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# Reversal of the Interferon-Sensitive Phenotype of a Vaccinia Virus Lacking E3L by Expression of the Reovirus S4 Gene

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**The vaccinia virus (VV) E3L gene, which encodes a potent inhibitor of the interferon (IFN)-induced, double-stranded RNA (dsRNA)-dependent protein kinase, PKR, is thought to be involved in the IFN-resistant phenotype of VV. The E3L gene products, p25 and p20, act as inhibitors of PKR, presumably by binding and sequestering activator dsRNA from the kinase. In this study we demonstrate that VV with the E3L gene specifically deleted (vP1080) was sensitive to the antiviral effects of IFN and debilitated in its ability to rescue vesicular stomatitis virus from the antiviral effects of IFN. Infection of L929 cells with E3L-minus virus led to rRNA degradation typical of activation of the 2′-5′-oligoadenylate synthetase/RNase L system, and extracts of infected cells lacked the PKR-inhibitory activity characteristic of wild-type VV. The reovirus S4 gene, which encodes a dsRNA-binding protein (σ3) that can also inhibit PKR activation by binding and sequestering activator dsRNA, was inserted into vP1080. The resultant virus (vP1112) was partially resistant to the antiviral effects of IFN in comparison with vP1080. Further studies demonstrated that transient expression of the reovirus σ3 protein rescued E3L-minus VV replication in HeLa cells. In these studies, rescue by σ3 mutants correlated with their ability to bind dsRNA. Finally, vP1112 was also able to rescue the replication of the IFN-sensitive virus vesicular stomatitis virus in a manner similar to that of wild-type VV. Together, these results suggest that the reovirus S4 gene can replace the VV E3L gene with respect to interference with the IFN-induced antiviral activity.**

Vaccinia virus (VV) has been shown to be relatively resistant to the antiviral effects of interferon (IFN) in most cell lines tested (43). VV is also known to rescue the replication of IFN-sensitive viruses, such as vesicular stomatitis virus (VSV) and encephalomyocarditis virus (EMCV), upon coinfection (39, 40). Both IFN resistance and rescue are thought to be due to inhibition of either of two IFN-induced, double-stranded RNA (dsRNA)-dependent, antiviral pathways: the 2′-5′-oligoadenylate (2-5A) and dsRNA-dependent protein kinase (PKR) pathways (23, 24, 28, 29, 40). The 2-5A synthetase is activated in the presence of dsRNA, catalyzing the formation of oligoadenylate molecules of various lengths linked in a 2′-to-5′ fashion (5, 17, 29, 34). The 2-5A molecules activate a latent endonuclease, RNase L, which cleaves single-stranded RNA, thus preventing viral protein synthesis (5, 34). PKR is also activated in the presence of dsRNA and undergoes an autophosphorylation reaction followed by phosphorylation of its endogenous substrate, eukaryotic protein synthesis initiation factor 2 (eIF-2), on its α subunit (12, 33). Phosphorylation of eIF-2α prevents the eIF-2B-mediated exchange of GDP for GTP, resulting in cessation of protein synthesis (31).

As a means to better understand the IFN-mediated antiviral system, recent work has focused on identifying viral genes which are involved in virus-mediated inhibition of the IFN-induced antiviral activities. Virus-induced functions identified in adenovirus, influenza virus, human immunodeficiency virus, poliovirus, and VV have been implicated in interference with the antiviral effects of IFN (reviewed in reference 15).

Previous studies have demonstrated that VV encodes activities which specifically block PKR activation and the 2-5A pathway (1, 23, 28, 29, 41). More recently, deletion of the K3L open reading frame, which has significant sequence similarity to the amino-terminal region of eIF-2α, was shown to render VV sensitive to the antiviral effects of IFN. It was therefore proposed that the K3L-specified protein functioned as a pseudosubstrate for PKR, thus blocking PKR-mediated phosphorylation of eIF-2α (3).

Additional reports have described the isolation of a dsRNA-binding protein from VV-infected cells (38). This 25-kDa protein and a related 20-kDa protein were mapped to the E3L locus (4). On the basis of the dsRNA-binding activity of p20/25, it was proposed that E3L encoded the SKIF activity previously described for VV-infected cells (1, 41). Further evidence for the involvement of E3L in abrogating the antiviral effects of IFN came from studies demonstrating that transient expression of E3L blocked activation of PKR (4) and allowed expression of a reporter gene that had reduced expression levels upon PKR activation (7). Interestingly, it was also shown that E3L-encoded products are localized to both the cytoplasm and nucleus of VV-infected cells (14a, 44) and occur in two forms, i.e., 25 and 20 kDa (4, 38, 44). These proteins were shown to be translated from the same transcript, initiating at the first and second methionine residues, respectively (44).

To further study the E3L gene in relation to IFN resistance, the gene was specifically deleted from the wild-type VV Copenhagen strain, VC-2 (described previously [37]), to form vP1080. Initial studies with vP1080 demonstrated that the E3L gene was not only involved in IFN resistance but also constituted a host range regulatory function, since specific deletion of E3L resulted in a phenotype debilitated for replication on several cell culture systems, including Vero and HeLa cells, but retaining the ability to replicate with wild-type efficiency on primary chicken embryo fibroblasts (CEF) (2a).

