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# Activation of Caspases and p53 by Bovine Herpesvirus 1 Infection Results in Programmed Cell Death and Efficient Virus Release

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# Activation of Caspases and p53 by Bovine Herpesvirus 1 Infection Results in Programmed Cell Death and Efficient Virus Release

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**Programmed cell death (PCD), or apoptosis, is initiated in response to various stimuli, including virus infection. Bovine herpesvirus 1 (BHV-1) induces PCD in peripheral blood mononuclear cells at the $G_0/G_1$ phase of the cell cycle (E. Hanon, S. Hoornaert, F. Dequiedt, A. Vanderplasschen, J. Lyaku, L. Willems, and P.-P. Pastoret, Virology 232:351–358, 1997). However, penetration of virus particles is not required for PCD (E. Hanon, G. Meyer, A. Vanderplasschen, C. Dessy-Doize, E. Thiry, and P. P. Pastoret, J. Virol. 72:7638–7641, 1998). The mechanism by which BHV-1 induces PCD in peripheral blood mononuclear cells is not understood, nor is it clear whether nonlymphoid cells undergo PCD following infection. This study demonstrates that infection of bovine kidney (MDBK) cells with BHV-1 leads to PCD, as judged by terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling, DNA laddering, and chromatin condensation. p53 appears to be important in this process, because p53 levels and promoter activity increased after infection. Expression of proteins that are stimulated by p53 ($p21^{Waf1}$ and Bax) is also activated after infection. Cleavage of Bcl-$x_L$, a protein that inhibits PCD, occurred after infection, suggesting that caspases (interleukin-1β-converting enzyme-like proteases) were activated. Other caspase substrates [poly(ADP-ribose) polymerase and actin] are also cleaved during the late stages of infection. Inhibition of caspase activity delayed cytotoxic activity and virus release but increased the overall virus yield. Taken together, these results indicate that nonlymphoid cells undergo PCD near the end of productive infection and further suggest that caspases enhance virus release.**

Bovine herpesvirus 1 (BHV-1) belongs to the *Alphaherpesvirinae* subfamily and is a significant viral pathogen of cattle (reviewed in reference 39). Acute infection leads to conjunctivitis, tracheitis, and upper respiratory tract infections and can induce a complex upper respiratory infection called shipping fever. Although BHV-1 is not the sole infectious agent associated with shipping fever, it initiates the disorder by immunosuppressing infected cattle (reviewed in reference 84). Consequently, secondary bacterial infections can cause pneumonia. Like other members of the *Alphaherpesvirinae* subfamily, BHV-1 establishes a latent infection in sensory ganglionic neurons (reviewed in reference 39). Viral DNA persists in sensory neurons for the lifetimes of infected cattle but can periodically reactivate. Infection of permissive cells by BHV-1 leads to rapid cell death and virus spread. Viral gene expression is temporally regulated by two immediate-early (IE) transcription units (75, 77, 92, 93). IE gene expression is stimulated by a virion component (bTIF) which interacts with a cellular transcription factor (Oct-1), and subsequently this protein complex binds TAATGARAT motifs present in IE promoters (52). It is generally agreed that tissue-specific factors mediate latency and pathogenesis by influencing viral gene expression.

There are two types of cell death, necrosis and programmed cell death (PCD) (apoptosis), and they differ in their morphological and biochemical characteristics (reviewed in reference 48). PCD occurs during embryogenesis, tissue differentiation, aging, or tumor regression (reviewed in reference 86). PCD can be triggered by a variety of stimuli, including viral infection (reviewed in reference 79), growth factor withdrawal (26), and DNA damage (reviewed in reference 25). Cells exhibit nuclear fragmentation, chromatin condensation, cleavage of DNA into nucleosomal oligomers, cell shrinkage, and appearance of apoptotic bodies which are engulfed by surrounding cells (reviewed in references 91 and 95).

The tumor suppressor protein p53 monitors cell cycle checkpoints (40), senses DNA damage (42), assembles DNA repair machinery (89), modulates gene amplification (96), and activates PCD (78, 97). The ability of p53 to induce PCD is crucial for tumor suppressor function (15, 28, 47, 81, 97). However, whether p53's ability to activate gene expression is required for PCD is controversial (6, 70, 88). A p53-inducible gene product, $p21^{Waf1}$, a component of p53-dependent $G_1$ arrest, is dispensable for p53-dependent PCD but is required for cell cycle arrest (reviewed in references 1, 17, 22, 41, and 49). A link between p53 induction and activation of interleukin-1β-converting enzyme-like proteases, or caspases, following DNA damage has also been established (9, 45, 71). p53-independent mechanisms of PCD also exist, and they usually lead to caspase activation.

Many viruses induce PCD when cultured cells are infected (reviewed in references 67, 79, and 82a). Premature PCD of infected cells reduces burst size and thus prevents spread. Members of the *Alphaherpesvirinae* subfamily, e.g., herpes simplex virus type 1 (HSV-1), varicella-zoster virus, or BHV-1, induce PCD after infection of cultured cells (24, 30–32, 43, 72). BHV-1-induced PCD occurs at the $G_0/G_1$ phase of the cell cycle in cultured peripheral blood mononuclear cells (PBMC) (24, 31). Since inactivated BHV-1 induced PCD of PBMC (30,

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TABLE 1. Primers used in this study

| Transcript | Primers |
|---|---|
| p53 | 5′ CCGAGGCCGGCTCTGAGTATACCACCATCC 3′ (+)<br>5′ CTCATTCAGCTCCCGGAACATCTCGAAGCG 3′ (−) |
| Bcl-2 | 5′ GTCGCTACCGTCGTGACTT 3′ (+)<br>5′ CAGCCTCCGTTATCCTGGA 3′ (−) |
| Actin | 5′ GTGGGCCGCTCTAGGCACCAA 3′ (+)<br>5′ CTCTTTGATGTCACGCACGATTTC 3′ (−) |

32), it is possible that PCD was a result of PBMC activation (90) and not a direct result of infection. Furthermore, it is not clear whether PCD occurs when fibroblasts or epithelial cells are infected with BHV-1.

In this study, we analyzed BHV-1-induced PCD in Madin-Darby bovine kidney (MDBK) cells. Following infection of MDBK cells, PCD occurred during the late stages of infection. In contrast to the case for PBMC, PCD did not occur when virus preparations were inactivated by Psoralen or heat. Virus-induced PCD appeared to occur in a p53-dependent manner. Processing and activation of caspase 2 and cleavage of poly-(ADP-ribose) polymerase (PARP), actin, and Bcl-$x_L$ occurred after infection, suggesting that caspases were activated. Inhibition of caspase activity increased virus yield but delayed or inhibited virus release.

## MATERIALS AND METHODS

**Cells and virus.** MDBK cells or CV-1 cells (African green monkey kidney cells; American Type Culture Collection, Rockville, Md.) were grown in Earle's modified Eagle's medium supplemented with 10% fetal calf serum. The Cooper strain of BHV-1 was obtained from the National Veterinary Services Laboratory, Animal and Plant Health Inspection Services (Ames, Iowa). MDBK cells were infected with 5 PFU of BHV-1/cell.

BHV-1 was inactivated by photochemical treatment as described previously (30). Briefly, Psoralen (catalogue no. 8399; Sigma) was dissolved in dimethyl sulfoxide (DMSO) to a final concentration of 200 μg/ml. This stock was added to the viral suspension ($10^7$ 50% tissue culture infective doses/ml) to a final concentration of 1 μg/ml. This viral suspension was then irradiated with 254-nm-wavelength UV (UVP Products, San Gabriel, Calif.). Alternatively, virus was inactivated by boiling the viral suspension for 10 min.

**Antibodies and plasmids.** Antibodies for p53 (sc-99 and sc-100), Bcl-2 (sc-783), Bax (sc-526), $p21^{Waf1/Cip1}$ (sc-528), Bcl-$x_L$ (sc-1690), actin (sc-1616), and caspase 2 (sc-626) were purchased from Santa Cruz Biotechnology (Santa Cruz, Calif.). PARP antibodies (SA252) were purchased from BioMol (Plymouth Meeting, Pa.).

