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Isolation and Characterization of a Chinese Hamster Ovary Mutant Cell Line with Altered Sensitivity to Vaccinia Virus Killing

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The Chinese hamster ovary (CHO) cell line is nonpermissive for vaccinia virus, and translation of viral intermediate genes was reported to be blocked (A. Ramsey-Ewing and B. Moss, Virology 206:984–993, 1995). However, cells are readily killed by vaccinia virus. A vaccinia virus-resistant CHO mutant, VV5-4, was isolated by retroviral insertional mutagenesis. Parental CHO cells, upon infection with vaccinia virus, die within 2 to 3 days, whereas VV5-4 cells preferentially survive this cytotoxic effect. The survival phenotype of VV5-4 is partial and in inverse correlation with the multiplicity of infection used. In addition, viral infection fails to shut off host protein synthesis in VV5-4. VV5-4 was used to study the relationship of progression of the virus life cycle and cell fate. We found that in parental CHO cells, vaccinia virus proceeds through expression of viral early genes, uncoating, viral DNA replication, and expression of intermediate and late promoters. In contrast, we detect only expression of early genes and uncoating in VV5-4 cells, whereas viral DNA replication appears to be blocked. Consistent with the cascade regulation model of viral gene expression, we detect little intermediate- and late-gene expression in VV5-4 cells. Since vaccinia virus is known to be cytolytic, isolation of this mutant therefore demonstrates a new mode of the cellular microenvironment that affects progression of the virus life cycle, resulting in a different cell fate. This process appears to be mediated by a general mechanism, since VV5-4 is also resistant to Shope fibroma virus and myxoma virus killing. On the other hand, VV5-4 remains sensitive to cowpox virus killing. To examine the mechanism of VV5-4 survival, we investigated whether apoptosis is involved. DNA laddering and staining of apoptotic nuclei with Hoechst 33258 were observed in both CHO and VV5-4 cells infected with vaccinia virus. We concluded that the cellular pathway, which blocks viral DNA replication and allows VV5-4 to survive, is independent of apoptosis. This mutant also provides evidence that an inductive signal for apoptosis upon vaccinia virus infection occurs prior to viral DNA replication.

Vaccinia virus (VV) is the prototype of poxviruses, which cause severe cytopathological effects (CPE) in their hosts. VV usually produces a local lesion at the site of inoculation, and other poxviruses such as mousepox virus or malignant myxoma virus can kill hosts in several days after infection (11, 17). In tissue culture cells, VV causes cell rounding early after infection (1, 36). As infection progresses, the architecture of cells degenerates, with alteration of membrane permeability and leakage of lysosomal enzymes. These are often viewed as passive cellular responses leading to necrotic death of the infected cells. Cell death and detachment can occur as soon as 24 h postinfection (p.i.) (36).

Some aspects of poxvirus-induced cytotoxicity are modulated by host cells. For example, abortive infection of molluscum contagiosum (MC) virus causes only a transient cell rounding (39, 52). In addition, strain-specific resistance to poxvirus infection has been observed in rabbits and mice (5, 44, 47, 51, 59). Furthermore, cytotoxicity may not be linked to virus replication, since human leukocytes support VV replication only after phytohemagglutinin stimulation, whereas unstimulated leukocytes still show CPE (40, 42). It has long been thought that virus infection leads to a fast and irreversible shutoff of host transcription and translation. However, transcriptional activation of a host heat shock protein was recently described (32). Lastly, studies of viral host range genes revealed that apoptosis may also play a role in virus and host interactions. Apoptosis could act as an important cellular mechanism that defends against poxvirus propagation (6, 31). In conclusion, it seems conceivable that diversity in virus virulence and pathogenicity is influenced by expression of various cellular genes which affect the extent of virus replication and cytotoxicity in different cell types.

We are interested in the mechanisms of cytotoxic response to VV infection. To understand the role of cellular genes in VV-induced cytotoxicity, we took a genetic approach to isolate cells which are resistant to VV killing. Our rationale, shown in Fig. 1A, is that during virus infection, some host proteins are involved in virus life cycle progression leading to cell death. It is clear that productive infection is not a prerequisite for cell death, since nonpermissive cells are readily killed by VV. However, it is not clear mechanistically how virus progression and cell fate are mutually affected. Therefore, a cytotoxicity-resistant phenotype could be derived from an alteration of host environment affecting virus life cycle progression in cells. Since VV is tightly regulated in a cascade manner, interruption of any of these steps such as virus entry or viral gene expression and regulation may lead to virus life cycle arrest, resulting in cell survival.

On the basis of the theory stated above, we chose to inactivate expression of a candidate cellular factor by retrovirus insertional mutagenesis in a large pool of cells. We then selected mutants with the desired phenotype by subsequent VV infection. Technically, it is known that retroviruses, when used as insertional mutagens, have very low efficiency (20). Usually

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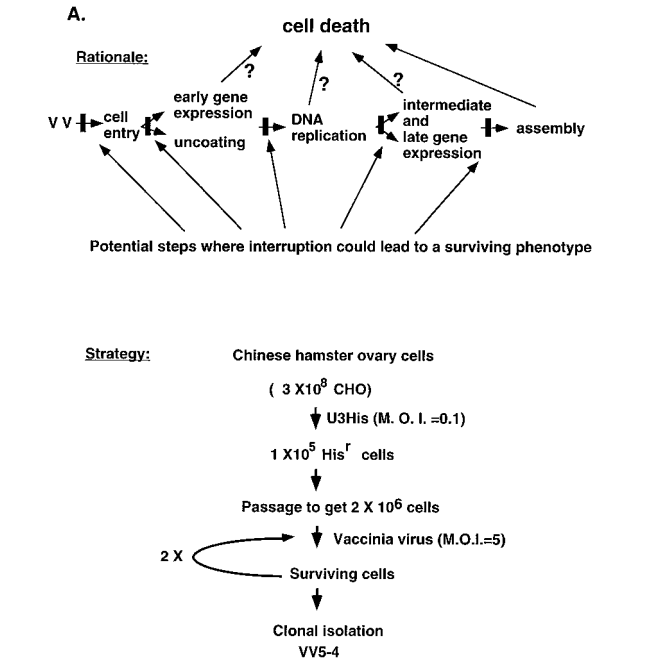


FIG. 1. (A) Rationale and schematic representation of isolation of CHO cells resistant to VV-induced cell death by using promoter trap retroviral vectors. (B) Staining of CHO and VV5-4 cells after VV infection. Cells were infected with VV at three MOIs, and the medium was changed every 3 days. Cells were stained with 0.1% crystal violet at 8 days p.i. and photographed.

10^6 to 10^7 integration events were required to inactivate a single allele of genes in mammalian cells (18, 35, 61). To improve the efficiency of insertional mutagenesis, we used a retrovirus promoter trap vector, U3His, as described previously (63, 64). The strategy, shown in Fig. 1A, is to infect cells with promoter trap retrovirus U3His and select histidinol-resistant (His^r) cells in which an individual virus is integrated downstream of a cellular promoter. Selection for marker gene expression results in inactivation of a cellular gene by interruption of cellular transcription. The collective numbers of His^r clones in a library could be very large, including most, if not all, active promoters in a cell. If a cellular gene plays a role in VV cytotoxicity, mutant cells defective for its expression will be selected from this library by VV infection with a higher efficiency, around 10^{-4} to 10^{-5} (8). In addition, the gene is readily accessible for cloning, since it is next to the promoter trap virus integration site.

Using promoter trap retrovirus vector U3His, we have isolated a Chinese hamster ovary (CHO) mutant clone, VV5-4, which appears to be resistant to the necrotic effect of VV. We demonstrated that entry of VV into these mutant cells and expression of viral early genes are intact. However, survival of mutant cells seems to correlate with a deficiency of viral DNA replication.

MATERIALS AND METHODS

Reagents, cell lines, and virus. Fluorescein di-β-D-galactopyranoside was purchased from Molecular Probes Inc. and used as the manufacturer suggested. Histidinol, *o*-nitrophenyl-β-D-galactopyranoside (ONPG), and Hoechst 33258 were purchased from Sigma Inc. ^{3}H-acetyl coenzyme A (4.3 Ci/mmol) was purchased from Amersham Inc. CHO cells were derived from CHO-K1 cells and express a mouse ecotropic receptor for retrovirus infection (30). CHO cells were grown in F12 medium supplemented with 5% fetal bovine serum. BSC40 cells and VV strain WR were provided by S. Pennathur. Recombinants VRE-βCAT, VP30LacZ, and VMJ360 were described before (12, 48) and provided by B. Moss. VRE-βCAT expresses two marker genes, the chloramphenicol acetylphosphotransferase (CAT) gene from an early promoter of the VV growth factor (VGF) gene and the *lacZ* gene from a late (F17R) promoter. VMJ360 and VP30LacZ express the *lacZ* gene from early and intermediate (G8R) promoters, respectively. Another recombinant, β-VV expressing the *lacZ* gene from a fowlpox virus C1a promoter, was described before (7). [^{3}H]thymidine-labeled VV (3,000 PFU/cpm) and [^{35}S]methionine-labeled VV (23 PFU/cpm) were prepared and purified from sucrose gradients as described elsewhere (3, 68).

Retroviral mutagenesis and selection of a CHO mutant resistant to VV cytotoxicity. Promoter trap virus U3His, which was used for making a promoter trap cell library, was described before (63, 64). The details of construction of a CHO cellular library that contains most of the promoter integration events will be published elsewhere. In brief, 3×10^8 CHO cells were grown in eight-story cell factories and infected with U3His at a multiplicity of infection (MOI) of 0.05 to 0.1 PFU per cell. One day after infection, cells were selected in 250 μM histidinol in histidine-free Dulbecco modified Eagle medium supplemented with 34 μg of proline per ml and 5% fetal bovine serum. Medium was changed every 3 days for 2 weeks, and all His^r colonies were pooled.

