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Vaccinia Virus Serpin-1 Deletion Mutant Exhibits a Host Range Defect Characterized by Low Levels of Intermediate and Late mRNAs

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Orthopoxviruses encode three serpin homologs—SPI-1, SPI-2 and SPI-3—of which SPI-2 has been well characterized as an inhibitor of ICE-like proteases. A rabbitpox virus SPI-1 deletion mutant exhibited a host range restriction in human lung A549 and pig kidney 15 cell lines that was attributed to apoptosis. Here we report that replication of a vaccinia virus SPI-1 deletion mutant (ΔSPI-1) was restricted in primary human keratinocytes as well as A549 cells. Although chromatin condensation was detected in some A549 cells, other morphological or biochemical signs of apoptosis including DNA fragmentation, cleavage of poly(ADP-ribose)polymerase or nuclear mitotic apparatus protein, or caspase 3 activation were not found. Moreover, ΔSPI-1 protected A549 cells from apoptosis induced by tumor necrosis factor, whereas the corresponding ΔSPI-2 mutant did not. Further studies indicated undiminished amounts of vaccinia virus early mRNA and replicated DNA in the absence of the SPI-1 product. However, there were reduced amounts of viral intermediate and late mRNAs, viral late proteins, cleaved core proteins, and virus particles. These data suggested that apoptosis is not the determining factor in the host range restriction of ΔSPI-1 and that the SPI-1 gene product is needed to allow efficient expression of intermediate and late genes in A549 cells.

INTRODUCTION

The best characterized poxviruses, of which vaccinia virus is the prototype, belong to the orthopoxvirus genus (Moss, 1996). Although the host ranges of orthopoxviruses are relatively narrow in nature, they are quite broad in cultured cells and laboratory animals. Some mutations, however, restrict the replication of orthopoxviruses in specific cell types. In each case, the restriction occurs at a stage after the penetration of virus particles into the cytoplasm, pointing to important intracellular virus–host interactions. Deletion of the E3L gene, which encodes a double-stranded RNA binding protein, impairs replication of vaccinia virus in human HeLa, monkey Vero, and murine L929 cells but not in rabbit kidney 13 cells or chick embryo fibroblasts (Chang *et al.,* 1992, 1995; Beattie *et al.,* 1996). Defective orthopoxvirus replication in Chinese hamster ovary (CHO) cells, caused by deletion of the cowpox CHO *hr* gene, and in rabbit kidney 13 or human cells, caused by mutation of the vaccinia virus K1L gene, are correlated with defects in viral gene expression (Gillard *et al.,* 1985; Spehner *et al.,* 1988; Ramsey-Ewing and Moss, 1995, Ramsey-Ewing and Moss, 1996). Modified vaccinia virus Ankara has multiple gene defects (Meyer *et al.,* 1991; Antoine *et al.,* 1998; Wyatt *et al.,* 1998) that prevent replication at the level of virus morphogenesis (Sutter and Moss, 1992) in many cell lines except BHK-21 and in primary chick embryo fibroblasts (Carroll and Moss, 1997; Drexler *et al.,* 1998). Apoptosis has been associated with some orthopoxvirus host range mutations. Although apoptosis was delayed in CHO cells infected with vaccinia virus containing an intact cowpox CHO *hr* gene, cell death is unlikely to play a significant role in host restriction of vaccinia virus lacking this gene (Ink *et al.,* 1995; Ramsey-Ewing and Moss, 1998). Recently the viral serine protease inhibitor (serpin)-1 (SPI-1) gene was found to be necessary for replication of rabbitpox virus in human lung A549 and pig kidney 15 cell lines, and subsequent studies suggested that its role in these cells was to prevent apoptosis (Ali *et al.,* 1994; Brooks *et al.,* 1995).

SPI-1 is one of three related orthopoxvirus genes with sequence motifs that place them in the serpin superfamily. Serpins also are present in myxoma virus (Macen *et al.,* 1993; Petit *et al.,* 1996; Messud-Petit *et al.,* 1998; Nash *et al.,* 1998) and a murine gamma herpesvirus (Bowden *et al.,* 1997; Simas *et al.,* 1998). Typically, serpins irreversibly bind via their reactive center loop to serine proteases and inhibit the activity of the latter (Whisstock *et al.,* 1998). Serpins have multiple roles and some are involved in the regulation of immune and inflammatory responses and cell death (Bird, 1998). The best-characterized orthopoxvirus serpin is SPI-2, also known as CrmA, which efficiently inhibits aspartic acid-specific

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cysteine proteases (caspases) and less efficiently inhibits the aspartic acid-specific serine protease granzyme B and prevents IL1-β formation and cytokine-induced apoptosis (Ray *et al.,* 1992; Quan *et al.,* 1995; Tewari and Dixit, 1995; Ray and Pickup, 1996; Dobbelstein and Shenk, 1996; Kettle *et al.,* 1997). The SPI-1 gene encodes a protein that is 44% identical to SPI-2 but with a different reactive center (Kotwal and Moss, 1989; Smith *et al.,* 1989). Both proteins are expressed early in infection and reside intracellularly (Kettle *et al.,* 1995). SPI-1 can act synergistically with SPI-2 to block granule-mediated cell killing pathways (Macen *et al.,* 1996). The SPI-3 gene is distantly related to SPI-1 and SPI-2 (Boursnell *et al.,* 1988). Deletion of SPI-3 causes syncytium formation at neutral pH, but the fusion-inhibitory role of the protein does not depend on serpin motifs (Law and Smith, 1992; Turner and Moyer, 1992, 1995; Zhou *et al.,* 1992).

For the present study, we constructed a vaccinia virus mutant with nearly all of the SPI-1 gene deleted. This mutant was unable to replicate efficiently in either primary human keratinocytes or human A549 cells. Although some morphological signs of apoptosis were observed, there were no biochemical correlates of apoptosis, and the replication defect was associated with diminished levels of viral intermediate and late mRNAs, reduced viral late protein synthesis, and an interruption of virion morphogenesis.

RESULTS

Host range restriction of a vaccinia virus SPI-1 deletion mutant

Homologous recombination was used to replace a 585-bp segment of the SPI-1 open reading frame of vaccinia virus strain WR with a guanine phosphoribosyl transferase (*gpt*) expression cassette. Plaques that formed on BS-C-1 cells in the presence of mycophenolic acid (Falkner and Moss, 1988) were purified repeatedly and virus stocks were prepared. The expected genomic alterations were confirmed by Southern blotting (data not shown), and the recombinant vaccinia virus was named ΔSPI-1. Another recombinant vaccinia virus named ΔSPI-2, with a 300-bp deletion in the SPI-2 gene, was constructed and used for comparative purposes.

The ability of the mutant viruses to propagate in several different cell lines was determined by staining with vaccinia virus antiserum. On BS-C-1 monolayers, both ΔSPI-1 and ΔSPI-2 formed large immunostaining plaques that were indistinguishable from those of wild-type vaccinia virus WR (Fig. 1). WR and ΔSPI-2 also formed large plaques on A549 cells, although the plaques increased in size more slowly than on BS-C-1 cells (Fig. 1). In contrast, only small clusters of immunostaining A549 cells were detected with ΔSPI-1. A specific defect in ΔSPI-1 propagation also was found in primary human keratinocytes (Fig. 1). Similar results were obtained with independently derived keratinocytes from different individuals, indicating that this host range defect was a consistent phenomenon. However, when the keratinocytes were allowed to differentiate in medium containing calcium and serum, the cells rapidly became permissive for ΔSPI-1. In addition, a continuous human keratinocyte cell line (HaCaT) was permissive for ΔSPI-1 (data not shown). Because of the tendency of primary human keratinocytes to convert to a permissive state, further detailed studies were carried out with A549 cells.