A chloramphenicol acetyltransferase (CAT) plasmid containing the murine p53 promoter, p0.7CAT (p53CAT), was provided by V. Rotter (University of South Carolina). Plasmid pA10CAT contains the simian virus 40 early promoter and was obtained from B. Howard (National Institutes of Health). The plasmid containing the intact human $p21^{Waf1/Cip}$ promoter, pWWPCAT, was obtained from B. Vogelstein (Johns Hopkins University). pSV2-β-gal was purchased from Clontech, Palo Alto, Calif.

**TUNEL assay.** The terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end-labeling (TUNEL) technique detects endonucleolytically cleaved DNA by the addition of labeled dUTP to DNA ends by terminal transferase. MDBK cells grown on coverslips were infected with 5 PFU/cell and stained with an in situ cell death detection kit (catalogue no. 1684 809; Boehringer Mannheim) according to the manufacturer's instructions.

**Hoechst 33342 staining.** Monolayers of MDBK cells grown on glass coverslips were infected with BHV-1 at 5 PFU/cell. At various times postinfection (p.i.), cells were washed with phosphate-buffered saline (PBS), fixed with ice-cold

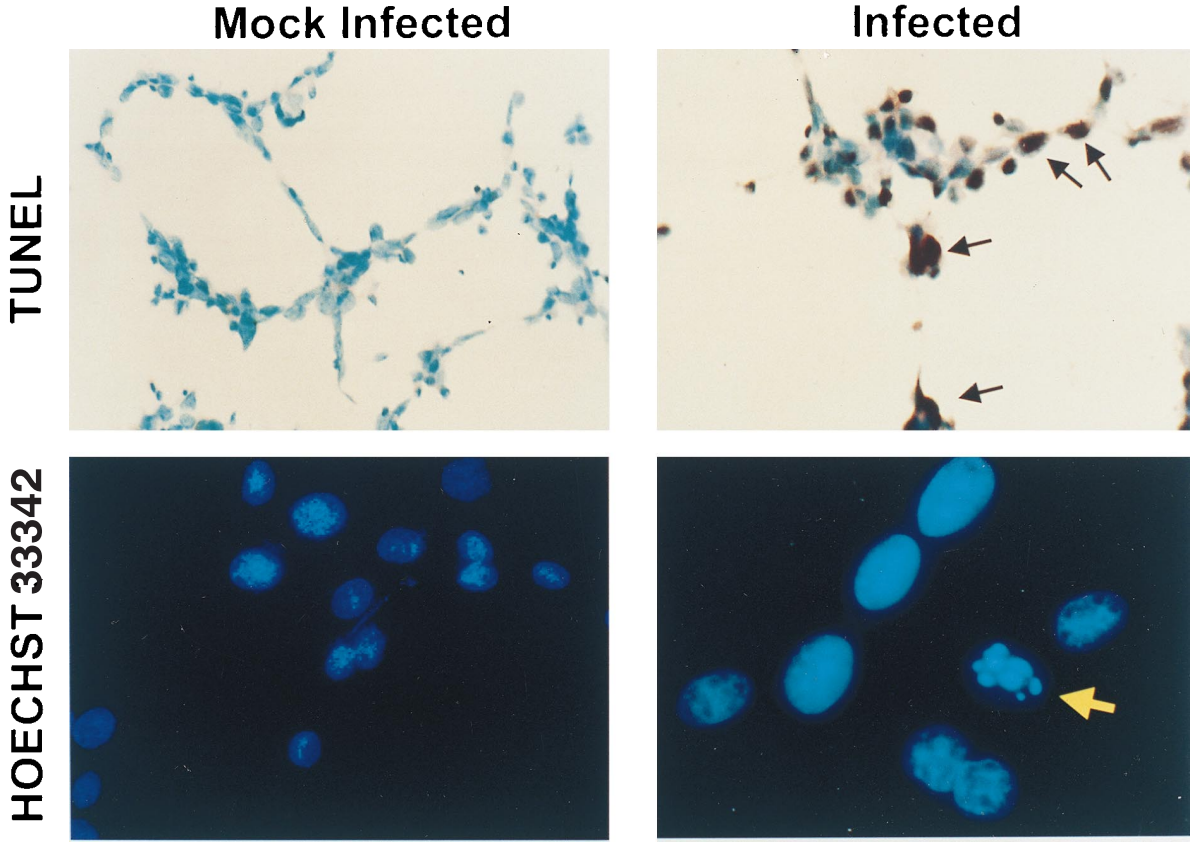


FIG. 1. BHV-1 induces PCD in MDBK cells. MDBK cells were infected with BHV-1 for 48 h. Mock-infected cells served as a control. Hoechst 33342 staining was observed under UV light. Magnifications, ×800 for Hoechst staining and ×200 for TUNEL assay. Arrows point to TUNEL-positive cells or to nuclei that contain condensed chromatin.

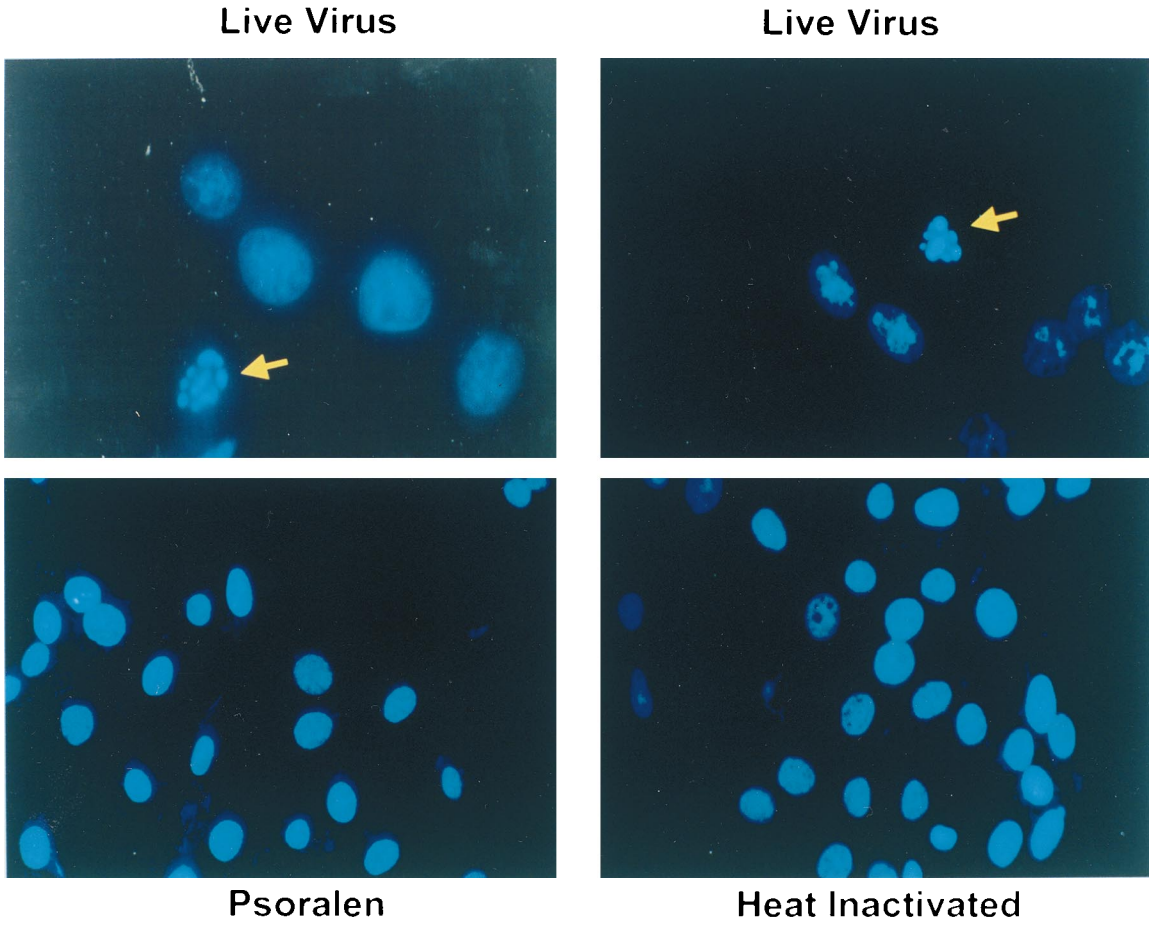


FIG. 2. Inactivated BHV-1 does not induce chromatin condensation in MDBK cells. BHV-1 was treated with Psoralen or was heat inactivated as described in Materials and Methods (magnification, ×400). As a positive control, a similar amount of untreated BHV-1 was used to infect cultures of MDBK cells (magnification, ×800). At 48 h p.i., Hoechst 33342 staining was performed and the cells were observed under UV light. Arrows point to a nucleus that contains condensed chromatin.

methanol-acetone (1:1, vol/vol) for 10 min at −20°C, and then washed once with PBS. Nuclei were stained with Hoechst 33342 (catalogue no. B-2261; Sigma) (0.5 μg/ml in PBS) for 10 min in the dark, washed once with PBS, and mounted on slides in glycerol-citric acid phosphate buffer (9:1, vol/vol), pH 4.1. Cells were visualized under UV with a fluorescence microscope (Microphot; Nikon, Mellville, N.Y.).

