are several possible explanations for why IE1 might not induce apoptosis during *p35* mutant infection in the presence of aphidicolin. One possibility is that IE1 levels must reach a threshold level before apoptosis is triggered. Although *ie-1* is expressed early in infection, IE1 accumulates to much higher levels during the late and very late phases of infection (6, 20). The observation that the amount of IE1 produced in transfected cells is similar to the IE1 levels found late in infection (8) is consistent with this explanation. A more plausible explanation is that IE1 induces apoptosis in combination with active DNA replication; IE1 might induce unscheduled cellular DNA synthesis which is interpreted by the cell as a signal for apoptosis, or IE1 might alter the cell cycle position such that initiation of DNA replication is interpreted as a signal to initiate apoptosis.

Another observation which suggests that IE1 is not the sole viral signal which induces apoptosis in *p35* mutant-infected cells is that tsB821, a temperature sensitive (ts) mutant of *ie-1*, induces apoptosis during infection of SF-21 cells at the restrictive temperature (40). tsB821 exhibits temperature sensitivity in binding to hr sequences in vitro (8) and in transactivating early and late promoters in transient-expression assays (40). tsB821 is also temperature sensitive in its ability to induce apoptosis in transient assays (data not shown). The fact that apoptosis is induced during tsB821 infections suggests that other features of Ac*M*NPV infection are responsible for the induction of apoptosis. However, tsB821 does eventually express viral genes and replicates its DNA at the nonpermissive temperature, and initiation of apoptosis coincides with the delay in DNA synthesis (40). There are several interpretations of these results: *ie-1* may not be absolutely essential for viral gene activation, DNA replication, and apoptosis; a small amount of active IE1 may be made at the nonpermissive temperature; or the tsB821 protein may retain partial function at the restrictive temperature. Our current experiments do not distinguish among these possibilities.

The actual mechanism by which IE1 induces apoptosis in transfected cells remains unknown. IE1 might transregulate cellular genes which could adversely affect a number of cellular pathways, such as cell cycle regulation, and thereby indirectly trigger apoptosis. Alternatively, IE1 may interact more directly in cellular pathways or may catalyze the formation of replication complexes which SF-21 cells perceive as aberrant DNA replication and/or DNA damage. The rate of induction of apoptosis by IE1 in transfected SF-21 cells was slower than the rate of induction by several known pro-apoptotic genes under identical promoter control and transfection conditions (45b). Thus, IE1 is likely to induce apoptosis indirectly, possibly by transactivating cellular genes or initiating aberrant DNA replication in relationship to the cell cycle position.

We have also shown that IE1-induced apoptosis was blocked by both P35 and antiapoptotic IAPs. P35 is a general inhibitor of the phylogenetically conserved ICE family proteases (4, 46) and is expected to block apoptosis induced by almost all signals. Less is known concerning the IAP family of inhibitors;