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In this paper we describe the phenotype of E3L-minus VV (vP1080) and the effect of expression of the S4 gene from reovirus type 3 by this E3L-minus virus. The S4 gene encodes a 41-kDa protein, designated σ3, which binds specifically to dsRNA (13) and can inhibit activation of PKR by sequestering dsRNA (14). Similar to what is seen with the E3L gene, transient expression of the S4 gene rescues protein synthesis of a reporter gene in a system in which protein synthesis is reduced by activation of PKR (19). Expression of the S4 gene in an E3L-minus VV background restored an IFN-resistant phenotype to the E3L-minus virus, including the ability to rescue VSV and EMCV from the antiviral effects of IFN. Transient expression of the S4 gene rescued replication of the E3L-minus vaccinia virus in HeLa cells. The observed rescue by transient expression of the reovirus σ3 protein was dependent on its dsRNA-binding activity, since mutant forms of the protein that were unable to bind dsRNA did not rescue, whereas mutants which retained dsRNA-binding activity were able to rescue E3L-minus virus replication in HeLa cells. Together, the results presented in this paper further define the role of the E3L gene product in virus-mediated inhibition of the IFN system and demonstrate that the reovirus S4 gene product, σ3, is a *trans*-acting IFN resistance function.

## MATERIALS AND METHODS

**Cell lines.** Vero cells were grown as monolayers at 37°C in minimal essential medium (MEM) supplemented with 5% fetal bovine serum (FBS). L929 cells were grown as monolayers at 37°C in Dulbecco's MEM supplemented with 5% FBS and 50 μg of gentamicin sulfate per ml. HeLa cells were grown as monolayers at 37°C in MEM supplemented with 10% FBS and 50 μg of gentamicin sulfate per ml. Primary CEF from 10- to 11-day-old embryos of specific pathogen free origin were generously provided by Select Laboratories (Gainesville, Ga.). CEF were grown as monolayers at 37°C in Dulbecco's MEM supplemented with 10% FBS, 100 U of penicillin G per ml, 100 μg of streptomycin sulfate per ml, and 0.25 μg of amphotericin B per ml.

**Virus and virus constructs.** The VV Copenhagen strain, VC-2, was propagated in CEF as previously described (37). The E3L-minus deletion mutant was engineered in the following manner. The 5′ and 3′ E3L flanking regions were generated by PCR. For generation of the E3L 5′ flanking arm, oligonucleotides MPSYN425 (5′ CCC GGA TCC TTT TTA GAG AGA ACT AAC AC 3′) and MPSYN427 (5′ ATC ATC TCT AGA CCG AAA AAG AAT TCC TTC GC 3′) were used as primers with plasmid pSD401VC, containing the VV *Hin*dIII E fragment, as the template. The resultant 0.5-kb fragment was digested with *Bam*HI and *Xba*I and inserted into pBS-BG (pBS SK+ [Stratagene, La Jolla, Calif.] deleted of β-galactosidase sequences between the *Nae*I and *Kpn*I sites) that had been previously digested with the same enzymes, resulting in plasmid pMPE3RA. For generation of the E3L 3′ flanking arm, oligonucleotides MPSYN426 (5′ CCC CTG CAG TGA TTC TAG TTA TCA ATA AC 3′) and MPSYN428 (5′ ATC ATC GTT AAC GAA ATA ATC ATG TAA GGC 3′) were used with plasmid pSD401VC as the template. The resultant 0.5-kb fragment was digested with *Hin*cII and *Pst*I and inserted into plasmid pMPE3RA that had been previously digested with the same enzymes, resulting in plasmid pMPE3DEL. This plasmid was digested with *Bam*HI and ligated with a *Bgl*II fragment containing the β-galactosidase gene under control of the VV 11K promoter, resulting in plasmid pMPE3DELBG. This plasmid was used in standard in vivo recombination (26) with VC-2 as the rescue virus. Recombinants were identified by isolation of blue plaques on monolayers of primary CEF stained with X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), as previously described (26). The resultant recombinant, designated vP1080, was plaque purified three times, and integrity was verified by hybridization to a radiolabeled β-galactosidase probe, by the inability to hybridize to a radiolabeled E3L-specific probe, and by restriction analysis.

A VV recombinant expressing the reovirus S4 gene was generated as follows. A 1.2-kb fragment containing the reovirus S4 gene was inserted downstream of the VV H6 promoter (27) previously inserted into plasmid pSPHA for insertion into the hemagglutinin site of VV. This plasmid, designated pH6-S4, was used in standard in vivo recombination experiments (26) with the VV E3L-minus deletion mutant (vP1080) as the rescuing virus. Recombinant vP1112 was identified by hybridization to a reovirus S4 probe. The resultant recombinant was plaque purified three times, and integrity was verified by restriction analysis and by immunoprecipitation of the S4 gene product.

Stocks of recombinant viruses were prepared by growth in CEF, and titers were determined on CEF, as previously described (37). The abilities of wild-type and recombinant VV to form plaques were monitored by diluting virus stocks to 500 PFU/ml and plating onto monolayers of Vero cells as previously described (26). Monolayers were stained 3 days later with crystal violet.