**Analysis of high-molecular-weight DNA after infection.** Monolayers of MDBK cells were grown in 100-mm-diameter plates and then infected with 5 PFU of BHV-1 per cell. DNA from uninfected or infected cells was extracted (43). Five micrograms of each DNA sample was electrophoresed on a 1.5% agarose gel containing 0.1 μg of ethidium bromide per ml. The DNA was visualized under UV light, and the sizes of the respective amplified products were estimated by comparing the mobilities with a 100-bp ladder (catalogue no. 15628-019; GIBCO-BRL).

**Western blot analysis.** Extract from infected or uninfected cells was prepared as described previously (33). Approximately 20 μg of extract was resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto Immobilon (Millipore) membranes according to the manufacturer's instructions. Membranes were probed with the indicated antibodies, and Western blots were developed by chemiluminescence (ECL+ plus, RPN 2132; Amersham). The sizes of the proteins on SDS-PAGE were estimated by using Amersham molecular weight markers (catalogue no. RPN 756).

**RNA PCR analysis.** Total RNA from uninfected or infected cells was extracted by using RNAgents (total RNA isolation system from Promega; catalogue no. Z5110) according to the manufacturer's instructions. Three micrograms of total RNA was reverse transcribed by using a random primer (Invitrogen) with reverse transcriptase from GIBCO-BRL according to the manufacturer's instructions. The first-strand cDNA was amplified by using the primers described in Table 1. The cDNA was amplified in a Hybaid thermal cycler under the following conditions: 95°C for 1 min, 60°C for 1 min, 72°C for 2 min, and 72°C for 7 min to allow for final extension. Amplified products were resolved on 2% agarose gels (NuSieve). The sizes of the amplified products were estimated by using ϕX174 replicative DNA cleaved with *Hae*III DNA (New England Biolabs).

**Immunoprecipitation.** Uninfected or infected MDBK cells were rinsed twice with methionine-free Dulbecco modified Eagle medium (DMEM) (GIBCO-BRL) and incubated in methionine-free DMEM at 37°C for 30 min. The cells were then incubated (37°C for 4 h) with 200 μCi of [$^{35}$S]methionine (Amersham) in 2 ml of methionine-free DMEM supplemented with 1% dialyzed fetal bovine serum. The labeled cells were then washed with ice-cold PBS. One milliliter of lysis buffer (50 mM Tris-HCl [pH 7.3], 75 mM NaCl, 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride [PMSF], 1 mM leupeptin, 1 mM apopain) was added directly to the dish and rocked for 30 min at 4°C. Cells were scraped into a microcentrifuge tube and centrifuged at 10,000 × *g* for 10 min at 4°C.

Cell lysate (500 μl) prepared from metabolically labeled cells was precleared with normal mouse serum for 30 min, followed by precipitation with a p53 or actin antibody overnight at 4°C. Twenty microliters of protein A-agarose beads (Santa Cruz) was incubated for 30 min at 4°C, and the beads were subsequently washed three times (each) with 1 ml of PBS containing 1, 0.5, or 0.05% Triton X-100. The beads were then suspended in 50 μl of 2× SDS loading buffer and boiled for 10 min. Proteins were separated by SDS-PAGE and visualized by fluorography with En$^3$Hance (Amersham).

**Extraction of PARP.** Infected or uninfected cells were washed twice with ice-cold PBS and then once with ice-cold buffer A (100 mM Tris-HCl [pH 7.4], 10 mM $MgSO_4$, 500 mM sucrose, 10 mM PMSF, 0.5 μg of leupeptin per ml, 0.75 μg of pepstatin per ml, and 5 μg of antipain per ml). Cells were permeabilized by incubation on ice for 20 min with buffer B (100 mM Tris-HCl [pH 7.4], 10 mM $MgSO_4$, 500 mM sucrose, 10 mM PMSF, 1% Nonidet P-40; 0.25 μg of leupeptin per ml, 0.35 μg of pepstatin per ml, and 50 μg of antipain per ml). PARP was extracted from permeabilized cells by incubation with cold buffer C (200 mM $K_2HPO_4$, 100 mM Tris-HCl [pH 7.4], 10 mM $MgSO_4$, 500 mM sucrose, 10 mM PMSF, 0.5 μg of leupeptin per ml, 0.75 μg of pepstatin per ml, and 5 μg of antipain per ml) on ice for 20 min. The extract was centrifuged at 2,000 × *g* for 10 min at 4°C, and the supernatant was collected and mixed with 4 volumes of urea loading buffer (62.5 mM Tris-HCl [pH 6.8], 6 M urea, 10% glycerol, 2% SDS, 0.00125% bromophenol blue, and 5% β-mercaptoethanol).

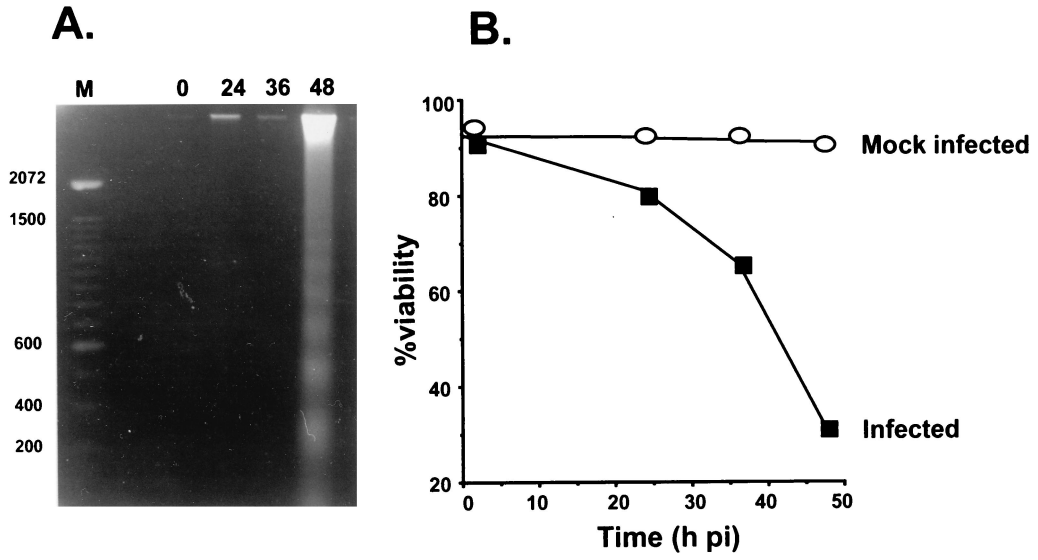


FIG. 3. Infection induces DNA degradation and reduces cell viability. (A) Agarose gel electrophoresis of DNA extracted from MDBK cells at 24, 36, or 48 h p.i. Lane 0, DNA prepared from mock-infected cells; lane M, 100-bp ladder. Numbers on the left indicate base pairs. (B) Measurement of cell viability as judged by trypan blue staining. Data from one of two experiments is shown.