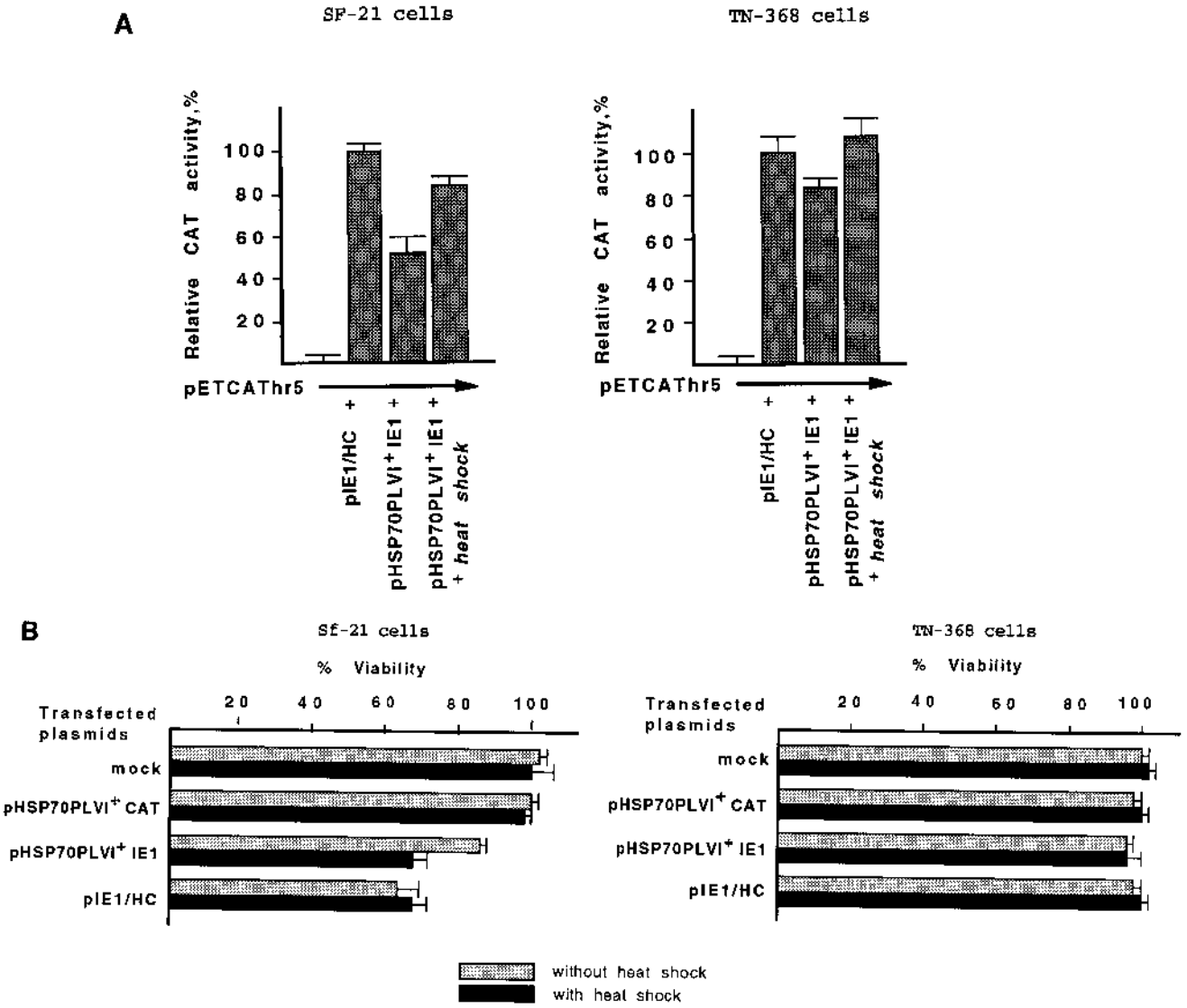


FIG. 4. Analysis of the transregulatory and apoptotic activities of *ie-1* under the control of the hsp70 promoter in SF-21 and TN-368 cell lines. (A) Transient-expression assays showing the level of CAT activity from the *etl* promoter in SF-21 and TN-368 cells. SF-21 or TN-368 cells (2.0×10^6 cells per 60-mm-diameter dish) were transfected with the reporter plasmid pETCAThr5 alone or cotransfected with pETCAThr5 and pIE1/HC or pHSP70PLVI$^+$IE1 containing the *ie-1* gene under hsp70 promoter control. Transfected cells were harvested at 24 h posttransfection. Cells transfected with pHSP70PLVI$^+$IE1 were heat shocked at 18 h posttransfection and then harvested 6 h after heat shock. CAT activity in cell lysates was determined by enzyme assays. (B) Percentage of viable SF-21 cells remaining 18 h after transfection with *ie-1* containing plasmid pIE1/HC or pHSP70PLVI$^+$IE1 or with the CAT control plasmid pHSP70PLVI$^+$CAT. For heat-shocked samples, cells were heat shocked and stained with trypan blue 8 to 12 h later. The numbers of viable cells were determined as described in Materials and Methods.

these inhibitors may be more limited in their ability to protect against apoptotic signals. Thus, the pattern of inhibition of IE-1-induced apoptosis is consistent with *p35* mutant-induced apoptosis but this pattern has been observed for other nonviral apoptotic inducers too (45a).