To select for CHO mutant cells resistant to VV cytotoxicity, these His^r colonies were infected by VV at an MOI of 5 PFU per cell, and surviving cells were collected and expanded for another round of VV infection. This infection-selection procedure was performed a total of three times, and a single resistant clone, VV5-4, was isolated.

Molecular analysis of the retrovirus integration site in VV5-4. For Southern blot analysis, genomic DNA was isolated from VV5-4 cells and digested with *Hin*dIII or *Eco*RI. Digested DNA (10 μg per lane) was separated on a 0.7% agarose gel and transferred to a nitrocellulose blot. The blot was probed with a ^{32}P-labeled 1-kb *Nhe*I fragment containing the *his* (histidinol dehydrogenase) gene and exposed for autoradiography as described elsewhere (63, 64). For cloning of the cellular promoter upstream of U3His in VV5-4 cells, genomic DNA prepared from VV5-4 cells was digested with *Eco*RI, packaged into lambda Zap vector (Stratagene), and screened with a ^{32}P-labeled 1-kb *Nhe*I fragment containing the *his* gene, and the positive clones were plaque purified. The 4.4-kb *Eco*RI fragment containing the 5′ long terminal repeat (LTR) and flanking cellular sequences in these lambda clones were rescued into pBluescript plasmid by in vivo excision as suggested by the manufacturer. After restriction enzyme digestion analyses, a 1-kb *Eco*RI-*Nhe*I fragment containing genomic flanking sequences upstream of the 5′ LTR was excised and subcloned in front of the CAT gene. The resulting CAT plasmid was transfected into CHO cells, and CAT assays were performed 2 days after transfection as described previously (21, 22).

Assays for cell survival after VV infection. CHO and VV5-4 cells were infected with VV at an MOI of 2, 4, or 8 PFU per cell at 37°C for 60 min. After removal of the inoculum, the cells were incubated in normal medium and fixed for cell staining at 8 days p.i. by 0.1% crystal violet. For survival curve analysis, multiple 60-mm-diameter dishes of CHO and VV5-4 cells (8×10^5 per dish) were infected with VMJ360 at an MOI of 2, 4, 8, or 20 PFU per cell. At days 1, 2, 3, 5, and 7 p.i., one dish of each cell line was trypsinized, and attached viable cells were counted by trypan blue staining. In addition, for cells infected at an MOI of 4, extra plates were taken out at 4, 8, 24, 72, and 120 h p.i. for 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) staining as described previously (7).

For long-term survival analysis, CHO and VV5-4 cells were infected with β-VV at an MOI of 5, and cells were analyzed with fluorescein di-β-D-galactopyranoside by fluorescence-activated cell sorting (FACS) at 0, 3, 6, 9, 12, and 15 days p.i. as described previously (7). These cells were simultaneously analyzed for the presence of viral DNA by PCR with primers made from VGF 5′ and 3′ sequences: 5′ATGTCGATGAAATATCTG3′ and 5′AGTTCGTCGAGTGAA CCT3′. A standard curve was included in the PCR analysis to quantitate the sensitivity of the assay, which could detect a single copy of the virus genome per cell.

One-step growth curve for VV. BSC40, CHO, or VV5-4 cells were infected with VV at an MOI of 5 PFU per cell. Cell lysates were then harvested at 0, 6,

12, 24, 48, and 72 h p.i., and virus titers were determined on BSC40 cells as described previously (7).

Radioactive amino acid labeling for cellular protein synthesis. CHO and VV5-4 cells were either mock infected or infected with VV at an MOI of 2.5. At 0, 1, 2, 4, 8, and 23 h p.i., 2 ml of labeling medium containing [^{35}S]methionine (200 μCi/ml) was added to cells for 15 min of short pulse-labeling. After removal of labeling medium, cells were washed with phosphate-buffered saline (PBS) and lysed with sodium dodecyl sulfate (SDS)-containing sample buffer. Equal amounts of cell lysates were analyzed on an SDS–10% polyacrylamide gel as described previously (48).

Virus binding assay. Cells were infected with [^{35}S]methionine-labeled VV at an MOI of 2 or 4 PFU per cell at 4°C for 10, 20, 40, 60, 90, and 120 min. At each time point, cells were washed three times with PBS and lysed in detergent-containing buffer. Fractions of the cell lysates were counted in a scintillation counter.

Expression of marker genes from viral early, intermediate, and late promoters. For early and late gene expression, BSC40, CHO, and VV5-4 cells were infected with VRE-βCAT at an MOI of 5 PFU per cell at 37°C for 60 min. After removal of the virus inoculum, the cells were further incubated in normal medium, and cell lysates were prepared at 0, 1, 2, 3, 4, 6, 8, 10, 12, and 24 h p.i. Protein concentration was determined by the Bradford method (4) (Bio-Rad). Normalized amounts of cell lysates were used either for CAT assays with ^{3}H-acetyl coenzyme A or for β-galactosidase (β-Gal) assay with ONPG as described previously (15, 26). For intermediate-gene expression analyses, cells were infected by VP30LacZ at an MOI of 5 PFU per cell and harvested at 0, 4, 8, 12, and 24 h p.i., and β-Gal activity was determined by the ONPG assay as described previously (26).

Virus uncoating assays of infected cells. Dishes (25 by 100 mm) of cells were infected with [^{3}H]thymidine-labeled VV at an MOI of 2.5 PFU per cell and incubated with medium containing 40 μg of cytosine arabinoside per ml as described previously (45). In a control experiment, cells were treated with cycloheximide (100 μg/ml) for 30 min prior to VV infection. Cells were harvested at 2 h p.i. and lysed by 80 strokes in a Dounce homogenizer. Lysates were centrifuged at 200 rpm for 10 min to remove nuclei. Cytosolic fractions were layered on top of a 25 to 40% sucrose gradient and centrifuged at 13,500 rpm for 45 min. Each fraction (1 ml) was collected from top of the gradient and counted in a scintillation counter as described previously (28, 45, 68).

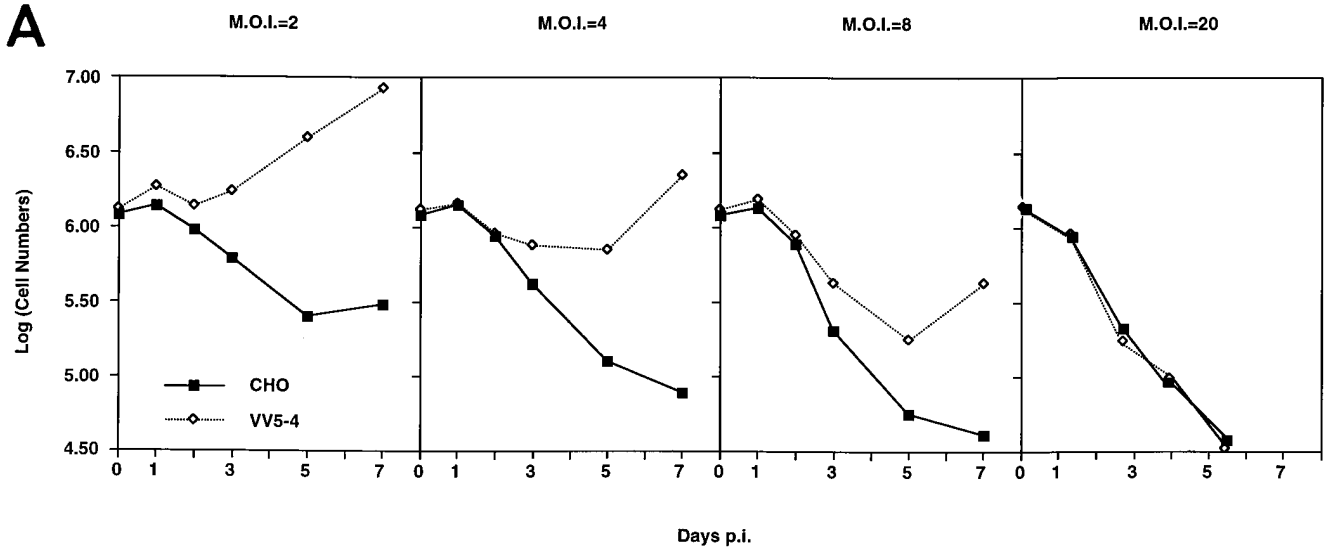
Measurement of viral DNA replication by slot blot hybridization. Our method for detecting viral DNA replication was modified from established procedures described before (16, 29). In brief, cells were infected by VV at an MOI of 5 PFU per cell and harvested at various times. Cells were washed in PBS and lysed. After proteinase K (50 μg/ml) digestion, the lysate was used for phenol-chloroform extraction and total DNA was isolated by ethanol precipitation. Portions of DNA samples were loaded onto a nitrocellulose paper by using a microsample filtration manifold (Schleicher & Schuell, Inc.). The filter was hybridized with a 0.5-kb ^{32}P-VGF DNA fragment, washed, and autoradiographed. The experiment was repeated three times, and resulting blots were scanned and quantitated with a PhosphorImager (Molecular Dynamics, Inc.).