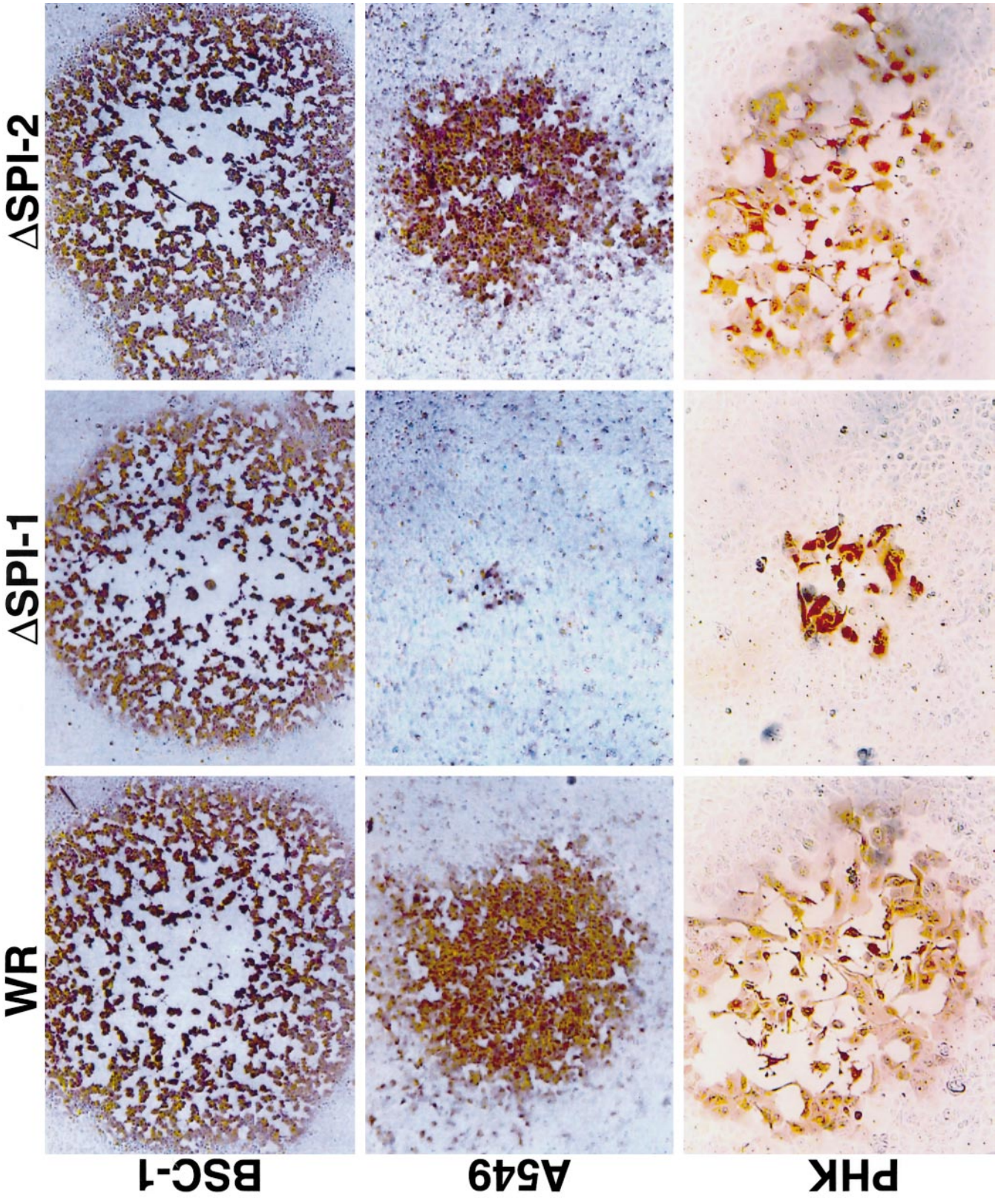
Replication of ΔSPI-1 is inhibited in A549 cells

A small plaque phenotype could result from a defect in vaccinia virus replication or spread. Virus yields first were determined in BS-C-1 and A549 cells infected with WR or ΔSPI-1 at 0.01 plaque forming units (PFU) per cell. At this multiplicity, replication and spread of WR and ΔSPI-1in BS-C-1 cells were virtually identical because similar peak infectivity titers were reached between 48 and 72 h after infection (Figs. 2A and 2C). WR replicated more slowly in A549 cells than in BS-C-1 cells but reached a similar titer by 96 h (Fig. 2A). In contrast, the titer of ΔSPI-1 at 96 h was >4 logs lower in A549 cells than in BS-C-1 cells (Fig. 2C).

One-step growth curves were constructed by infecting BS-C-1 and A549 cells with 10 PFU/cell of WR or ΔSPI-1. In BS-C-1 cells, similar maximal titers of WR and ΔSPI-1 were reached between 24 and 36 h after infection (Figs. 2B and 2D). A similar titer also was obtained in A549 cells infected with WR (Fig. 2B). However, the titer of ΔSPI-1 was nearly 2 logs lower in A549 cells than in BS-C-1 cells (Fig. 2D). We concluded from these infectivity studies that the ΔSPI-1 has a specific replication defect in A549 cells.

Transmission electron microscopy of permissive and nonpermissive cells infected with ΔSPI-1

Electron microscopic images of thin sections of BS-C-1 cells infected with ΔSPI-1 (Figs. 3A and 3C) or WR (Figs. 3B and 3D) were indistinguishable, with abundant immature and mature forms of virus particles in each case. Replication also appeared normal in A549 cells infected with WR (Figs. 3F and 3H), whereas mature virus particles were rare in A549 cells infected with ΔSPI-1, and there was a reduction in particles at all stages of morphogenesis (Figs. 3E and 3G). In addition, at 24 h after infection with ΔSPI-1, ~7.5% of the A549 cells had condensed chromatin and an invaginated nuclear membrane suggestive of apoptosis (Fig. 3E). These nuclear changes were less prominent at 2, 6, and 12 h after infection of A549 cells with ΔSPI-1. Other classical morphological features of apoptosis, such as nuclear fragmentation, cytoplasmic vacuolization, and membrane blebbing, or formation of apoptotic bodies were not evident. Similar though less pronounced nuclear changes