**Virus infections.** To assess the effects of IFN treatment on viral protein synthesis, L929 (ATCC CCL1) cell monolayers were pretreated for 24 h with 0, 10, 100, or 1,000 international reference units (IRU) of mouse alpha/beta IFN (IFN-α/β) (Lee Biomolecular Research, Inc., San Diego, Calif.) per ml. Cell monolayers were infected with wild-type or recombinant VV at a multiplicity of infection (MOI) of 10. Following infection, the inoculum was aspirated, fresh medium was added, and monolayers were incubated at 37°C. At 7 h postinfection (hpi), the medium was aspirated and replaced with 1 ml of methionine-free medium (Gibco-BRL, Grand Island, N.Y.) supplemented with 50 μCi of [$^{35}$S]methionine per ml. After 1 h, the cells were scraped into the medium and pelleted. The supernatant was aspirated, the pellet was washed with phosphate-buffered saline (PBS), and cells were resuspended in 100 μl of PBS. Lysates were prepared by three cycles of freezing and thawing followed by clarification. Total protein concentrations in the lysates were determined with the Bio-Rad protein assay kit, and 50 μg of each sample was fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and visualized by fluorography.

**Immunoprecipitation.** Virus infections and immunoprecipitations were performed as previously described (37), using rabbit antiserum generated against VV p25 (38) or reovirus σ3 (14). Immunoprecipitated proteins were fractionated by SDS-PAGE and visualized by fluorography.

**Isolation of RNA.** IFN-treated L929 cells were infected with wild-type or recombinant VV at an MOI of 10, as described above, and at 7 hpi RNA was isolated by the guanidinium isothiocyanate method (18). Equivalent volumes of each RNA preparation were fractionated by formaldehyde-agarose electrophoresis in 10 mM sodium phosphate buffer (pH 6.5) as previously described (32). Briefly, equivalent volumes of total RNA were adjusted to 50% formamide, 2 M formaldehyde, and 10 mM sodium phosphate, pH 7.2. Samples were heated at 65°C for 15 min, placed on ice, and then spun to concentrate the samples. RNA loading dyes were added, and the samples were fractionated overnight at 30 V. Gels were stained with ethidium bromide, photographed, and transferred to Hybond as described previously (32). The membrane was probed with 18S rRNA probes (a generous gift of Ira G. Wool, University of Chicago) and visualized by autoradiography.

**PKR inhibition assay.** L cells were infected with wild-type or recombinant VV at an MOI of 10, and then cytoplasmic extracts were prepared. Briefly, at 6 hpi the medium was replaced with 1.5 ml of Eagle's MEM (Sigma) without methionine. After 30 min of incubation at 37°C, 150 μl of Eagle's MEM containing 1% dialyzed FBS and 50 μCi of [$^{35}$S]methionine per ml was added. Cells were incubated for 30 min at 37°C, washed, scraped into cold PBS, pelleted by centrifugation at 1,000 × *g*, and resuspended in Nonidet P-40 (NP-40) lysis buffer (20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid] [pH 7.5], 120 mM KCl, 5 mM magnesium acetate, 1 mM dithiothreitol, 10% [vol/vol] glycerol, 0.5% NP-40) (100 μl/$10^7$ cells) as previously described (2). Cells were sonicated for 10 s (Bransonic 52), and nuclei were pelleted by centrifugation at 10,000 × *g* for 10 min at 4°C. Levels of kinase-inhibitory activity were determined by adding cytoplasmic extracts prepared from infected cells to kinase reaction buffer (20 mM HEPES [pH 7.5], 120 mM KCl, 5 mM magnesium acetate, 1 mM dithiothreitol, 100 mM [γ-$^{32}$P]ATP [1 Ci/mmol]) containing various concentrations of reovirus dsRNA in a final volume of 20 μl. Partially purified PKR from IFN-treated, uninfected HeLa cells was added as a source of kinase. The kinase reaction mixture was incubated at 30°C for 15 min, and the reaction was stopped by adding an equal volume of 2× SDS-PAGE sample buffer. Samples were boiled for 5 min, separated by SDS-PAGE, and analyzed by autoradiography.

**Rescue experiments with the E3L-minus VV, vP1080.** HeLa cells were transfected with pMTVa-based vectors containing full-length, deletion mutant, or point mutant S4 genes (8, 16). Briefly, 10 μg of DNA in a 250-μl volume of 248 mM $CaCl_2$ was added dropwise to 250 μl of 2× HBS (274 mM NaCl, 10 mM KCl, 1.5 mM $NaH_2PO_4 \cdot 7H_2O$, 12 mM glucose, 42 mM HEPES, pH 7.1) while an airstream was applied to the surface of the liquid. The DNA mixture was incubated for 20 min at room temperature and then added to a 60-mm-diameter plate of subconfluent HeLa cells that had been prefed with MEM–10% FBS, pH 7.2. At 48 h posttransfection, the cells were infected with wild-type VC-2 or with E3L-minus vP1080 at an MOI of 30 and incubated for 6 h at 37°C. Cells were placed in methionine-free medium for 30 min and then pulse-labeled with 50 μCi of [$^{35}$S]methionine per ml for 30 min as described above. Following the 30-min pulse-labeling period, cells were harvested and NP-40 lysates were prepared as previously described (4). Proteins were solubilized by boiling in 2× SDS-PAGE sample buffer for 5 min, fractionated by SDS-PAGE, and visualized by fluorography.

