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Double-Stranded RNA Is a Trigger for Apoptosis in Vaccinia Virus-Infected Cells

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The vaccinia virus E3L gene codes for double-stranded RNA (dsRNA) binding proteins which can prevent activation of the dsRNA-dependent, interferon-induced protein kinase PKR. Activated PKR has been shown to induce apoptosis in HeLa cells. HeLa cells infected with vaccinia virus with the E3L gene deleted have also been shown to undergo apoptosis, whereas HeLa cells infected with wild-type vaccinia virus do not. In this report, using virus recombinants expressing mutant E3L products or alternative dsRNA binding proteins, we show that suppression of induction of apoptosis correlates with functional binding of proteins to dsRNA. Infection of HeLa cells with *ts*23, which leads to synthesis of increased dsRNA at restrictive temperature, induced apoptosis at restrictive but not permissive temperatures. Treatment of cells with cytosine arabinoside, which blocks the late buildup of dsRNA in vaccinia virus-infected cells, prevented induction of apoptosis by vaccinia virus with E3L deleted. Cells transfected with dsRNA in the absence of virus infection also underwent apoptosis. These results suggest that dsRNA is a trigger that can initiate a suicide response in virus-infected and perhaps uninfected cells.

The interferon (IFN) system is the primary defense mechanism against viral infection in mammalian cells. It has been proposed that the cell is alerted to viral infection by the presence of double-stranded RNA (dsRNA), which is not found in detectable amounts in most uninfected cells, but which is present in most virus-infected cells (33). This signal results in induced expression of IFN and subsequent secretion of IFN to neighboring cells. Within the neighboring cells, IFN induces expression of several enzymes, of which 2′-5′-oligoadenylate synthetase (2′-5′A synthetase) and a protein kinase (PKR) are best characterized (64, 72). Activation of these enzymes is dependent on the presence of dsRNA. Activated 2′-5′A synthetase polymerizes oligoadenylates with 2′,5′ linkages (35). These oligoadenylates then activate a latent cellular enzyme, RNase L, which cleaves single-stranded RNAs (ssRNAs) (1, 9, 27, 59, 71, 73, 84). Activated PKR phosphorylates the α subunit of the eucaryotic translation initiation factor, eIF-2α (25, 50, 70), as well as the NF-κB inhibitor, IκB (39, 53, 61). Phosphorylation of eIF-2α leads to alteration of function of this translation factor. Phosphorylation of IκB leads to its dissociation from NF-κB, allowing this transcription factor to migrate to the nucleus and stimulate transcription of certain genes. Both 2′-5′A synthetase and PKR activation result in an inhibition of viral, and at times, host protein synthesis.

Some viruses are resistant to the effects of IFN and are able to circumvent IFN-induced host cell defenses. Influenza virus inhibits activation of PKR by activating a cellular protein, P58 (46, 47, 49). The adenovirus VAI RNA binds to PKR, preventing PKR from binding to dsRNA, but does not itself activate PKR (54). The vaccinia virus (VV) E3L gene has been shown to be necessary for the VV IFN-resistant phenotype (6, 14). E3L encodes two dsRNA binding proteins, p25 and p20 (15, 81, 85), which have been identified in both the nucleus and cytoplasm of infected and transfected cells (14, 85). These proteins prevent activation of PKR, presumably by sequestering dsRNA, thereby making it unavailable to activate PKR (15, 20). Another VV gene, K3L, encodes a protein that has homology to eIF-2α (8, 28). The K3L product is a competitive inhibitor of eIF-2 phosphorylation (12, 21, 34). Other viruses, including reovirus and rotavirus, express proteins, σ3 (31) and p8 (43), respectively, that bind to and likely sequester dsRNA similarly to E3L. The E3L gene products and the rotavirus p8 protein contain a conserved dsRNA binding domain found in several proteins known to bind to dsRNA (13, 15, 55, 74).

VV with the E3L gene deleted (VVΔE3L) is sensitive to the effects of IFN. This virus also demonstrates a host range in that it cannot replicate in HeLa cells but exhibits nearly wild-type replication in rabbit kidney RK-13 cells (6, 7, 14). Replication of this virus can be rescued in HeLa cells by transient expression of a plasmid containing a gene coding for one of several dsRNA binding proteins, including the E3L gene (14), the reovirus S4 gene (coding for σ3) (6), and the rotavirus NSP3 gene (coding for p8) (43). In transient transfections with E3L mutant products, rescue of replication correlated with the ability of the protein to bind dsRNA (14). This suggested that a functional dsRNA binding protein was necessary to promote replication of VVΔE3L in HeLa cells.

Recently, Lee and Esteban reported that cells expressing activated PKR undergo apoptosis (45). Apoptosis is a physiological process of cellular suicide (36) which can be induced by a number of events detrimental to the cell, including DNA damage, bacterial and viral infections, or serum starvation. By committing suicide, the cell presumably can prevent replication of a damaged cellular genome or prevent replication and spread of infectious agents. Many viruses, including influenza virus (77), Sindbis virus (51), human immunodeficiency virus (44), herpes simplex virus (16), chicken anemia virus (60), measles virus (24), reovirus (80), and varicella-zoster virus

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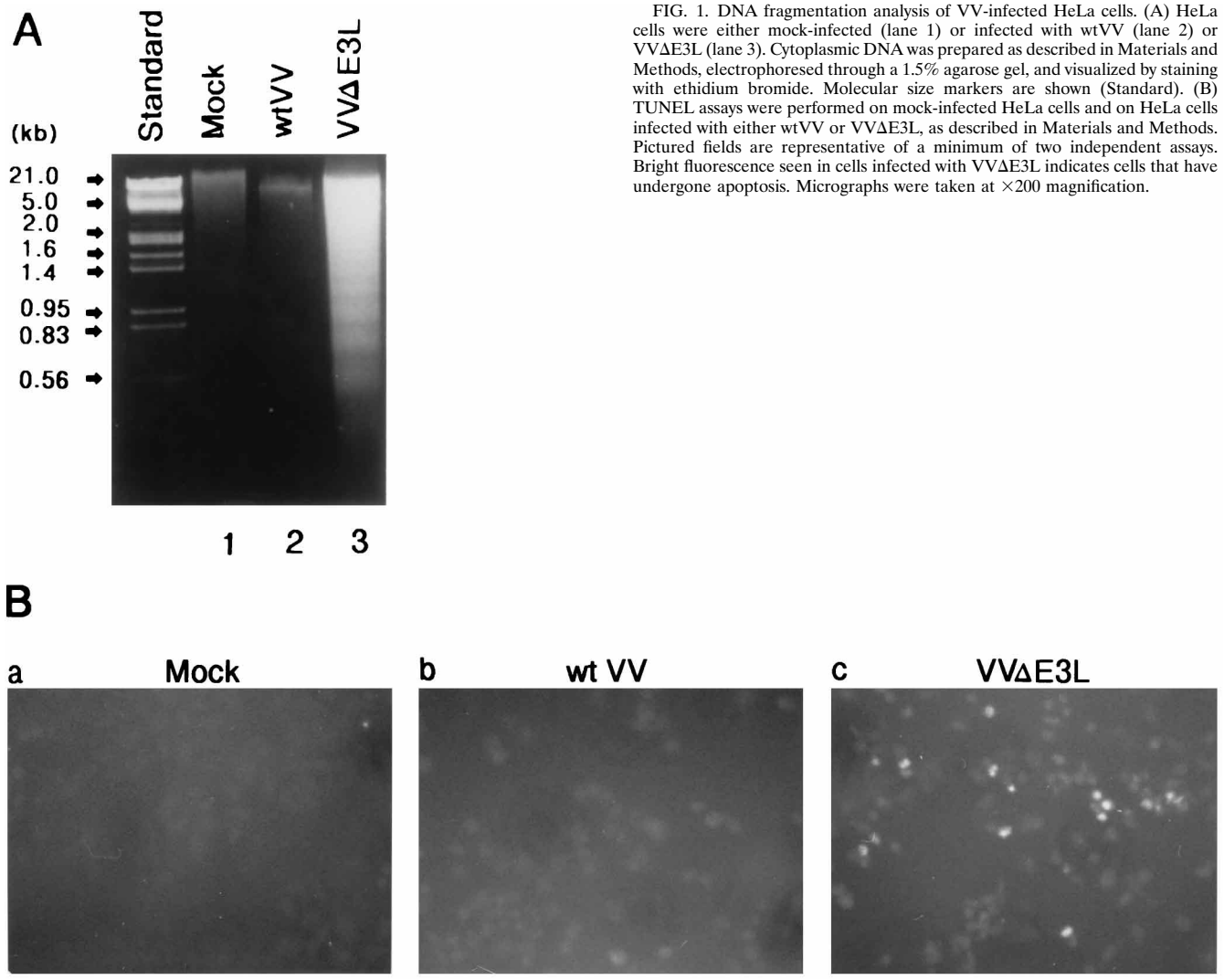

FIG. 1. DNA fragmentation analysis of VV-infected HeLa cells. (A) HeLa cells were either mock-infected (lane 1) or infected with wtVV (lane 2) or VVΔE3L (lane 3). Cytoplasmic DNA was prepared as described in Materials and Methods, electrophoresed through a 1.5% agarose gel, and visualized by staining with ethidium bromide. Molecular size markers are shown (Standard). (B) TUNEL assays were performed on mock-infected HeLa cells and on HeLa cells infected with either wtVV or VVΔE3L, as described in Materials and Methods. Pictured fields are representative of a minimum of two independent assays. Bright fluorescence seen in cells infected with VVΔE3L indicates cells that have undergone apoptosis. Micrographs were taken at ×200 magnification.