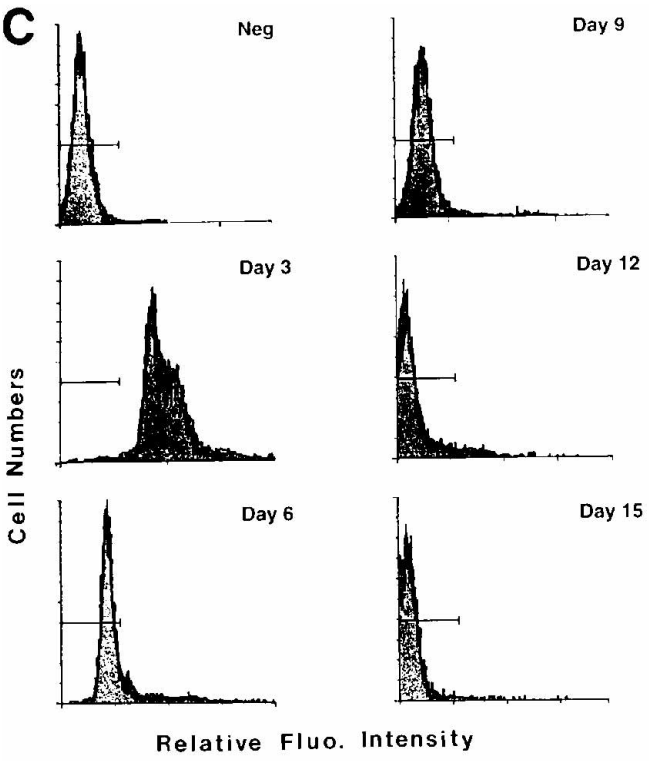
Apoptosis assays. For DNA laddering analysis, 10^{6} CHO and VV5-4 cells were either mock infected or infected with VV at an MOI of 2.5 or 5 PFU per cell. Infected cells were harvested at 24 h p.i., and total DNA was isolated as described previously (55). Total DNA was incubated with RNase A (6 μg/ml) at 50°C for 60 min and immediately loaded on a 2% agarose gel. After electrophoresis, the gel was stained with ethidium bromide and photographed. Alternatively, cells were seeded onto coverslips and infected as described above. At 6 h p.i., the cells were fixed, permeabilized, stained with Hoechst 33258, and photographed as described previously (37).

RESULTS

Isolation of a CHO mutant, VV5-4, that is resistant to VV cytotoxicity. Retroviral promoter trap vector U3His was used to select for integrations next to cellular promoters (63). Previous studies have shown that these integration sites function as cellular promoters in vitro and in vivo (64). In addition, when these viruses are used as insertional mutagens, 2×10^{4} to 1×10^{5} promoter integrations are required in order to inactivate a single allele gene in cells (8). The CHO cell line was chosen for this study for two reasons. (i) It is a hypodiploid cell line. The average number of chromosomes in CHO is 16 when $n = 21$. (ii) It exhibits substantial functional hemizygocity at many different loci (23, 24, 54). Numerous genetic mutants were isolated from CHO cells at a frequency representing genes with a single allele (53, 57, 58). Loss or silencing by methylation of the second allele in isolated CHO mutants has been reported (27, 67). Therefore, CHO cells appear to be feasible for genetic mutant isolation using retroviruses as insertional mutagens. Although chemical mutagenesis was used in most of these cases, retroviral insertions by promoter trapping could provide more advantages. The promoter trapping approach has been shown to be successful for mutant identification in vitro and in vivo (8, 13, 19, 30, 62). Moreover, in these situations, retrovirus serves as a useful tag for gene isolation (8, 13, 19, 30, 43, 62). Details of promoter trapping methodologies will be described elsewhere. Figure 1A illustrates the selection protocol used. To facilitate large-scale screening, we infected CHO cells with retroviral promoter trap vector U3His and selected 10^{5} His^{r} clones. We then repeatedly infected these pools of cells with VV to select cells that would survive VV killing. One cell clone, VV5-4, enriched by this procedure was isolated for further studies. This promoter trap selection appears to be specific since no spontaneous resistant clone was isolated from parental CHO cells without retroviral mutagenesis. Resistance of mutant clone VV5-4 to VV was compared with that of parental CHO cells. As shown in Fig. 1B, VV5-4 cells produced more viable colonies than parental CHO cells at an MOI of 2. VV5-4 cells still survived better than CHO cells at MOIs of 4 and 8. We noticed that the resistant phenotype of VV5-4 was partial and that all cells died when an MOI of 20 was used. Survival of VV5-4 at different MOIs is quantitated in Fig. 2A; in this analysis, we monitored survival of VV5-4 cells after VV infection in comparison with that of CHO cells for a period of 7 days. Consistent with Fig. 1B, a reverse correlation of viable VV5-4 cells versus MOI was demonstrated. At MOIs of 2 and 4, VV5-4 cells showed the greatest phenotypic difference from CHO cells. At an MOI of 2, roughly 50% of the originally seeded VV5-4 cells survived whereas most CHO cells were killed. Some CHO cells survived after 7 days, but many fewer than VV5-4 cells. At an MOI of 4, we could not detect any live CHO cells after 7 days, at which time VV5-4 cells grew to almost confluency. The sensitivity of VV5-4 to virus killing increased when an MOI of 8 was used, and all cells were killed at an MOI of 20. Surviving VV5-4 cells all seemed to arrest for at least 24 h immediately after infection, before they recovered to proliferate again. To ensure that these growing VV5-4 cells were derived from the virus-infected population and were not cells escaping infection, we investigated viral marker gene expression in these surviving cells by staining cells for β-Gal expression from an viral early promoter (12). As shown in Fig. 2B, both CHO and VV5-4 cells express β-Gal as early as 4 h p.i. The blue color remained intense and uniform in both cell lines during the first 24 h p.i. Afterwards, CHO cells started dying whereas the culture of VV5-4 was maintained; β-Gal expression in these cells became weaker and heterogeneous in 3 to 5 days. To assess VV5-4 survival on a longer time scale, we monitored infected VV5-4 cells for a period of 15 days and determined β-Gal expression by FACS analysis as shown in Fig. 2C. β-Gal expression was readily detected in VV5-4 at 3 days p.i., suggesting again that the initial infection was complete. The level of β-Gal in VV5-4 remained detectable at 6 days p.i. and was reduced to the background level at 9 days p.i. PCR analysis of the viral genome from these infected cells (Fig. 2D) indicated that VV DNA was initially present and was diluted out in the growing cell population. The data in Fig. 2 demonstrate that VV infects but cannot persist in VV5-4 cells.

CHO cells are nonpermissive for VV and produce few viruses (14, 56). We therefore compared VV5-4 with CHO for virus growth in a one-step growth experiment. As shown in Fig. 3, in BSC40 cells, which are permissive for VV, the virus titer increases about 1,000-fold in one round of infection, whereas in CHO cells, the virus titer increases only 10-fold. VV5-4





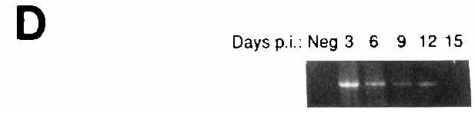


FIG. 2. (A) Survival curves for VV5-4 after VV infection. CHO and VV5-4 cells were infected by VV at MOIs of 2, 4, 8, and 20 PFU per cell. At different time points, cells were trypsinized and the numbers of viable cells were determined by trypan blue exclusion. (B) β-Gal expression in virus-infected cells detected by X-Gal staining. CHO and VV5-4 were infected with VMJ360 at an MOI of 4 and fixed for β-Gal expression by X-Gal staining at the times indicated at the left side. (C) β-Gal expression in virus-infected VV5-4 cells analyzed by FACS as described previously (7). VV5-4 cells were infected with β-VV at an MOI of 5, and cells were maintained in normal medium for β-Gal analysis by FACS every 3 days for a period of 15 days. The background staining of mock-infected cells (Neg) is represented in each panel by a horizontal bar extending from the *y* axis. (D) Detection of viral DNA from infected VV5-4 cells at different times as described above. Total DNA was isolated for PCR analysis with primers made from VGF sequences as described in Materials and Methods.

remained nonpermissive for VV, and even fewer viruses were detected.

Since VV is known to shut off host protein synthesis, which is thought to be inhibitory for cell survival, we performed a pulse-labeling experiment to detect new protein synthesis in virus-infected cells. As shown in Fig. 4, cellular protein synthesis in CHO cells was greatly decreased at 4 h p.i. Later, almost all cellular protein synthesis was shut off, and viral protein synthesis was observed. In contrast, cellular protein synthesis in VV5-4 cells, though slightly reduced, remains active throughout 24 h. This result suggests that VV5-4 is able to antagonize the inhibitory effect of host protein synthesis by VV infection.

Mutant cell lines escaping killing by other viruses such as herpesvirus or alphaviruses have been described (2, 25, 41, 66). In one case, when proteoglycan modification of the cell surface was changed and the cells were rendered resistant to herpes simplex virus killing, virus entry into cells was affected (2, 25). In other cases, when cells were resistant to alphavirus killing, defects of endocytic vesicle acidification and progeny maturation were reported (41, 66). These mutants provide useful information for understanding various cellular mechanisms controlling virus-induced cytotoxicity. With the isolation of VV5-4 cells, we sought to determine the survival mechanism of VV5-4 in relationship to progression of the VV life cycle.

VV5-4 allows VV entry and expression of viral early promoters. VV infection in CHO cells is abortive and was reported to be blocked at the stage of translation of viral intermediate genes (48). Cellular protein synthesis was shut off, and cells died within few days. Since VV5-4 is derived from CHO and survives VV infection better, we performed a series of comparative studies to determine if differences in the progression of the viral life cycle in CHO and VV5-4 result in different cell fates.

