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SHORT COMMUNICATION

Is Governed by the Expression of the *crmA* Gene

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Pig kidney cells (LLC-PK_1) were infected with one of three viruses: wild-type cowpox virus (Brighton red strain) expressing the *crmA* gene; recombinant cowpox virus A602, lacking the *crmA* gene; or cowpox virus A604, a revertant of virus A602, expressing the *crmA* gene. The wild-type virus and virus A604 produced identical cytopathic effects consistent with death by necrosis. In these cells, the structural features of the plasma membrane, the nuclear membrane, and the chromatin were maintained until lysis of the cells. In contrast, cowpox virus A602 produced cytopathic effects consistent with death by apoptosis. These effects included loss of microvilli on the cell surface, margination and condensation of the chromatin, progressive convolution of the nuclear membrane, release of dense chromatin masses on disintegration of the nucleus, fragmentation of the DNA, and the generation of apoptotic bodies. These results suggest that the *crmA* gene is necessary to inhibit processes of apoptosis induced in LLC-PK_1 cells by infection with cowpox virus. Thus in cells of certain types, the *crmA* gene can act with other viral genes to control the mode of death of the virus-infected cell. This capability may be advantageous to virus replication *in vivo,* potentially facilitating both virus trafficking and interference with antiviral immune defenses.

The cowpox virus *crmA* gene encodes a serpin that is a potent inhibitor of mammalian interleukin-1β converting enzyme (ICE) and other ICE-like cysteine proteinases (*1–4*). The CrmA protein is also an inhibitor of granzyme B, a serine proteinase that shares a similar substrate specificity with ICE (*5, 6*).

The *crmA* gene was the first poxvirus gene implicated in the direct inhibition of the process of apoptosis. In 1993, Yuan *et al.* (*7*) showed that the *ced-3* gene, one of the genes essential for apoptosis in *Caenorhabditis elegans,* encoded a protein sharing significant amino acid sequence similarity to the precursors of mammalian ICE. This suggested that ICE, or a similar cysteine proteinase, might be involved in the induction of apoptosis in mammalian cells. It also suggested that inhibitors of ICE, such as the CrmA protein, might inhibit ICE-mediated apoptosis. These predictions were confirmed by Miura *et al.* (*8*) who showed both that the expression of the murine ICE in Rat-1 cells led to the induction of apoptosis and that ICE-induced apoptosis could be inhibited by the expression of either the cowpox virus *crmA* gene or the human *bcl-2* gene, an inhibitor of apoptosis in a number of systems (reviewed in *9*).

The CrmA protein has subsequently been shown to inhibit apoptosis induced by several different stimuli in a variety of cells. Gagliardini *et al.* (*10*) demonstrated that the expression of the *crmA* gene could prevent cell death induced by the deprivation of nerve growth factor from chicken dorsal root ganglion neurons. Boudreau *et al.* (*11*) showed that *crmA* gene expression could inhibit apoptosis in mammary epithelial cells. The CrmA protein has also been shown to inhibit apoptosis mediated by the following: tumor necrosis factor α (*12–15*); Fas (*12, 14, 16, 17*); FADD, a death domain containing protein that interacts with the death domain of Fas (*18*); TRADD, a protein that binds to the intracellular domain of the type I TNF receptor (*19*); and Yama/CPP32β, a mammalian homologue of Ced-3, which cleaves poly(ADP-ribose)polymerase (*4*). These results suggest that the CrmA protein is capable of inhibiting endogenous ICE-like proteinases involved in pathways of apoptosis. In addition, the CrmA-mediated inhibition of granzyme B, which is involved in apoptosis induced by cytotoxic lymphocytes (*20–22*), indicates that the CrmA protein may be capable of inhibiting processes of apoptosis mediated by exogenous proteinases delivered to a target cell by cytotoxic lymphocytes (*5*). Though as yet, there is evidence only to support CrmA-mediated inhibition of cytotoxic lymphocyte killing primarily mediated by Fas ligand (*23*).

These observations suggested that the CrmA protein might contribute to the inhibition of apoptosis induced in a cell infected with cowpox virus. Cowpox virus normally causes the death of infected cells by necrosis, consistent

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with this proposal. Furthermore, evidence that one other gene of cowpox virus can function in a this manner was recently provided by Ink *et al.* (*24*), who showed that expression of the *CHOhr* gene, a host-range gene, could delay the onset of apoptosis in Chinese hamster ovary cells infected with vaccinia virus. The importance of this type of viral countermeasure to this host response is underscored by the fact that DNA-containing viruses of several families, including the adenoviruses, the herpesviruses, and the baculoviruses, each encode proteins that can inhibit the induction of apoptosis in virus-infected cells (*25–31*).