(68), are known to induce apoptosis in infected cells. Many other viruses have mechanisms to inhibit induction of apoptosis: two genes from the baculoviruses, *p35* and *iap*, inhibit apoptosis (11, 17, 18); the DNA tumor viruses adenovirus (26, 67, 75, 83), human papillomavirus (62), and simian virus 40 (63) inactivate the p53 pathway in order to block apoptosis; Epstein-Barr virus upregulates expression of *bcl-2* and codes for a *bcl-2* homolog (29, 30, 78); cowpox virus blocks apoptosis induced by Fas and by tumor necrosis factor alpha through expression of the *crmA* gene product, which is a protease inhibitor (79).

Though much has been learned about apoptosis and the circumstances that induce it, the pathways that induce apoptosis in virus-infected cells in many cases are still not clear. VVΔE3L has been reported to induce apoptosis in HeLa cells, while wild-type VV (wtVV) does not lead to apoptosis in HeLa cells (45). To determine the domain(s) of the E3L gene that is responsible for the inhibition of apoptosis, we used VV recombinants to express mutated E3L products. Analysis of the results showed that induction of apoptosis by mutant VV constructs correlated with the lack of a functional dsRNA binding protein. Further, we found that alteration of the level of dsRNA that is synthesized in VV-infected cells had a proportional effect on apoptosis: *ts*23, a temperature-sensitive mutant of VV, synthesizes excess dsRNA at restrictive temperature (5), and infection by this virus induced apoptosis at restrictive temperature only; treatment of HeLa cells with cytosine arabinoside (araC), which decreases the amount of dsRNA synthesized in VV-infected cells (19), resulted in a decrease in the number of apoptotic cells. Finally, we have shown that HeLa cells transfected with dsRNA independently of viral infection underwent apoptosis in an IFN-dependent manner, while cells transfected with ssRNA did not. These results suggest that it is dsRNA that initiates the apoptotic response to VV infection and that this cellular response is blocked in wtVV infections because the E3L gene products prevent a cellular protein-dsRNA interaction.

MATERIALS AND METHODS

Cells. RK-13 cells were maintained in minimum essential medium supplemented with 0.1 mM nonessential amino acids (GIBCO), 50 μg of gentamycin sulfate/ml, 292 μg of glutamate/ml (complete RK-13 medium), and 5% fetal bovine serum (FBS) (Hyclone). HeLa cells were maintained in Dulbecco's modified minimum essential medium (high glucose; GIBCO) supplemented with 50 μg of gentamycin sulfate/ml, 292 μg of glutamate/ml (complete HeLa medium), and 5% FBS (Hyclone). All cell lines were maintained in a 37°C, 5% CO_2 environment. IFN treatment was done 20 to 24 h prior to harvest: HeLa cells

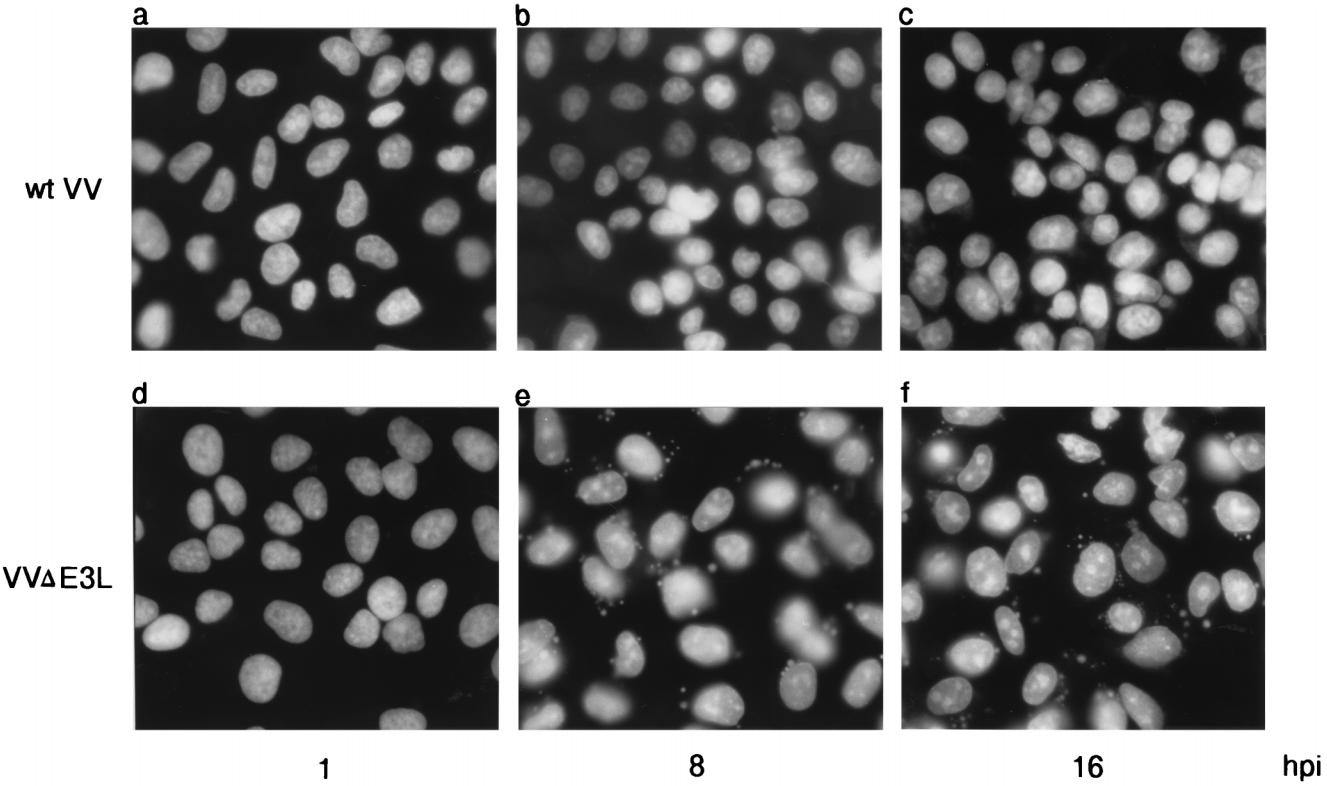

FIG. 2. Staining of infected HeLa cells with Hoechst dye. Cells were infected with either wtVV (panels a to c) or VVΔE3L (panels d to f) at an MOI of 5 PFU/cell. Cells were fixed at 1 (panels a and d), 8 (panels b and e), or 16 (panels c and f) h postinfection. Micrographs were taken at ×630 magnification.

were treated at 50% confluency with 1,000 U of human alpha IFN (IFN-α)/ml (Lee Biomolecular), and RK-13 cells were treated at 85% confluency with 1,000 U of rabbit IFN-α/ml (Lee Biomolecular).