**CAT assay.** MDBK cells grown in 60-mm-diameter dishes were transfected with 1 μg each of p0.7CAT or pWWPCAT and pSV2-β-gal with Superfect reagent (Qiagen) according to the manufacturer's instructions. Cells were infected with BHV-1 (5 PFU/cell) at 24 h after transfection. Cells were harvested at 24, 36, or 48 h p.i., and CAT assays were performed as previously described (19). The amounts of extract used to measure CAT activity were adjusted based on β-galactosidase (β-gal) activity. β-gal activity was measured by using *ortho*-nitrophenyl-β-D-galactopyranoside (ONPG) (Sigma) as a substrate in colorimetric assays (57).

**In vitro reconstitution of PCD. (i) Preparation of cell extracts.** Uninfected or infected MDBK cells were washed once with PBS and then with cell extract buffer [50 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES) (pH 7.4), 50 mM KCl, 5 mM EGTA, 2 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 10 μM cytochalasin B, and 1 mM PMSF]. The cells were harvested, swelled by treatment with an equal volume of cell extract buffer on ice for 20 min, and then lysed in a Dounce homogenizer (Wheaton) by 20 strokes with a type B pestle. The homogenate was centrifuged at 4°C for 20 min at 14,000 rpm in a

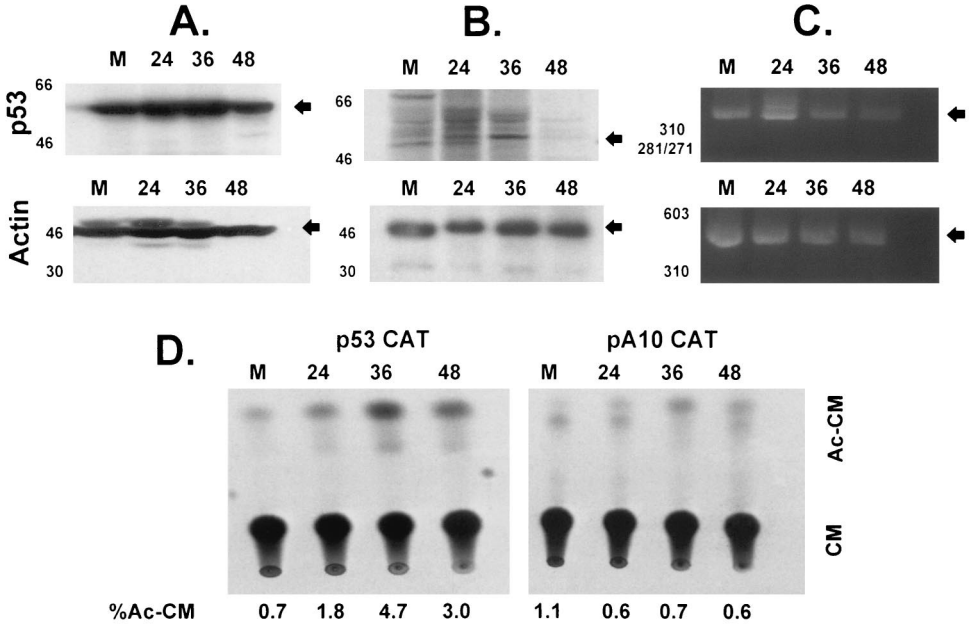


FIG. 4. Analysis of p53 after infection. MDBK cells were infected with BHV-1 for the indicated times (hours p.i.). Mock-infected cells (lanes M) served as a control. (A and B) Western blot analysis of p53 and actin in infected cells (A) and fluorography of $^{35}$S-labeled p53 and actin (B). Immunoprecipitation was carried out with an antibody that specifically recognizes p53 or actin. Arrows on the right of each panel indicate the position of p53 or actin. The numbers to the left of each panel represent the positions of protein markers (in kilodaltons). (C) RNA PCR analysis with primers specific for p53 or β-actin. Three micrograms of total RNA was reverse transcribed with Superscript reverse transcriptase, and the cDNA was amplified by PCR. The arrows on the right represent the positions of the amplified cDNA. Numbers on the left are the positions of ϕX174 DNA digested with *Hae*III (in base pairs). (D) A mouse p53 promoter construct (p53CAT) or pA10CAT was cotransfected with pSV2-β-gal into MDBK cells with Superfect transfection reagent from Qiagen. Twenty-four hours after transfection, the cells were infected with 5 PFU/cell. Cells were harvested at the indicated times p.i., and CAT activity was measured. The amount of extract used to measure CAT activity was adjusted based on β-gal activity in the respective samples. Ac-CM, acetylated chloramphenicol.

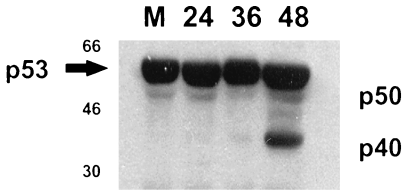


FIG. 5. Cleavage of p53 after infection. Western blot analysis of p53 with extracts prepared from mock-infected (lane M) or infected (24, 36, or 48 h p.i.) cells is shown. The blots were probed with an antibody that detects the N terminus of p53 (Pab 240). Cleaved products are designated p50 and p40. The numbers to the left represent the positions of protein markers (in kilodaltons).

Beckman (Palo Alto, Calif.) Avanti 30 centrifuge, and the supernatant was stored at −70°C.

**(ii) Preparation of nuclei.** Confluent monolayers of CV-1 cells were washed with PBS, and nuclei were prepared (35). The nuclei were suspended in storage buffer (10 mM PIPES [pH 7.4], 80 mM KCl, 20 mM NaCl, 250 mM sucrose, 5 mM EGTA, 1 mM DTT, 0.5 mM spermidine, 0.2 mM spermine, 1 mM PMSF, and 50% glycerol) and stored in aliquots at −70°C.

**(iii) Cell-free apoptosis.** Cell extract (75 μg) was incubated with $2 \times 10^5$ nuclei in 10 mM HEPES (pH 7.4)–50 mM NaCl–2 mM $MgCl_2$–5 mM EGTA–1 mM DTT and incubated at 37°C for 1 h. The caspase inhibitor Z-VAD-FMK (25 μM) was added to extracts prior to incubation.

**(iv) Hoechst staining of nuclei.** Four or five microliters of the cell-free apoptosis reaction mixture was stained with 2 μl of 10 μM Hoechst 33342 (Sigma) in formalin on a glass slide for 2 to 3 min in the dark. The slides were washed for 2 min with water, air dried, and baked for 60 min at 50°C. Slides were mounted with 0.5 μg of Canada balsam (catalogue no. C-1795; Sigma) per ml and observed under UV light with a fluorescence microscope (Diaphot; Nikon).

**Effect of caspase inhibitors on virus release and yield.** The following caspase inhibitors were purchased from Calbiochem (La Jolla, Calif.) and used for these studies: caspase 3 inhibitor II (Z-DEVD-FMK; catalogue no. 264155), caspase 1 inhibitor I (Ac-YVAD-CHO; catalogue no. 400010), and caspase inhibitor I (Z-VAD-FMK; catalogue no. 627610).

For the virus production assay, 25 μM Z-VAD-FMK was added at 6 and 24 h p.i., due to the short half-life of the peptide. Cells and medium were harvested at 48 h p.i. Nearly all cells which were infected but not treated with Z-VAD-FMK were rounded and detached from the plate at 48 h p.i. For total virus yield, cells plus medium were frozen and thawed three times to release virus particles. The yield of infectious virus was determined by estimating the 50% end points (68).

For the virus release assay, cells and medium were separated by centrifugation (1,000 rpm for 3 min) in a CR412 centrifuge (Jouan, St. Herblain Cedex, France). The resulting cell pellet was frozen, thawed three times, and suspended in 1 ml of DMEM. Infectious virus yields in the medium or in the cell pellet were determined separately. The percent virus release was calculated by dividing the amount of infectious virus released into the medium by the total virus yield.