The effects of the *crmA* gene on modes of cell death were examined in the context of infections with either wild-type cowpox virus (Brighton red strain) or one of two recombinant viruses, a Δ*crmA* cowpox virus (A602) and a revertant (A604) of this virus. These viruses were constructed by standard methods (*32*), except that precautions were taken to minimize alterations to genetic loci other than the *crmA* gene. This was done by use of insertion vectors whose only regions of homology with the virus genome were contained within the 1.4-kb *Nco*I–*Hae*III fragment spanning the *crmA* gene. This method prevented the potential replacement of several kilobases of viral genome with DNA from the plasmid, thereby reducing the possibility that alterations were introduced into the viral DNA in addition to those targeted to the *crmA* gene. Accordingly, the Δ*crmA* cowpox virus A602 was made using insertion plasmid p1853, comprising a 2-kb *Xho*I–*Bam*HI fragment of DNA containing the *p7.5/gpt* gene (the *Escherichia coli* guanosine phosphoribosyltransferase gene under the control of the vaccinia virus p7.5 promoter) derived from plasmid p1761. Plasmid p1761 comprises a 2.1-kb *Eco*RI fragment containing the *p7.5/gpt* gene from pTK61-*gpt* (*32*) inserted into the *Eco*RI site of pGEM7zf+ (Promega Corp., Madison, WI). The revertant of virus A602, cowpox virus A604, was constructed using insertion vector p1843, comprising the 1.4-kb *Nco*I–*Hae*III fragment spanning the cowpox virus *crmA* gene, inserted into the *Eco*RI site of a pUC19 plasmid lacking the polylinker region except for the *Eco*RI and *Hin*dIII sites. DNA hybridization analyses (*33*), using p1843 as the probe of viral DNAs digested with *Eco*RI, *Pst*I, and *Cla*I, were used to confirm the deletion or reinsertion of the *crmA* gene in the recombinant viruses A602 and A604. Rabbit antibody against the CrmA protein was used in immunoprecipitation analyses to confirm both the lack of expression of the *crmA* gene by virus A602 and the expression of the *crmA* gene by virus A604. The infection of the chorioallantoic membranes of chick embryos, as described (*34*), confirmed that virus A602 produced the expected white pocks (*1, 35*), whereas virus A604 produced red pocks identical to those generated by the wild-type virus (data not shown).

The effects of the *crmA* gene on cell death during virus infection were first examined by monitoring the cytopathic effects of cowpox virus replication in cells of several types. Wild-type cowpox virus and cowpox virus A602, which lacks the *crmA* gene, each replicated in human 143 cells (*36*), Chinese hamster ovary cells (*37*), Rat-1 cells (*8*), RK13 rabbit kidney cells (*38*), or LLC-PK_1 pig kidney cells (*39*). In cells of each of these types, the wild-type virus produced cytopathic effects which included a change of the cell morphology from an elongated spindle-shaped form to a rounded form. These cytopathic effects were maintained up to 24 hr after infection, at which time the cells began to disintegrate. Cowpox virus A602 produced cytopathic effects similar to those of the wild-type virus in cells of each type except the LLC-PK_1 cells (Fig. 1). In these cells, within 8 hr of infection, virus A602 produced multiple protuberances on the surfaces of most if not all the cells. As the infection progressed, the protuberances separated from the cells, producing spherical or ovoid-shaped bodies that were released into the medium. Significantly, these cytopathic effects were not produced in cells infected with either the original wild-type virus or the revertant virus A604, demonstrating that these effects, which are characteristic features of cells undergoing apoptosis (reviewed in *40*), were the result of the deletion of the *crmA* gene.