ΔSPI-2
ΔSPI-1
WR
BSC-1
A549
PHK

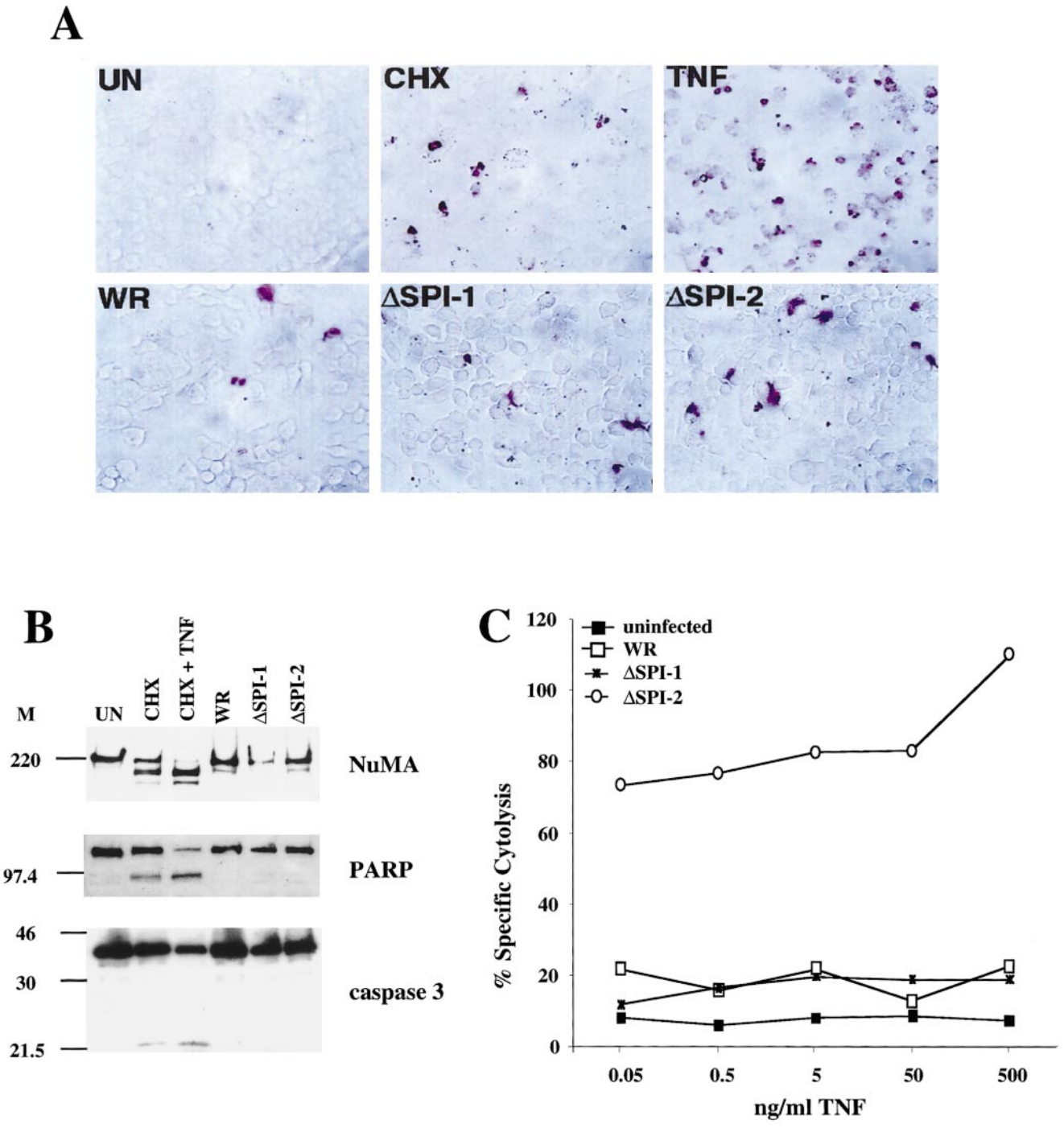


FIG. 4. Biochemical correlates of apoptosis. (A) 10,000 A549 cells per chamber of an eight-well chamber slide were untreated (UN); treated with 50 μg/ml of CHX, 50 μg/ml of CHX, and 100 ng/ml of TNF; and infected with 10 PFU/cell of WR, ΔSPI-1, or ΔSPI-2. After 12 h, the cells were fixed and stained to reveal nicked DNA. Slides were viewed under an inverted light microscope and photographed. (B) Analysis of protein cleavage products by Western blot analysis. A549 cells were treated or infected as in (A). After 24 h, the cells were lysed and a portion of each lysate was analyzed by Western blotting with antibody to NuMA (top), PARP (middle), or caspase-3 (bottom) followed by a horse radish peroxidase conjugated secondary antibody. Antibodies were detected by chemiluminescence. (C) After 12 h, mock-infected or infected A549 cells were incubated with indicated concentrations of TNF and percentage specific cytolysis was determined by measuring the release of lactate dehydrogenase.

FIG. 1. Immunostained plaques formed by vaccinia virus WR, ΔSPI-1, and ΔSPI-2 in BS-C-1, A549, and primary human keratinocytes (PHK). After 48 h, infected cells were fixed, stained with an anti-vaccinia virus polyclonal antibody followed by a horse radish peroxidase-conjugated second antibody and then photographed.

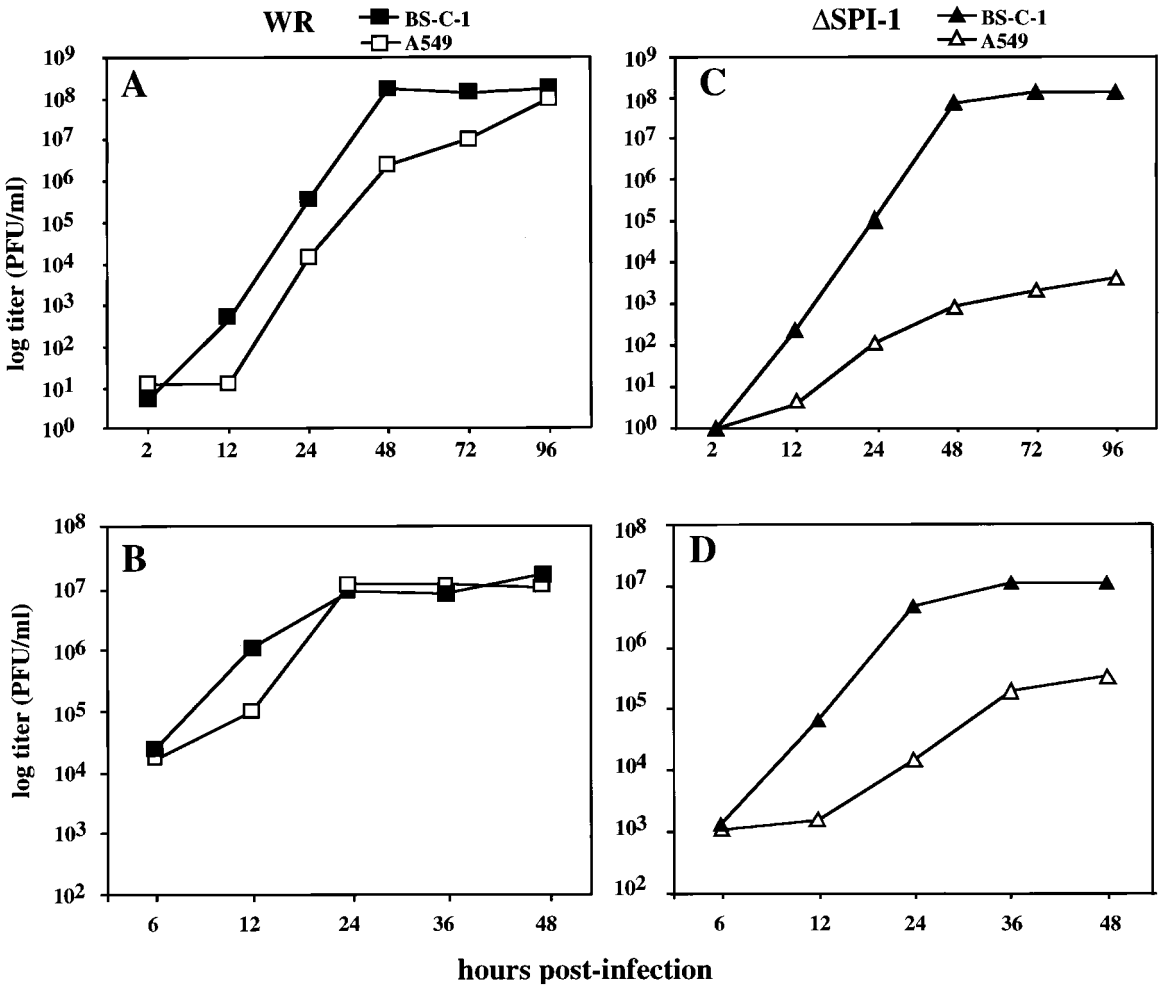


FIG. 2. Replication of vaccinia virus WR and ΔSPI-1 in BSC-1 and A549 cells. BSC-1 and A549 cells were infected with 0.01 (A) or 10 (B) PFU/cell of WR or 0.01 (C) or 10 (D) PFU/cell of ΔSPI-1. The cells were harvested at the indicated times and the virus titers in the lysates were determined by plaque assay on BS-C-1 cells.

were present in 2% of the A549 cells at 24 h after infection with WR (Fig. 3F) but were not noted in uninfected A549 cells (not shown) or in BS-C-1 cells infected with WR or ΔSPI-1 (Figs. 3A and 3B). Brooks and coworkers (Brooks *et al.,* 1995) had reported that a high percentage of A549 cells infected with a rabbitpox virus SPI-1 mutant exhibited morphological signs of apoptosis as well as chromatin degradation determined by TUNEL assay.

Biochemical correlates of apoptosis

Because microscopy only provides a snapshot of apoptotic events, we looked for biochemical correlates of apoptosis. DNA fragmentation was not detected by gel electrophoresis of A549 cells prelabeled with [^{125}I]deoxyuridine and then infected with ΔSPI-1 (data not shown), although this technique was previously used to demonstrate DNA degradation in vaccinia-virus-infected CHO cells (Ramsey-Ewing and Moss, 1998). The more sensitive TUNEL assay showed dark, punctate staining indicative of nicked DNA in some A549 cells infected with ΔSPI-1; however, the number of such stained cells was similar in A549 cells infected with WR and ΔSPI-2 or simply treated with cycloheximide (CHX) and much less than in cells treated with CHX and tumor necrosis factor (TNF; Fig. 4A). Thus there was no correlation between host restriction and detection of DNA cleavage in A549 cells infected with wild-type or mutant vaccinia viruses.