## RESULTS

**BHV-1 induces PCD in cultured MDBK cells.** Although BHV-1 induces PCD in lymphoid cells (31, 32), it is not clear whether infection of permissive fibroblasts or cells of epithelial origin leads to PCD. The most commonly used markers of PCD are (i) fragmentation of chromosomal DNA into oligonucleosomal fragments, which can be detected by TUNEL assays or by agarose gel electrophoresis, and (ii) chromatin condensation, which is identified by DNA staining agents, Hoechst stain, or DAPI (4′,6-diamidino-2-phenylindole), for example (29). In addition, cell viability was assessed by trypan blue exclusion.

TUNEL-positive infected cells, but no TUNEL-positive mock-infected cells, were readily detected at 48 h p.i. (Fig. 1). Condensed chromatin, as judged by Hoechst 33342 staining, was also detected in many infected cells but not in mock-infected cells (Fig. 1). In mock-infected cells, chromatin was diffusely stained, whereas staining in infected cells was punctate and marginated. Chromatin condensation was observed in 3 to 5% of infected cells at 24 h p.i. and increased at 36 and 48 h p.i. Since previous studies demonstrated that inactivated BHV-1 initiates PCD in PBMC (30), we tested whether inactivated virus could induce PCD in MDBK cells. In contrast to the case for PBMC, we were unable to detect higher levels of PCD after inactivated virus was added to MDBK cells (Fig. 2). Regardless of whether the virus was inactivated by Psoralen cross-linking or boiling, chromatin condensation was not readily detected at 48 h p.i.

High-molecular-weight DNA prepared from cells infected for 48 h was degraded and exhibited the characteristic laddering observed after PCD (Fig. 3A). Degraded DNA was not readily detected in mock-infected cells or in cells infected for 24 h. As expected, the percentage of viable cells decreased as a function of time as determined by using trypan blue to monitor viable cells (Fig. 3B). Taken together, these studies indicated that PCD occurred in MDBK cells during the late stages of infection.

**Examination of p53 after infection.** The p53 protein inhibits cell cycle progression after DNA damage, thus facilitating DNA repair (69), or it can induce PCD if extensive DNA damage occurs (97). During p53-dependent PCD, p53 levels generally increase (reviewed in reference 2). Although steady-state levels of p53 protein were not dramatically induced after infection (Fig. 4A), higher levels of $^{35}$S-labeled p53 protein were present at 24 or 36 h p.i. (Fig. 4B). The antibody used for immunoprecipitation recognizes wild-type p53 but not mutated forms, suggesting that MDBK cells contain wild-type p53. Compared to actin RNA levels, p53 RNA levels were slightly higher at 24 h p.i. (Fig. 4C). At 36 h p.i., a sixfold increase in p53 promoter activity (p53CAT) was observed (Fig. 4D). In contrast, the simian virus 40 early promoter (pA10CAT) was not stimulated during the late stages of infection.

Overexposure of p53 Western blots revealed a band that migrated with an apparent molecular mass of 40 kDa at 48 h p.i. (Fig. 5). The 40-kDa band was detected in several different experiments. Following DNA damage, p53 binds specifically to damaged DNA and is cleaved into 40- and 50-kDa products

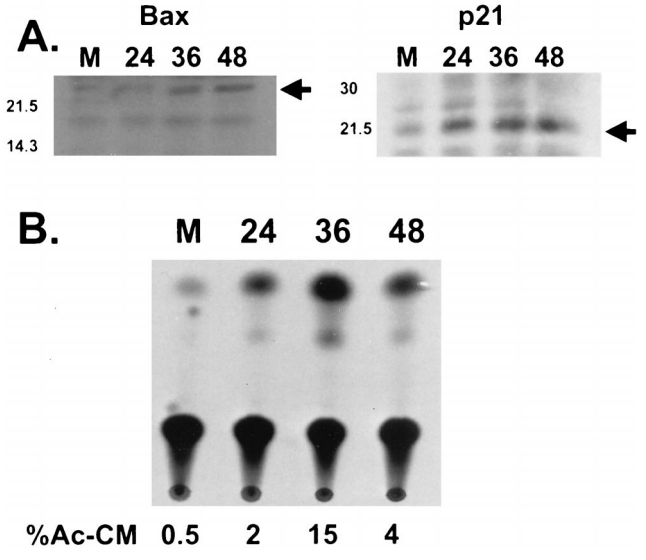


FIG. 6. Analysis of $p21^{Waf1/Cip1}$ and Bax after infection. (A) Whole-cell lysate was prepared from mock-infected cells (lane M) or infected cells (24, 36, or 48 h p.i.), and Western blot analysis was performed with an antibody which specifically recognizes Bax or p21. Arrows indicate the positions of the respective proteins. The numbers to the left represent the positions of protein markers (in kilodaltons). (B) A human $p21^{Waf1}$ promoter construct (pWWPCAT) was cotransfected with pSV2-β-gal into MDBK cells with Superfect transfection reagent from Qiagen. Twenty-four hours after transfection, cells were infected with 5 PFU/cell. Cells were harvested at the indicated times p.i., and CAT activity was measured. The amount of extract used to measure CAT activity was adjusted based on β-gal activity in the respective samples. Ac-CM, acetylated chloramphenicol.

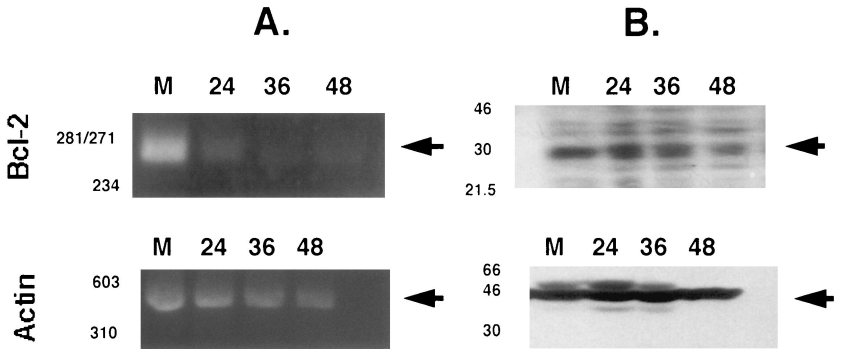


FIG. 7. Analysis of Bcl-2 after infection. (A) RNA PCR analysis of Bcl-2 or actin mRNA in mock-infected cells (lane M) or infected cells (24, 36, or 48 h p.i.). Three micrograms total RNA was reverse transcribed with Superscript reverse-transcriptase, and the cDNA was amplified by PCR. Numbers on the left are the positions of ϕX174 DNA digested with *Hae*III (base pairs). (B) Whole-cell lysate was prepared from mock-infected cells (lane M) or infected cells (24, 36, or 48 h p.i.), and Western blot analysis was performed with an antibody which specifically recognized Bcl-2 or actin. The arrows indicate the position of Bcl-2 or actin. The numbers to the left represent the positions of protein markers (in kilodaltons).

(p40 or p50, respectively) (61). Cleavage of p53 occurs at the C terminus by autoproteolysis and is important for p53-dependent PCD. In summary, these studies demonstrated that infection increased p53 protein levels slightly and that p53 was cleaved.

**Examination of Bax, Bcl-2, and p21$^{Waf1/Cip1}$ after infection.** Several genes that regulate the cell cycle or apoptosis are transactivated by p53. These include the genes encoding p21$^{Waf1/Cip1}$ (21), GADD45 (40), Mdm2 (62), cyclin G (60), and Bax (53). Since p53 protein expression increased after infection, the levels of the p21 and Bax proteins were examined by Western blot analysis. Bax and p21$^{Waf1/Cip1}$ levels increased as a function of time after infection (Fig. 6A). Following infection, the p21$^{Waf1/Cip1}$ promoter (pWWPCAT) was activated more than 30-fold (Fig. 6B), which agreed with an increase in p21$^{Waf1/Cip1}$ protein levels.