**Rescue of VSV and EMCV.** VSV and EMCV rescue experiments were performed as previously described (39, 41). Briefly, monolayers of mouse L929 cells were pretreated with 0 or 100 IRU of mouse IFN-α/β (Lee Biomolecular Research, Inc.) per ml. After 24 h, the monolayers were washed with PBS and infected with wild-type or recombinant VV at an MOI of 1 for 2 h. The monolayers were washed with PBS and infected with EMCV or VSV (Indiana strain) at an MOI of 10 in the presence of 5 μg of actinomycin D per ml for 1 h. Monolayers were washed with PBS and overlaid with medium containing 5 μg of actinomycin D per ml. At 6 hpi, cells were pulse-labeled with [$^{35}$S]methionine as

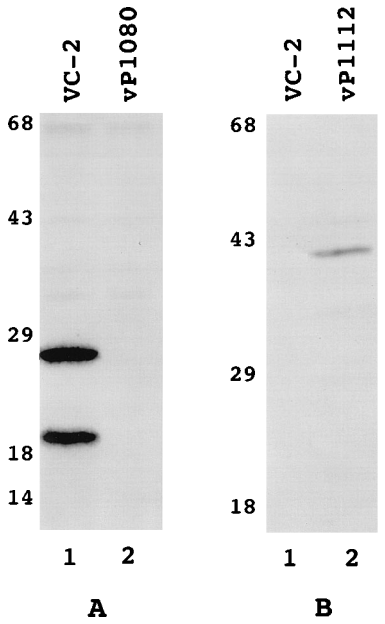


FIG. 1. Analysis of protein expression in cells infected with VV constructs. CEF were infected with wild-type virus (VC-2) (lanes 1), the E3L-minus virus (vP1080) (panel A, lane 2), or the E3L-minus virus expressing the reovirus S4 gene (vP1112) (panel B, lane 2) at an MOI of 10, pulsed with [$^{35}$S]methionine immediately after absorption, and harvested after 24 h. Extracts were prepared and proteins were immunoprecipitated with rabbit antiserum to VV p25 protein (A) or reovirus σ3 protein (B), as described in Materials and Methods. Proteins were fractionated by SDS-PAGE and visualized by fluorography.

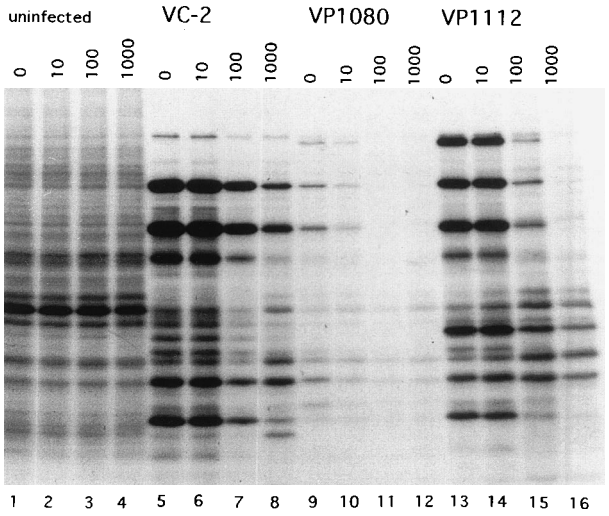


FIG. 2. IFN sensitivity of VV constructs. Monolayers of L929 cells were pretreated for 24 h with increasing amounts of mouse IFN-α/β (shown above the lanes, in IRU per milliliter) and infected with wild-type or recombinant VV as described in Materials and Methods. Cells were mock infected (lanes 1 to 4), or infected with wild-type virus (VC-2) (lanes 5 to 8), the E3L-minus deletion mutant (vP1080) (lanes 9 to 12), or the E3L-minus virus expressing the reovirus S4 gene (vP1112) (lanes 13 to 16) and pulsed for 1 h with [$^{35}$S]methionine at 7 hpi. Lysates were prepared and equivalent quantities of total protein were fractionated by SDS-PAGE and visualized by fluorography, as described in Materials and Methods.

described above. NP-40 lysates were prepared as previously described (4). Proteins were solubilized by boiling in 2× SDS-PAGE sample buffer for 5 min, fractionated by SDS-PAGE, and visualized by fluorography.

## RESULTS

**Expression analysis of wild-type and recombinant VVs.** In order to test the in vivo role of the reovirus σ3 protein as a PKR inhibitor, the S4 gene, under control of the VV H6 promoter, was inserted into the hemagglutinin locus of E3L-minus VV (vP1080), resulting in recombinant vP1112. To assess expression of endogenous E3L protein or the reovirus σ3 protein, immunoprecipitation analyses were performed with lysates derived from VC-2-, vP1080-, and vP1112-infected CEF by using antiserum specific to the VV E3L-specified proteins or the reovirus σ3 protein (Fig. 1). The E3L-specific antiserum was shown to precipitate two species of 20 and 25 kDa from lysates derived from VC-2-infected cells (Fig. 1A, lane 1), in agreement with previous reports (4, 38, 44). The 25-kDa protein is thought to be the full-length gene product, while the 20-kDa protein is missing amino-terminal sequences, presumably as a result of leaky scanning during initiation of translation (4, 34a, 38, 44). These proteins, however, were not precipitated from E3L-minus virus (vP1080)-infected cells, supportive of deletion of the E3L open reading frame in deriving vP1080 (Fig. 1A, lane 2). Figure 1B demonstrates expression of the 41-kDa reovirus σ3 protein in vP1112-infected cells (lane 2) but not in parental virus (VC-2)-infected cells (lane 1).