Other characteristic features of apoptosis include the condensation and margination of the chromatin and the indentation of the nuclear membrane. Subsequently, the lobes of the nucleus fragment into the cytoplasm or into apoptotic bodies. Effects of these types were also evident in the LLC-PK_1 cells infected with the Δ*crmA* virus A602, but not in cells infected with either the wild-type virus or the *crmA* revertant virus A604. Fragmentation of the nuclei of cells infected with virus A602 was most clearly resolved by electron microscopy of sections of infected cells. Electron microscopy enabled mature and immature virus particles to be detected in the cytoplasm of each cell, providing a means of confirming that virus replication had occurred in a given cell. Only cells in which such particles were detected were evaluated for morphological features resulting from virus replication. Sections through cells harvested 8 hr after infection with the Δ*crmA* virus A602 showed cells possessing morphologies typical of cells undergoing apoptosis (Fig. 2). These cells had lost the microvilli present on the surface of uninfected cells. They possessed marginated chromatin, often localized into lobes. With increasing time after infection, their nuclei became more convoluted, progressively degenerating into fragments of chromatin within the cytoplasm or within apoptotic bodies. In contrast, cells infected with either wild-type cowpox virus or the revertant A604 maintained the normal structure of their nuclei until lysis of the cell. There was no evidence of margination of the chromatin, indentation of the nuclei, fragmentation of the nuclei, or the generation of apoptotic bodies, in cells infected with either of these two viruses at any time after infection.