Approximately 25% of SF-21 cells underwent apoptosis when transfected with the genomic library lacking the HK5 clone containing *p35*. Only the IE15 clone was observed to induce apoptosis when each genomic clone was tested individually, and the proportion of cells undergoing apoptosis was similar to that observed with the genomic library lacking HK5. The level of apoptosis observed with the IE1 subclone alone could account for the level of apoptosis observed with the genomic library lacking p35. Furthermore, when we tested each of the genomic clones in the presence of IE1 in order to provide IE1 transregulatory activity, no increase in the level of apoptosis over that of IE1 alone was observed (data not shown). However, we have not eliminated the possibility that other viral genes may also have apoptotic activity; only 25% of the cells may be at a point in the cell cycle where induction of apoptosis occurs upon expression of viral genes under these assay conditions.

In summary, we have demonstrated that IE1 can induce apoptosis in SF-21 cells. The activity of this gene could account for the fact that *p35* mutants induce apoptosis during virus infection of this cell line. Like adenovirus E1A, which also induces apoptosis (16, 39), IE1 acts as a strong transcriptional activator in transient-expression assays. However, IE-1 is likely to have other functions, including a role in the initiation of DNA replication. Independent data suggest that DNA repli-

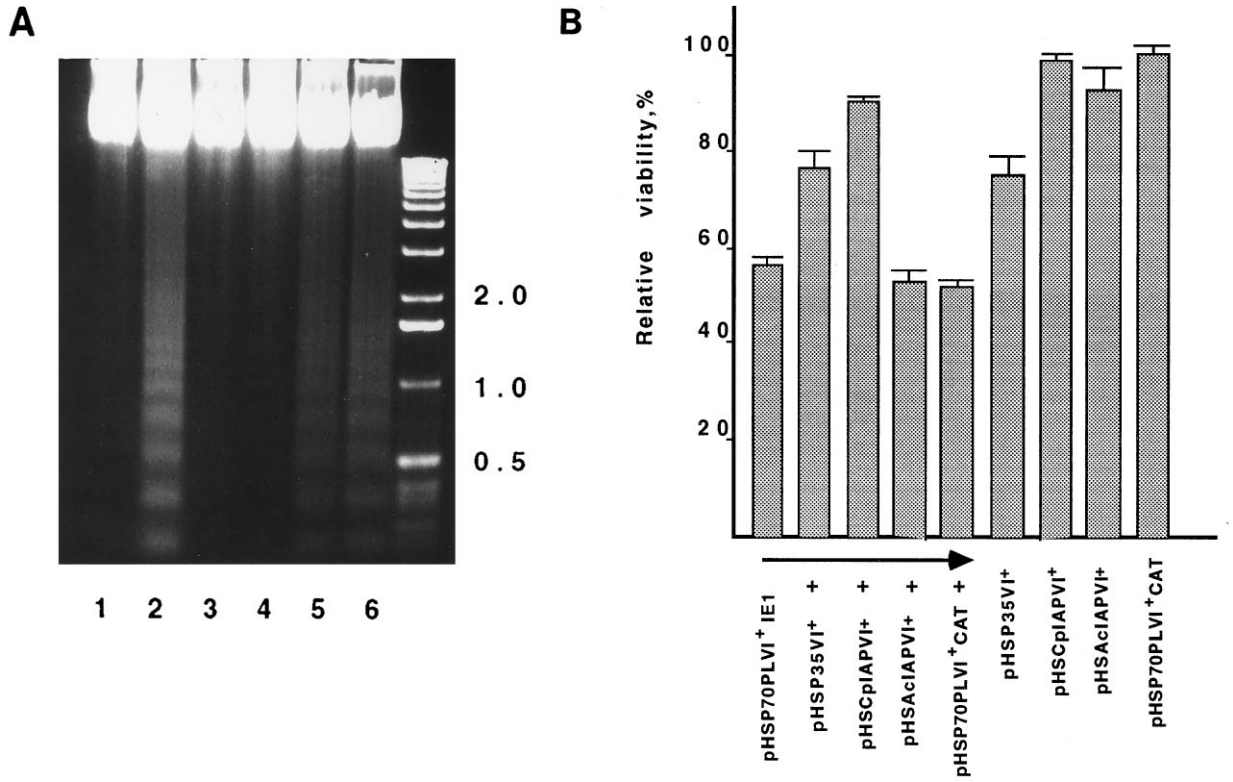


FIG. 5. Inhibition of IE1-induced apoptosis by *p35* and Cp-*iap*. (A) The effects of gene expression on DNA fragmentation. SF-21 cells (0.5×10^6 cells per 35-mm-diameter dish) were transfected with pHSP35VI$^+$ (lane 3), pHSPCpIAPVI$^+$ (lane 4), pHSAcIAPVI$^+$ (lane 5), or pHSP70PLVI$^+$CAT (lane 6) (3.0 μg of plasmid DNA) and transfected 3 h later with pHSP70PLVI$^+$IE1 (0.5 μg of plasmid DNA). At 18 h after the second transfection, cells were heat shocked and then harvested 12 h later. Total cellular DNA was extracted from transfected cells and examined by agarose (1.5%) gel electrophoresis. Lane 1, total cellular DNA from cells transfected with pHSP70PLVI$^+$CAT (negative control); lane 2, DNA fragmentation in pHSP70PLVI$^+$IE1-transfected cells. DNA molecular weight markers (size in kilobase pairs) are indicated at the right. (B) The effect of gene expression on cell viability. Cells were transfected as described above except that they were analyzed for cell viability rather than DNA fragmentation. A control set of transfections were also performed in which the cells were mock transfected during the second transfection rather than receiving pHSP70PLVI$^+$IE1.

cation may directly or indirectly play a role in the induction of apoptosis in baculovirus-infected cells. Transcriptional activation of cellular genes, interference with a cellular process such as the cell cycle, or initiation of unscheduled DNA replication could all account for the ability of IE1 to induce apoptosis. The cellular pathway used in IE1-induced apoptosis involved one or more P35-inhibitable cysteine proteases and was also inhibitable by the IAP class of apoptotic inhibitors.

ACKNOWLEDGMENTS

We thank Grigori G. Prikhod'ko, Somasekar Seshagiri, and Jeanne McLachlin for technical advice.