Plasmids. The plasmid pMPE3ΔGPT (kindly provided by James Tartaglia, Virogenetics) was used for recombining genes into the E3L locus of VV vp1080. The plasmid is a derivative of pBSIISK that has had the β-galactosidase sequences (327 bp between the *Nae*I and *Kpn*I sites) deleted and that contains the left and right arms flanking the VV E3L gene but lacks the E3L gene itself. The deletion junction contains unique *Pst*I, *Sma*I, and *Bam*HI sites for the insertion of foreign genes. The plasmid also contains an *ecogpt* gene whose expression is driven by an entomopox virus 42-kDa gene promoter inserted at a unique *Xho*I site at the pBS/VV left arm junction, allowing for selection of transfected cells by treatment with mycophenolic acid. pMPE3ΔGPT was altered by the addition of a multiple cloning site inserted into the existing unique *Pst*I and *Bam*HI sites via a double-stranded synthetic oligonucleotide with overhanging ends (5′GATCC AAAAAGCTTAAAGTCAACAAGTCGACAACTGCA3′ and 3′GTTTTTCG AATTTCAGTTGTTCAGCTGTTG5′), creating pMPE3ΔGPTMCS. The multiple cloning site includes unique *Sal*I, *Hin*cII, and *Hin*dIII sites. The E3L mutant genes Δ7C, Δ26C, and G164V (13) were removed from pBSIIKS+ by treatment with *Bam*HI and *Hin*dIII and subcloned into pMPE3ΔGPTMCS by ligation into the same sites. The porcine group C rotavirus p8 gene was obtained by PCR from pMT-NSP3 (43) by using primers that added *Bam*HI sites at both ends of the gene. *Bam*HI-restricted products were cloned into *Bam*HI-restricted pMPE3ΔGPT.

Transfections and in vivo recombinations. In vivo recombination was performed in rabbit kidney RK-13 cells. Transfections were performed via LipofectACE (GIBCO) per the manufacturer's directions, in conjunction with infections. Confluent RK-13 cells were infected at a multiplicity of infection (MOI) of 10 with E3L-deleted VV. VVΔE3L [formerly called vP1080(6)] contains a *lacZ* gene in the locus at which E3L was deleted, resulting in the formation of blue plaques by this virus. After 30 min, 2 ml of antibiotic-free complete medium plus 2% FBS was added to cells which were then placed in a CO_2 incubator at 37°C during preparation of the DNA to be transfected. DNA was mixed with LipofectACE (GIBCO) according to the manufacturer's protocol, and the mixture was added to the cells. The cells were incubated at 37°C for 36 h to allow recombination between the plasmid and virus to take place; the cells were then harvested, centrifuged for 10 min at 500 × *g* at 4°C, and resuspended in 1 ml of complete medium containing 2% FBS.

Selection of recombinants. Virus was extracted from transfected/infected cells by three rounds of freezing and thawing and used to infect confluent RK-13 cells that had been pretreated for 6 h with *ecogpt* selection medium: 40 ml of complete medium containing 2% FBS, 80 μg of mycophenolic acid (MPA), 10 mg of xanthine, and 600 μg of hypoxanthine. After a 1-h infection, 5 ml of *ecogpt* selection medium was added to the plates and cells were allowed to grow at 37°C until plaques were clearly visible (2 to 4 days). The cells were then harvested by being scraped into their growth medium, centrifuged for 10 min, resuspended in 1 ml of complete medium containing 2% FBS, and subjected to three rounds of freeze-thaw. This virus stock (0.3 ml) was then used to infect a 100-mm-diameter plate of confluent RK-13 cells which had been pretreated for 6 h with *ecogpt* selection medium. Again, cells were incubated at 37°C in *ecogpt* selection medium, and after the appearance of plaques, the cells were harvested, centrifuged, and resuspended as before. Following the freeze-thaw procedure, 200 μl was used to infect 100-mm plates of confluent RK-13 cells, which were then incubated in complete medium containing 2% FBS (no selection medium). During this incubation, a second recombination event takes place, which results in either recovery of the original virus (VVΔE3L) or of recombinant virus containing the inserted gene of interest. Two days after this infection, the medium was replaced with an overlay containing 0.5% agarose, 0.3% X-Gal (5-bromo-4-chloro-3-indolyl-β-galactoside), 0.004% neutral red, and 2% FBS in complete medium. Ten colorless plaques were picked from the overlaid plate and suspended in 1 ml of complete medium containing 2% FBS. Colorless plaques indicate the presence of virus containing the gene of interest (the *lacZ* gene has been replaced by the gene of interest).

Virus stocks. VC-2 (wtVV, Copenhagen strain), vp1080 (E3L-deleted VV), vp872 (K3L-deleted VV), and vp1112 (VV recombinant containing the S4 segment of reovirus [6]) were kindly provided by James Tartaglia, Virogenetics. Subsequent stocks of vp1080 were grown in CEF cells (chicken embryo fibroblasts). Recombinant virus stocks were grown in either RK-13 cells or CEFs according to the following procedure: plaques were suspended in 1 ml of complete medium containing 2% FBS, and titers were determined in RK-13 cells. The plaque suspension was then used to infect a 60-mm plate of confluent RK-13 cells at an MOI of 0.01 or a 100-mm plate of CEFs at an MOI of 0.1, and the cells were allowed to reach full cytopathic effect. Cells were then harvested, centrifuged at 500 × *g* for 10 min at 4°C, washed with cold phosphate-buffered saline (PBS), resuspended in 1 ml complete medium containing 2% FBS, and subjected to three rounds of freeze/thaw.

Agarose gel apoptosis assays. Apoptosis assays were performed on uninfected HeLa cells and cells infected with variants of VV at an MOI of 1 to 5. HeLa cells were harvested at 24 h postinfection or 16 h posttransfection, and assays were performed as described by Lee and Esteban (45), with the following modifica-

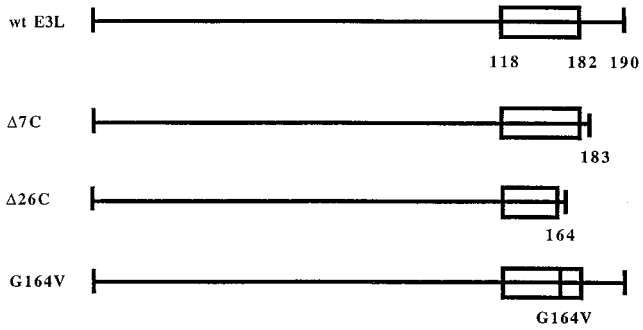

FIG. 3. Schematic diagram of mutant E3L gene products. Boxed region shows the position of the dsRNA binding domain (amino acids 118 to 182). For mutants of E3L the positions of the mutations are as indicated.

tions. Extracts were deproteinated by brief extraction with phenol-chloroform at room temperature; after precipitation with ethanol, DNA was resuspended in 19 μl of TE (Tris-EDTA) and treated with RNase (final concentration of 1 mg/ml) for 2 min at room temperature. DNA was resolved in a 1.5% agarose gel and visualized with ethidium bromide.

TUNEL assays. Terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) assays were performed on HeLa cells infected with variants of VV at an MOI of 1 to 5 and were carried out according to a modification of the manufacturer's protocol (Oncor; ApopTag In Situ Apoptosis CAP Detection kit, fluorescein). Briefly, cells were grown to 50% confluency in 35-mm plates (Nunc), infected for 1 h, and incubated for an additional 11 h; cells were fixed in the plates with 2% formaldehyde and postfixed with 70% ethanol. Fixed cells were treated with terminal transferase (4.8 U/plate) to attach deoxyuridines to 3′ OH groups of DNA. The deoxyuridines are covalently linked to digoxigenin molecules. The cells were then incubated with digoxigenin antibodies that had been conjugated to fluorescein. After the final washes with PBS, the cells were sealed with a glass coverslip and photographed under a fluorescence microscope.

Hoechst dye staining. HeLa cells were grown to near confluency in 8-well tissue culture slides (Nunc) and infected with either wtVV or VVΔE3L at an MOI of 5 PFU/cell. Cells were washed and fixed at 1, 8, or 16 h postinfection as described for TUNEL assays. The fixed cells were stained with 10 μg/ml of Hoechst 33258 in PBS for 10 min at room temperature. Cells were washed with PBS, and coverslips were mounted in 50% glycerol in PBS. Staining was visualized as described for TUNEL assays.

dsRNA transfection. HeLa cells were transfected with either dsRNA [poly(rI)-poly(rC)] or ssRNA [poly(rA)] by the calcium phosphate method (2). Briefly, 35 μg of dsRNA or ssRNA in 500 μl of 250 mM $CaCl_2$ was added dropwise to an equal volume of 2× Hanks balanced salt solution (HBS), pH 7.05, while continually applying a gentle air stream to the surface of the 2× HBS. After allowing the mixture to stand undisturbed for 10 to 20 min, the solution was layered under the surface of the complete growth medium containing 5% FBS (Hyclone), covering a just-confluent monolayer of HeLa cells. After 16 h the cells were harvested and assayed as described for infected cells. Cells were either pretreated for 24 h with 1,000 U of human IFN-α/ml or were untreated.