**Effect of IFN treatment on protein synthesis of wild-type and recombinant VVs.** To determine the effect of IFN treatment on viral protein synthesis, IFN-treated L929 cell monolayers were infected with wild-type or recombinant VV and pulsed with [$^{35}$S]methionine from 7 to 8 hpi. In agreement with previous reports (3, 23, 28), there was little effect of IFN on wild-type VV protein synthesis, except at very high concentrations of IFN (Fig. 2, lanes 5 to 8). Specific deletion of the E3L gene (vP1080) resulted in enhanced IFN sensitivity, with barely detectable levels of viral proteins in the presence of as little as 10 IRU of mouse IFN-α/β per ml (Fig. 2, lanes 9 to 12). Significantly, however, insertion of the S4 gene into the E3L-minus virus (vP1112) restored viral protein synthesis in the presence of 10 IRU of mouse IFN-α/β per ml (Fig. 2, lanes 13 to 16). These results demonstrate that expression of a heterologous dsRNA-binding protein in a replication-deficient E3L-minus deletion mutant was sufficient for partial rescue of viral protein synthesis in IFN-treated cells.

**RNA stability in wild-type and recombinant VV-infected cells.** The VV E3L and reovirus σ3 proteins have been shown to be dsRNA-binding proteins (4, 13), suggesting that they could abrogate IFN effects by inhibiting one or both of the IFN-induced dsRNA-dependent antiviral pathways, the 2-5A and PKR pathways. Activation of the 2-5A pathway in cells can be detected by analyzing degradation of rRNA into characteristic, discrete fragments (42). We assessed 2-5A activity in cells infected with VC-2, vP1080, and vP1112 by assessing rRNA integrity. L929 cells, pretreated with increasing amounts of mouse IFN-α/β, were mock infected or virus infected, and total RNA was isolated at 7 hpi. In mock-infected cells (Fig. 3, lanes 1 to 5) or wild-type VC-2-infected cells (Fig. 3, lanes 6 to 10), there was no detectable rRNA degradation in the presence or absence of IFN treatment. In marked contrast, deletion of the E3L open reading frame (vP1080) resulted in degradation of rRNA in the presence of IFN but not in its absence (Fig. 3, lanes 11 to 15 [degradation products are indicated by arrowheads]). Insertion of the reovirus S4 gene into E3L-minus virus restored rRNA stability (Fig. 3, lanes 16–18), again supporting the idea that the S4 gene product can functionally replace the E3L gene products in their ability to inhibit the IFN-induced

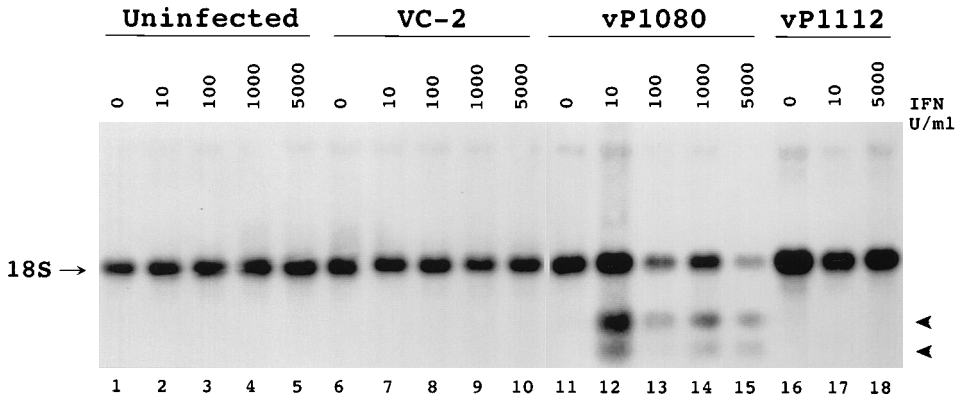


FIG. 3. Activation of 2-5A system in VV-infected cells. Monolayers of L929 cells were pretreated for 24 h with increasing amounts of mouse IFN-α/β, as shown above the lanes, and infected with wild-type or recombinant VV as described in Materials and Methods. Total RNA was isolated from mock-infected cells (lanes 1 to 5) or cells infected with wild-type virus (VC-2) (lanes 6 to 10), the E3L-minus virus (vP1080) (lanes 11 to 15), or the E3L-minus virus expression the reovirus S4 gene (vP1112) (lanes 16 to 18), fractionated by formaldehyde-agarose electrophoresis, and probed with 18S rRNA-specific probes (generously provided by Ira G. Wool, University of Chicago), as described in Materials and Methods. The migration position of intact 18S rRNA is indicated on the left, and the characteristic, discrete 18S rRNA degradation products are indicated by arrowheads on the right.

dsRNA-dependent 2-5A synthetase pathway in VV-infected cells.