This research was supported in part by Public Health Service grants AI38262 and AI23719 from the National Institute of Allergy and Infectious Diseases.

REFERENCES

1. **Ayres, M. D., S. C. Howard, J. Kuzio, M. L. Ferber, and R. D. Possee.** 1994. The complete DNA sequence of *Autographa californica* nuclear polyhedrosis virus. Virology **202:**586–605.
2. **Beidler, D. R., M. Tewari, P. D. Friesen, G. Poirier, and V. M. Dixit.** 1995. The baculovirus p35 protein inhibits Fas- and tumor necrosis factor-induced apoptosis. J. Biol. Chem. **270:**16526–16528.
3. **Birnbaum, M. J., R. J. Clem, and L. K. Miller.** 1994. An apoptosis-inhibiting gene from a nuclear polyhedrosis virus encoding a polypeptide with cys/his sequence motifs. J. Virol. **68:**2521–2528.
4. **Bump, N. J., M. Hackett, M. Hugunin, S. Seshagiri, K. Brady, P. Chen, C. Ferenz, S. Franklin, T. Ghayur, P. Li, P. Licari, J. Mancovich, L. Shi, A. Greenberg, L. K. Miller, and W. W. Wong.** 1995. Inhibition of ICE family proteases by baculovirus anti-apoptotic protein P35. Science **269:**1885–1888.
5. **Carson, D. D., M. D. Summers, and L. A. Guarino.** 1991. Transient expression of the *Autographa californica* nuclear polyhedrosis virus immediate-early gene, IE-N, is regulated by three viral elements. J. Virol. **65:**945–951.
6. **Chisholm, G. E., and D. J. Henner.** 1988. Multiple early transcripts and splicing of the *Autographa californica* nuclear polyhedrosis virus IE-1 gene. J. Virol. **62:**3193–3200.
7. **Choi, J., and L. A. Guarino.** 1995. The baculovirus transactivator IE1 binds to viral enhancer elements in the absence of insect cell factors. J. Virol. **69:**4548–4551.
8. **Choi, J., and L. A. Guarino.** 1995. A temperature-sensitive IE1 protein of *Autographa californica* nuclear polyhedrosis virus has altered transactivation and DNA-binding activities. Virology **209:**90–98.
9. **Choi, J., and L. A. Guarino.** 1995. Expression of the IE1 transactivator of *Autographa californica* nuclear polyhedrosis virus during viral infection. Virology **209:**99–107.
10. **Clem, R. J., M. Fechheimer, and L. K. Miller.** 1991. Prevention of apoptosis by a baculovirus gene during infection of insect cells. Science **254:**1388–1390.
11. **Clem, R. J., J. M. Hardwick, and L. K. Miller.** 1996. Anti-apoptotic genes of baculoviruses. Cell Death Differ. **3:**9–16.
12. **Clem, R. J., and L. K. Miller.** 1993. Apoptosis reduces both the in vitro replication and the in vivo infectivity of a baculovirus. J. Virol. **67:**3730–3738.
13. **Clem, R. J., and L. K. Miller.** 1994. Control of programmed cell death by the baculovirus genes *p35* and *iap*. Mol. Cell. Biol. **14:**5212–5222.
14. **Crawford, A. M., and L. K. Miller.** 1988. Characterization of an early gene accelerating expression of late genes of the baculovirus *Autographa californica* nuclear polyhedrosis virus. J. Virol. **62:**2773–2781.
15. **Crook, N. E., R. J. Clem, and L. K. Miller.** 1993. An apoptosis-inhibiting baculovirus gene with a zinc finger motif. J. Virol. **67:**2168–2174.
16. **Debbas, M., and E. White.** 1993. Wild-type p53 mediates apoptosis by E1A, which is inhibited by E1B. Genes Dev. **7:**546–554.
17. **Duckett, C. S., V. E. Nava, R. W. Gedrich, R. J. Clem, J. L. Van Dongen, M. C. Gilfallan, H. Shiels, J. M. Hardwick, and C. B. Thompson.** EMBO J., in press.
18. **Gorman, M. A., L. F. Moffat, and B. H. Howard.** 1982. Recombinant ge-