PKR autophosphorylation assay. Cell extracts were prepared as described previously (40). PKR was purified by affinity chromatography on dsRNA-agarose (42). An equal volume of buffer A (100 mM KCl, 1 mM benzamidine, 10% glycerol, 20 mM HEPES [pH 7.5], 5 mM MgOAc, 5 mM $MnCl_2$, 1 mM dithiothreitol) containing 100 μM [γ-^{32}P]ATP at 1.0 Ci/mmol (Du Pont-New England Nuclear) was added to the resin, and the reaction mixture was incubated at 30°C for 10 min. The ^{32}P-labeled PKR was analyzed by standard sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography.

RESULTS

The VV E3L gene has been previously reported to inhibit induction of apoptosis in VV-infected HeLa cells (45). We confirmed this result with an agarose gel apoptosis assay of cytoplasmic DNA extracts from cells infected by wtVV, E3L-deleted VV (VVΔE3L), or K3L-deleted VV (VVΔK3L). The K3L gene products share sequence homology to the α subunit of protein synthesis initiation factor eIF-2; these proteins reduce phosphorylation of the α subunit of eIF-2 by at least 10-fold when expressed transiently (20, 21, 34). Extracts from cells infected with VVΔE3L exhibited the characteristic nucleosome-sized banding pattern, indicating the presence of fragmented DNA in the cytoplasm (Fig. 1A, lane 3). Extracts from mock-infected cells (lane 1), wtVV-infected cells (lane 2), and VVΔK3L-infected cells (data not shown) showed no detectable DNA fragmentation. The TUNEL assay shown in Fig. 1B utilizes fluorescein-linked digoxigenin antibodies to detect the location of deoxyuridine residues that have been attached by terminal transferase to free 3′ OH groups of DNA (see Materials and Methods). Therefore, brightly fluorescing cells indicate the presence of fragmented DNA. Only the cells infected with VVΔE3L include many brightly fluorescing cells (Fig. 1B, panel c) indicative of apoptotic cells: few such cells were detected in mock-infected cells (Fig. 1B, panel a) or cells infected with wtVV (Fig. 1B, panel b). Finally, we have stained infected cells with the DNA-intercalating dye Hoechst 33258. As seen in Fig. 2, cytoplasmic vacuoles stained heavily with Hoechst dye in cells infected with VVΔE3L at either 8 (panel e) or 16 (panel f) h postinfection. Extranuclear staining with Hoechst dye is indicative of apoptosis (3). Very few cells infected with either wtVV (panels a to c) or with VVΔE3L at 1 h postinfection (panel d) showed evidence of cytoplasmic staining. We have quantitated these results by counting the number of apoptotic cells in 250 to 600 cells. In cells infected with wtVV, 1 to 6% of the cells showed signs of apoptosis. In cells infected with VVΔE3L, 82 and 77% of the cells showed clear signs of apoptosis at 8 and 16 h postinfection, respectively, while only 3% of the cells showed signs of apoptosis at 1 h postinfection. These results confirm that the E3L gene product is necessary to inhibit apoptosis in VV-infected HeLa cells.

To determine which domains of the E3L gene product are necessary and sufficient to inhibit apoptosis, VV recombinants containing various E3L mutant genes were constructed. The mutant E3L genes were inserted into E3L-deleted VV, using in vivo recombination with the plasmid pMPE3ΔGPTMCS. This procedure was used to construct VVE3LΔ7C (VV containing an E3L gene with the 7 C-terminal amino acids deleted), VVE3LΔ26C (VV containing an E3L gene with the 26 C-terminal amino acids deleted), and VVE3LG164V (VV containing an E3L gene with glycine 164 mutated to valine) (Fig. 3). Transient transfections done previously (14) have shown that carboxyl-terminal mutations either failed to bind to dsRNA and failed to rescue replication (E3LΔ26C and E3LG164V) or bound to dsRNA with reduced affinity and rescued virus replication only poorly (E3LΔ7C) (13).

To determine if a correlation existed between the E3L product's ability to bind to dsRNA and the induction of apoptosis, we first tested the virus constructs containing mutations in the carboxy terminus of the E3L product. As shown previously (13), E3LΔ7C has deletions to the carboxy boundary of the binding domain and binds only weakly to dsRNA (Table 1). E3LΔ26C terminates within the binding domain and does not

TABLE 1. Binding activity

Virus	dsRNA binding
wtVV	+[a]
VVΔE3L	−[a]
VVE3LΔ7C	+/−[a]
VVE3LΔ26C	−[a]
VVE3LG164V	−[a]
VVΔE3L/p8	+[b]
VVΔE3L/S4	+[c]

[a] Data from reference 13.
[b] Data from reference 43.
[c] Data from reference 31.

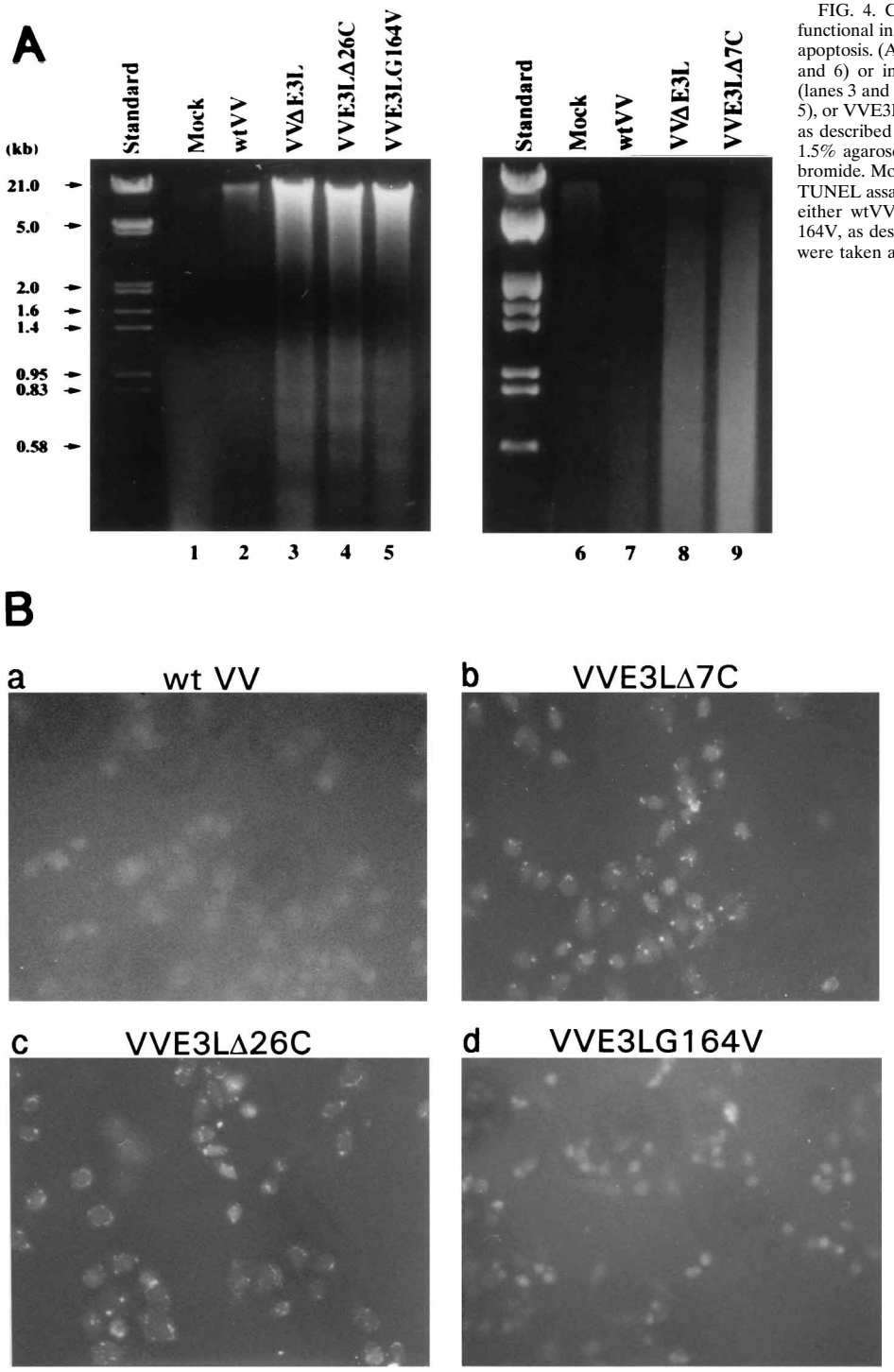

FIG. 4. C-terminal mutant products of E3L that are not functional in dsRNA binding also fail to inhibit induction of apoptosis. (A) HeLa cells were either mock infected (lanes 1 and 6) or infected with wtVV (lanes 2 and 7), VVΔE3L (lanes 3 and 8), VVE3LΔ26C (lane 4), VVE3LG164V (lane 5), or VVE3LΔ7C (lane 9). Cytoplasmic DNA was prepared as described in Materials and Methods, electrophoresed in 1.5% agarose gel, and visualized by staining with ethidium bromide. Molecular size markers are shown (Standard). (B) TUNEL assays were performed on HeLa cells infected with either wtVV or VVE3LΔ7C, VVE3LΔ26C, or VVE3LG 164V, as described in Materials and Methods. Micrographs were taken at ×200 magnification.