**Inhibition of PKR in wild-type and recombinant VV-infected cells.** Extracts from wild-type or recombinant VV-infected cells were assayed for levels of PKR-inhibitory activity present at 7 hpi. Extracts were preincubated with various concentrations of dsRNA and then mixed with partially purified PKR in the presence of [$\gamma$-$^{32}$P]ATP. Activation of PKR was monitored by PKR autophosphorylation as visualized by SDS-PAGE and autoradiography (Fig. 4). In extracts from mock-infected cells, PKR autophosphorylation was detected after incubation with dsRNA at a concentration of 0.1 μg/ml (Fig. 4A, lane 2). In extracts prepared from VC-2-infected cells, larger amounts of dsRNA were required in order to detect similar levels of PKR autophosphorylation. In this experiment, PKR autophosphorylation was detected only at dsRNA concentrations of at least 1 μg/ml (Fig. 4B), confirming the presence of a PKR inhibitor in VV-infected cells (23, 28, 38, 41). In contrast, extracts prepared from E3L-minus virus (vP1080)-infected cells did not contain detectable PKR-inhibitory activity (Fig. 4C). In fact, PKR was activated in vP1080-infected extracts to which no dsRNA was added (Fig. 4C, lane 1), suggesting that dsRNA was produced during infection in sufficient quantity to activate the exogenous PKR. Whereas extracts prepared from E3L-minus virus-infected cells contained no detectable PKR-inhibitory activity, extracts from cells infected with E3L-minus virus expressing the reovirus S4 gene (vP1112) had partial restoration of this activity. As can be seen in Fig. 4D, the amount of PKR-inhibitory activity present in extracts from vP1112-infected cells was intermediate between those in extracts from uninfected and VC-2-infected cells. These data demonstrate that VV expressing the dsRNA-binding proteins p20/p25 or σ3 contain PKR-inhibitory activity, although the efficiencies of this inhibition may differ.

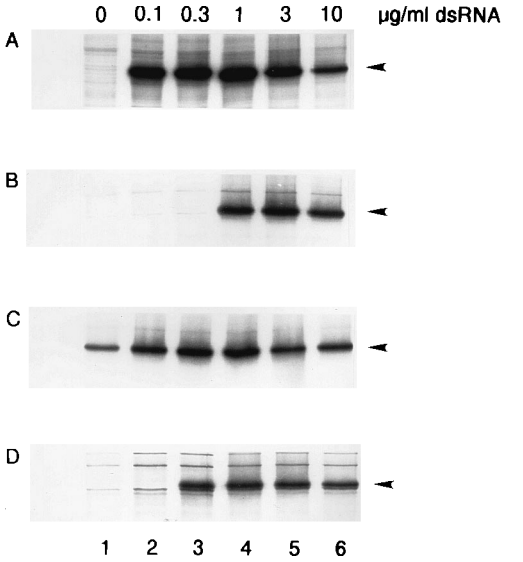


FIG. 4. PKR-inhibitory activity expressed in wild-type or recombinant VV-infected cells. L cells were mock infected (A) or infected with wild-type Copenhagen VV (B), VV with the E3L gene deleted (vP1080) (C), or E3L-minus, S4-expressing VV (vP1112) (D). NP-40 lysates of infected cells were prepared as described in Materials and Methods and mixed with various concentrations of dsRNA, and then partially purified PKR from IFN-treated HeLa cells was added as a source of kinase. The kinase assay was performed as described in Materials and Methods. Phosphorylated proteins were separated by SDS-PAGE and visualized by autoradiography. The concentrations of dsRNA present are indicated above the lanes. The arrow indicates the 72,000-$M_r$ dsRNA-dependent kinase.

**Rescue of VSV.** The abilities of wild-type and recombinant VVs to rescue protein synthesis of the IFN-sensitive virus VSV were tested by coinfection experiments. Mouse L929 cells were mock or IFN treated for 24 h prior to infection with wild-type or recombinant VV. After 2 h of infection with VV, the cells were infected with VSV in the presence of actinomycin D. Expression of VSV proteins was monitored by labeling cells for 1 h with [$^{35}$S]methionine at 6 hpi. Extracts were prepared and fractionated by SDS-PAGE, and proteins were visualized by fluorography. Seen in Fig. 5 is the typical protein-induced profile observed in VSV-infected L929 cells (lane 1). Coinfection with either wild-type VV (VC-2; lane 3), E3L-deficient virus (vP1080; lane 5), or E3L-minus virus expressing the reovirus σ3 protein (vP1112; lane 7) had no apparent effect on VSV protein synthesis. In the presence of IFN, however, VSV protein expression was markedly reduced (Fig. 5, lane 2), concomitant with restoration of host protein synthesis, as previously reported (39). Coinfection with wild-type VC-2 restored VSV protein synthesis (Fig. 5, lane 4); however, coinfection with E3L-minus deletion mutant vP1080 was un-

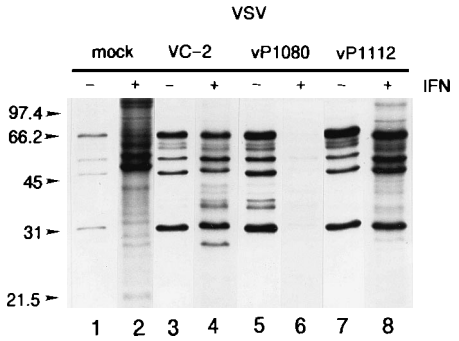


FIG. 5. VV-mediated rescue of VSV from the antiviral effects of IFN. L929 cells were treated with 0 (odd-numbered lanes) or 100 (even-numbered lanes) IRU of mouse IFN-α/β per ml. After 24 h, monolayers were mock-infected (lanes 1 and 2) or infected with wild-type virus (VC-2) (lanes 3 and 4), the E3L-minus virus (vP1080) (lanes 5 and 6), or the E3L-minus virus expressing the reovirus S4 gene (vP1112) (lanes 7 and 8) at an MOI of 1. After 2 h, monolayers were washed with PBS and infected with VSV at an MOI of 10 in the presence of 5 μg of actinomycin D per ml. Cells were pulse-labeled for 1 h with [$^{35}$S]methionine at 6 hpi, and extracts were prepared as described in Materials and Methods. Proteins were fractionated by SDS-PAGE and visualized by fluorography. Numbers on the left are molecular weights in thousands.