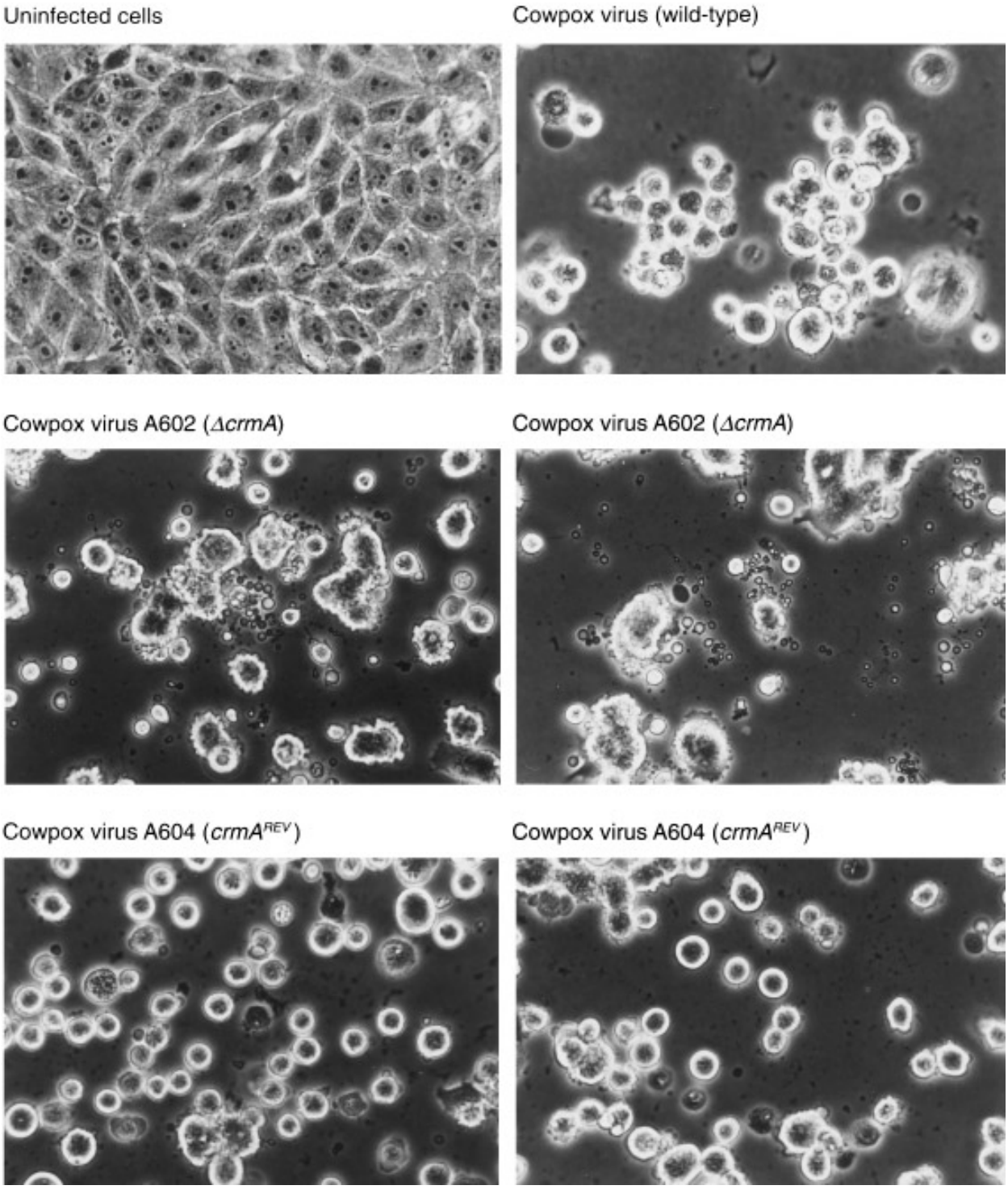

FIG. 1. Expression of the *crmA* gene affects the morphology LLC-PK_1 cells infected with cowpox virus. LLC-PK_1 cells were infected with wild-type cowpox virus, cowpox virus A602 (lacking the *crmA* gene), or cowpox virus A604 (a revertant of virus A602), at multiplicities of infection of 10 PFU per cell. After 8 hr of incubation at 37°, uninfected cells and the virus-infected cells were visualized by phase-contrast microscopy (using a Zeiss IM35 inverted microscope) and photographed on Kodak TMAX 100.

The quantitative nature of the differences in cytopathic effects induced by the three cowpox viruses upon infection of the LLC-PK_1 cells was further demonstrated by quantifying the proportions of populations of infected cells containing either fragmented nuclei or membranes permeable to propidium iodide (Fig. 3). As in other instances where apoptosis is induced in a population of cells (reviewed in *42*), apoptosis in the virus-infected