We first determined VV adsorption to both CHO and VV5-4 cells by using [^{35}S]methionine-labeled VV. As shown in Fig. 5, at MOIs of both 2 and 4, the amounts of VV binding to the two cell lines were comparable at up to 2 h of adsorption. In addition, virus binding to the two cell lines followed similar

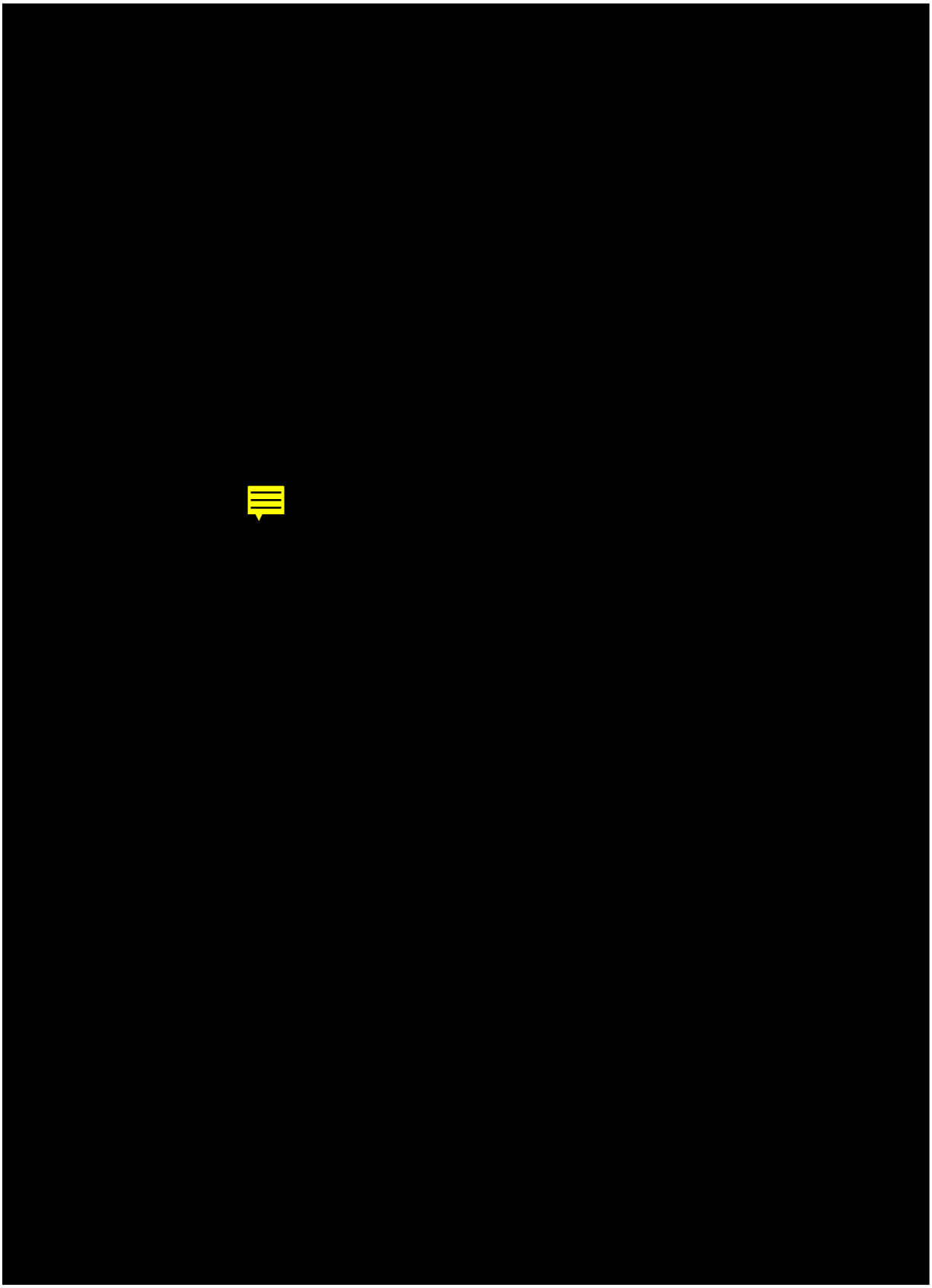


FIG. 2—*Continued.*

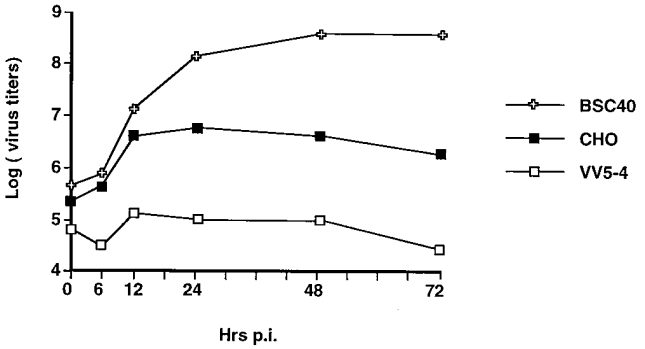


FIG. 3. One-step growth curve analysis. CHO and VV5-4 cells were infected with VV at an MOI of 5, and cell lysates were prepared at the indicated times. Virus titers were determined on BSC40 cells.

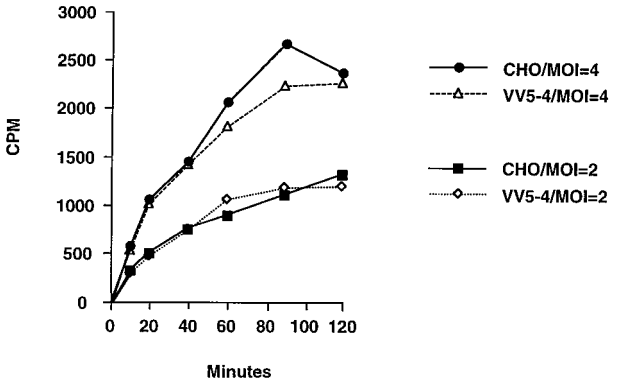


FIG. 5. Virus binding to CHO and VV5-4 cells. Cells were infected with [^{35}S]methionine-labeled VV at MOIs of 2 and 4. After incubation at 4°C for the times indicated, cells were washed with PBS and lysed. Cell-associated radioactivity was determined by scintillation counting. The experiments were repeated twice, and averages are shown.

kinetics. These results suggest that the initial event of virus entry into VV5-4 cells is not affected. Phenotypic differences between VV5-4 and CHO are not attributed to different extents of infection.

To analyze postbinding steps, we used two recombinant VVs for comparative studies to examine viral gene expression in CHO and VV5-4. One recombinant, VRE-βCAT, expresses the CAT gene from an early (VGF) promoter and the *lacZ* gene from a late (F17R) promoter (48). Another recombinant virus, VP30LacZ, expresses the *lacZ* gene from an intermediate (G8R) promoter (48). CHO and VV5-4 cells were infected with a virus, and cells were harvested at different times for various promoter assays as described in Materials and Methods. BSC40 cells were also used as a control in all experiments. Levels of expression of CAT from a viral early promoter in CHO and VV5-4 cells were nearly identical (Fig. 6A). It thus appeared that VV5-4 allows virus early-gene expression as efficiently as the parental CHO cell line. On the other hand, the patterns of intermediate- and late-gene expression were quite different in CHO and VV5-4 cells. β-Gal expression from an intermediate (Fig. 6B) or late (Fig. 6C) promoter was readily detected in CHO cells infected with the respective viruses. However, activities of these promoters were greatly reduced in VV5-4 (Fig. 6B and C). Since the resistant phenotype of VV5-4 is not absolute, some leaky expression of the intermediate gene was expected. These results suggested that in parental CHO cells, all three classes of viral genes are expressed, and CHO, being nonpermissive for VV, may block virus production at the assembly stage. In contrast, we found that virus progression is arrested at a much earlier stage in VV5-4 cells. This specific blockage resides between expression of early and intermediate genes, namely, either at the uncoating of incoming virions or at the viral DNA replication stage.

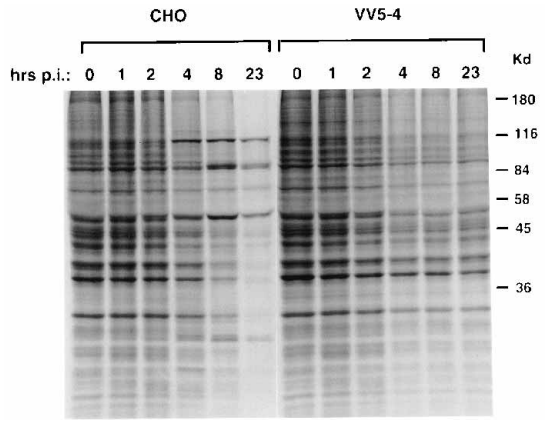


FIG. 4. Cellular protein synthesis remains active in VV5-4 cells after VV infection. CHO and VV5-4 cells were infected with VV at an MOI of 2.5 PFU per cell and pulse-labeled with [^{35}S]methionine at the times shown at the top. The cell lysates were analyzed on an SDS–10% polyacrylamide gel as described in Materials and Methods.

VV is able to uncoat in VV5-4 cells. It was shown that VV uncoats in cells rapidly after infection (50). Uncoating could be detected as early as 1 to 2 h p.i. and is blocked by cycloheximide treatment, which inhibits early-gene expression (28, 45). The influence of host cells on virus uncoating has been documented before. For example, infection of primate cells with MC virus produces transient cell rounding and aggregation. However, CPE is transient, and virus cores persist in cells for several days but never become uncoated (39). Moreover, rabbitpox viruses inactivated by UV, nitrogen mustard, and heat are not infectious and could not be uncoated in cells (33), and purification of a potential uncoating factor of cellular origin was described (45). It is plausible that uncoating of incoming virus is a critical step at which host factors could play a regulatory role.