bind to dsRNA at all (13) (Table 1). In the E3LG164V mutant, the glycine at residue 164, which is conserved among all the proteins that contain the conserved binding domain, has been mutated to a valine. This point mutation completely destroys the protein's ability to bind to dsRNA (13) (Table 1). Each of these three mutant E3L products fails to prevent activation of PKR in vitro (13). The results shown in Fig. 4 suggest that expression of E3L products that exhibit altered ability to bind to dsRNA correlated with the failure to inhibit induction of apoptosis. In the agarose gel assay (Fig. 4A), extracts from cells infected with VVE3LΔ7C (lane 9), VVE3LΔ26C (lane 4), and VVE3LG164V (lane 5) all clearly contain fragmented DNA. As seen in the TUNEL assay in Fig. 4B, cells infected with any of these three recombinant viruses (panels b, c, and d) include many brightly fluorescing cells, comparable to what is seen in cells infected by virus with the E3L gene deleted.

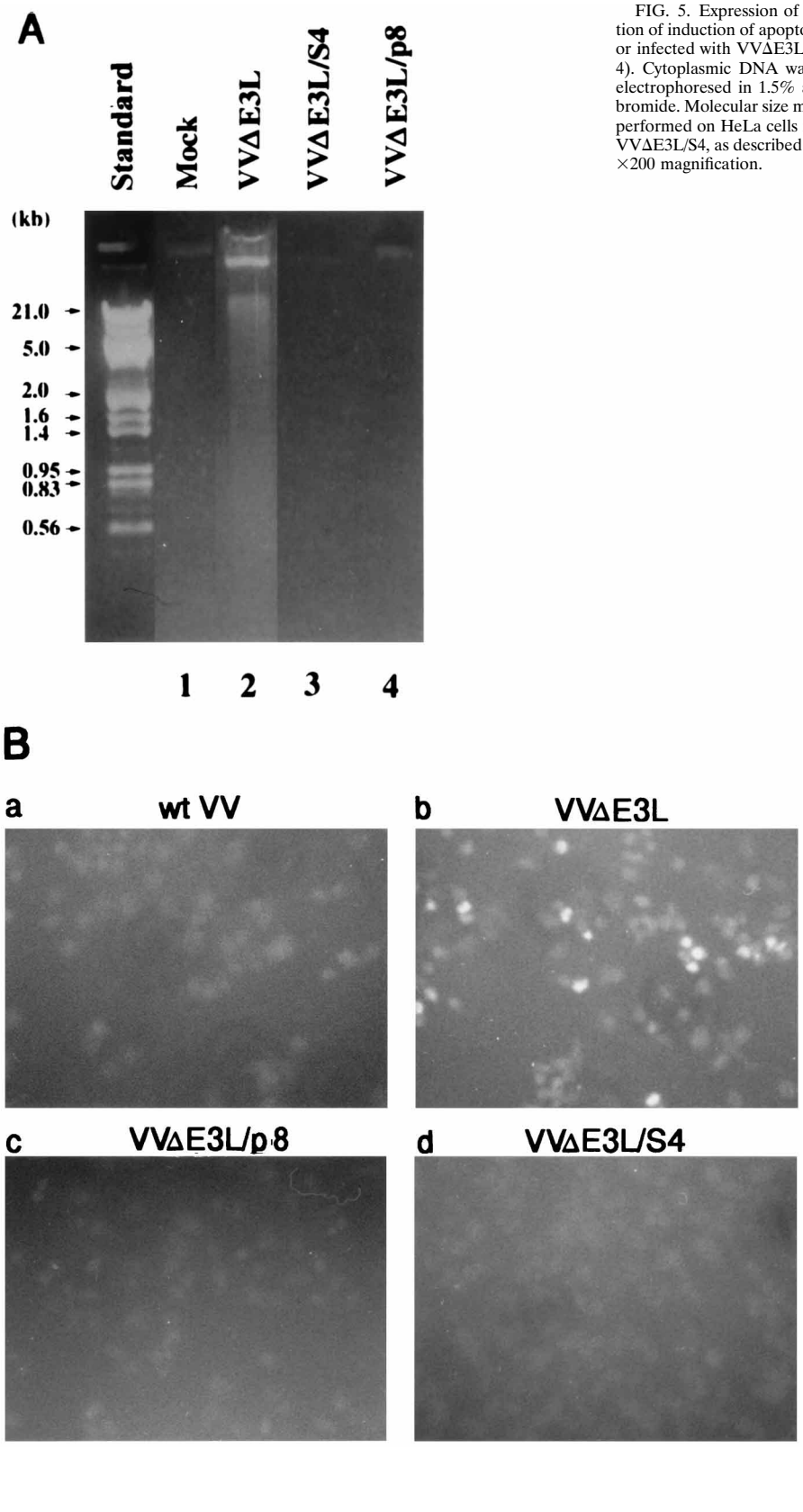

FIG. 5. Expression of alternative dsRNA binding proteins results in inhibition of induction of apoptosis. (A) HeLa cells were either mock infected (lane 1) or infected with VVΔE3L (lane 2), VVΔE3L/S4 (lane 3), or VVΔE3L/p8 (lane 4). Cytoplasmic DNA was prepared as described in Materials and Methods, electrophoresed in 1.5% agarose gel, and visualized by staining with ethidium bromide. Molecular size markers are shown (Standard). (B) TUNEL assays were performed on HeLa cells infected with either wtVV, VVΔE3L, VVΔE3L/p8, or VVΔE3L/S4, as described in Materials and Methods. Micrographs were taken at ×200 magnification.

To further test the importance of the presence of a functional dsRNA binding protein in inhibition of apoptosis, we used virus constructs expressing alternative dsRNA binding proteins. VVΔE3L/p8 has the E3L gene deleted but it contains a gene coding for the C-terminal (p8) portion of the porcine rotavirus group C NSP3 protein. This protein contains the conserved dsRNA binding domain found in the E3L product and at 69 amino acids is the smallest known natural protein to bind to dsRNA (43) (Table 1). VVΔE3L/S4 also has the E3L gene deleted, but it contains the reovirus S4 gene (6). The S4

gene product, σ3, shares no significant homology with the dsRNA binding domain found in E3L but has been shown to bind specifically to dsRNA (31) (Table 1). Both of these virus constructs inhibited the induction of apoptosis (Fig. 5A, lanes 3 and 4, and Fig. 5B, panels c and d).

These results suggest that dsRNA may be responsible for inducing apoptosis in HeLa cells. Further evidence in support of this conclusion was obtained from utilizing conditions that either increased or decreased the level of dsRNA synthesized during a VV infection. Bayliss and Condit (5) have shown that a mutant in the VV *ts*23 complementation group synthesizes excess dsRNA at the restrictive temperature. We have analyzed induction of apoptosis in cells infected with wtVV, VVΔE3L, VV*ts*23, and in mock-infected cells, at permissive (31°C) and restrictive (39°C) temperatures. Neither mock-infected cells (Fig. 6A, lanes 7 and 8) nor cells infected with wtVV (Fig. 6A, lanes 3 and 4, and Fig. 6B, panels e and f) showed evidence of apoptosis, using either the agarose gel assay or the TUNEL assay at either 31 or 39°C. Cells infected with VVΔE3L (Fig. 6A, lanes 5 and 6, and Fig. 6B, panels c and d) showed evidence of apoptosis at both temperatures. Cells infected with *ts*23 showed evidence of apoptosis by either assay at 39°C (Fig. 6A, lane 2, and Fig. 6B, panel a) but not at 31°C (Fig. 6A, lane 1, and Fig. 6B, panel b), consistent with induction of apoptosis by the increased dsRNA synthesized at the restrictive temperature. In contrast to the case with VV*ts*23, a decrease in dsRNA levels can be attained by treatment of the cells with araC (19), which prevents DNA synthesis. VV requires DNA synthesis to initiate transcription of late genes. It is during transcription of late genes that the majority of dsRNA is generated, so that there is an 85% reduction in dsRNA synthesis in HeLa cells treated with araC (19). Cells infected with VVΔE3L in the presence of araC (Fig. 6C, panel d) showed less evidence of apoptosis than cells infected in the absence of araC (Fig. 6C, panel c), suggesting that the reduced level of dsRNA is reducing the apoptotic response by the cells. araC had no effect on apoptosis in mock-infected (Fig. 6C, panels a and b) or wild-type-infected (Fig. 6C, panels e and f) cells.