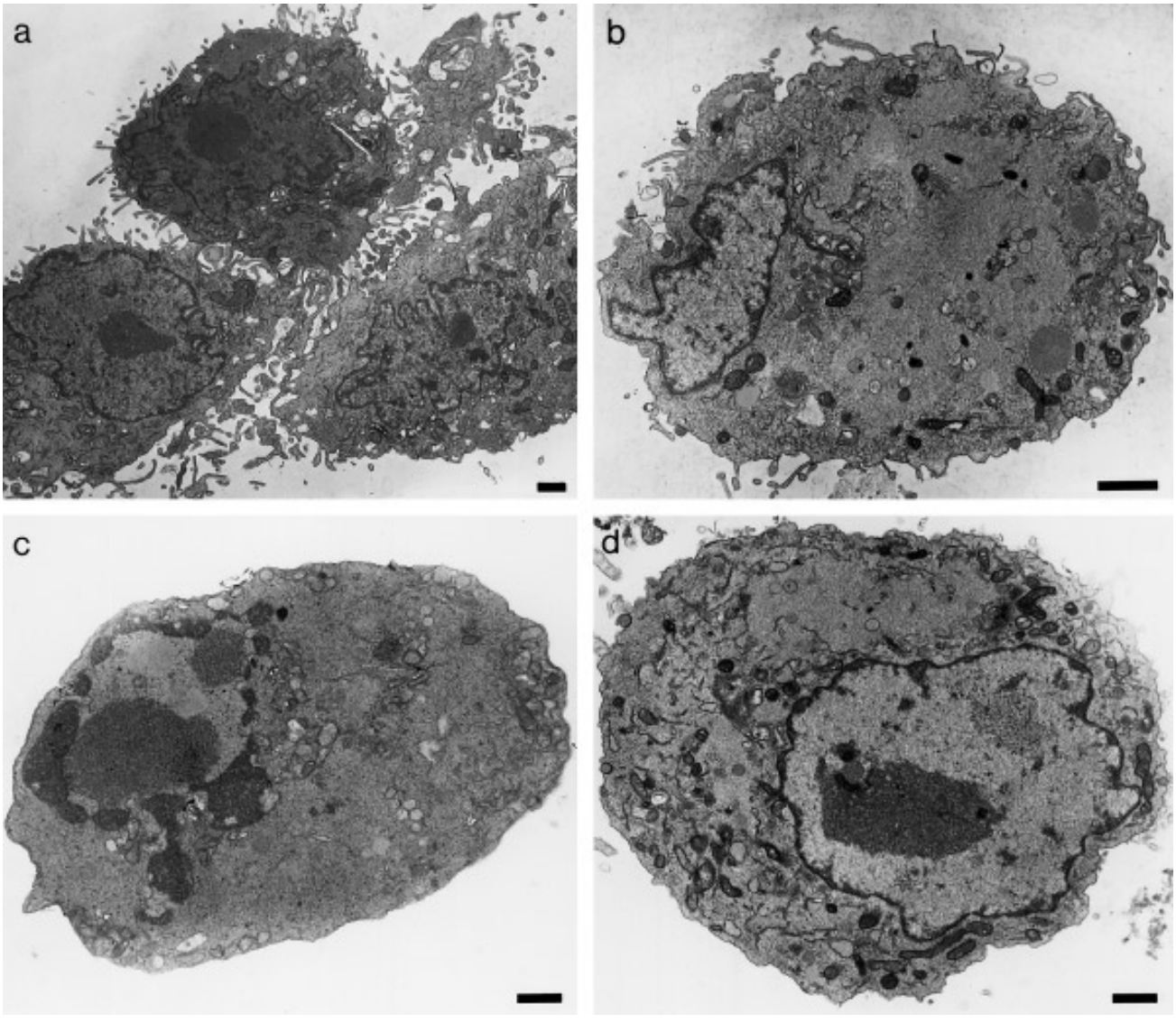

FIG. 2. LLC-PK_1 cells infected with cowpox virus exhibit morphological features associated either with death by necrosis, in the presence of CrmA protein, or death by apoptosis, in the absence of the CrmA protein. LLC-PK_1 cells were left uninfected (a) or infected with wild-type cowpox virus (b), Δ*crmA* virus A602 (c), or cowpox virus A604 (d), each at multiplicities of infection of 10 PFU per cell. After 8 hr of incubation at 37°, the cells were scraped from their dishes into the media. The cells were recovered by centrifugation (1000 rpm/20 min/4° in a Sorvall H1000B rotor), fixed in buffer containing 2% glutaraldehyde/0.1 *M* sodium cacodylate, pH 7.4/3.4% sucrose, and prepared for electron microscopy as described (*41*). Briefly, the cells were postfixed in osmium tetroxide and stained in uranyl acetate. The cells were embedded in Spurr resin, and sections were cut on a Reichert–Jung ultracut E microtome using a diamond knife. Sections were then poststained with uranyl acetate and lead citrate. After washing, the sections were viewed and photographed in a Jeol 100B electron microscope. Scale bars in each photograph represent 1 μm.

cells occurs asynchronously, perhaps because of the lack of cell-cycle synchrony among the cells at the time of viral infection. Nevertheless, marked differences were evident among the cell populations, especially by about 12 hr after infection, when most of the cells infected with viruses expressing the *crmA* gene have become permeable to propidium iodide. First, cells were stained with DAPI (4′,6-diamindino-2-phenylindole) to detect the cells passing through the late apoptotic phase of nuclear fragmentation, a phase that occurs prior to disintegration of the cell. About 16% of the cells infected with cowpox virus A602 (lacking the *crmA* gene) were going through this apoptotic phase. In contrast, fragmented nuclei were detected in less than 0.3% of cells infected with wild-type cowpox virus, and in less than 0.6% of cells infected with revertant virus A604. The latter populations of cells may have been irreversibly committed to apoptosis prior to infection, because 1.3% of uninfected cells also contained fragmented nuclei. Parenthetically, the percentage of uninfected cells containing fragmented nuclei may have been slightly higher than those of the populations of *crmA*-expressing virus-infected cells, because only the uninfected cells continued to divide and reach confluency levels capable of inducing contact inhibition of cell