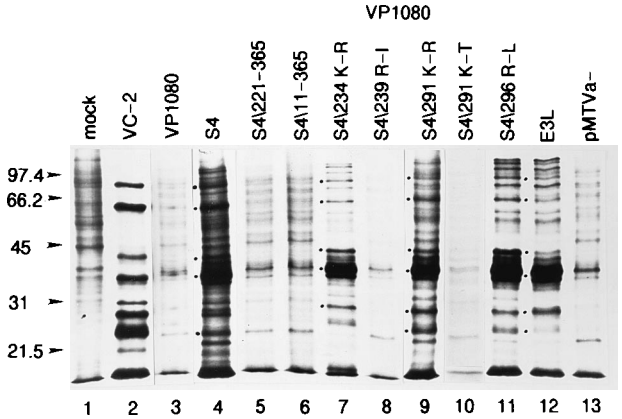


FIG. 6. Rescue of vP1080 replication in HeLa cells by transient expression of reovirus S4 constructs. HeLa cells were mock transfected (lanes 1 to 3) or transfected with plasmids expressing wild-type S4 (lane 4), amino-terminal deletion mutants of S4 (lanes 5 and 6), point mutants of S4 (lanes 7 to 11), wild-type E3L (lane 12), or the parental expression vector, pMTVa- (lane 13). After 48 h, cells were mock infected (lane 1) or infected with wild-type virus (VC-2) (lane 2) or the E3L-minus virus (vP1080) (lanes 3 to 13). Cells were pulsed with [$^{35}$S]methionine at 7 hpi, and NP-40 lysates were prepared as described in Materials and Methods. Proteins were fractionated by SDS-PAGE and visualized by autoradiography. See the text for a detailed description of the reovirus S4 deletion and point mutants. Numbers on the left are molecular weights in thousands.

able to rescue VSV-induced protein synthesis (lane 6). Significantly, E3L-minus virus expressing the reovirus σ3 protein (vP1112) was able to restore VSV-specific protein expression in a manner similar to that of VC-2 (Fig. 5, lane 8). Similar results were obtained for the rescue of EMCV in the presence of IFN (i.e., vP1112 rescued EMCV, while vP1080 did not [data not shown]). These results show that expression of a dsRNA-binding protein is required for the ability of VV to rescue protein synthesis of the IFN-sensitive viruses VSV and EMCV and that a protein from an RNA virus, the reovirus σ3 protein, can substitute for the endogenous E3L protein.

**Rescue of viral protein synthesis in VV vP1080-infected cells with S4 and mutants of S4.** Since insertion of the reovirus S4 gene has been shown to rescue the ability of E3L-minus VV to productively replicate on certain cell substrates, such as Vero and HeLa cells (2a), the requirement for a functional dsRNA-binding activity in the restoration of the putative host range phenotype was assessed in the following manner. HeLa cells were transfected with S4 expression plasmids and then infected with vP1080 at 48 h posttransfection. The infected cell cultures were pulse-labeled for 1 h with [$^{35}$S]methionine at 7 hpi to monitor late viral protein synthesis. Figure 6 shows that the reovirus S4 and VV E3L genes, both of which encode dsRNA-binding proteins, were capable of restoring the typical late VV protein profile in HeLa cells (lanes 4 and 12, respectively), which is not observed in nontransfected HeLa cells infected with vP1080 (lane 3).

A putative dsRNA-binding domain has been previously mapped to an 85-amino-acid region at the carboxyl terminus of the 365-amino-acid σ3 protein (8, 20, 21). Deletion of the first 221 amino acids resulted in a protein containing the complete dsRNA-binding domain and capable of binding nucleic acid in a Northwestern (RNA-protein) blot assay (21) although not in a soluble assay with native protein (8). Expression of this S4/221-365 deletion mutant or a nonfunctional mutant containing a 10-amino-acid deletion at the amino terminus of σ3 (S4/11-365 [8]) was unable to rescue vP1080 replication (Fig. 6, lanes 5 and 6, respectively). Previously constructed (8) S4 mutants with point mutations within the putative dsRNA-binding region were also tested for their ability to rescue vP1080 replication. Point mutants of S4 that expressed σ3 proteins unable to bind dsRNA (S4/239 R-I [residue 239, Arg to Ile] and S4/291 K-T) did not rescue vP1080 (Fig. 6, lanes 8 and 10, respectively). In contrast, point mutants of S4 that retained their dsRNA-binding ability (S4/234 K-R, S4/291 K-R, and S4/296 R-L) were able to rescue vP1080 replication (Fig. 6, lanes 7, 9, and 11, respectively). These results demonstrate that the abilities of S4 deletion and point mutations to rescue vP1080 replication correlate with their abilities to bind dsRNA as observed in a soluble binding assay (8).