To directly test if dsRNA could induce apoptosis, dsRNA was transfected into HeLa cells and extracts were assayed for DNA fragmentation. As can be seen in Fig. 7, transfection of dsRNA in the absence of viral infection (lanes 2, 3, 5, 6, 8, and 9) induced apoptosis in HeLa cells (most easily seen in lanes 8 and 9). This effect is not seen with ssRNA (lanes 1, 4, and 7). Pretreatment of the cells with IFN resulted in increased DNA fragmentation in cells transfected with dsRNA (lanes 3, 6, and 9), as evidenced by the extended laddering effect seen on the agarose gel (compare lanes 8 and 9). This suggests that dsRNA-induced apoptosis occurs via an IFN-induced, dsRNA-dependent protein, possibly PKR.

Since each of the treatments that we have described that induces apoptosis in HeLa cells (infection with VVΔE3L or with VV*ts*23 at restrictive temperature and transfection with dsRNA) inhibits protein synthesis in treated cells, we were concerned that induction of apoptosis was simply a response to inhibition of protein synthesis. To test this hypothesis, we treated HeLa cells with a concentration of cycloheximide that inhibits HeLa cell protein synthesis (86) and assayed induction of apoptosis in uninfected cells and cells infected with wtVV and VVΔE3L (Fig. 8). Not only did cycloheximide treatment not induce apoptosis in uninfected cells (compare panels a and b), it decreased the number of apoptosing cells in a culture infected with VVΔE3L (compare panels c and d). These results suggest that the dsRNA-mediated induction of apoptosis is not simply due to an inhibition of protein synthesis and that in virus-infected cells protein synthesis is required for induction of apoptosis.

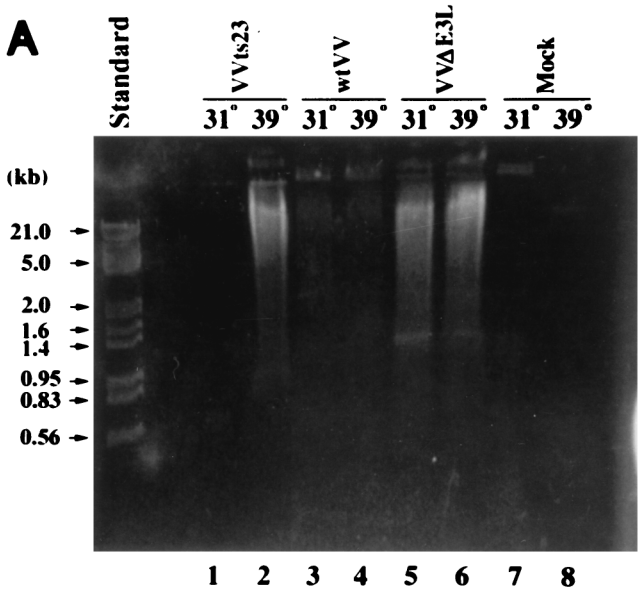

FIG. 6. Alteration of the level of dsRNA present in VV-infected HeLa cells and the resulting effect on induction of apoptosis. (A and B) HeLa cells were mock infected or infected with VV*ts*23, wtVV, or VVΔE3L. Cells for the odd-numbered lanes of panel A, and for fields b, d, and f of panel B were maintained at the permissive temperature for VV*ts*23 (31°C), while cells for the even-numbered lanes of panel A, and fields a, c, and e of panel B were maintained at the restrictive temperature for VV*ts*23 (39°C). Panel A is an agarose gel assay for cytoplasmic DNA, while panel B is a TUNEL assay for DNA fragmentation. (C) HeLa cells were either mock-infected or infected with VVΔE3L or wtVV and maintained in the absence or presence of araC. Fragmented DNA was visualized with a TUNEL assay. Micrographs were taken at ×200 magnification.

As mentioned previously, VVΔE3L does not replicate in HeLa cells (6, 7, 14) and infection by this virus resulted in apoptosis in HeLa cells. VVΔE3L does replicate in rabbit kidney RK-13 cells, but it is sensitive to the effects of IFN in these cells. This host range phenotype may be the result of differing levels of PKR activity in these cells, leading to differing levels of induction of apoptosis. To test this hypothesis, we prepared detergent lysates from both RK-13 and HeLa cells which had been either pretreated or not pretreated with IFN. PKR was isolated from the extracts by binding to dsRNA agarose. The bound PKR was phosphorylated with [γ-^{32}P]ATP, eluted with SDS sample buffer and boiling, and electrophoresed by SDS-PAGE. Figure 9A, lane 3, shows that in the absence of pretreatment with IFN, PKR activity could not be detected in extracts from RK-13 cells. Pretreatment with IFN induced PKR activity (lane 4) to a detectable level. Therefore, we predicted that in the absence of IFN treatment VVΔE3L would induce only low levels of apoptosis in RK-13 cells and that induction of apoptosis would be inducible by IFN treatment of these cells. This was the case, as shown in Fig. 9B, lanes 4 and 5. In contrast, in HeLa cells, which contain high levels of PKR activity even in the absence of IFN pretreatment, VVΔE3L induced apoptosis to a similar extent in treated and untreated cells (Fig. 9B, lanes 1 and 2). Since both IFN treatment and virus infection were necessary for induction of maximal apoptosis in RK-13 cells, the results again suggest that the cellular apoptotic response was prompted by the presence of dsRNA, via an IFN-induced dsRNA-dependent protein.