In contrast to that of Bax and p21$^{Waf1/Cip1}$, expression of the antiapoptotic protein Bcl-2 is negatively regulated by p53 (54). Bcl-2 protein levels in infected cells were slightly lower at 36 or 48 h p.i. than those in mock-infected cells (Fig. 7B). To confirm that Bcl-2 expression was repressed after infection, RNA PCR analysis was performed with total RNA prepared from mock-infected cells or cells infected with BHV-1. A 268-bp fragment corresponding to the Bcl-2 amplified product was detected in mock-infected cells but was not readily detected at 24, 36, or 48 h p.i. (Fig. 7A). As expected, actin RNA and protein levels were not altered dramatically. These studies demonstrated that Bax and p21$^{Waf1/Cip1}$ were induced after infection but that Bcl-2 levels decreased.

**Proteins that are cleaved during PCD undergo proteolysis after infection.** Caspases are cysteine proteases that play a pivotal role in PCD (65). Caspases 2, 3, and 7 are activated by cleavage during p53-dependent PCD (9, 45, 71). To test whether infection led to cleavage of caspase 2, an antibody that recognizes intact caspase 2 and its 12-kDa cleavage product

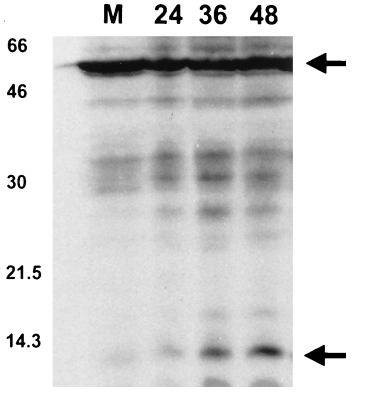


FIG. 8. Analysis of caspase 2 after infection. Whole-cell lysate was prepared from mock-infected cells (lane M) or infected cells (24, 36, or 48 h p.i.), samples were electrophoresed in a 12.5% gel, and Western blot analysis was performed with an antibody which specifically recognizes caspase 2. The arrow at 12 kDa indicates the cleaved form of caspase 2, and the one at 51 kDa shows the location of intact caspase 2. Molecular mass markers are indicated on the left and are expressed as kilodaltons.

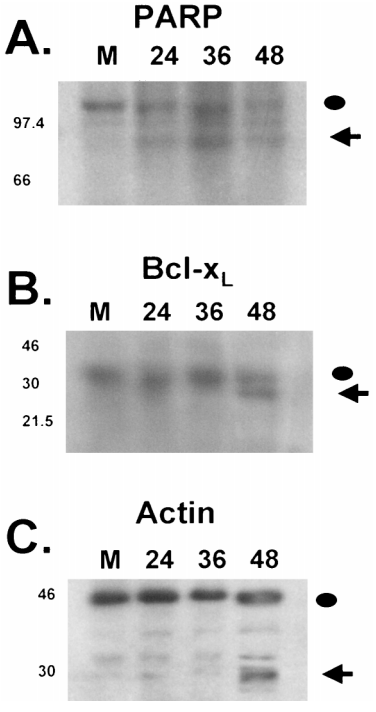


FIG. 9. Cleavage of PARP, Bcl-$x_L$, and actin after infection. Whole-cell lysate was prepared from mock-infected cells (lanes M) or infected cells (24, 36, or 48 h p.i.), and Western blot analysis was performed with an antibody which specifically recognized PARP (A), Bcl-$x_L$ (B), or β-actin (C). The arrows indicate the positions of the respective cleaved proteins, and the ovals indicate the intact proteins.

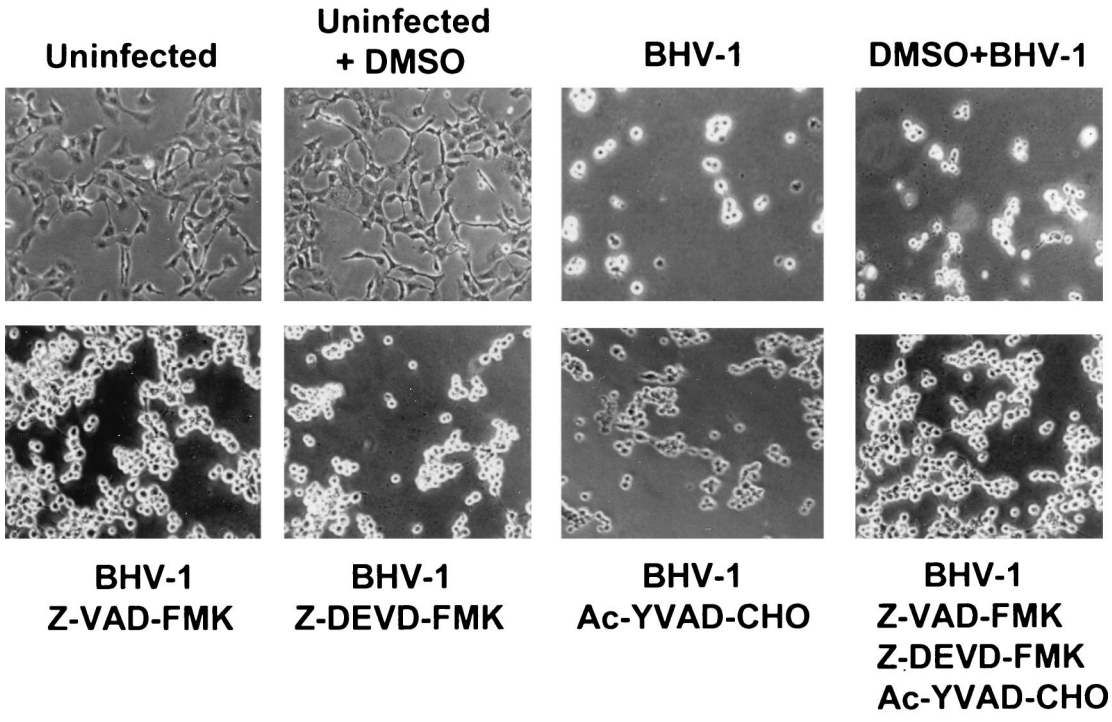


FIG. 10. Z-VAD-FMK delays cell death after infection. The indicated caspase inhibitor (25 μM) was added at 6 and 24 h p.i. Representative areas of the cultures were photographed (magnification, ×200) at 48 h p.i. Since the caspase inhibitors were suspended in DMSO, a control experiment was performed to test the effects of DMSO on PCD.

was utilized. At 36 or 48 h p.i., an increase in the 12-kDa cleavage product was detected (Fig. 8).

To determine whether other caspase substrates were cleaved following infection, Western blot analysis was performed with antibodies directed against Bcl-$x_L$, actin, or PARP. The Bcl-$x_L$ gene encodes two distinct proteins as a result of alternative splicing, a long form (Bcl-$x_L$) and a short form (Bcl-$x_S$ (4). Bcl-$x_L$ inhibits apoptosis, but Bcl-$x_S$ antagonizes the antiapoptotic action of Bcl-$x_L$ and Bcl-2 (51). Bcl-$x_L$ is cleaved during PCD by caspases (12). Actin is cleaved by caspase 3, and this appears to occur after cleavage of PARP (50). At 36 or 48 h p.i., cleavage of PARP was detected (Fig. 9A). Cleavage of Bcl-$x_L$ and actin was detected at 48 h p.i. (Fig. 9B and C). Thus, cleavage of caspase 2, PARP, actin, and Bcl-$x_L$ occurred during the late stages of infection, suggesting that the caspase cascade was activated.

**Inhibition of caspases delays BHV-1-induced cytopathogenic effects but enhances total virus yield.** To determine if caspases play a role during infection, MDBK cells were treated with caspase inhibitors at 6 h p.i., and the degree of cytopathology was monitored by light microscopy. For these studies, three different classes of caspase inhibitors were tested: (i) Z-VAD-FMK, which inhibits caspase 1-like proteases and prevents apoptosis mediated by a variety of stimuli (7, 80); (ii) Ac-YVAD-CHO, which inhibits interleukin-1β-converting enzyme (83); and (iii) Z-DEVD-FMK, which inhibits caspase 3-like enzymes (59). Since adherence of infected cells to the tissue culture dish is a reliable marker for viability of BHV-1-infected MDBK cells, we used this as a means to estimate what role, if any, caspase inhibitors played during infection. Relative to untreated infected cultures or cultures which were infected and treated with DMSO, more cells were attached to the plate when cultures were treated with Z-VAD-FMK at 48 h p.i. (Fig. 10). Although Z-DEVD-FMK or Ac-YVAD-CHO treatment increased the number of cells attached to plastic after infection, the effect was not as dramatic as that with Z-VAD-FMK. Furthermore, addition of all three caspase inhibitors did not dramatically enhance the protective effect. If cultures were incubated for longer periods of time, all cells were eventually released from the substrate and were not viable (20). The simplest explanation of this study was that caspases were not absolutely required for virus infection.