nomes which express chloramphenicol acetyltransferase in mammalian cells. Mol. Cell. Biol. **2:**1044–1051.
19. **Guarino, L. A., and M. D. Summers.** 1986. Interspersed homologous DNA of *Autographa californica* nuclear polyhedrosis virus enhances delayed-early gene expression. J. Virol. **60:**215–223.
20. **Guarino, L. A., and M. D. Summers.** 1987. Nucleotide sequence and temporal expression of a baculovirus regulatory gene. J. Virol. **61:**2091–2099.
21. **Guarino, L. A., and W. Dong.** 1994. Functional dissection of the *Autographa californica* nuclear polyhedrosis virus enhancer element hr5. Virology **200:** 328–335.
22. **Hay, B. A., D. A. Wassarman, and G. M. Rubin.** 1995. Drosophila homologs of baculovirus inhibitor of apoptosis proteins function to block cell-death. Cell **83:**1253–1262.
23. **Hay, B. A., T. Wolff, and G. M. Rubin.** 1994. Expression of baculovirus P35 prevents cell death in *Drosophila*. Development **120:**2121–2129.
24. **Hershberger, P. A., J. A. Dickson, and P. D. Friesen.** 1992. Site-specific mutagenesis of the 35-kilodalton protein gene encoded by *Autographa californica* nuclear polyhedrosis virus: cell line-specific effects on virus replication. J. Virol. **66:**5525–5533.
25. **Hink, W. F.** 1970. Established insect cell line from the cabbage looper, *Trichoplusia ni*. Nature (London) **226:**466–467.
26. **Kool, M., C. H. Ahrens, R. W. Goldbach, G. F. Rohrmann, and J. M. Vlak.** 1994. Identification of genes involved in DNA replication of the *Autographa californica* baculovirus. Proc. Natl. Acad. Sci. USA **91:**11212–11216.
27. **Kovacs, G. R., J. Choi, L. A. Guarino, and M. D. Summers.** 1992. Functional dissection of the *Autographa californica* nuclear polyhedrosis virus immediate-early 1 transcriptional regulatory protein. J. Virol. **66:**7429–7437.
28. **Liston, P., N. Roy, K. Tamai, C. Lefebvre, S. Baird, G. Cherton-Horvat, R. Farahani, M. McLean, and J. E. Ikeda.** 1996. Suppression of apoptosis in mammalian cells by NAIP and a related family of IAP genes. Nature (London) **379:**349–353.
29. **Lu, A., and L. K. Miller.** 1995. The roles of eighteen baculovirus late expression factor genes in transcription and DNA replication. J. Virol. **69:**975–982.
30. **Martinou, I., P. A. Fernandez, M. Missotten, E. White, B. Allet, R. Sadoul, and J. C. Martinou.** 1995. Viral protein E1B 19K and p35 protect sympathetic neurons from cell death induced by NGF deprivation. J. Cell Biol. **128:**201–208.
31. **Morris, T. D., and L. K. Miller.** 1992. Promoter influence on baculovirus-mediated gene expression in permissive and nonpermissive insect cell lines. J. Virol. **66:**7397–7405.
32. **Nissen, M. S., and P. D. Friesen.** 1989. Molecular analysis of the transcriptional regulatory region of an early baculovirus gene. J. Virol. **63:**493–503.
33. **O'Reilly, D. R., L. K. Miller, and V. A. Luckow.** 1992. Baculovirus expression vectors: a laboratory manual. W. H. Freeman & Co., New York.