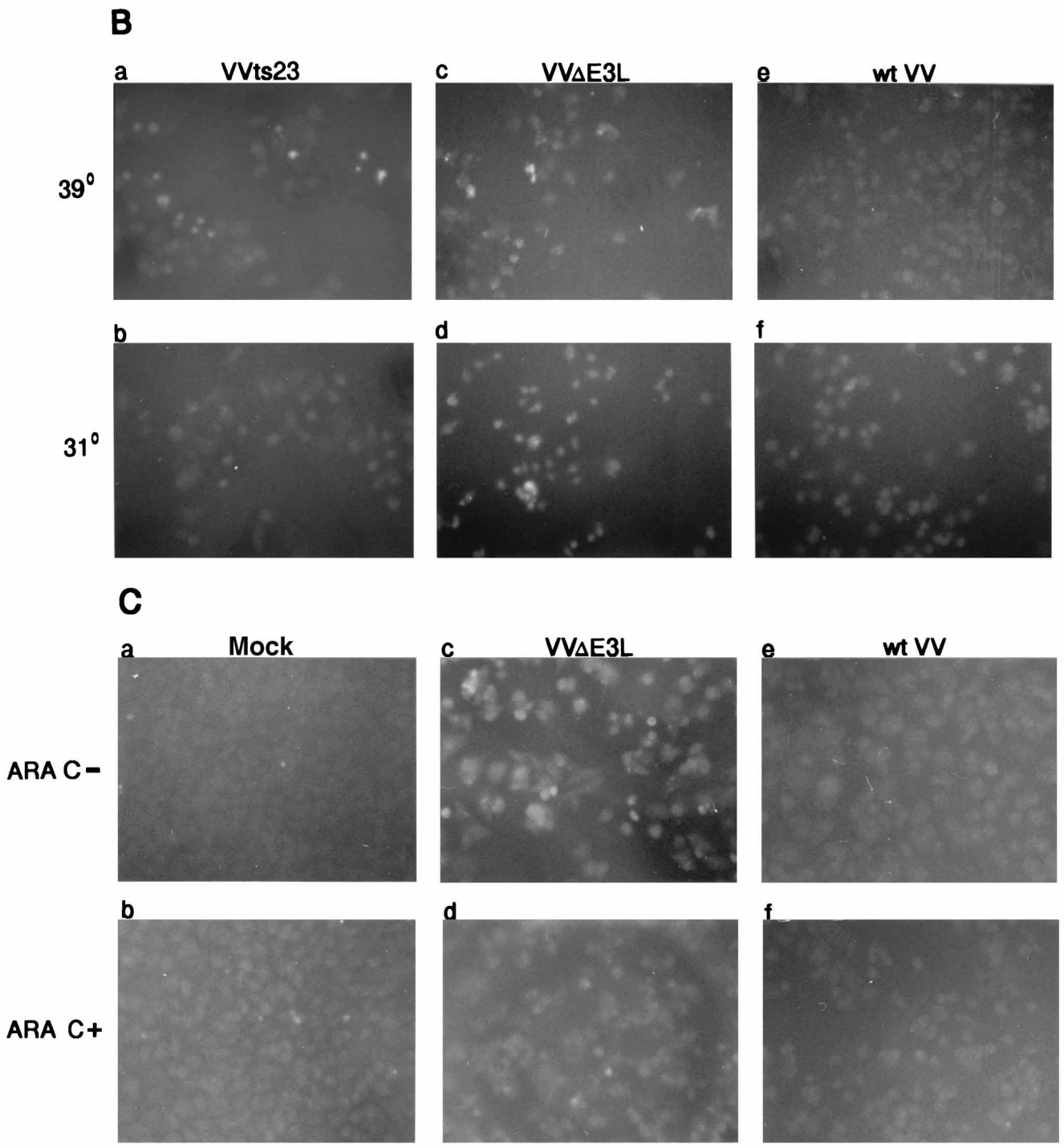

FIG. 6—*Continued.*

DISCUSSION

Previous studies have shown that HeLa cells infected by E3L-deleted VV undergo apoptosis, while cells infected by wtVV do not (45). One known function of the E3L gene products is binding to dsRNA. These dsRNA binding proteins have been shown to prevent activation of PKR and 2′-5′A synthetase in VV-infected cells (6), presumably by sequestering viral dsRNA and making it unavailable to activate these enzymes. This study characterizes the role of viral dsRNA in induction of apoptosis in VV-infected cells. The results reported here provide three lines of evidence that viral dsRNA is in fact the factor responsible for induction of apoptosis in VV-infected cells. First, in each of the mutants tested, the absence of apoptosis correlated with the presence of a functional dsRNA binding protein. The second line of support is the correlation between increased synthesis of dsRNA by the

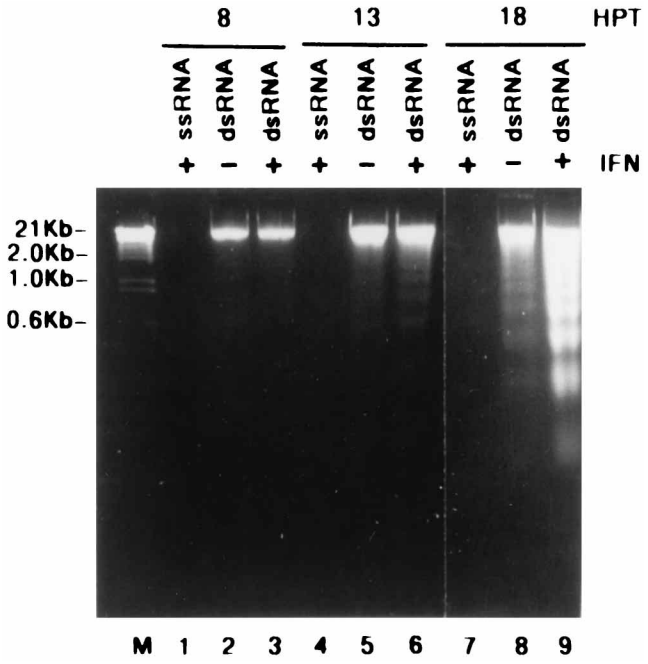

FIG. 7. dsRNA induces apoptosis in HeLa cells in the absence of viral infection. HeLa cells were either treated with IFN (lanes 1, 3, 4, 6, 7, and 9) or mock treated (lanes 2, 5, and 8) as described in Materials and Methods. Cells were transfected with dsRNA (lanes 2, 3, 5, 6, 8, and 9) or ssRNA (lanes 1, 4, and 7). After 16 h, cytoplasmic DNA was prepared as described in Materials and Methods, electrophoresed in 1.5% agarose gel, and visualized by staining with ethidium bromide. Molecular size markers are shown (lane M).

*ts*23 mutant virus (5), which resulted in apoptosis at the restrictive temperature only, and the decreased synthesis of dsRNA in the araC-treated, VVΔE3L-infected cells (19), which resulted in decreased apoptosis. Third, transfection of dsRNA into HeLa cells, in the absence of virus infection, led to induction of apoptosis in a dsRNA-inducible manner.

Our results confirm the previously reported results that the VV E3L gene products block induction of apoptosis in VV-infected HeLa cells and extend those results to determine what functions of the E3L gene products are responsible for inhibition of induction of apoptosis. Clearly, the dsRNA binding function of the carboxy terminus of the E3L gene products is required for inhibition of induction of apoptosis. Deletion of seven C-terminal amino acids led to a protein that binds dsRNA only weakly (13) and that rescues replication of VVΔE3L in *trans* only weakly (14). Also, VVE3LΔ7C does not replicate in HeLa cells and does not inhibit induction of apoptosis in infected HeLa cells. In fact, among all of the genes that we have inserted into VV we have found that only genes encoding a functional dsRNA-binding protein inhibit induction of apoptosis.

All of our results are consistent with a model in which a cytoplasmic dsRNA-activated enzyme, perhaps PKR, plays a key role in signalling induction of cell death. PKR is further implicated by the host cell specificity of induction of apoptosis. HeLa cells contain constitutively high levels of PKR activity, and infection with E3L-deleted VV induces apoptosis even without IFN treatment. RK-13 cells, on the other hand, do not contain detectable PKR activity in the absence of IFN treatment, and infection with E3L-deleted VV leads to apoptosis only after IFN treatment. The low levels of PKR activity in untreated RK-13 cells may be either the consequence of low levels of PKR expression in these cells or the presence of a PKR inhibitor in these cells. Our preliminary results suggest that RK-13 cells may in fact contain an inhibitor of PKR, since extracts of RK-13 cells decreased PKR activity when mixed with extracts from HeLa cells (41).

PKR has already been implicated in several signalling pathways. Activated PKR has been shown to have tumor suppressor function: expression of catalytically inactive mutants of

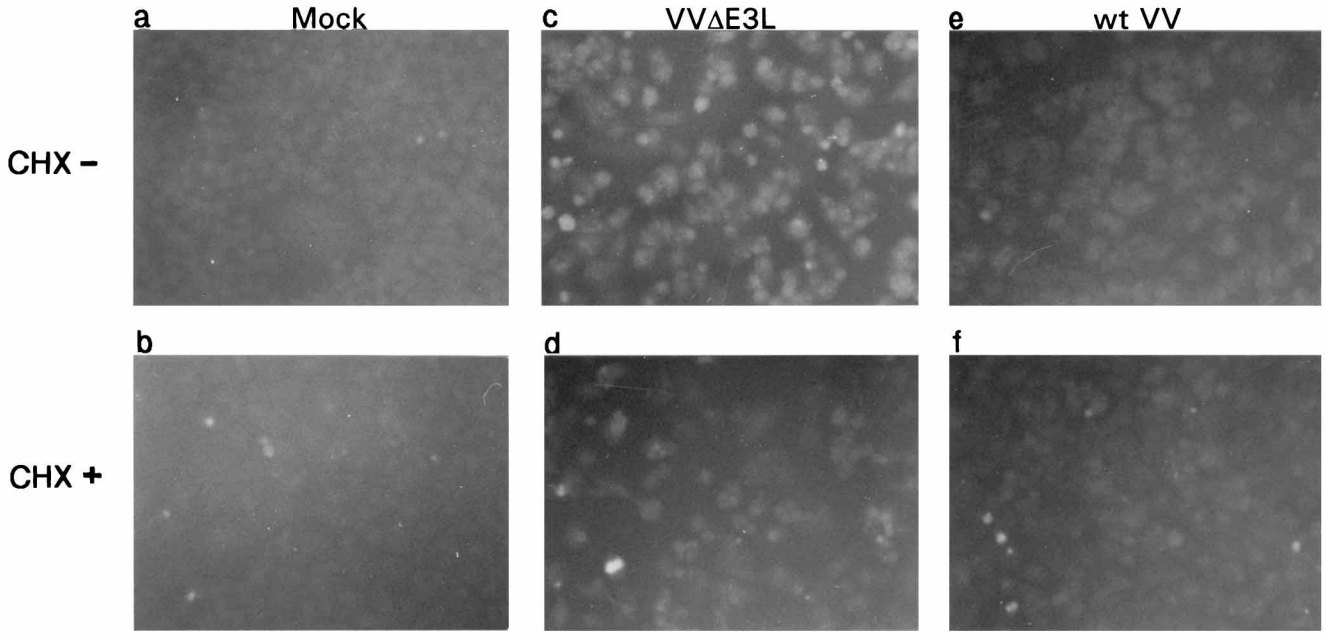

FIG. 8. Treatment of cells with cycloheximide does not induce apoptosis. HeLa cells were treated with 50 μg of cycloheximide per ml (CHX +) or left untreated (CHX −). Cells were mock infected or infected with VVΔE3L or wtVV. TUNEL assays were performed as described in Materials and Methods. Micrographs were taken at ×200 magnification.