To test whether inhibition of caspases by Z-VAD-FMK treatment had an effect on virus release, the amount of virus released from infected cells was measured in cultures treated with Z-VAD-FMK and compared to that for untreated cultures. Since Z-VAD-FMK has a short half-life (14) and the medium obtained from infected cells was diluted more than 1,000-fold for measuring virus, it was unlikely that residual levels of Z-VAD-FMK were active or affected the study. When cultures were subjected to three cycles of freeze-thawing (−70 to 37°C) to estimate the total amount of virus in infected cells, a twofold increase in virus titers was observed following treatment with Z-VAD-FMK (Fig. 11B). Twofold-less virus was present in the medium that was removed at 48 h p.i. when cells were treated with Z-VAD-FMK (Fig. 11A). In summary, these studies suggested that Z-VAD-FMK treatment enhanced the amount of total infectious virus but reduced or delayed virus release.

**In vitro reconstitution of PCD.** Experiments were performed to confirm the importance of caspases during virus-induced PCD and to prove that the components necessary to initiate PCD of healthy nuclei were present in infected cells. Several studies have demonstrated that cell extracts prepared from cells undergoing PCD mimic the intracellular environment of a dying cell and thus can induce nuclear changes which are observed during PCD (chromatin condensation or DNA laddering, for example) (34, 44, 58). Cell extract was prepared from infected cells (24, 36, or 48 h p.i.) and added to nuclei prepared from uninfected cells, and alterations in nuclear

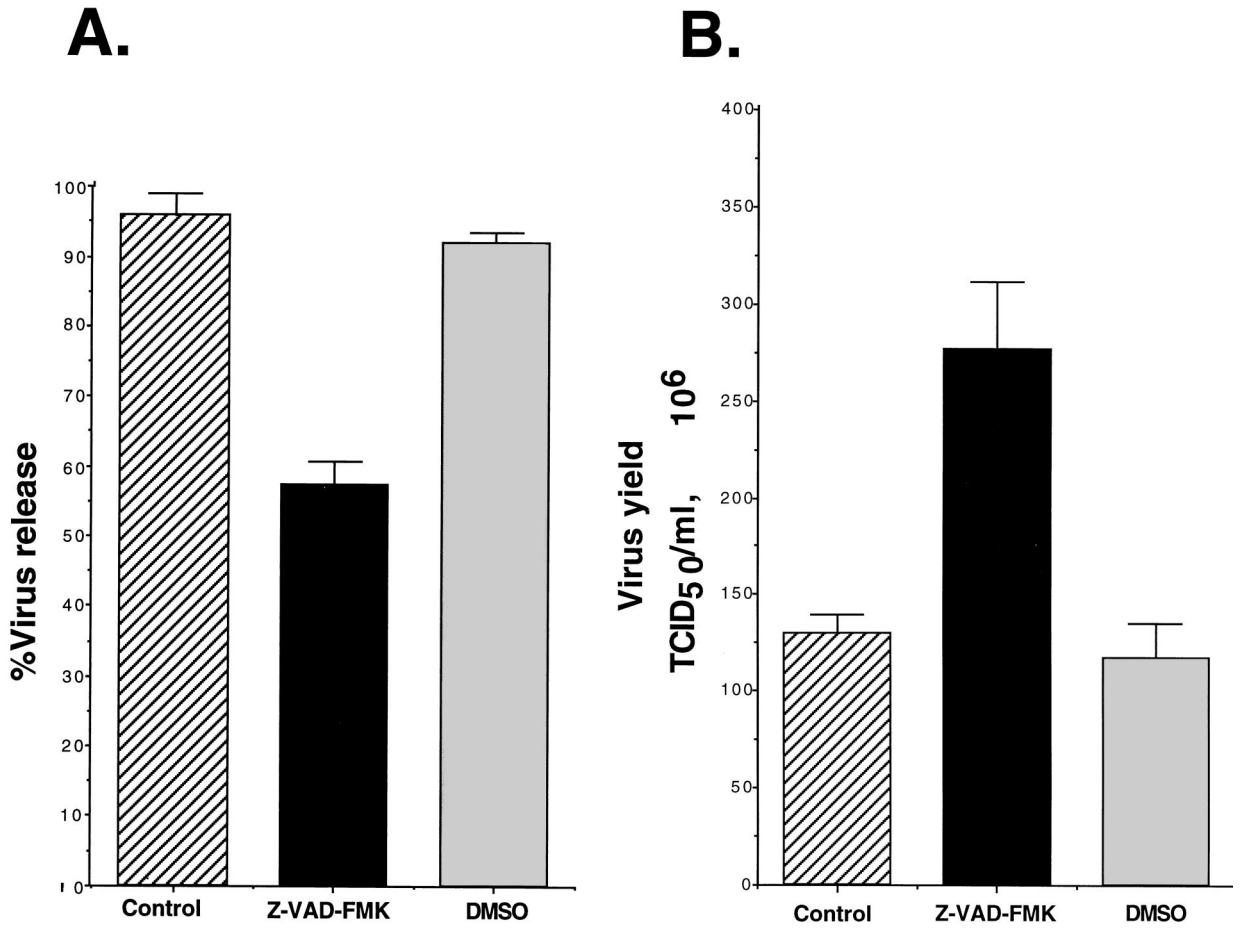


FIG. 11. Z-VAD-FMK enhances virus yield but reduces virus release at 48 h p.i. Z-VAD-FMK (25 μM) was added to cultures 6 and 24 h p.i. Cells were removed from the dish at 48 h p.i. (A) Cell-free virus release was measured by collecting the medium from infected cells at 48 h p.i. after removal of cells by centrifugation. (B) Cells plus culture medium were subjected to three freeze-thaw cycles (−70 to 37°C), and cells were removed by centrifugation. The amount of infectious virus was measured as described in Materials and Methods. Data are the means and standard deviations from three independent experiments. $TCID_{50}$, 50% tissue culture infective dose.

DNA were monitored by Hoechst 33342 staining. Nuclei from CV-1 cells were used because these cells have a low frequency of PCD when actively growing and because CV-1 cells are not permissive for BHV-1 infection. Incubation of healthy CV-1 nuclei with extract prepared from BHV-1-infected cells (24, 36, or 48 h p.i.) induced chromatin condensation (Fig. 12). A higher percentage of nuclei contained condensed chromatin when the nuclei were incubated with extracts prepared from cells infected for 48 versus 24 h p.i. The same extract prepared from mock-infected cells did not efficiently induce chromatin condensation. Incubation of the extract prepared from infected cells (48 h p.i.) with Z-VAD-FMK inhibited chromatin condensation. These studies indicated that extract prepared from infected cells can induce chromatin condensation of nuclei prepared from nonpermissive cells and that active caspases play a role during this process.

## DISCUSSION

Although PCD occurred in MDBK cells after infection, inactivated virus did not efficiently induce PCD (Fig. 2). This finding is in contrast to those from previous studies using PBMC (30–32) or activated $CD4^+$ T cells (24). In general, lymphoid cells are prone to PCD, whereas fibroblasts or epithelial cells are more resistant (90), suggesting that inactivated virus can induce PCD in cells which easily undergo PCD. It is also possible that novel virus-host interactions may be important for inactivated virus to initiate PCD in lymphocytes. For example, a novel member of the tumor necrosis factor (TNF) NGF receptor family (HVEM) (56) mediates HSV-1 entry into activated T cells. Conversely, entry of HSV-1 and BHV-1 into epithelial or other nonlymphoid cells is mediated by an unrelated membrane glycoprotein which resembles the poliovirus receptor (27). If BHV-1 entry into PBMC is mediated by binding of a viral glycoprotein to a TNF-like receptor, this interaction could lead to PCD in the absence of active infection, because binding of ligand to TNF receptors can initiate PCD (reviewed in references 26, 82a, and 91). Since PCD was not observed until late after infection, the early events of productive infection may inhibit PCD, as previously described for HSV-1 (43). The possibility that BHV-1 encodes positive and negative regulators of PCD supports the hypothesis that cell-type-specific interactions are important during PCD.