The cleavage of poly(ADP-ribose)polymerase (PARP) and nuclear mitotic apparatus protein (NuMA) commonly occurs in apopotic cells (Casciola-Rosen *et al.,* 1996; Weaver *et al.,* 1996). Thus when A549 cells were treated with CHX or with CHX and TNF, the 116-kDa PARP present in control cells was diminished and the 89-kDa product appeared (Fig. 4B). In contrast, only uncleaved PARP was detected in A549 cells infected with WR, ΔSPI-1, or ΔSPI-2. Similarly, the 240-kDa NuMA protein present in control A549 cells was cleaved into smaller species when treated with CHX and TNF (Fig. 4B). Less cleavage occurred when the A549 cells were treated

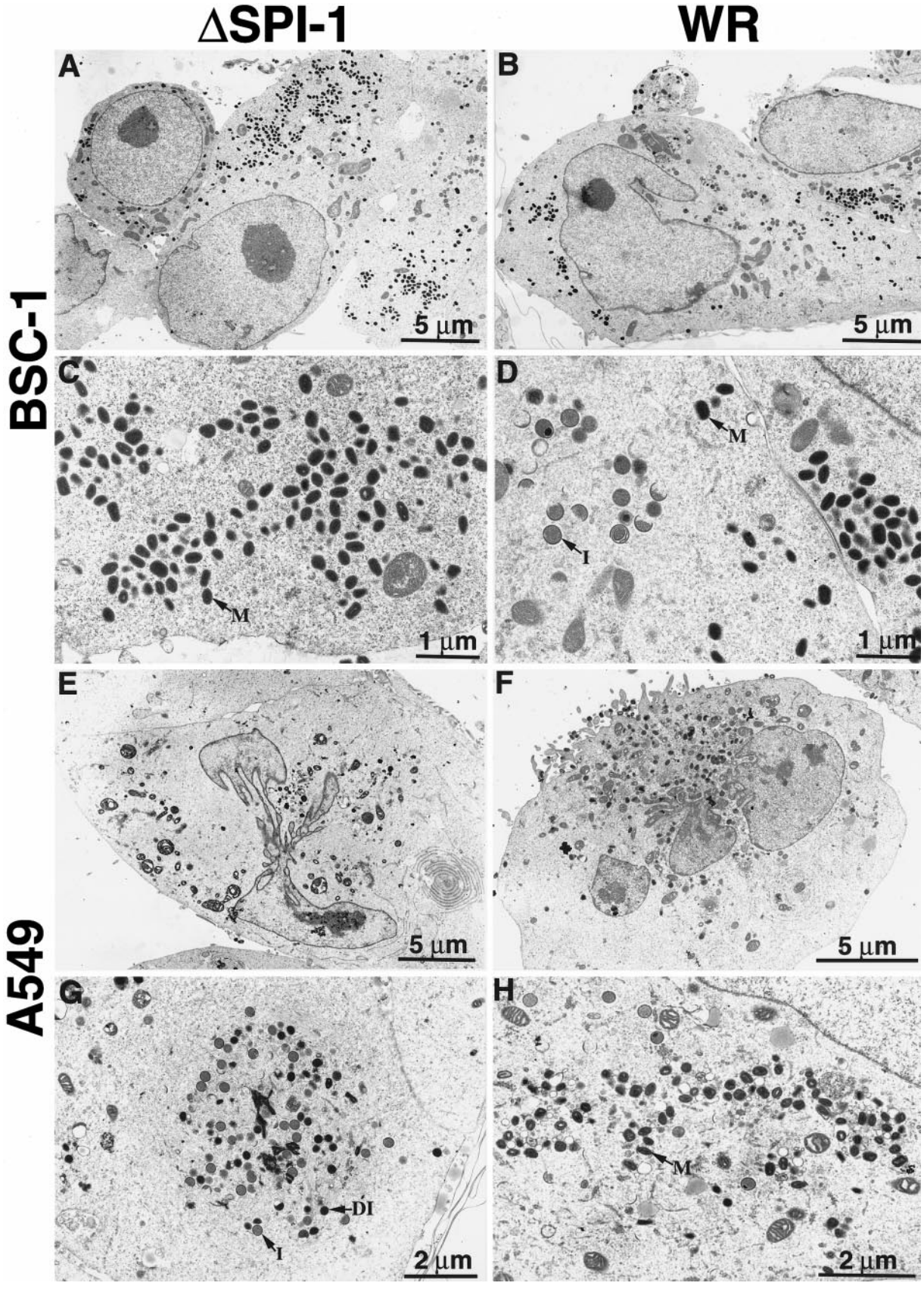


FIG. 3. Electron micrographs of cells infected with WR or ΔSPI-1. BS-C-1 and A549 cells, infected for 24 h with WR or ΔSPI-1 were fixed, embedded, sectioned, and examined by transmission electron microscopy. The magnification in each panel is indicated by a bar in the lower right corner. M, mature intracellular virion; I, immature virion; DI, dense immature virion.

with CHX alone and still less when infected with WR, ΔSPI-1, or ΔSPI-2 (Fig. 4B).

We also looked for earlier signs of apoptosis. PARP is cleaved by caspase-3, which is formed from a 32-kDa precursor by cleavage at multiple aspartic acid sites (Keane *et al.,* 1997). In normal A549 cells, only pro-caspase-3 was detected, whereas in cells induced to undergo apoptosis by incubation with TNF and CHX, pro-caspase-3 was reduced and caspase-3 was detected (Fig. 4B). Some caspase-3 was detected in cells treated with CHX alone but only pro-caspase-3 was detected in cells infected with WR, ΔSPI-1, or ΔSPI-2 (Fig. 4B).

As ΔSPI-1 did not induce apoptosis in the majority of infected A549 cells, we determined whether ΔSPI-1 could protect A549 cells from death induced by TNF. Over a wide concentration range, TNF only slightly enhanced cytolysis of A549 cells infected with WR or ΔSPI-1 (Fig. 4C). By contrast, A549 cells infected with ΔSPI-2 were very sensitive to TNF-mediated cytolysis (Fig. 4C). Thus the absence of SPI-2 not SPI-1 rendered A549 cells sensitive to TNF-induced apoptosis, and this did not correlate with host range restriction.

Reduced viral protein synthesis in A549 cells infected with ΔSPI-1

Although A549 cells infected with ΔSPI-1 exhibited some morphological signs of apoptosis, no biochemical correlates were found. We therefore sought to determine whether the low yield of virus particles resulted from diminished viral protein synthesis. BS-C-1 and A549 cells were labeled with [^{35}S]methionine at various times after infection with WR, ΔSPI-1, or ΔSPI-2. After each labeling period, lysates were prepared and analyzed by SDS–PAGE and autoradiography. In BS-C-1 cells infected with WR, the onset of viral late protein synthesis and shut off of host protein synthesis occurred between 8 and 12 h after infection as revealed by the appearance of new prominent bands (Fig. 5A). A similar pattern of viral protein synthesis occurred in BS-C-1 cells infected with ΔSPI-1 or ΔSPI-2 (Fig. 5A). In A549 cells, the onset of viral late protein synthesis and the profound shut-off of cell protein synthesis occurred between 12 and 18 h after infection with WR (Fig. 5B), consistent with the slower production of infectious virus. A similar pattern also was found in A549 cells infected with ΔSPI-2 (Fig. 5B). Although viral late protein bands were detected in A549 cells infected with ΔSPI-1, their intensity was far less than in cells infected with WR. The difference did not result from a delay in viral late protein synthesis because the intensities of the bands were even lower at 36 and 48 h than at 24 h (data not shown).