We decided to assay for VV uncoating in CHO and VV5-4 cells. We infected these cells with VV at an MOI of 2.5 and harvested cells at 2 h p.i. as described before (45). Cytoplasmic fractions were separated on sucrose gradients, and each fraction was collected for uncoating of the virus genome. As shown in Fig. 7, purified virions with intact membranes were sedimented at fractions 21 to 24. When viruses entered CHO cells and uncoating was blocked by cycloheximide addition, radioactivity of incoming virus cores was detected at fractions 13 to 18. This peak disappeared when cycloheximide was omitted from the CHO cell culture, with concomitant appearance of free DNA at the top of the gradient. This finding suggested that virus uncoating occurs quickly in CHO cells. Uncoating of VV in VV5-4 cells appeared similar to that in CHO cells in that viral cores were accumulated only with cycloheximide treatment, suggesting that viral cores are uncoated equally efficiently in VV5-4 cells. However, we were not able to detect free DNA in infected VV5-4 cells in repeated attempts. Despite the presence of small peaks of viral cores in fractions 13 to 15 in both CHO and VV5-4 cells, these peaks represented less than 15% of input cores. We therefore concluded that

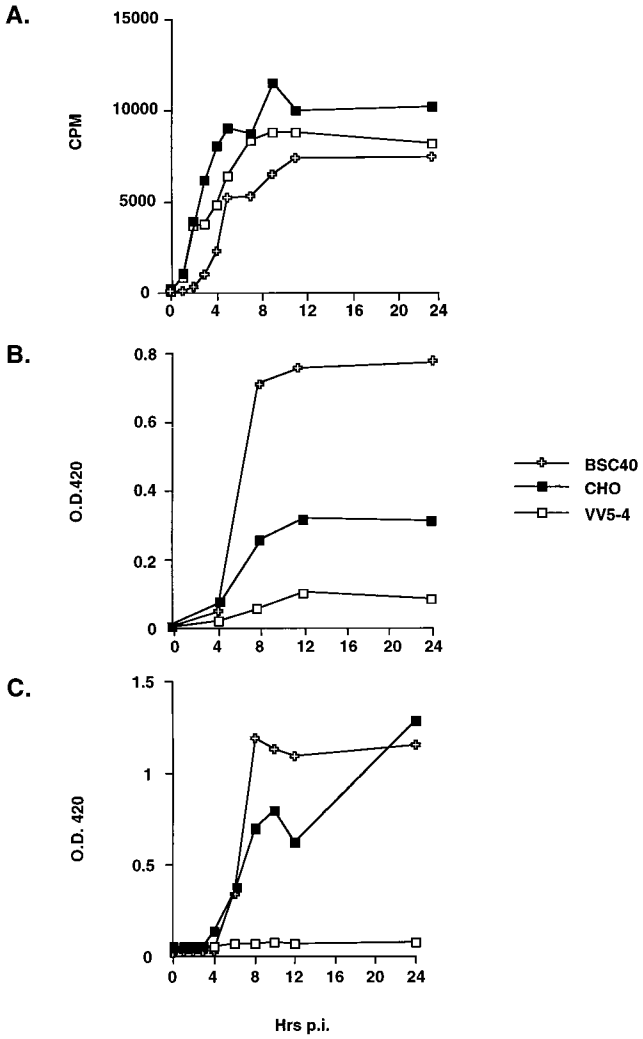


FIG. 6. Expression of viral promoters in VV5-4 cells. (A) Early-promoter analysis. BSC40, CHO, and VV5-4 cells were infected with VRE-βCAT at an MOI of 5, and cell lysates were harvested at different times for CAT assays as described previously (9). (B) Intermediate-promoter analysis. BSC40, CHO, and VV5-4 cells were infected with VP30LacZ at an MOI of 5 PFU per cell, and cell lysates were harvested at different times for β-Gal assays. (C) Late-promoter analysis. Cell lysates were prepared from infected cells used for panel A, and β-Gal activity was determined (26). O.D.420, optical density at 420 nm.

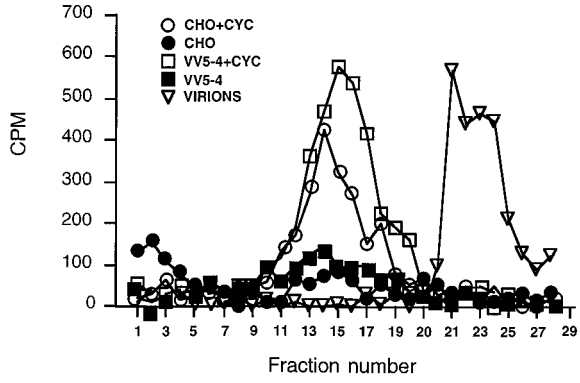


FIG. 7. Uncoating of VV in VV5-4 cells. CHO and VV5-4 cells were infected with [^{3}H]thymidine-labeled VV at an MOI of 2.5 PFU per cell and incubated with medium containing cytosine arabinoside as described previously (45). Open symbols represent control experiments in which cells were pretreated with cycloheximide (CYC; 100 μg/ml) prior to VV infection to block virus uncoating. These infected cells were harvested at 2 h p.i., and cytosolic fractions were isolated and fractionated as described in Materials and Methods. Triangles represent sedimentation profiles of purified virions in sucrose gradients. Fraction 1 indicates the top of the gradient, and each fraction was collected and counted as described previously (45).

most of the incoming viruses are uncoated in both CHO and VV5-4 cells.

Viral DNA fails to replicate in VV5-4 cells. All of the experiments described above indicate that viral DNA replication could be the earliest blockage for VV life cycle progression in VV5-4 cells. To investigate viral DNA replication, CHO and VV5-4 cells were infected with VV at an MOI of 5 PFU per cell, and total DNA was isolated at various times. DNA was applied to a slot blot, and viral DNA replication was analyzed with a 0.5-kb ^{32}P-labeled viral VGF DNA probe as described previously (16, 29). As shown in Fig. 8A, viral DNA is rapidly accumulated in CHO cells from 4 to 10 h p.i., whereas only a minimal increase is observed in VV5-4 cells. Quantitation of the results (Fig. 8B) demonstrates that viral DNA replication occurs in CHO cells but is largely blocked in VV5-4 cells. Since the resistant phenotype of VV5-4 is leaky, we expect that there may have been some hybridization to the viral probe. Previous studies by several groups suggested that viral DNA replication is necessary for expression of viral intermediate and late genes (34, 65). The pattern of VV life cycle blockage in VV5-4 cells is consistent with these observations.

Apoptosis is detected in VV5-4 cells. VV5-4 is resistant to VV killing, and this phenotype could arise by different mechanisms. It is possible that the VV life cycle is tightly regulated and once viral DNA replication is arrested, cells remain viable. As a consequence, input viruses are diluted out once cells resume proliferation. An alternative explanation could involve apoptosis, which was previously found in CHO cells infected with VV (31). It is also possible that apoptosis signaling somehow is defective in VV5-4 cells and leads to greater survival of cells. A similar phenotype of fibroblast cells transfected with the *bcl-2* gene has been reported (38). These cells overexpress the *bcl-2* gene and survive alphavirus killing. If a similar mechanism occurs in VV5-4, it would suggest that VV5-4 survives VV cytotoxicity by suppressing a cell suicidal mechanism.

Parental CHO and clone VV5-4 cells were infected with VV at MOIs of 2.5 and 5, and total DNA was prepared at 24 h p.i. for DNA laddering analysis. As shown in Fig. 9, comparable amounts of DNA degradation were observed in the two cell lines. Apoptosis was also illustrated by staining cells with the fluorescent dye Hoechst 33258 at 6 h p.i. (Fig. 10). Mock-infected VV5-4 and CHO cells had intact nuclei (Fig. 10A and B), whereas infected cells showed extranuclear apoptotic staining after VV infection (Fig. 10C and D). The extents of apoptotic nuclei were comparable (bottom of Fig. 10). Consequently, the survival phenotype of VV5-4 is not derived from interruption of apoptosis. We also concluded that inductive signals for apoptosis upon VV infection must exist prior to viral DNA replication. In contrast, apoptosis induced by rabbitpox virus infection in PK15 cells requires signals at a later phase of virus infection (6).

VV5-4 survives SFV and myxoma virus infection but is killed by cowpox virus. CHO is not permissive for VV or for other poxviruses such as Shope fibroma virus (SFV) and myxoma virus. On the other hand, cowpox virus grows very well on CHO cells. All four viruses readily kill CHO cells regardless of host restriction. Since VV5-4 is resistant to VV killing, we wished to determine whether it survives infections by other

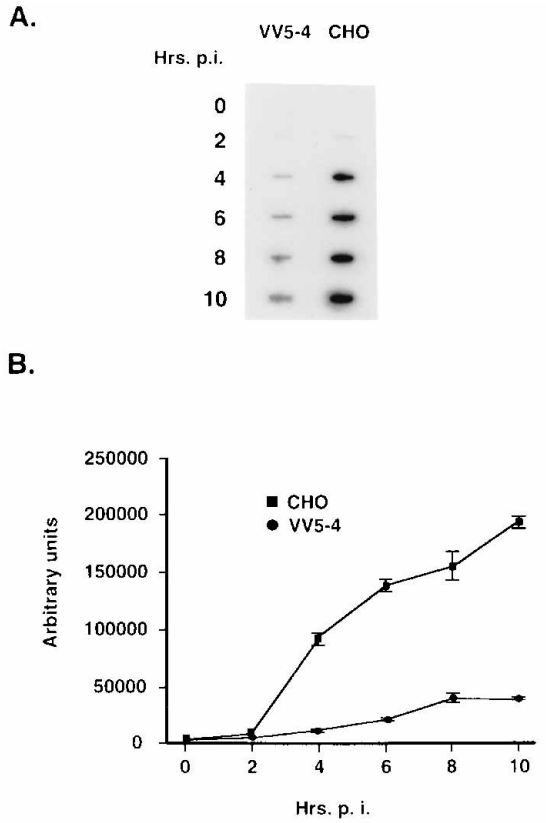


FIG. 8. Viral DNA replication is blocked in VV5-4 cells. (A) Slot blot analysis of viral DNA replication in VV5-4 and CHO cells. Cells were infected with VV at an MOI of 5 PFU per cell, and total DNA was isolated at the times indicated. A portion of the DNA samples was applied to nitrocellulose paper by using a filtration manifold and hybridized with a ^{32}P-labeled 0.5-kb VGF DNA fragment as described in Materials and Methods. (B) Quantitative analysis of viral DNA replication in VV5-4 and CHO cells. Nitrocellulose filters as shown in panel A were scanned with a PhosphorImager and quantitated by computer program analysis as described by the manufacturer (Molecular Dynamics). The data shown are averaged from three independent experiments, and the vertical bars represent standard deviations.