The levels of newly synthesized p53 protein and promoter activity increased after infection (Fig. 4), suggesting that p53 plays a role in virus-induced PCD. Genes that are regulated by p53 (those for Bax, $p21^{Waf1/Cip1}$, and Bcl-2, for example) were also induced or repressed in a p53-dependent fashion (Fig. 6 and 7). Cleavage of p53 occurs in response to DNA damage,

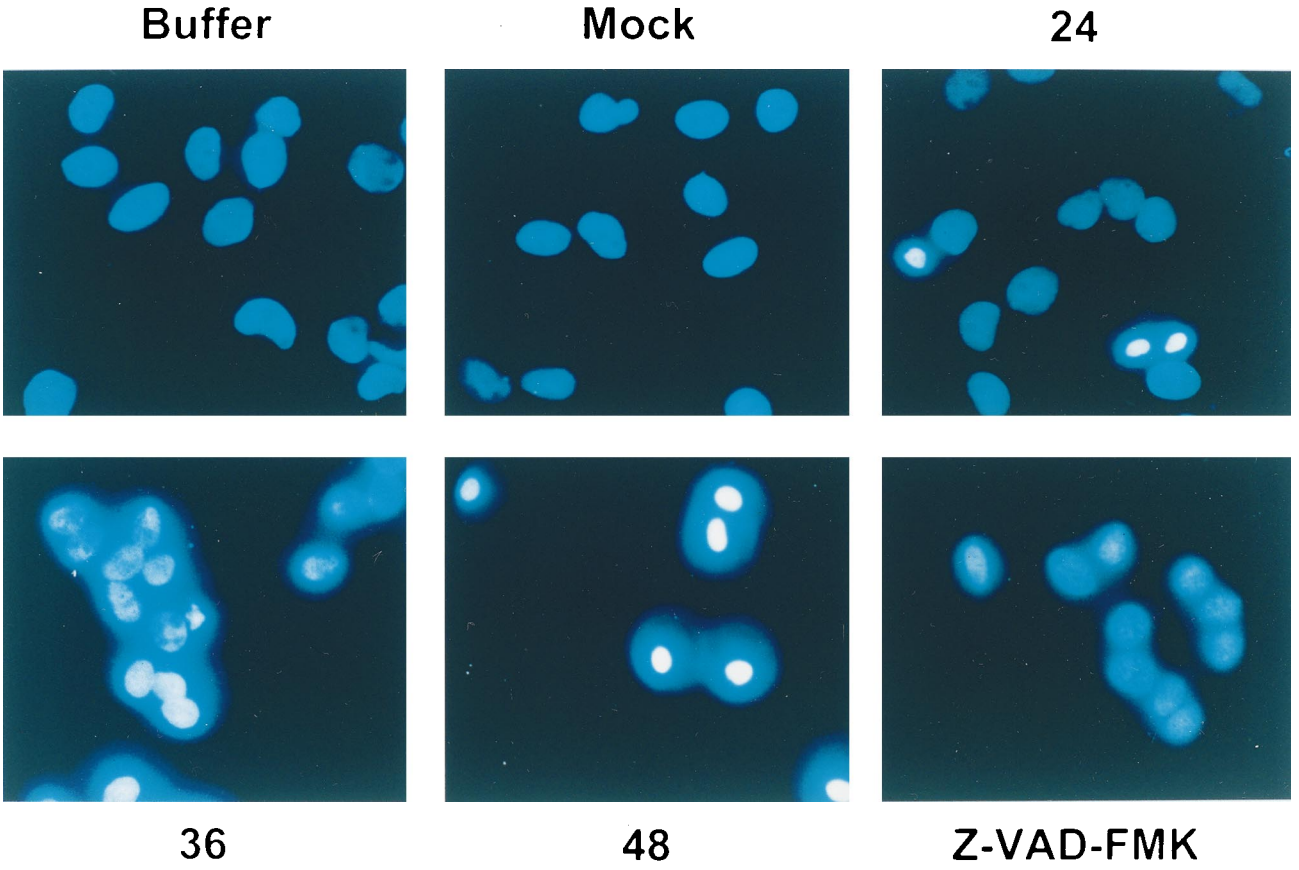


FIG. 12. Extracts prepared from infected cells induce chromatin condensation. Nuclei prepared from CV-1 cells ($2 \times 10^5$) were incubated with 75 μg of extract prepared from mock-infected or infected cells. For caspase inhibition, 25 μM Z-VAD-FMK was added prior to addition of nuclei. Nuclei were incubated with the respective cell extracts for 2 h at 37°C. Nuclei were stained with Hoechst 33342 as described in Materials and Methods, and the samples were observed under a UV microscope. Magnification, ×400.

generating p50 or p40 (55), and consequently alters the transcriptional regulatory activity of p53 (11, 36, 37). HSV-1 and HSV-2 induce DNA damage after infection (64, 74), suggesting that infection by BHV-1 leads to DNA damage and p53 induction. E1A, E2F-1, or c-Myc can also sensitize cells to undergo p53-dependent PCD (3, 7, 18, 26, 46, 66, 94). Myc-mediated PCD may have relevance to BHV-1 infection, because c-Myc levels are elevated after infection (20, 31). Despite the findings that link p53 to BHV-1-induced PCD, p53-independent mechanisms may also be important. This hypothesis is supported by the finding that p53 induction was modest compared to induction by adenovirus infection (82) and that adenovirus infection induces PCD by p53-dependent and -independent mechanisms (82, 82a). A $p53^{-/-}$ primary bovine cell line is not available, making it difficult to directly address this issue.

The importance of caspase activation during PCD is well established (87). The finding that caspase 2 is cleaved after infection indicated that caspases are activated. A number of substrates for caspases have been identified: protein kinases (8, 23), Rb (38), Sp1 (63), Bcl-2 (12), ICAD (73), Bcl-$x_L$ (16), and cytoskeletal proteins (lamin $B_1$ and actin) (5, 9, 50). Bcl-$x_L$ protects cells from p53-dependent PCD (51, 76), and cleavage of Bcl-$x_L$ inactivates this function or induces PCD (16), suggesting that cleavage of Bcl-$x_L$ after infection is important. Although cleavage of PARP, Bcl-$x_L$, and actin occurred after infection, cleavage of PARP was detected earlier. In general, PARP cleavage is an early event during PCD, while cytoplasmic proteins are necessary for maintaining the structural integrity of the cell and thus are cleaved later (25, 91).

PCD is an important host cell defense that limits viral infection. Prevention of PCD enhances production of human immunodeficiency virus (13), simian immunodeficiency virus (10), and adenoviruses (14). Conversely, induction of PCD by specific viral genes near the end of infection may promote cell-to-cell spread and virus release and interfere with inflammatory responses. The adenovirus E3 gene promotes virus release and enhances virus growth in cultured cells by promoting cell death that is distinct from PCD (85). The finding that Z-VAD-FMK enhanced total infectious virus production at 48 h p.i. implied that prevention of PCD enhanced virus yield. Since virus release was reduced at 48 h p.i. when cells were treated with Z-VAD-FMK (Fig. 10), we hypothesized that caspase activation or other events during PCD led to virus assembly or release. Infection of cattle typically leads to upper respiratory tract infections and transient immunosuppression, thus allowing opportunistic bacterial infections (reviewed in reference 84). If extensive lymphocyte PCD occurs after infection, immunosuppression would occur. PCD of nonlymphoid cells in the upper respiratory tract could also lead to virus spread and interfere with an inflammatory response. To understand the role that PCD plays with respect to BHV-1 pathogenesis, it will be necessary to determine what cell types undergo PCD in infected cattle and correlate these findings to symptoms associated with infection.

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