The above results were confirmed by Western blotting using a broadly reactive antiserum that had been prepared from a rabbit repeatedly immunized with purified infectious WR virions. The number and intensities of viral proteins from BS-C-1 cells infected with WR, ΔSPI-1, or ΔSPI-2 were similar (Fig. 6A). By contrast, the intensities of the bands were lower in A549 cells that were infected with ΔSPI-1 than with WR or ΔSPI-2 (Fig. 6B). These results were extended by using antibodies to specific viral late proteins: an RNA-polymerase-associated factor (RAP94), a core component (4b), and a membrane protein (A17L). In each case, the intensities of the protein bands were similar in BS-C-1 cells (Fig. 6C), whereas the corresponding protein bands were specifically reduced in A549 cells that were infected with ΔSPI-1 (Fig. 6D). The A17L and 4b proteins were chosen because they undergo proteolytic cleavage at different stages of virus maturation. Cleavage of the A17L protein precedes that of the 4b protein (Betakova *et al.,* 1999). Even though the amount of A17L was less in A549 cells infected with ΔSPI-1 than WR or ΔSPI-2, the predominant species was cleaved (Fig. 6D). The predominant 4b species, however, was the uncleaved form (Fig. 6D), suggesting a block in virion maturation as well as an inhibition of viral late protein synthesis.

Viral DNA replication in permissive and nonpermissive cells

Because viral late protein synthesis is dependent on viral DNA replication, it was important to measure the latter. Viral DNA was determined by applying lysates of infected cells to a membrane, which then was hybridized with a labeled viral DNA probe. DNA accumulation was similar in BS-C-1 and A549 cells whether they were infected with WR, ΔSPI-1, or ΔSPI-2 (Fig. 7). Thus the diminished viral late protein synthesis was not correlated with a gross defect in viral DNA replication.

Reduced reporter gene expression in A549 cells infected with ΔSPI-1

As the form of the viral DNA that serves as the template for intermediate or late gene transcription is unknown, we could not rule out the possibility that the replicated ΔSPI-1 DNA was functionally less active than WR DNA. To bypass the genome replication requirement for vaccinia virus intermediate and late gene expression, transfection assays were performed using plasmids containing the *Escherichia coli lac*Z gene regulated by a viral intermediate or late promoter. Because intermediate transcription factors are synthesized before viral DNA replication, transfection assays were carried out in the presence and absence of cytosine arabinoside, an inhibitor of DNA replication. Under both conditions, the level of intermediate promoter regulated β-galactosidase was lower in A549 cells infected with ΔSPI-1 than with WR (Fig. 8A). Because DNA replication is required for synthesis of late transcription factors, transfection with the late promoter plasmid was only carried out in the ab-

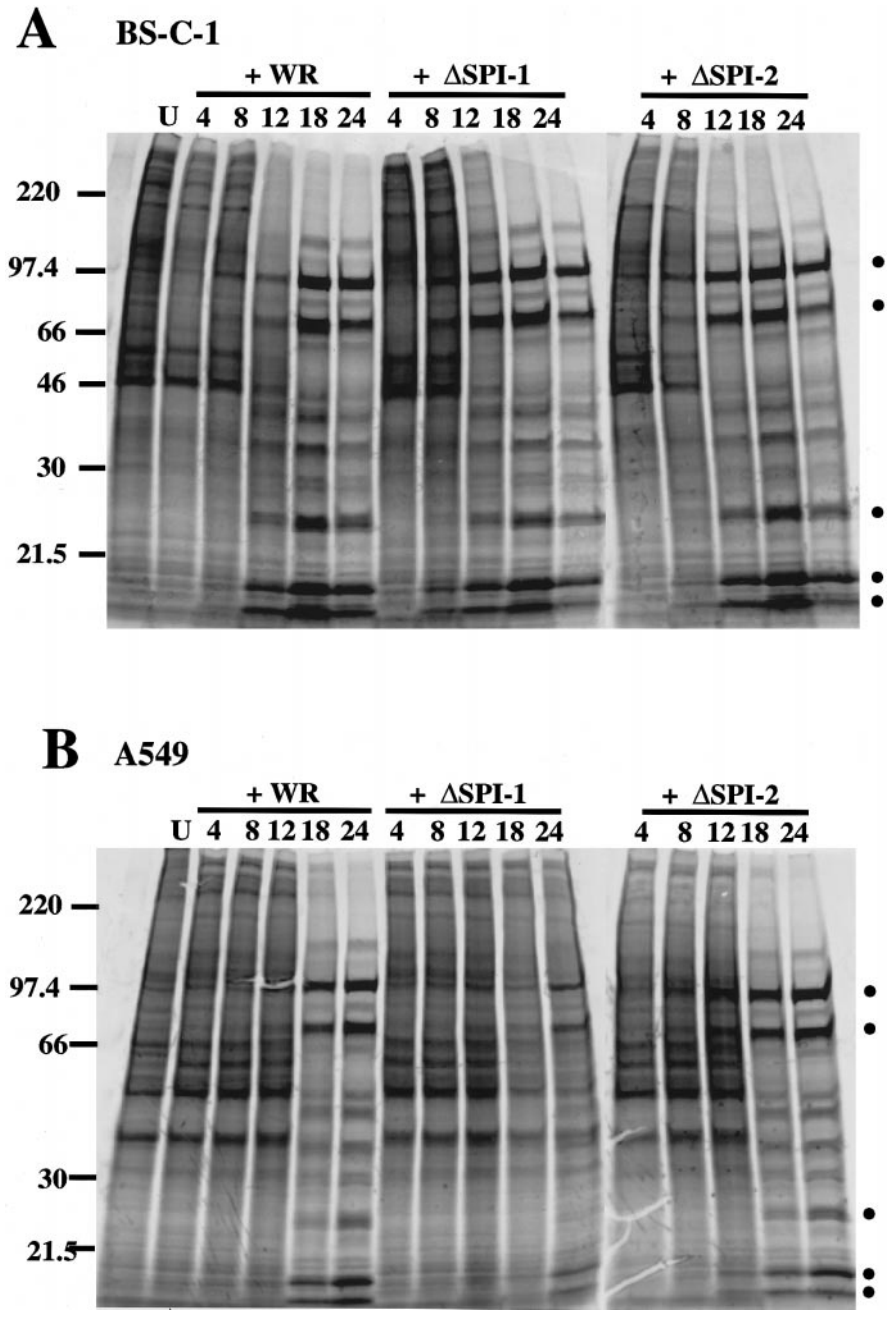


FIG. 5. Metabolic labeling of viral proteins under permissive and nonpermissive conditions. BS-C-1 (A) and A549 (B) cells were infected with 10 PFU/cell of WR, ΔSPI-1, or ΔSPI-2. At the indicated hours after infection, the cells were labeled with [^{35}S]methionine, and a portion of each lysate was analyzed by SDS polyacrylamide gel electrophoresis. Labeled proteins were detected with a PhosphorImager. The positions of marker proteins and their masses in kDA are indicated on the left. The dots on the right indicate prominent viral late proteins.

sence of cytosine arabinoside. Under these conditions, expression was also lower in A549 cells that were infected with ΔSPI-1 than with WR (Fig. 8B).