34. **Passarelli, A. L., and L. K. Miller.** 1993. Three baculovirus genes involved in late and very late gene expression: *ie-1*, *ie-n*, and *lef-2*. J. Virol. **67:**2149–2158.
35. **Pearson, M., R. Bjornson, G. Pearson, and G. Rohrmann.** 1992. The *Autographa californica* baculovirus genome: evidence for multiple replication origins. Science **257:**1382–1384.
36. **Pearson, M. N., and G. F. Rohrmann.** 1995. *Lymantria dispar* nuclear polyhedrosis virus homologous regions: characterization of their ability to function as replication origins. J. Virol. **69:**213–221.
37. **Rabizadeh, S., D. J. LaCount, P. D. Friesen, and D. E. Bredesen.** 1993. Expression of the baculovirus p35 gene inhibits mammalian neural cell death. J. Neurochem. **61:**2318–2321.
38. **Rankin, C., B. G. Ooi, and L. K. Miller.** 1988. Eight base pairs encompassing the transcriptional start point are the major determinant for baculovirus polyhedrin gene expression. Gene **70:**39–50.
39. **Rao, L., M. Debbas, P. Sabbatini, D. Hockenbery, S. Korsmeyer, and E. White.** 1992. The adenovirus E1a protein induces apoptosis, which is inhibited by the E1B 19-kDa and Bcl-2 protein. Proc. Natl. Acad. Sci. USA **89:**7742–7746.
40. **Ribeiro, B. M., K. Hutchinson, and L. K. Miller.** 1994. A mutant baculovirus with a temperature-sensitive IE1 transregulatory protein. J. Virol. **68:**1075–1084.
41. **Rodems, S. M., and P. D. Friesen.** 1995. Transcriptional enhancer activity of *hr5* requires dual-palindrome half sites that mediate binding of a dimeric form of the baculovirus transregulator IE1. J. Virol. **69:**5368–5375.
42. **Rothe, M., M. G. Pan, W. J. Henzel, T. M. Ayres, and D. V. Goeddel.** 1995. The TNFR2-TRAF signaling complex contains 2 novel proteins related to baculoviral-inhibitor of apoptosis proteins. Cell **83:**1243–1252.
43. **Sugimoto, A., P. D. Friesen, and J. H. Rothman.** 1994. Baculovirus p35 prevents developmentally programmed cell death and rescues a ced-9 mutant in the nematode Caenorhabditis elegans. EMBO J. **13:**2023–2028.
44. **Toeroek, I., and F. Karch.** 1980. Nucleotide sequences of heat shock activated genes in Drosophila melanogaster. I. Sequences in the regions of the 5′ and 3′ ends of the hsp70 gene in the hybrid plasmid 56h8. Nucleic Acids Res. **8:**3105–3123.
45. **Vaughn, J. L., R. H. Goodwin, G. J. Tompkins, and P. McCawley.** 1977. The establishment of two cell lines from the insect *Spodoptera frugiperda* (Lepidoptera: Noctuidae). In Vitro (Rockville) **13:**213–217.
45a. **Vucic, D., A. J. Harvey, and L. K. Miller.** Unpublished data.
45b. **Vucic, D., S. Seshagiri, A. J. Harvey, and L. K. Miller.** Unpublished data.
46. **Xue, D., and H. R. Horvitz.** 1995. Inhibition of the Caenorhabditis elegans cell-death protease ced3 by a ced-3 cleavage site in baculovirus p35 protein. Nature (London) **377:**248–251.