JOURNAL OF VIROLOGY, Oct. 2007, p. 10924–10932 Vol. 81, No. 20
0022-538X/07/$08.00+0 doi:10.1128/JVI.01239-07

Replication-Competent Herpes Simplex Virus 1 Isolates Selected from Cells Transfected with a Bacterial Artificial Chromosome DNA Lacking Only the U_L49 Gene Vary with Respect to the Defect in the U_L41 Gene Encoding Host Shutoff RNase[▽]

Maria Teresa Sciortino,[1] Brunella Taddeo,[2] Maria Giuffrè-Cuculletto,[1] Maria Antonietta Medici,[1] Antonio Mastino,[1] and Bernard Roizman[2]*

Department of Microbiological, Genetic and Molecular Sciences, University of Messina, Salita Sperone 31, 98166 Messina, Italy,[1] *and The Marjorie B. Kovler Viral Oncology Laboratories, The University of Chicago, Chicago, Illinois 60637*[2]

Received 6 June 2007/Accepted 23 July 2007

To generate a null U_L49 gene mutant of herpes simplex virus 1 (HSV-1), we deleted from the viral DNA, encoded as a bacterial artificial chromosome (BAC), the U_L49 open reading frame and, in a second step, restored it. Upon transfection into Vero cells, the BAC-ΔU_L49 DNA yielded foci of degenerated cells that could not be expanded and a few replication-competent clones. The replication-competent viral clones derived from independent transfections yielded viruses that expressed genes with some delay, produced smaller plaques, and gave lower yields than wild-type virus. A key finding is that the independently derived replication-competent viruses lacked the virion host shutoff (*vhs*) activity expressed by the RNase encoded by the U_L41 gene. One mutant virus expressed no *vhs* protein, whereas two others, derived from independent transfections, produced truncated *vhs* proteins consistent with the spontaneous in-frame deletion. In contrast, cells infected with the virus recovered upon transfection of the BAC-U_L49R DNA (R-U_L49) accumulated a full-length *vhs* protein, indicating that in the parental BAC-ΔU_L49 DNA, the U_L41 gene was intact. We conclude that expression of the *vhs* protein in the absence of U_L49 protein is lethal, a conclusion bolstered by the evidence reported elsewhere that in transfected cells *vhs* requires both VP16 and VP22, the product of U_L49, to be neutralized.

Of the 84 different known proteins encoded by herpes simplex virus 1 (HSV-1), at least 4 proteins, all located in the tegument of the virion, interact with mRNAs. Of these, the proteins encoded by the U_S11, U_L47, and U_L49 open reading frames (ORFs) bind RNAs, whereas the fourth, encoded by the U_L41 ORF, acts as an RNase (reviewed in reference 44). Apart from potential regulatory functions, interest in the RNA binding proteins stemmed from the observation that virions package mRNAs (40, 42). Moreover, in the course of studies of this phenomenon, our laboratories reported that VP22, the product of the U_L49 ORF, transports the mRNA from infected to uninfected cells for expression prior to viral infection (41). The studies reported here were initially designed to determine the contribution of VP22 to the packaging of mRNAs in the virion. We show that a series of ΔU_L49 mutants derived from independent transfections of viral DNA lacking the U_L49 ORF yielded recombinant viruses defective in the U_L41 gene. On the basis of the results reported in this article and in parallel studies published by Taddeo et al. (52), we conclude that VP22 and VP16 are both required for the replication of viruses encoding functional U_L41 protein. Relevant to this report are the following.

The shutoff of cellular protein synthesis, a function designated virion host shutoff (*vhs*), was first identified and mapped to the U_L41 ORF by Frenkel and colleagues (25, 26, 47). Intensive studies carried out over 2 decades demonstrated that the U_L41 product is a γ_2 protein, that it is packaged in the virion, and that it mediates the degradation of mRNAs during the early phases of infection by endonucleolytic cleavage (reviewed in reference 44). Furthermore, several lines of evidence indicate that *vhs* degrades mRNA in a selective manner (15, 16, 49). In recent studies, this laboratory unambiguously demonstrated that the U_L41 protein is an endoribonuclease with a substrate specificity similar to that of RNase A (50, 51). At late stages of infection the U_L41 protein is no longer active, even though it accumulated in large amounts in the course of synthesis of the late protein. Studies carried out primarily by Smiley and associates demonstrated that the U_L41 product binds VP16, giving rise to the speculation that VP16 neutralizes the RNase activity at late times after infection (27, 39, 43). Attempts to express and accumulate the U_L41 protein by transfection of cells in the absence of both VP16 and VP22 failed. In contrast, a plasmid encoding a U_L41 ORF in which 3 codons were replaced to inactivate the enzymatic activity was readily expressed in the absence of both U_L48 and U_L49. These studies also demonstrated that the U_L41 protein binds VP22, but only in the presence of VP16 (52). The studies presented here extend this observation by showing that ΔVP22 viruses contain disabling mutations in the U_L41 ORF.