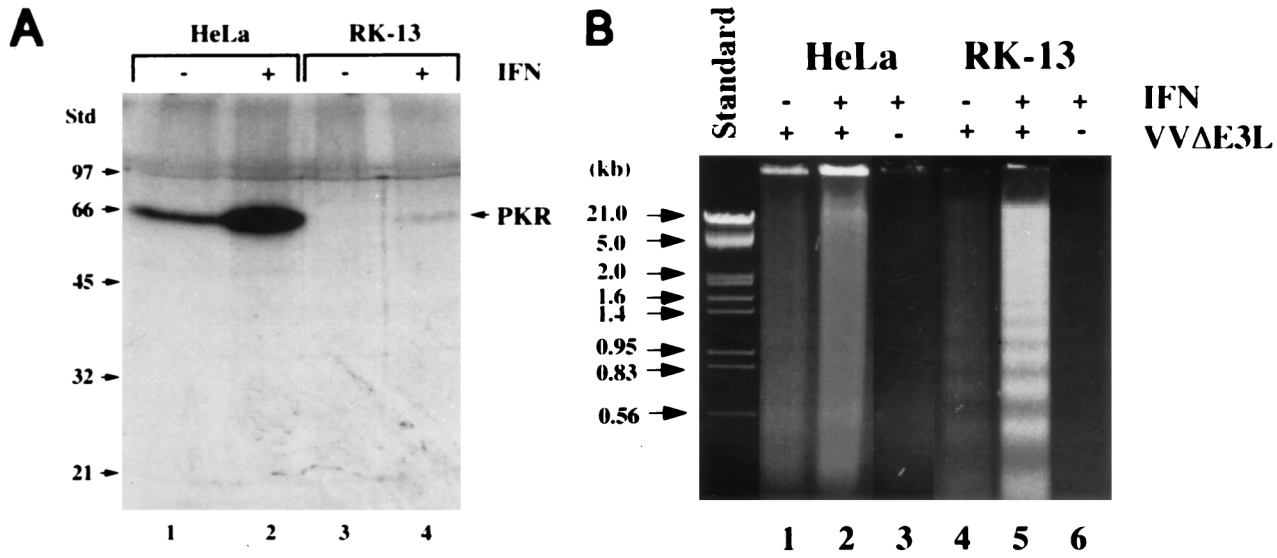

FIG. 9. Pretreatment of RK-13 cells with IFN induces PKR and increases the level of induction of apoptosis by VVΔE3L. (A) Autophosphorylation of PKR bound to poly(rI)-poly(rC) agarose was assayed in extracts prepared from IFN-treated (lanes 2 and 4) or mock-treated (lanes 1 and 3) HeLa cells (lanes 1 and 2) and RK-13 cells (lanes 3 and 4). Labeled proteins were resolved by SDS-PAGE and visualized by autoradiography. Molecular size markers (in kilodaltons) are shown. (B) HeLa cells (lanes 1 to 3) and RK-13 cells (lanes 4 to 6) were pretreated with IFN (lanes 2, 3, 5, and 6) or left untreated (lanes 1 and 4) and were either mock infected (lanes 3 and 6) or infected with VVΔE3L (lanes 1, 2, 4, and 5). Cytoplasmic DNA was prepared as described in Materials and Methods, electrophoresed in 1.5% agarose gel, and visualized by staining with ethidium bromide. Molecular size markers are shown (Standard).

PKR results in malignant transformation of NIH 3T3 cells (57). Transformation is possibly the result of PKR mutant inhibition of eIF-2α phosphorylation (23). Expression of functional PKR in VV led to induction of apoptosis in infected cells (45). PKR has been implicated in Fas antigen gene activation by influenza virus (76, 77). The transcription factor NF-κB is activated by PKR (39, 53, 61). Koromilas et al. (38) found that PKR mediated the transcriptional activation of the immunoglobulin κ gene when a pre-B-lymphoma cell line was treated with either lipopolysaccharide or IFN-γ. These results are consistent with the finding that tumor growth factor-β inhibited both PKR expression and activity (69) and inhibited lipopolysaccharide-induced transcriptional activation of the κ gene (38). Activated PKR has also been reported to be an essential component of the platelet-derived growth factor signal transduction pathway, which leads to transcriptional activation of several growth regulation genes (58).

Whether or not the apoptosis signal is mediated by PKR, the initiator of the signal appears to be dsRNA. Since most viruses induce the synthesis of dsRNA at some time during their life cycle (33) it is possible that this is a major pathway that organisms have evolved to limit virus infection in the organism. Nonetheless, clearly there are examples of virus-induced apoptosis that do not involve dsRNA. Human immunodeficiency virus appears to induce apoptosis through CD4-Env interactions (52). The Fas antigen pathway has been implicated in influenza virus-induced apoptosis (76, 77). In fact, Takizawa et al. (76) concluded that dsRNA was likely not the inducer of apoptosis in influenza virus-infected cells, since addition of synthetic dsRNA to the medium did not induce apoptosis. However, under our similar experimental conditions, dsRNA simply added to the medium rather than transfected into the cells did not induce apoptosis in HeLa cells either (data not shown). The adenovirus E1A gene product is known to induce apoptosis, and induction is blocked by the E1B gene products (65). The reovirus attachment protein σ1 has been shown to be required for induction of apoptosis and may be responsible for the differences in pathogenicity of various strains (80).

In each of the cases mentioned above, the virus also has a mechanism to inhibit activation or action of PKR, perhaps inhibiting dsRNA-mediated induction of apoptosis. McMillan et al. (56) reported that human immunodeficiency virus type 1 Tat directly interacts with PKR and that the one-exon form of the protein inhibited activation of PKR. Influenza virus activates a cellular inhibitor of PKR activation, P58 (4, 46–49), that can transform cells when overexpressed. Adenovirus blocks PKR activation through binding of the VAI RNA to PKR (54). Reovirus encodes a viral dsRNA binding protein (31) capable of inhibiting PKR activation (32). It is possible that other examples of dsRNA-induced apoptosis will be found, since so many viruses have a mechanism in place to prevent a cellular response to dsRNA. It should be noted, however, that not all inhibitors of PKR can prevent the dsRNA-mediated induction of apoptosis. VVΔE3L still contains a functional K3L gene that can act as an inhibitor of eIF-2α phosphorylation. Whether inhibition of eIF-2α phosphorylation is not sufficient to prevent induction of apoptosis or whether the effects of the K3L gene products are overcome in VVΔE3L-infected cells is at present unclear.

In addition to E3L, the VV serpin family of proteins has been shown to regulate induction of apoptosis. Cowpox virus with the *crmA* gene (SPI2) deleted (66) and rabbitpox virus with the related SPI1 gene deleted (10) have been shown to induce apoptosis in certain cells. The VV (WR strain) *crmA* gene has also been shown to be necessary for inhibition of Fas-mediated induction of apoptosis (22). The interplay between the serpin genes and E3L is at present unclear. Rabbitpox virus-infected cells contain functional E3L proteins (82), as does the WR strain of VV (15). Interestingly, the strain of VV used in this study (Copenhagen) has been shown not to make a functional SPI2 protein, although it does make detectable SPI1 protein (37).

Though further studies are required to investigate the precise mechanism by which apoptosis occurs in VV-infected HeLa cells, our results suggest that activation of an IFN-induced dsRNA-dependent protein is a likely possibility. Proceeding from the premise that dsRNA induces cellular suicide in HeLa cells, we can now investigate the mechanism(s) by which the response is implemented.

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