Steady-state levels of viral mRNAs in permissive and nonpermissive cells

To further analyze the expression defect, steady-state levels of representative early (VGF), intermediate (G8R), and late (11K) viral RNAs (Baldick and Moss, 1993) were determined in A549 cells infected with WR or ΔSPI-1. Equal amounts of total RNA were hybridized to a molar excess of radioactively labeled antisense riboprobes, and the products were digested with RNases and analyzed by electrophoresis and autoradiography. VGF RNA was detected between 2 and 8 h after infection of A549 cells with either WR or ΔSPI-1 (Fig. 9A). G8R RNA was detected at 12 and 18 h after infection, but the amounts were much less in A549 cells infected with ΔSPI-1 than

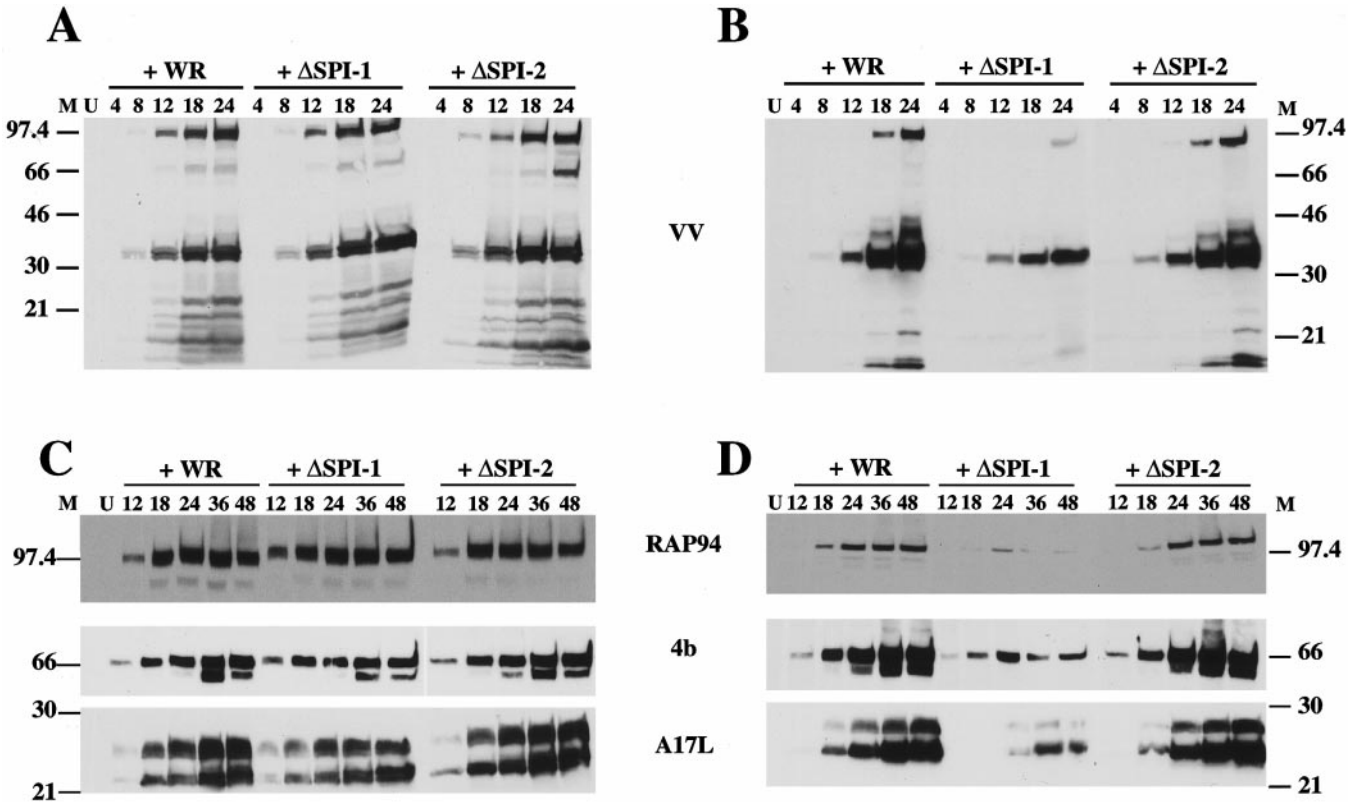


FIG. 6. Western blot analysis of viral proteins in BS-C-1 and A549 cells infected with WR or ΔSPI-1. At the indicated hours after infection, the cells were lysed and the proteins subjected to SDS–PAGE. The proteins from BS-C-1 (A and C) and A549 (B and D) were transferred to a membrane and incubated with rabbit polyclonal antibodies to: infectious vaccinia virus (VV) (A and B) or to the RNA polymerase-associated protein RAP94, the 4b core protein, or the membrane protein A17L as indicated (C and D). A horse radish peroxidase-conjugated anti-rabbit antibody was used for detection of antigen–antibody complexes.

WR (Fig. 9B). The 11K RNA was measured between 12 and 24 h after infection. Whereas it increased during this period in cells infected with WR, with ΔSPI-1 only low levels were detected at 12 and 18 h and was undetectable at 24 h (Fig. 9C). Thus the defects in viral intermediate and late gene expression and virion formation in

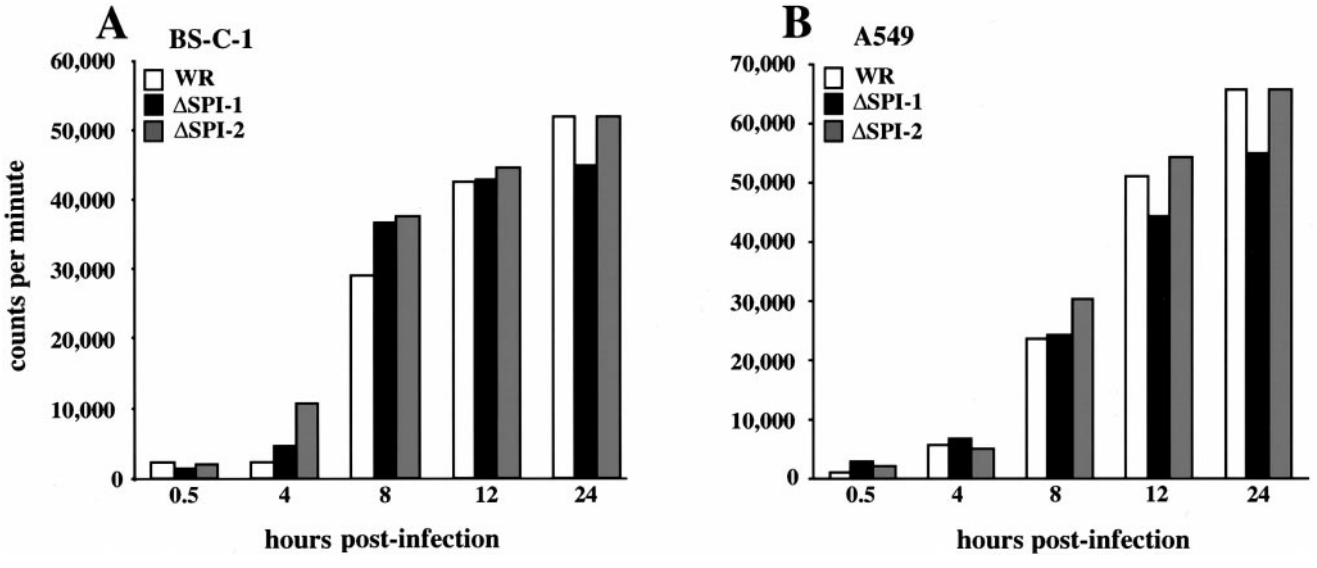


FIG. 7. Viral DNA replication under permissive and nonpermissive conditions. (A) BS-C-1 or (B) A549 cells were infected with 10 PFU of WR, ΔSPI-1, ΔSPI-2 per cell. At the indicated times, cells were harvested and lysed and DNA samples were applied to a nylon membrane using a slot blot manifold. The membranes were hybridized with ^{32}P-labeled vaccinia virus DNA and radioactivity was quantitated using a PhosphorImager.

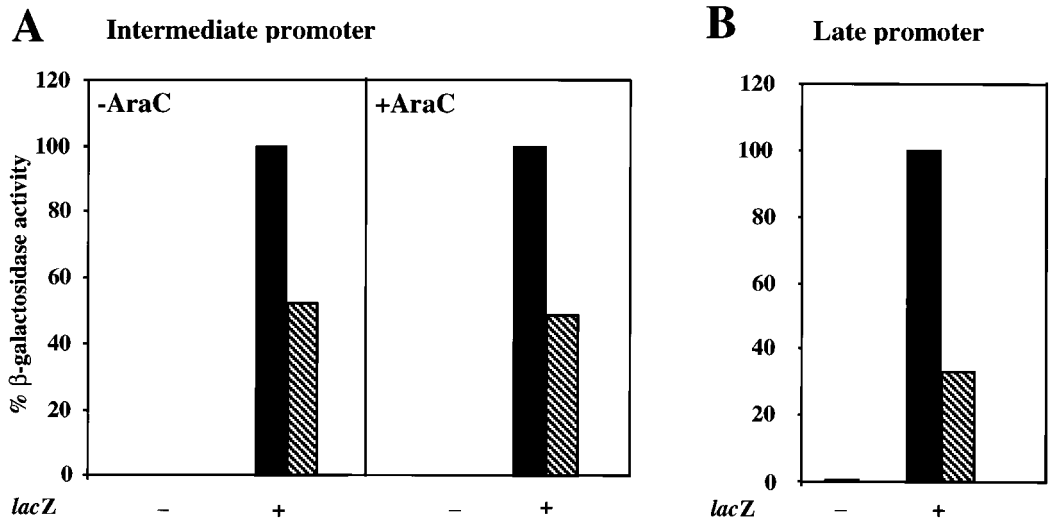


FIG. 8. Expression of intermediate and late genes from transfected plasmids. A549 cells were infected with WR (filled bars) or ΔSPI-1 (hatched bars) and mock transfected (−) or transfected (+) with plasmids containing the *lac*Z open reading frame regulated by the intermediate G8R promoter (A) or the late 11K promoter (B). After 24 h, β-galactosidase was measured. AraC, cytosine arabinoside.