VP22 is a 301-residue γ_1 protein capable of forming higher-order structures consisting of dimers or tetramers (31). The protein is nucleotidylylated by casein kinase II (3) and phosphorylated by other enzymes, although the isoform incorporated into the virion is hypophosphorylated (11, 12, 19, 36).

* Corresponding author. Mailing address: University of Chicago, Viral Oncology Laboratory, 910 East 58th St., Chicago, IL 60637. Phone: (773) 702-1898. Fax: (773) 702-1631. E-mail: bernard.roizman@bsd.uchicago.edu.

▽ Published ahead of print on 1 August 2007.

The many functions attributed to VP22 include (i) binding to chromatin, microtubules, and membranes (24, 29, 57); (ii) interaction with template activating factor 1 (TAF-1) and impairment of nucleosome assembly on entering viral DNA (53); (iii) mediation of hyperacetylation and bundling of microtubules to render them more resistant to depolymerization (9); (iv) interaction with membranes, and more specifically with membranes of the acidic compartments of cells (5); (v) interaction with several tegument and envelope proteins, including VP16 and gD (6, 13, 21, 54). Although it has been postulated that VP22 plays a key role in virus assembly, the ΔU_L49 mutants reported to date do not appear to be defective in this function (34). Perhaps the single most important function attributed to VP22 is the ability to spread to adjacent cells after infection or transfection solely with a plasmid encoding VP22. With regard to recipient cells, VP22 was detected by immunofluorescence in the nuclei of cells fixed with paraformaldehyde or organic fixatives but to a much reduced level in cells fixed with methanol.

The transport of chimeric proteins consisting of VP22 covalently bound to green fluorescent protein (GFP) or other proteins from cell to cell has been disputed (1, 2, 4, 10, 17, 20, 22, 28, 33, 38, 55, 58, 59). The conflicting reports remain to be resolved, but it should be pointed out that some proteins are readily extracted during methanol fixation of cells and that covalently bound polypeptides of a size nearly equivalent to or larger than VP22 could be expected to change the properties of the protein.

The significance of the studies reported here stems from three observations: (i) none of the isolated viruses expressed functional *vhs* activity; (ii) at least two different kinds of mutations were responsible for the loss of activity; and (iii) the mutants were selected and amplified after transfection of the DNA.

MATERIALS AND METHODS

Cells and viruses. Vero, HeLa, and HEp-2 cell lines (American Type Culture Collection) were propagated in Dulbecco's modified Eagle's medium supplemented with 5% newborn calf serum. HSV-1(F) is a limited-passage prototype HSV-1 used in our laboratories (8). The ΔU_L41 mutant virus has been reported elsewhere (35). Virus stocks were titered on Vero cells. Serial 10-fold dilutions of the lysates were assayed on monolayers of Vero cells in 6-well dishes. After 1 h, cell monolayers were covered with Dulbecco's modified Eagle's medium containing 0.3% methylcellulose. After 3 days of incubation at 37°C, the medium was aspirated from the wells, and the cells were then fixed, stained with crystal violet, and visualized at 10× magnification with an inverted microscope for plaque detection.

Antibodies. The HSV-1 proteins were detected with the anti-ICP0 monoclonal antibody purchased from the Goodwin Institute (Plantation, FL), an anti-glutathione *S*-transferase (GST)-U_S3 polyclonal antibody (32), an anti-VP16 mouse monoclonal antibody (LP1; a kind gift of A. Minson), an anti-GST-U_L49 polyclonal antibody (3), an anti-U_S11 monoclonal antibody (37), and anti-GST-U_L41 rabbit polyclonal antiserum (51). A mouse monoclonal antibody against actin was purchased from Sigma (St. Louis, MO).