## DISCUSSION

VV has been shown to encode several proteins which serve to evade the host defense systems (35). Among this class of proteins are those which serve to abrogate the antiviral effects of IFN. VV is known to be relatively resistant to the antiviral effects of IFN in most cell lines (23, 28, 41) and additionally has been shown to be capable of rescuing IFN-sensitive viruses from the antiviral effects of IFN (39, 40). Recent studies have identified genes implicated in the IFN-resistant phenotype of VV; among these are K3L (3) and D11L (10). Additionally, the A18R gene may be involved in inhibiting the IFN-induced dsRNA-dependent 2-5A synthetase pathway, since a temperature-sensitive mutant with an abortive-late phenotype characterized by increased levels of dsRNA, 2-5A molecules, and concomitant rRNA degradation has been identified (2, 6, 22). Unlike K3L, D11L and A18R are essential genes for VV replication in tissue culture. These two genes, encode functions which may indirectly affect sensitivity to IFN by their involvement in virus-specific transcriptional control (2, 9). This level of control would therefore maintain lower levels of dsRNA in infected cells.

Recent studies have identified the E3L-encoded protein as a potential IFN resistance gene. The E3L products function as dsRNA-binding proteins (4) which show in vitro and in vivo PKR-inhibitory activity (4, 7, 38). E3L is particularly interesting in that it is one of the few VV-encoded proteins which are detected in the nuclei of infected cells (14a, 44). Interestingly,

deletion of E3L from wild-type VV resulted in a conditionally defective phenotype (2a); that is, the E3L-minus virus (vP1080) could productively replicate on certain cell substrates (CEF and RK-13 cells) but not on others (Vero and HeLa cells). This is reminiscent of other host range regulatory functions (K1L, C7L, and CP77kDa) encoded by orthopoxviruses (11, 25, 36). The mechanism by which the E3L-encoded protein acts as a host range function is not known. Whether this biological property relates to the dsRNA-binding activity or to an unknown cytoplasmic or nuclear activity needs to be determined.

Here we report that specific E3L deletion renders VV sensitive to IFN and unable to rescue IFN-sensitive viruses from the antiviral effects of IFN (Fig. 2 and 5, respectively). This is likely due to accumulation of viral dsRNA, leading to the activation of the PKR (15) and 2-5A (17) pathways. The E3L-encoded protein was also previously shown to interfere in vitro and in vivo with PKR activation (4, 7, 38). Sequestration of dsRNA in infected cells therefore increases the effective dsRNA concentrations required for activation of these antiviral pathways, a characteristic previously reported for wild-type VV-infected cells (1, 23, 28, 41). Consequently, E3L deletion provided a phenotype lacking the putative SKIF activity previously reported (Fig. 4), in addition to causing activation of the 2-5A pathway (Fig. 3).

We also report in this communication that expression of a heterologous viral dsRNA-binding protein, the reovirus σ3 protein (14), by an E3L-minus VV mutant partially restored an IFN-resistant phenotype, including the ability to rescue IFN-sensitive viruses from the antiviral effects of IFN (Fig. 2 and 5, respectively). In support of these observations, reovirus σ3 expression was also shown to provide a PKR-inhibitory activity (Fig. 4) as well as a 2-5A-inhibitory activity (Fig. 3).

These results demonstrate that both the VV E3L and reovirus S4 genes can function as IFN resistance genes. Significantly, both genes encode dsRNA-binding proteins, p20/25 (4) and σ3 (13), respectively, which inhibit PKR activation in vitro (4, 14) and allow expression of a reporter gene in a system in which protein synthesis is reduced by activation of PKR (7, 19). Although both p20/25 and σ3 are dsRNA-binding proteins, p20/25 has slightly greater dsRNA-binding activity as determined by poly(rI) · poly(rC)-agarose/dsRNA competition experiments (data not shown). One possible explanation for the partial IFN-resistant phenotype of an E3L-minus virus expressing the S4 gene is that σ3 is not as efficient an inhibitor of PKR as is p25 by virtue of its reduced ability to sequester dsRNA. Alternatively, the difference in the IFN sensitivities of VC-2 and vP1112 could be due to reduced expression of S4 in a VV background relative to endogenous E3L expression in wild-type VC-2.

Expression of σ3 in the VV E3L-minus mutant background resulted in a wild-type phenotype with respect to the ability to productively replicate in Vero and HeLa cells (2a). Data presented in Fig. 6 support the idea that the dsRNA-binding activity of σ3 was responsible for restoring the ability to replicate in and express typical VV-induced protein profiles in HeLa cells. Results from these studies illustrate that deletion and point mutations in the reovirus S4 gene (8) that result in a loss of dsRNA-binding activity of σ3 did not rescue the VV E3L-minus mutant (Fig. 6). Point mutations which did not cause loss of the dsRNA-binding activity of σ3 also retained the ability to rescue VV-induced protein synthesis in HeLa cells.

In summary, this communication provides evidence that the reovirus S4 gene product, σ3, is capable of interfering with the IFN-induced antiviral state. This has been problematic to test in the past, since attempts to insert variant S4 genes back into reovirus have been unsuccessful (30). Therefore, analysis of the role of σ3 in the IFN-resistant phenotype of reovirus awaits analysis of recombinants between IFN-sensitive and IFN-resistant virus isolates. Further, the data suggest the importance of the dsRNA-binding activity, and not an alternate putative E3L function, for the host range activity of the E3L gene. These results thus support the ideas that VV is able to control the level of dsRNA in infected cells as has been described previously (2, 6) and that virus sensitivity to dsRNA levels provides an important host range criterion in virus-host interactions.

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