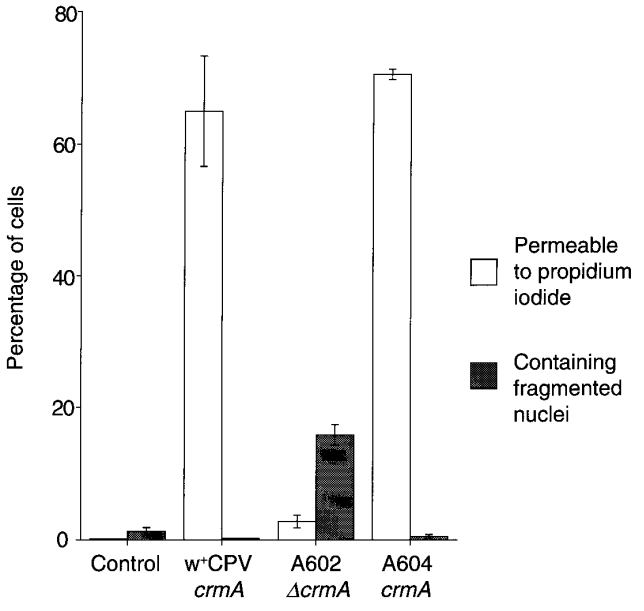

FIG. 3. The membranes of LLC-PK_1 cells infected with cowpox viruses expressing the *crmA* gene become permeable to propidium iodide while maintaining unfragmented nuclei, whereas cells infected with cowpox virus lacking the *crmA* gene retain membrane impermeability to propidium iodide while their nuclei undergo fragmentation. LLC-PK_1 cells in preconfluent monolayers were infected with wild-type cowpox virus, cowpox virus A602 (lacking the *crmA* gene), or cowpox virus A604 (a revertant of virus A602), at multiplicities of infection of 10 PFU per cell. After 12 hr of incubation at 37°, uninfected cells and the virus-infected cells were stained with either DAPI (final concentration of 5 μg/ml) or propidium iodide (final concentration of 5 μg/ml) as described (*43*). Cells were visualized by ultraviolet light and phase-contrast microscopy (using a Zeiss IM35 inverted microscope). A minimum of 500 cells per sample were analyzed. The bar graphs show the percentages of cells whose membranes were permeable to propidium iodide (white bars) and the percentages of cells containing fragmented nuclei (shaded bars). The bars show means of triplicate infection assays with error bars showing plus and minus one sample standard deviation of the data. Error bars are not shown in the instances where the bars are too small to be resolved on the graph.

division, which renders the cells liable to subsequent apoptotic cell death. It should also be noted that quantification of fragmented nuclei only gives an underestimate of the total number of cells undergoing apoptosis, because this technique cannot score cells in earlier or later phases of apoptosis. Second, to assess the status of the cell membranes, cells were stained with propidium iodide as described (*43*). Figure 3 shows that only 2.8% of cells infected with cowpox virus A602 were permeable to propidium iodide, despite extensive cytopathology in all cells, similar to that depicted in Fig. 1. In contrast, 65% of cells infected with wild-type cowpox virus and 71% of cells infected with revertant cowpox virus A604 were permeable to propidium iodide, with all cells exhibiting cytopathology (similar to that shown in Fig. 1). Thus the results of staining with DAPI and with propidium iodide show the induction of nuclear fragmentation and the conservation of membrane integrity in cells infected with virus A602 (lacking the *crmA* gene), both features which are consistent with the induction of apoptosis in these cells (reviewed in *43*). Whereas, more than 65% cells infected with either virus expressing the *crmA* gene show membrane permeability to propidium iodide with more than 99% of cells containing unfragmented nuclei, both features which are consistent with virus-induced death by necrosis.

Another characteristic feature of cells undergoing apoptosis is the fragmentation their DNAs, often to fragments whose lengths are in increments of about 180 base pairs. The latter results from intranucleosomal cleavages (reviewed in *44* and *45*). To determine if infection of LLC-PK_1 cells with cowpox virus resulted in the cleavage of chromosomal DNA, the structures of DNAs extracted from cells infected with wild-type cowpox virus, virus A602, or revertant virus A604 were examined. Figure 4 shows that the chromosomal DNA remains intact in uninfected cells (lanes a), and in cells infected either with the wild-type virus (lanes b) or the revertant virus A604 (lanes d), consistent with the lack of morphological changes in the chromatin of these cells. However, DNA fragmentation, generating fragments of 2 kilobases or more, did occur in cells infected with the Δ*crmA* virus A602 (lanes c), consistent with both the margination of chromatin and the nuclear fragmentation occurring in these cells. Smaller fragments of chromosomal DNA, similar to those produced by actinomycin D treatment of the cells (lanes e), were not detected in cells infected with cowpox virus A602. This shows that DNA fragmentation in the virus-infected cells does not progress to the extensive intranucleosomal cleavage evident in some cells during apoptosis. Similar DNA fragmentation has been described in cells of a number of types during apoptosis (*44, 46–50*).

Collectively, the pronounced blebbing of the plasma membrane, the separation of the blebs from the cell surface, the loss of surface microvilli, the preservation of membrane impermeability to propidium iodide, the margination of the chromatin, the fragmentation of the nuclei, and the fragmentation of the chromosomal DNA suggest that LLC-PK_1 cells infected with cowpox virus lacking the *crmA* gene undergo death by apoptosis. In contrast, cells infected with cowpox virus expressing the *crmA* gene display none of these features, suggesting that they undergo death by necrosis. This induction of apoptosis in cells infected with virus A602 is not the result of expression of the *gpt* gene that was used to replace the *crmA* gene. Infection of LLC-PK_1 cells by cowpox virus A555 (*51*), in which both copies of the *crmB* gene have been replaced by copies of the same *p7.5/gpt* gene used to inactivate the *crmA* gene in virus A602, produced cytopathic effects identical to those of the wild-type cowpox virus (data not shown).