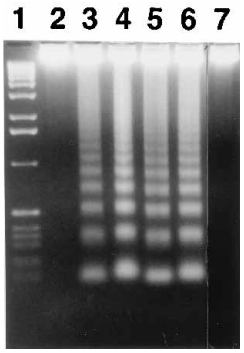


FIG. 9. DNA laddering analysis of apoptosis in VV5-4 cells. CHO (lanes 2, 3, and 5) and VV5-4 (lanes 4, 6, and 7) cells were either mock infected (lanes 2 and 7) or infected with VV at an MOI of 2.5 (lanes 3 and 4) or 5 (lanes 5 and 6). Cells were harvested at 24 h p.i., and total DNA was isolated (55). DNA samples prepared from equivalent numbers of cells were loaded onto a 2% agarose gel for electrophoresis and photographed. Lane 1 is a 1-kb ladder purchased from Bethesda Research Laboratories.

viruses as well. In addition, it is possible that the early blockage in VV5-4 could impose a new hurdle for cowpox virus and result in host restriction. We therefore infected VV5-4 with individual viruses at an MOI of 5 and stained cells at 7 days p.i. As shown in Fig. 11, the control CHO line is sensitive to all four viruses. On the other hand, VV5-4 is resistant to VV, SFV, and myxoma virus but is sensitive to cowpox virus. It is possible that VV5-4 imposes a common blockage to VV, SFV, and myxoma virus, although we have not investigated the point at which SFV and myxoma virus are arrested in VV5-4 cells. It was also interesting to find that cowpox virus is able to kill VV5-4. Cowpox gene CP77 was shown to enable VV to overcome host restriction in CHO cells (56). Our analysis of virus blockage in CHO cells suggests that CP77 functions at a later stage for virus assembly to overcome host restriction. On the other hand, the blockage in VV5-4 is at a much earlier step and does not change the host range of VV. Therefore, we would have predicted that the presence of CP77 would have no effect on the VV5-4 phenotype. To our surprise, VV5-4 was killed by cowpox virus. Furthermore, the ability of cowpox virus to kill VV5-4 was found to be mediated through CP77, since a recombinant VV expressing CP77 also killed VV5-4 after infection (data not shown). This result can be explained only if CP77 plays an additional role in an early phase prior to DNA replication. We speculate that CP77 upregulates virus early signals, mimicking a high-MOI situation which overcomes the blockage in VV5-4. It is possible that CP77 plays some roles at the early phase, since CP77 is an immediate-early gene and transcription is limited to the early phase (10). In addition, apoptosis induced prior to DNA replication is suppressed by CP77 (31). Furthermore, CP77 could substitute for other *hr* genes in cells that pose an early block of virus replication (46). Taking all of these data together, we could only speculate that CP77 is multifunctional and plays roles in different stages of the virus life cycle. The precise mechanism of CP77 in VV5-4 is not clear.

Molecular cloning of the cellular gene targeted by U3His in VV5-4. Since VV5-4 was generated by retrovirus insertional mutagenesis, it will be important to isolate the cellular gene targeted by U3His and to demonstrate its role in VV killing by complementation analysis. As an initial effort, we performed some preliminary analyses of virus integration in VV5-4 cells. As shown in Fig. 12A, Southern blot analysis of *Hin*dIII-digested VV5-4 DNA probed with a ^{32}P-labeled *his* gene revealed a single band on blots. Since U3His contains no *Hin*dIII site in viral sequences, this result suggested that VV5-4 contains a single integrated copy of U3His. On the other hand, digestion with *Eco*RI, which cuts U3His once, revealed a doublet in the blot that contains both 5′ and 3′ LTR sequences. We cloned both 5′ and 3′ LTR sequences as well as genomic DNA flanking either site as described in Materials and Methods. We subsequently located a 1-kb *Eco*RI-*Nhe*I genomic sequence flanking the 5′ end of U3His and cloned it into a CAT vector. As shown in Fig. 12B, this genomic DNA fragment exhibits promoter activity and drives CAT expression in transfected CHO cells. This result suggests that U3His serves as a promoter trap vector and integrates at the 3′ end of a cellular promoter in VV5-4 as predicted. It also indicates that the gene of interest is now located at the 3′ end of the U3His integration site.

DISCUSSION

We are interested in the contribution of the host to the VV life cycle. We designed experiments involving insertional mutagenesis of mammalian cells with a promoter trap retrovirus and selected for mutant cells that survived VV killing. Our

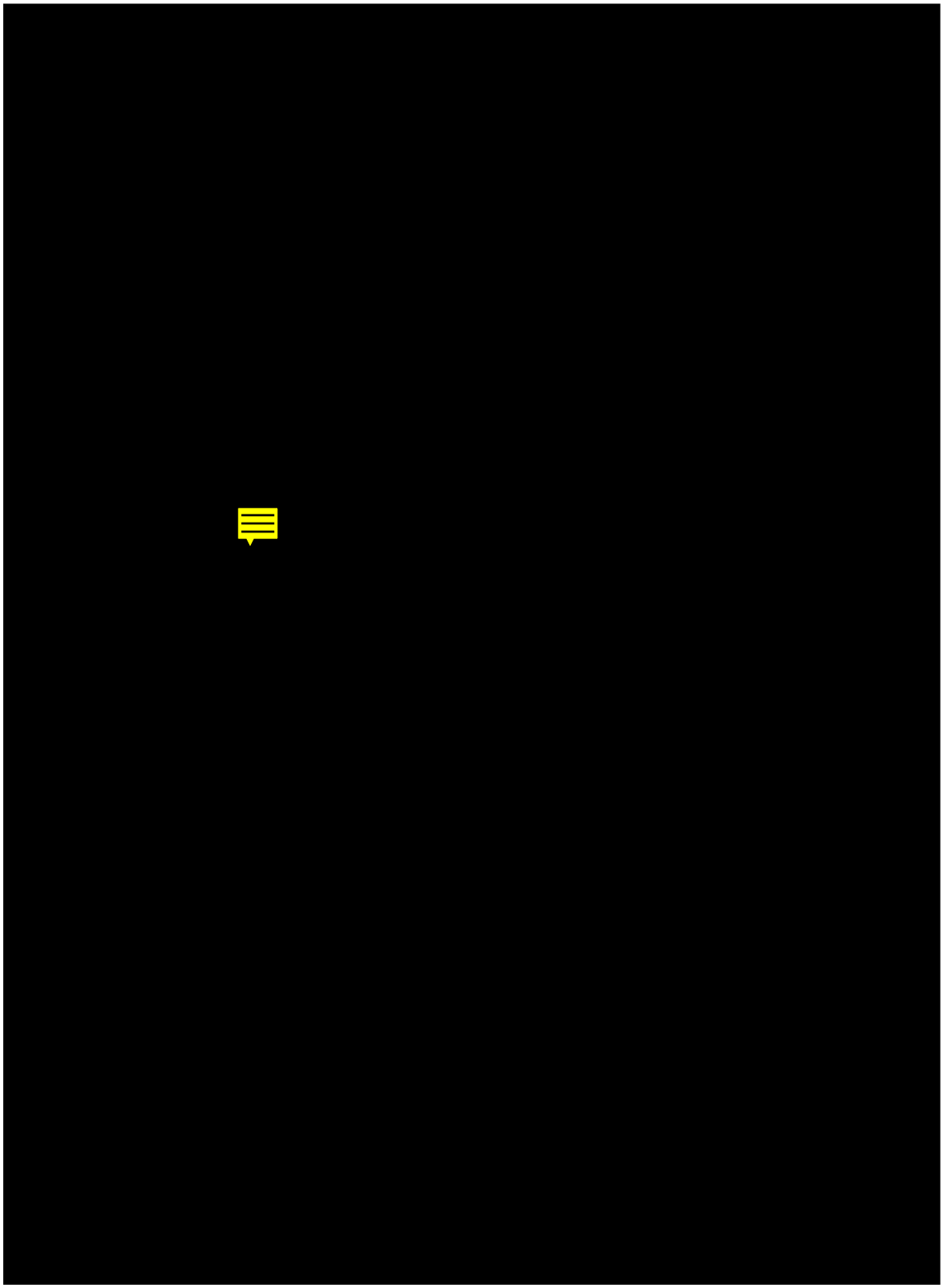


FIG. 10. Quantitation of apoptosis in VV5-4 cells. VV5-4 (A and C) and CHO (B and D) cells were either mock infected (A and B) or infected at an MOI of 5 (C and D). Cells were fixed at 24 h p.i., permeabilized, and stained with Hoechst 33248 as described in Materials and Methods. Three photographs of each sample were randomly taken, and the percentage of apoptotic cells was determined. Arrowheads identify extranuclear apoptotic staining. Data from two independent experiments are presented at the bottom.