A549 cells infected with ΔSPI-1 could be attributed to the low levels of viral mRNA.

DISCUSSION

The present study arose from our interest in identifying and characterizing genes that encode inhibitors of apoptosis. We planned to remove endogenous apoptosis inhibitor genes from vaccinia virus and then test the ability of heterologous genes to functionally substitute for them. A recent report, attributing the host range defect resulting from deletion of the rabbitpox virus SPI-1 gene to apoptosis (Brooks *et al.,* 1995), suggested to us the possibility of using virus replication in a complementation assay for apoptosis inhibitors. A vaccinia virus ΔSPI-1 mutant was constructed and found to replicate nearly 2 logs less efficiently than wild-type virus in human A549 cells, similar to what had been found for a rabbitpox virus ΔSPI-1 mutant (Ali *et al.,* 1994). The host range defect was specific because the vaccinia virus ΔSPI-1 mutant replicated normally in BS-C-1 and other cell lines and an independently isolated mutant had the same phenotype (J.L.S, unpublished data). By contrast, vaccinia virus with a deletion of the nearby SPI-2 gene, had no replication defect in A549 cells. Although nuclear invagination and chromatin condensation were noted at late times in some ΔSPI-1-infected A549 cells, other classical morphological features of apoptosis were not apparent. Also, none of the examined biochemical correlates of apoptosis, e.g., DNA fragmentation or cleavage of PARP, NuMA, or caspase-3 were associated with the host range defect. Indeed, the ΔSPI-1 mutant effectively prevented TNF-induced apoptosis of A549 cells, whereas a ΔSPI-2 mutant did not. It remains possible that SPI-1 interferes with other apoptotic pathways in A549 cells that were not examined. However, our original plan to use the host restriction of ΔSPI-1 for screening novel apoptosis inhibitors was untenable.

The basis for the host range defects caused by deletion of the SPI-1 genes of rabbitpox virus and vaccinia virus is likely to be similar in view of the close relationship of the two viruses and the common restriction for A549 cells. Although the SPI-1 host range defect of rabbitpox virus appeared to be more strongly associated with apoptosis (Brooks *et al.,* 1995) than described here for vaccinia virus, subsequent studies failed to confirm the association of DNA fragmentation with morphological changes and no other biochemical correlates of apoptosis were found in rabbitpox virus infected A549 cells (R. W. Moyer, personal communication). Moreover, even though apoptosis was demonstrated in pig kidney LLC-PK_1 cells infected with a SPI-2 deletion mutant of cowpox virus, there was no reduction in the virus yield (Ray and Pickup, 1996). Furthermore the occurrence of apoptosis in Chinese hamster ovary cells infected with vaccinia virus is not causally related to the host range defect in those cells (Ink *et al.,* 1995; Ramsey-Ewing and Moss, 1995). Thus a strong case has not been made for apoptosis causing any of the known host range defects of orthopoxviruses. Where observed, apoptosis may be unrelated to host restriction or actually a consequence of it.

The above findings led us to undertake a more detailed analysis of the host range restriction of the vaccinia virus ΔSPI-1 mutant in A549 cells. Our results may be summarized as follows: (1) viral early RNA synthesis and DNA replication were unaffected; (2) there were decreased amounts of viral intermediate and late mRNAs; (3) viral late protein synthesis was severely

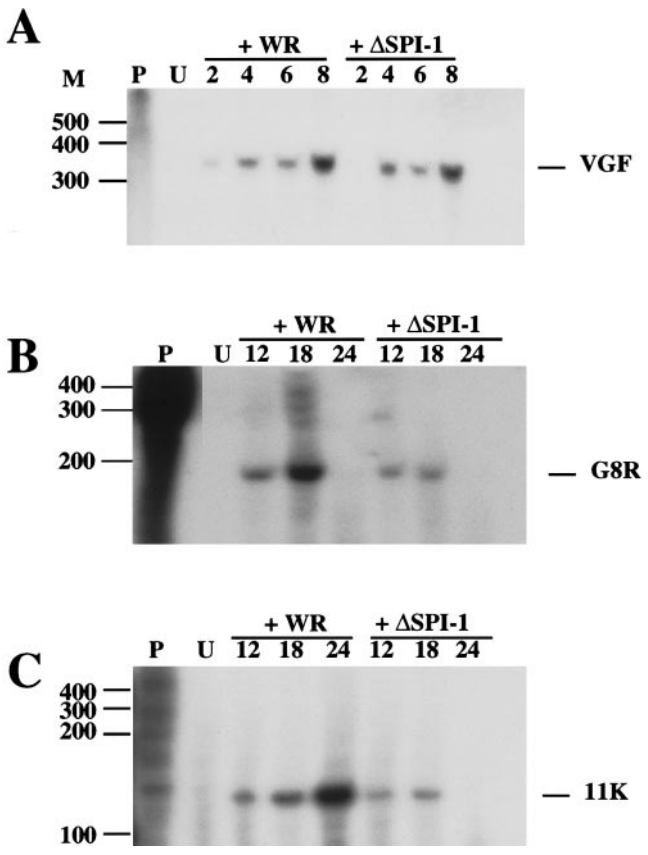


FIG. 9. Detection of viral early, intermediate, and late mRNAs. Total RNA from A549 cells infected with WR or ΔSPI-1 for the indicated hours was hybridized with ^{32}P-labeled riboprobes specific for VGF early (A), G8R intermediate (B) or 11K late (C) mRNAs. The samples were digested with RNase, analyzed by gel electrophoresis, and autoradiographed.

reduced; and (4) few mature virus particles were detected. The defect at the stage of intermediate and late gene expression was corroborated by experiments in which reporter genes under control of intermediate and late promoters were transfected into cells infected with ΔSPI-1 or wild-type vaccinia virus. The effect on late gene expression, as determined by RNA analysis and transfection, was more severe than that on intermediate gene expression. This does not necessarily mean, however, that late transcription is intrinsically more sensitive to the host range defect than intermediate transcription because a cascade effect could result from the requirement for intermediate proteins as late transcription factors. Moreover because many late proteins are needed to assemble virus particles, it is easy to see why virus particle formation was most severely inhibited of all parameters examined.

The K1L host range defect in rabbit kidney 13 cells also occurs at the level of intermediate gene expression. In this case, the role of the K1L gene product has been related to an unidentified host factor required for transcription of intermediate genes (Rosales *et al.,* 1994; Sutter *et al.,* 1994). A host factor is also necessary for transcription of late genes (Gunasinghe *et al.,* 1998; Zhu *et al.,* 1998). Our working hypothesis is that expression of some viral genes (e.g., K1L, CHO *hr*, SPI-1) modulates the activities of host proteins that serve as viral transcription factors. The activities of these host factors may vary in different cell lines and under different physiological conditions, as suggested by our finding that the host range defect of vaccinia virus ΔSPI-1 in primary keratinocytes can be overcome by inducing cell differentiation. It will be important to determine whether the role of SPI-1 in allowing orthopoxvirus replication in A549 cells is dependent on proteinase inhibition and if so on which proteinase. To address this question, we mutated SPI-1 by simply exchanging the amino acids at the P1 and P1′ sites predicted to be critical for serpin activity. Although the recombinant vaccinia virus exhibited a host range defect in A549 cells, further analysis indicated that the mutation destabilized SPI-1, thereby preventing us from drawing any specific conclusions regarding the mechanism of action of SPI-1 (J. L. Shisler and B. Moss, unpublished results).