The results suggest that the CrmA protein exerts its effects on cell death by inhibiting processes of apoptosis

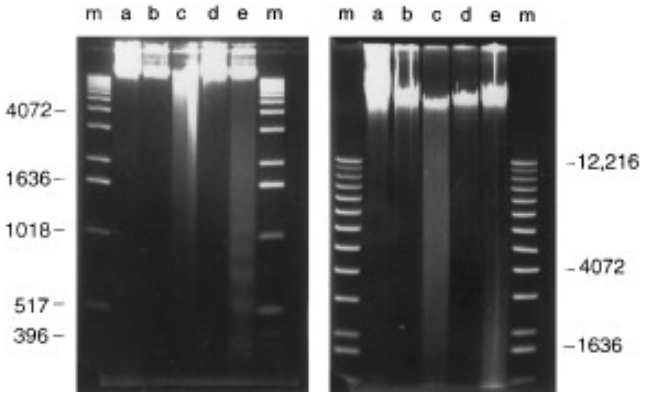

FIG. 4. The DNA of LLC-PK$_1$ cells remains intact during infection with cowpox viruses expressing the *crmA* gene, but becomes fragmented during infection with cowpox virus lacking the *crmA* gene. LLC-PK$_1$ cells (1.2×10^6) were either mock-infected or infected with cowpox virus at a multiplicity of 10 PFU/cell. After 12 hr of incubation at 37°, the cells were scraped from the dishes into the media. The cells were recovered by centrifugation (1000 rpm/10 min/4° in a Sorvall H1000B rotor) and resuspended in 0.3 ml of lysis buffer containing 20 mM HEPES, pH 7.2, 5 m*M* KCl, 0.5% NP-40, 0.1% sodium deoxycholate, and 10 m*M* EDTA. The suspensions were each divided into two and incubated on ice for 10 min. Proteinase K (Amersham, Arlington Heights, IL) was added to each suspension to a final concentration of 0.5 mg/ml, and the suspensions were then incubated at 37° for 2 hr. Residual protein was removed from each suspension by phenol–chloroform extraction. The nucleic acids in each sample were recovered by ethanol precipitation, and resuspended in 15 μl of TE buffer (10 m*M* Tris–HCl, pH 8.0, 1 m*M* EDTA). RNAs were removed by digestion with RNase A (USB, Cleveland, OH) at 37° for 30 min. DNAs in one of the duplicate samples were resolved by electrophoresis through a 2% agarose gel (left). DNAs in the other duplicate sample were resolved by electrophoresis through a 0.5% agarose gel (right). Resolved DNAs were stained with ethidium bromide and visualized by fluorescence under ultraviolet light. The lanes of the gels contain DNAs of (a) uninfected cells; (b) cells infected with wild-type cowpox virus; (c) cells infected with cowpox virus A602 (lacking the *crmA* gene); (d) cells infected with cowpox virus A604, the revertant of the A602 virus; and (e) uninfected cells treated with actinomycin D (1 μg/ml for 24 hr), to provide a positive control for apoptosis. Lanes designated (m) contain marker DNAs (lengths indicated in base pairs).

induced by cowpox virus infection of LLC-PK$_1$ cells. This interpretation is consistent with the known properties of the CrmA protein, which include the abilities to inhibit apoptosis induced by various stimuli in a variety of cells (*8, 10–18, 23*) and to inhibit ICE and ICE-like enzymes such as Yama, implicated in the process of apoptosis (*2–4*). However, we cannot formally exclude the possibility that the *crmA* gene in some way promotes processes leading to cell death by necrosis. This might occur in addition to CrmA-mediated inhibition of processes leading to apoptosis.