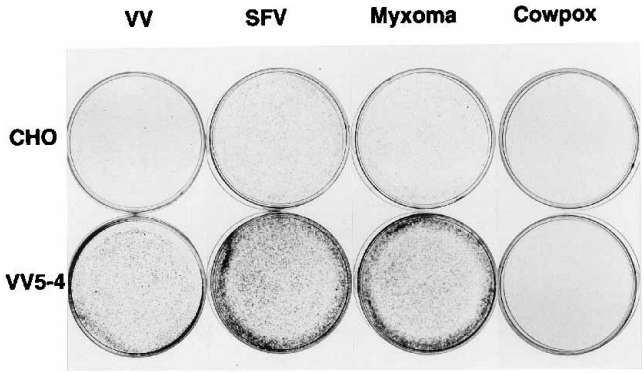


FIG. 11. Resistance of VV5-4 cells to other poxviruses. CHO and VV5-4 cells were infected with the viruses shown at top at an MOI of 5 and were maintained in normal medium for 8 days. Attached cells were washed, fixed, stained with 0.1% crystal violet, and photographed.

finding that the frequency at which VV5-4 arises is low, about 10^{-5}, nevertheless supports our prediction of functional hemizygosity. In addition, we demonstrated that a single copy of the retrovirus was integrated in VV5-4 and that the genomic sequences upstream of the viral integration site could direct CAT expression in cell transfection experiments. These data suggested that the retrovirus indeed serves as a promoter trap and that the gene of interest is located downstream of the viral integration site as predicted. In the future, U3His will serve as a useful tag for gene isolation.

We have reported in this paper the isolation and characterization of the survival phenotype of VV5-4. The CHO cell line is nonpermissive for VV, and the viral life cycle was reported to be blocked at the translation of intermediate genes (48). In our comparative studies, CHO cells support early, intermediate, and late classes of viral gene expression. Reasons for the difference between our data and those of Ramsey-Ewing and Moss (48) are not clear. We have tested different sources of CHO cells, and expression of all three classes of viral genes was detected, though the level was somewhat variable among these lines. Regardless of the discrepancy mentioned above, all CHO cells are readily killed by VV. The mutant clone VV5-4, on the other hand, is able to antagonize the cytotoxic effects of VV at low MOIs. In these situations, VV enters VV5-4 but does not progress through DNA replication. We monitored VV5-4 closely after VV infection to ensure that all cells were infected. We also monitored expression of viral β-Gal and the status of viral DNA in surviving VV5-4 cells for a period of 15 days. β-Gal and viral DNA in infected VV5-4 were both initially present and then reduced to undetectable levels in 2 weeks. These data further support our conclusion that VV infects VV5-4 but is blocked at DNA replication and as a consequence, viral DNA was lost from the surviving cell population. Therefore, we do not believe that VV5-4 survives in cells which have escaped viral infection since, to our assay's limit, VV5-4 expressed the viral marker gene as soon as several hours after infection, and expression persisted for several days, as detected both at the single-cell level by microscopic examination and in cell populations analyzed by FACS. Cultures in which cells were recovered from VV infection could be challenged with a fresh inoculum of VV once or several times. At each reinfection, they responded with the same characteristics as described here. Therefore, we believe that VV5-4 maintains a stable clonal phenotype and that we are not selecting for additional mutations from our original clone. Together, the data indicate that even for VV, which has been regarded as an autonomous

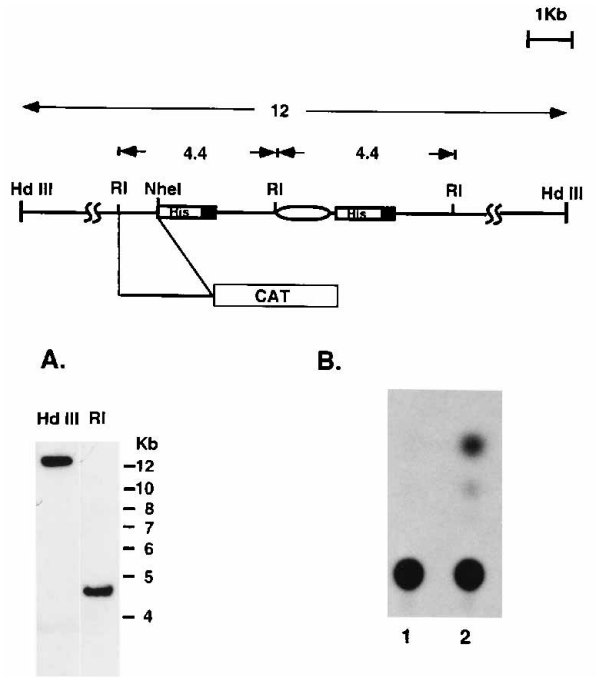


FIG. 12. Molecular analysis of retrovirus integration site in VV5-4 cells. (A) Southern blot analysis of U3His in VV5-4 cells. Genomic DNA was isolated from VV5-4 cells and digested with *Hin*dIII (Hd III) or *Eco*RI (RI), separated on a 0.7% agarose gel, transferred to a blot, and hybridized with a ^{32}P-labeled His fragment as described in Materials and Methods. Results from these and other enzyme digestions are summarized at the top. (B) Promoter analysis of 5′ flanking genomic DNA upstream of U3His in VV5-4 cells. A 1-kb *Eco*RI-*Nhe*I flanking genomic DNA fragment, shown at the top, was cloned into a plasmid containing CAT. The plasmid was transfected into CHO cells by using Lipofectamine (Bethesda Research Laboratories). Cells transfected with a control CAT plasmid with no promoter (lane 1) or an experimental plasmid (lane 2) were harvested at 2 days later, and CAT activity was measured as described previously (21, 22).

virus and relies little on hosts, the cellular microenvironment still influences progression of the viral life cycle, which in turn determines cell fate. Recently, it was reported that VV treated with psoralen and long-wave UV light could express early genes but not late genes (60). Interestingly, the treated VV does not kill infected cells, and the virus life cycle is blocked at DNA replication. These data, though based on an experimental design different from ours, revealed a similar interplay between virus and host cells and led to conclusions similar to ours.

Uncoating was previously reported to play a role in determining CPE of cells infected by MC virus (39). Tissue culture cells infected by MC virus showed early signs of CPE such as transient rounding and clumping but never died. Instead, they gradually recovered from infection in several days and continued to proliferate. An uncoating factor of cellular origin has been postulated to regulate virus core transition in studies with MC virus and other poxviruses denatured with various agents (33, 39, 50). An uncoating factor of 23 kDa was reported, but its function in the uncoating process remains unclear (45). From these data, it was clear to us that VV5-4 did not block uncoating of incoming viruses. Core accumulation was detected only when cycloheximide was added to the VV5-4 culture. Furthermore, in CHO cells, in which VV must have uncoated in order to advance to late gene expression, the uncoating profile of virus cores was indistinguishable from that in VV5-4 cells, supporting the view that uncoating is likely to occur in VV5-4 cells.

Apoptosis is another cellular response that affects cell fate in VV infection of certain cell types, including CHO cells (6, 31). As we noticed, although apoptosis is not involved in the generation of VV5-4, the phenotype of VV5-4 is affected by apoptosis. In the first 2 days after VV infection 30 to 50% of VV5-4 cells died from apoptosis. The extents of apoptosis in CHO and VV5-4 cells, as judged by the intensity of DNA laddering and quantitated by Hoechst 33258 staining, are comparable, suggesting that the signal pathway for apoptosis induction is intact in both cell lines. How do we explain the apparently contradictory observation for VV5-4? We think that cells infected by VV respond by more than one mechanism. In the case of CHO cells, apoptosis is triggered quickly after viral infection, probably by early-gene expression, to effect cell death. However, apoptosis could be detected in only 30 to 50% of the population. The rest of the infected cells die later from necrosis, perhaps as a result of host protein synthesis shutoff. On the other hand, VV5-4 cells, while capable of transmitting apoptosis signals triggered by VV infection, escape the necrotic effects of VV infection. Isolation of VV5-4 therefore allows us to separate these two cellular death pathways that occur simultaneously after viral infection. There are other data consistent with our explanation. The adenovirus E1B 19K gene reduced apoptosis of CHO cells infected by VV. However, it could not reverse inhibition of cellular protein synthesis (31). Similarly, the *bcl-2* gene also reduced VV-induced apoptosis of CHO cells, but no cells were rescued to survive as for VV5-4 (data not shown). Therefore, it is unlikely that VV5-4 arises by inhibition of apoptosis. Interestingly, this is different from what was reported for Sindbis virus by Levine et al. (38), who found that cells overexpressing *bcl-2* were resistant to virus-induced apoptosis and became persistently infected.

The isolation of VV5-4 therefore identified a new mode of the contribution of cellular factors to virus-host interactions. This finding could be interpreted in several ways. It is possible that VV5-4, as a recessive mutant, is deficient for a host factor which normally participates in viral DNA replication. Cellular activity in viral DNA replication has never been reported, although a cellular activity was implicated in transcription of viral intermediate genes (49). It is also possible that failure to shut off host protein synthesis by VV causes some indirect interference with viral DNA replication, and the cellular factor of interest may regulate cellular events related to viability rather than viral DNA replication (14). At this point, we have no data to support either hypothesis.

The resistant phenotype of VV5-4 extends to other poxviruses such as SFV and myxoma virus but not to cowpox virus. SFV and myxoma virus grow poorly on CHO cells; cowpox virus, on the other hand, grows well in both cell lines. The survival phenotype of VV5-4 therefore does not change the host ranges of these viruses. Host range and cell survival appear to be influenced by different factors. For example, the survival phenotype of VV5-4 is virus dosage dependent, and while the mechanism is still unclear, cells are killed with more viruses. Host restriction, on the other hand, is not influenced by the amount of input virus. Rather, it is a characteristic that is dependent on a viral gene, CP77, present in cowpox virus (31). This gene was isolated, and DNA sequences did not reveal homology with other known genes in databases except for the presence of two ankyrin-like repeats (56). The mechanism by which CP77 overcomes VV restriction in CHO cells remains unclear, although several possibilities exist. First, CP77 could relieve host restriction by suppressing apoptosis of VV-infected cells (31). Expression of CP77 RNA was observed early after virus infection, and the promoter has characteristics common to early genes (10, 56). Suppression of apoptosis was observed for CP77, but it may not be adequate to overcome host restriction (31). Alternatively, CP77 may act late to help virus assembly, since expression of all three classes of genes was detected in CHO cells. Consistent with this view, CP77 protein is quite stable and is accumulated in virus-infected cells. It is possible that CP77 plays more than one role in the virus life cycle. Besides its complicating roles in host range, the effect of CP77 on VV5-4 killing is intriguing and mimics that of a high-MOI infection. It seems to us that CP77 somehow could upregulate levels of early signals in VV5-4 to a level comparable to that produced by a high-MOI infection and result in phenotypic change. The precise mechanism of action of CP77 in VV5-4 is not known and will be investigated further in the future.