METHODS

Cells and virus

Primary human epidermal keratinocytes were isolated from neonatal foreskins and cultured in defined keratinocyte serum-free medium (Life Technologies). A549 and BS-C-1 cells were obtained from American Type Culture Collection and grown in DMEM (Quality Biologicals) supplemented with 10% fetal calf serum (FCS) and glutamine. The medium was supplemented with mycophenolic acid (25 μg/ml), xanthine (250 μg/ml), and hypoxanthine (15 μg/ml) as described (Falkner and Moss, 1988) for *gpt* selection.

Deletion mutants were prepared by homologous recombination essentially as described (Earl *et al.,* 1998b) using plasmids with a *gpt* cassette inserted between the *Spe*I and *Spl*I sites of SPI-1 or the *Eco*RV sites of SPI-2 to produce deletions of 585 and 300 bp, respectively .

Immunostaining of virus plaques and virus yield determinations were carried out essentially as described (Earl *et al.,* 1998a).

TNF cytolysis assay

Monolayer of A549 cells in six-well plates were infected with virus at of 10 PFU per cell in medium containing 2.5% FCS. After a 60-min absorption, cells were rinsed once and then incubated with medium containing 10% FCS for 12 h. Uninfected and infected cells were added to standard 96-well microtiter plates (10,000 cells/well) that contained either medium alone (to determine spontaneous release) or increasing concentrations of TNF (Boehringer Mannheim). After 18 h, lysis solution

was added to one set of wells to determine the maximum release of lactate dehydrogenase (LDH) using the CytoTox96 kit from Promega Corp. Supernatants were harvested and also tested for LDH. The percent specific cytolysis was determined by the following formula: [(experimental release − spontaneous release)/(maximum release − spontaneous release)] x 100. The spontaneous release for all experiments was no more than 40% of the maximum. All determinations were done in triplicate and presented as the mean value.

Western blot analysis of cell proteins

A549 cells (10^6) were infected with vaccinia virus at a multiplicity of 10 PFU per cell or treated with 50 μg/ml of CHX (Sigma), 100 ng/ml of TNF (Boerhinger Mannheim) or both CHX and TNF. At 24 h after infection, cells were collected by centrifugation and lysed in 35 mM glucose, 16 mM Tris–HCl (pH 8), 7 mM EDTA, 0.6 mM PMSF, 17 mM Tris–HCl (pH 6.8), 2 M urea, 2% 2-mercaptoethanol, 1% SDS, 0.003% bromphenol blue. DNA was sheared using a 21-gauge needle, and a portion of each lysate was boiled for 5 min and loaded on a SDS 4–20% polyacrylamide gel. Proteins were transferred electrophoretically to PVDF membranes (Millipore Corporation) and blocked with TTBS [1M Tris–HCl (pH 7.5), 5M NaCl, Tween 20] containing 2.5% non-fat dry milk in solution. Blots were incubated for 1 h with one of the following primary antibodies in TTBS containing 0.5% milk: polyclonal rabbit anti-PARP (Boehringer Mannheim) diluted 1:2000; polyclonal rabbit anti-caspase-3 (Pharmingen) diluted 1:1000; mouse monoclonal anti-NuMA (Calbiochem) diluted 1:40. After incubation with primary antibody, blots were rinsed three times with TTBS containing 0.5% milk and then incubated with a 1:10000 dilution of the appropriate HRPO-conjugated anti-rabbit or anti-mouse antibodies (Amersham) for 45 min. Blots were washed again in TTBS + 0.5% milk, incubated with the Pierce SuperSignal Chemiluminescent Kit, and exposed to Kodak film.

TUNEL assay

A549 cells were seeded at 10^4 cells/chamber on an eight-well chamber slide. Cells were infected at a m.o.i. of 10 PFU/cell in medium with 2.5% FCS for 60 min. After absorption, the infected cell monolayer was rinsed once with phosphate-buffered saline and incubated with medium with 10% FCS for 12 h. Monolayers were fixed and stained using the AP *In Situ* Cell Death Detection Kit (Boerhinger Mannheim) and photographed under an inverted light microscope.

Preparation of samples for electron microscopy

A549 cells (10^6) were seeded in 60-mm dishes and infected with 10 PFU per cell of vaccinia virus. At 24 h after infection, the cells were fixed, dehydrated, embedded, sectioned, collected on grids, and stained with lead citrate as previously described (Wolffe *et al.,* 1993).

Western blot analysis of viral proteins

Infected cells (10^6) were collected and incubated in 0.5 ml of hypotonic lysis buffer (0.06 M Tris–HCl (pH 6.8), 3% SDS, 10% glycerol, 5% fresh 2-mercaptoethanol, 0.002% bromphenol blue). The lysate was cleared by centrifugation at 12,000 *g* and a portion of each sample was mixed with SDS/2-mercaptoethanol sample buffer and boiled for 5 min. After polyacrylamide gel electrophoresis, the proteins were transferred electrophoretically to PVDF membranes (Millipore) and blocked with TTBS containing 2.5% non-fat dry milk. Blots were incubated for 1 h with a 1:2000 dilution of a rabbit polyclonal anti-vaccinia virus antibody (raised against purified infectious virus) in TTBS containing 0.5% milk. Rabbit antibody to RAP94 (Ahn *et al.,* 1994), the N terminus of the A17L protein (Betakova *et al.,* 1999), and the 4b core protein were used at a 1:1000 dilution. After incubation with each primary antibody, the blots were incubated with HRPO-conjugated secondary antibody as described above.

Metabolic labeling of viral proteins

Approximately 10^6 cells were seeded into six-well plates and infected with wild-type or mutant viruses at a m.o.i. of 10 PFU/cell. At 15 min before each labeling period, the cells were washed twice and incubated with prewarmed methionine-free medium. The cells then were incubated with 100 μCi of [^{35}S]methionine in 0.5 ml methionine-free medium for 30 min. The labeling medium was removed and the cells were washed twice with PBS and then incubated at 37°C for 5 min with 0.5 ml of hypotonic lysis buffer [20 mM Tris–HCl (pH 8.0), 10 mM NaCl, 0.5% Nonidet-P40 (NP-40)]. The lysate was collected and centrifuged for 2 min at 12,000 *g* to pellet nuclei. A portion of each sample was mixed with SDS/2-mercaptoethanol sample buffer, boiled for 5 min and resolved by electrophoresis in a SDS 4–20% polyacrylamide gel. Radioactive proteins were visualized using a PhosphorImager.

Analysis of viral DNA

Cells in 12-well plates were infected with 10 PFU/cell of vaccinia virus. At various times after infection, the cells were collected by centrifugation, suspended in 0.2 ml of PBS, and lysed by three freeze–thaw cycles and sonication. The lysates (0.1 ml) containing DNA were applied to a nylon transfer membrane (Amersham) using a slot blot apparatus. The membrane was treated sequentially with 0.5 M NaOH, 1 M Tris–HCl (pH 7.5) and 2[mult] SSC (0.3 M NaCl, 0.03 M Na citrate) and UV cross-linked. The membrane was incubated with a ^{32}P-labeled cloned vaccinia virus DNA probe in Rapi-Hybe Solution (Amer-

sham). Radioactivity was quantitated using the Phosphoimager.

Analysis of viral RNA

Monolayers of A549 cells in six-well tissue culture trays (10^6 cells/well) were infected with 10 PFU/cell of vaccinia virus. At various times, total cellular RNA was extracted using the RNAqueous kit (Ambion) and 1 μg was hybridized to a molar excess of ^{32}P-labeled riboprobes prepared as previously described (Baldick and Moss, 1993). RNase protected probe fragments were analyzed by electrophoresis on a 5% polyacrylamide–8 M urea gel and visualized by autoradiography as described (Baldick and Moss, 1993).

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