Although apoptosis occurs in LLC-PK$_1$ cells infected with cowpox virus A602, the yield of progeny virus obtained is similar to that obtained during infection of the LLC-PK$_1$ cells with the wild-type virus (Table 1). This finding is consistent with the relative kinetics of virus replication and death in these cells. Analyses of metabolically labeled proteins synthesized in virus-infected PK cells indicated that the late phase of virus replication begins about 4 hr postinfection (data not shown), several hours before cell disruption by either mode of cell death. As noted above, electron microscopy showed that by 8 hr after infection immature and mature virus particles were present in cells infected with either cowpox virus A602 or the wild-type virus. And, as Fig. 3 shows, if necrotic cell death is assessed by the criterion of membrane permeability to propidium iodide, then death in cells infected with *crmA*-expressing viruses occurs at about the same time that the major morphological features of apoptosis appear in cells infected with cowpox virus A602. Thus for either *crmA*-minus or *crmA*-plus viruses the major phases of the virus replication cycle are complete before the morphological features associated with either end-stage apoptosis or necrosis become apparent. Furthermore, the sensitivity of cowpox virus and other poxviruses to processes of apoptosis may be reduced by the cytoplasmic site of replication of the poxvirus, the limited viral dependence on enzymes encoded by the cell, and the potential ability of the virus to encode several inhibitors of apoptosis.

The LLC-PK$_1$ cells provide an indicator cell in which the antiapoptotic effect of the *crmA* gene is evident. In cells of the other types examined in this study, the lack of expression of the *crmA* gene did not result in apoptosis of the virus-infected cells. This suggests either that other

TABLE 1

Effect of *crmA* Gene Expression Upon the Yield of Progeny Virus from LLC-PK$_1$ Cells Infected with Cowpox Virus

Virus	Hours postinfection	Titer of virus (PFU/ml) in replicate infections (A–C)		
		A	B	C
CPV-w$^+$	5	7.8×10^5	7.5×10^5	1.0×10^6
	10	8.4×10^6	1.3×10^7	1.5×10^7
	24	6.8×10^6	7.3×10^6	7.8×10^6
CPV-A602	5	5.9×10^5	7.7×10^5	6.2×10^5
	10	8.5×10^6	8.6×10^6	6.0×10^6
	24	7.3×10^6	8.6×10^6	7.0×10^6
CPV-A604	5	1.5×10^6	1.4×10^6	1.8×10^6
	10	1.5×10^7	1.6×10^7	1.4×10^7
	24	1.9×10^7	2.2×10^7	2.2×10^7

Note. LLC-PK$_1$ cells were infected with wild-type cowpox virus, cowpox virus A602, or cowpox virus A604, at multiplicities of 10 PFU/ml. After 1 hr of incubation at 37° the inoculum was removed, and fresh medium was added to each culture. At 5, 10, or 24 hr after infection the cells in each culture were scraped into the medium. The cells were then disrupted by freezing, followed by thawing and sonication. The titers of virus in each sample were determined by plaque assay on human 143 cells. Titers of progeny virus were determined from three replicate infections of LLC-PK$_1$ cells with viruses of each of the three types.

viral genes may be sufficient to prevent apoptosis in cells of these types or that processes of apoptosis are not induced in these cells by the viral infection. The implication is that cowpox virus may be capable of inhibiting or delaying apoptosis to various degrees in a cell-type-specific manner.

This study suggests that the *crmA* gene is necessary to inhibit a process leading to apoptosis in LLC-PK_1 cells infected with cowpox virus. The probable target of the CrmA protein is one or more of the ICE-like cysteine proteinases or granzyme B-like serine proteinases implicated in apoptosis (*8, 52–57*). Thus, the *crmA* gene is another member of the family of poxvirus genes, including the cowpox virus *CHOhr* gene (*24*), the vaccinia virus *E3L* gene (*58*), and a second serpin gene, the *SPI-1* gene of rabbitpox virus (*59*), whose gene products may each contribute to the inhibition of apoptosis in poxvirus-infected cells. Together, members of this family of viral proteins may act to delay or inhibit processes of apoptosis that might restrict the replication of the virus in the cell. Clearly, such a function would be advantageous to the virus if either cell death by apoptosis or processes of apoptosis that interfere with viral replication can occur before the completion of virus replication.

The significance of the viral control of the mode of cell death extends beyond the issue of whether such controls can enable a virus to replicate efficiently in cells of a particular type. As discussed above, the ability to control the mode of cell death *in vivo* may allow the virus to interfere with several immune processes directed against the virus infection. In addition, because death by necrosis is usually associated with inflammatory responses, whereas cells that have undergone apoptosis are usually removed by phagocytosis without major ensuing inflammatory responses (reviewed in *40* and *60*), the mode of cell death is also likely to affect the way in which the virus is trafficked in the infected animal. Therefore, viral controls over the mode of cell death may be major factors in determining both the extent of a virus infection *in vivo* and the success of the immune defenses against that infection.

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