Mutant cell lines escaping killing by other viruses have been reported (2, 25, 38, 41, 66). In one case, when a mutant cell resistant to herpesvirus killing was analyzed, it was found that mutations in surface proteoglycan modification rendered cells resistant to virus entry (2, 25). In other cases, when cells were resistant to alphavirus killing, defects of postentry processing and progeny maturation were found (41, 66). These mutants provide new insight into virus-host interactions and allow us to dissect various cellular mechanisms that control virus-induced cytotoxicity. Since mutant VV5-4 was isolated by retrovirus insertions, we have begun to investigate the cellular locus that might be involved in its phenotype. Delineating the function of the gene will advance our understanding of the mechanism underlying VV-induced cell killing.

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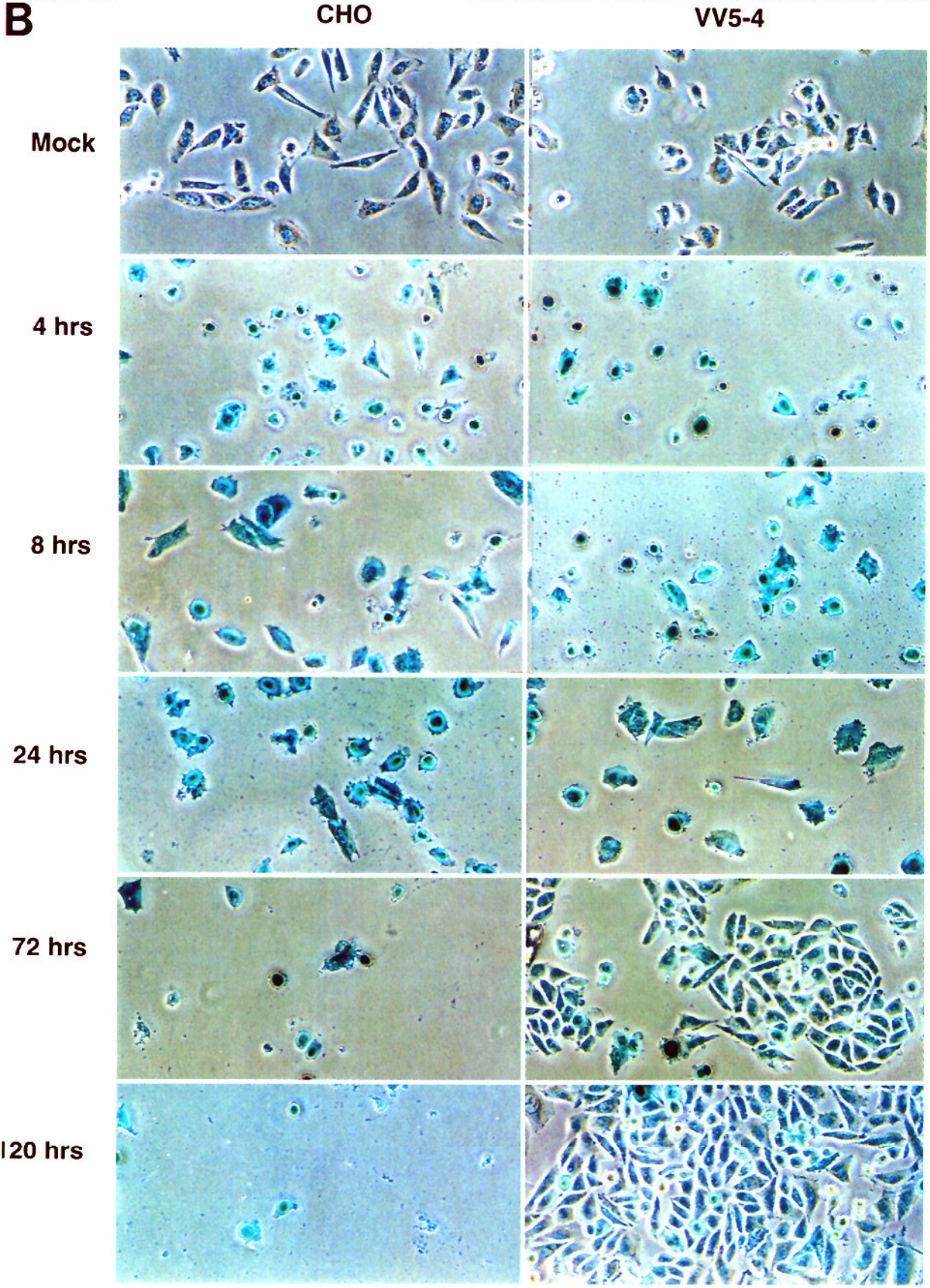
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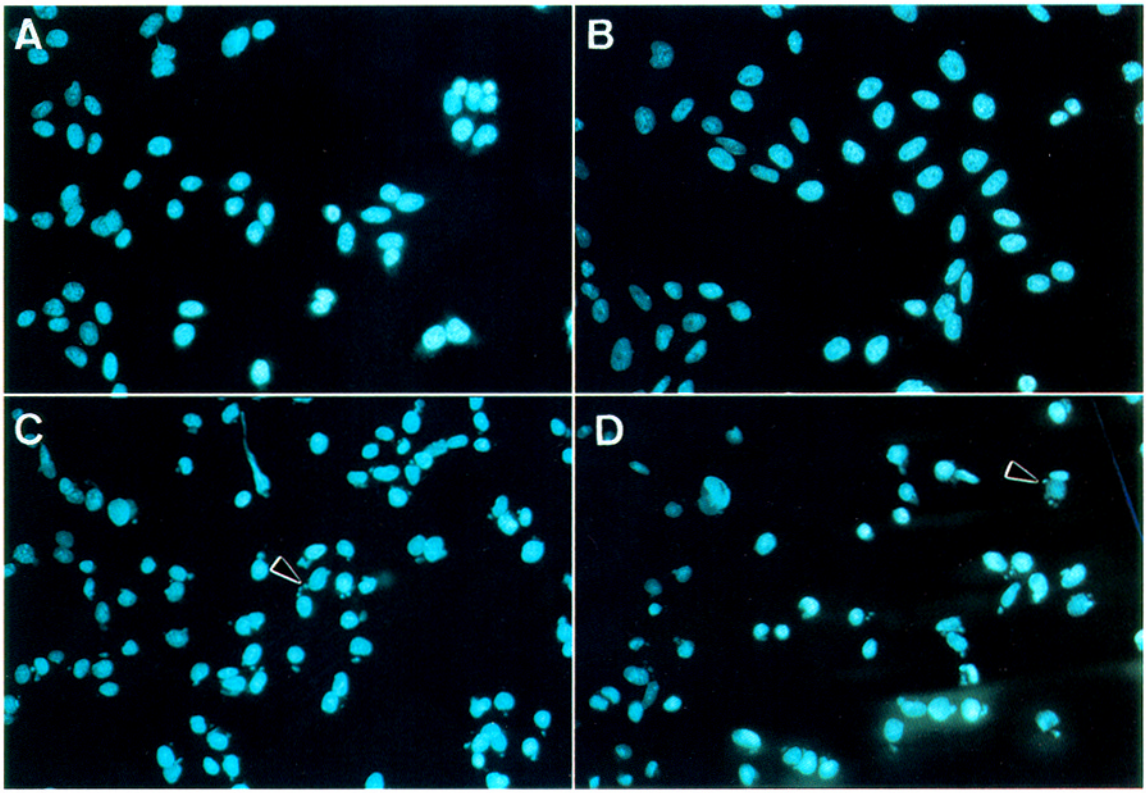
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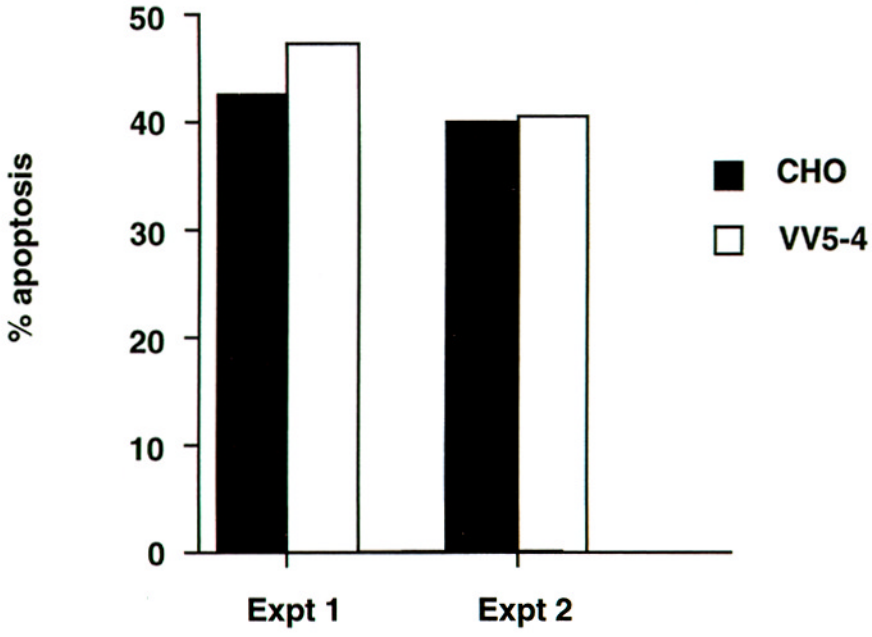
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