Construction of the BAC HSV-1 DNA from which the U_L49 gene was deleted and subsequently restored. The construction and properties of the BAC-HSV-1(F) DNA have been described elsewhere (23, 56, 60). The HSV-1 mutant lacking the U_L49 gene was constructed by using the HSV-1(F) bacterial artificial chromosome (BAC-HSV-1) with transfer plasmid pKo5Y (pRB5708), as schematically illustrated in Fig. 1. To construct the pMTS2 transfer plasmid, cosmid CS69, containing the HSV-1 sequence spanning nucleotides 77933 to 116016, was digested with BstBI restriction enzyme, generating a 4,615-bp fragment containing the U_L48, U_L49, and $U_L49.5$ ORFs and a portion of the U_L50 ORF. The 4,615-bp fragment was subcloned into pBluescript II KS(1) at a compatible ClaI restriction site to yield pMTS2. pMTS2 was cleaved with AatII/StuI to remove an 862-bp fragment encompassing amino acids 1 to 266 of the U_L49 ORF. Removal of this fragment generated a frame shift of the residual 35 codons of U_L49. The 862-bp fragment was replaced with a polylinker containing the following restriction sites: StuI, PacI, XbaI, BamHI, SmaI, PstI, EcoRI, and AatII (pMTS3). The plasmid obtained was then partially digested with XhoI, and the resulting 3,653-bp fragment was cloned into the pKo5Y shuttle vector to yield pMTS4. The procedure for construction of the mutant virus has been described elsewhere (56). Briefly, RR1 competent cells that harbored the BAC-HSV-1 (RR1-HSV-1) were transformed with 0.6 μg of transfer plasmid pMTS3 DNA, plated onto zeocin (Zeo; 20 μg/ml)-plus-chloramphenicol (Cm; 20 μg/ml) plates, and incubated overnight at 43°C. After incubation, 8 colonies were picked, plated onto Cm–10% sucrose (Suc) Lennox broth (LB) plates, and further incubated at 30°C overnight. To confirm the loss of the replacement vector, 20 Cm^r Suc^r colonies were restreaked in duplicate onto Cm Suc Zeo LB plates separately and then incubated at 30°C overnight. The Suc^r Cm Zeo^r colonies were further screened by PCR (95°C for 4 min; then 35 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min). The primers used were Δ49 forward (5′-CCCACAT TGGCTCCTGTCACG-3′; from nucleotide 105326 to 105346) and Δ49 reverse (5′-CCTTCCTCGCGGAAACCGAGAC-3′; from nucleotide 106665 to 106644). PCR-confirmed colonies were grown in LB Cm medium, and the recombinant BAC-HSV-ΔU_L49 DNA was prepared with a high-speed QIAGEN (Chatsworth, CA) plasmid purification kit. For the generation of U_L49-repaired virus (R-U_L49), the above-described 4,615-bp fragment derived from CS69 digestion with BstBI restriction enzyme was directly cloned into the pKo5Y shuttle vector to yield pMTS5. RR1 competent cells harboring BAC-ΔU_L49 DNA were transformed with 0.6 μg of pMTS5 transfer plasmid DNA, and the recombinant BAC-U_L49R DNA was obtained as described above.

Viral DNA extraction from RR1 bacterial cells. BAC-HSV-1, BAC-ΔU_L49, and BAC-U_L49R DNAs were extracted from RR1-HSV-1, RR1-ΔU_L49, and RR1-U_L49R bacterial cells, respectively, by using a QIAGEN Max extraction kit according to the manufacturer's instructions.

Transfection of cell lines with recombinant BAC-HSV DNAs. Subconfluent cultures of Vero, HEp-2, or HeLa cells were transfected with 1.6 μg of the recombinant BAC-HSV-ΔU_L49 or BAC-HSV-U_L49R DNA by use of Lipofectamine reagent according to the manufacturer's instructions (Life Technologies, Grand Island, NY). BAC-derived ΔU_L49 viruses obtained from three independent transfections of Vero cells were identified as V0, V1, and V3, respectively. One ΔU_L49 mutant virus obtained from HEp-2 transfected cells was identified as H-3. No replicating ΔU_L49 virus was obtained from HeLa cells. The same procedure was also used to generate U_L49R virus. The viruses were collected and titered as described above.

Southern blot analyses of viral DNA. Equal amounts (10 μg) of BAC-HSV-1, BAC-ΔU_L49, and BAC-U_L49R DNAs, purified from RR1 bacterial cells, were digested with EcoRV enzyme, electrophoretically separated on a 1% agarose gel, and transferred to a nylon membrane (Bio-Rad, Hercules, CA). The hybridization procedures were carried out as recommended by the manufacturer (Bio-Rad). Plasmid pAc-NH2, containing the amino-terminal sequence of U_L49 spanning nucleotides 1 to 576 (data not shown), was used to generate a biotin-16-UTP-labeled (Roche Diagnostics, Germany) probe by using a nick translation kit (Roche Diagnostics).

Immunoblotting of electrophoretically separated proteins from cell lysates. Confluent Vero cell monolayers were either mock infected or infected with 10 PFU/cell of HSV-1(F), ΔU_L41 mutant virus, or a ΔU_L49 BAC-derived virus and were collected 18 h after infection. The procedures for harvesting, solubilization, protein quantification, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transfer to nitrocellulose membranes were performed as previously reported (49). The membrane was probed for *vhs*, VP22, U_S3, ICP0, U_S11, VP16, and actin using the antibodies listed above.

Immunoblotting of proteins from purified virions. Wild-type and recombinant virus virions were purified as described elsewhere (40, 45). Briefly, Vero cells grown in 300-cm^2 flasks were exposed to 5 PFU of virus per cell. The cells were harvested 22 to 24 h after infection, resuspended in 1 mM phosphate buffer, and disrupted in a glass homogenizer with four strokes. Cytoplasmic fractions were individually layered in dextran-10 gradients (1.04 to 1.09 g/cm^3) in 1 mM phosphate buffer. The gradients were centrifuged, and virion-containing bands were collected and diluted in 10 mM phosphate buffer. Purified virions were concentrated and resuspended in 100 μl of 10 mM phosphate buffer and stored at −20°C before processing. The purified virions were resuspended in 4× loading buffer (50 mM Tris-HCl [pH 6.8], 10 mM dithiothreitol, 2% sodium dodecyl sulfate, 0.1% bromphenol blue, 10% glycerol), subjected to electrophoresis on a 10% denaturing polyacrylamide gel, and then transferred to a nitrocellulose membrane. The membrane was blocked for 1 h with 5% nonfat dry milk and

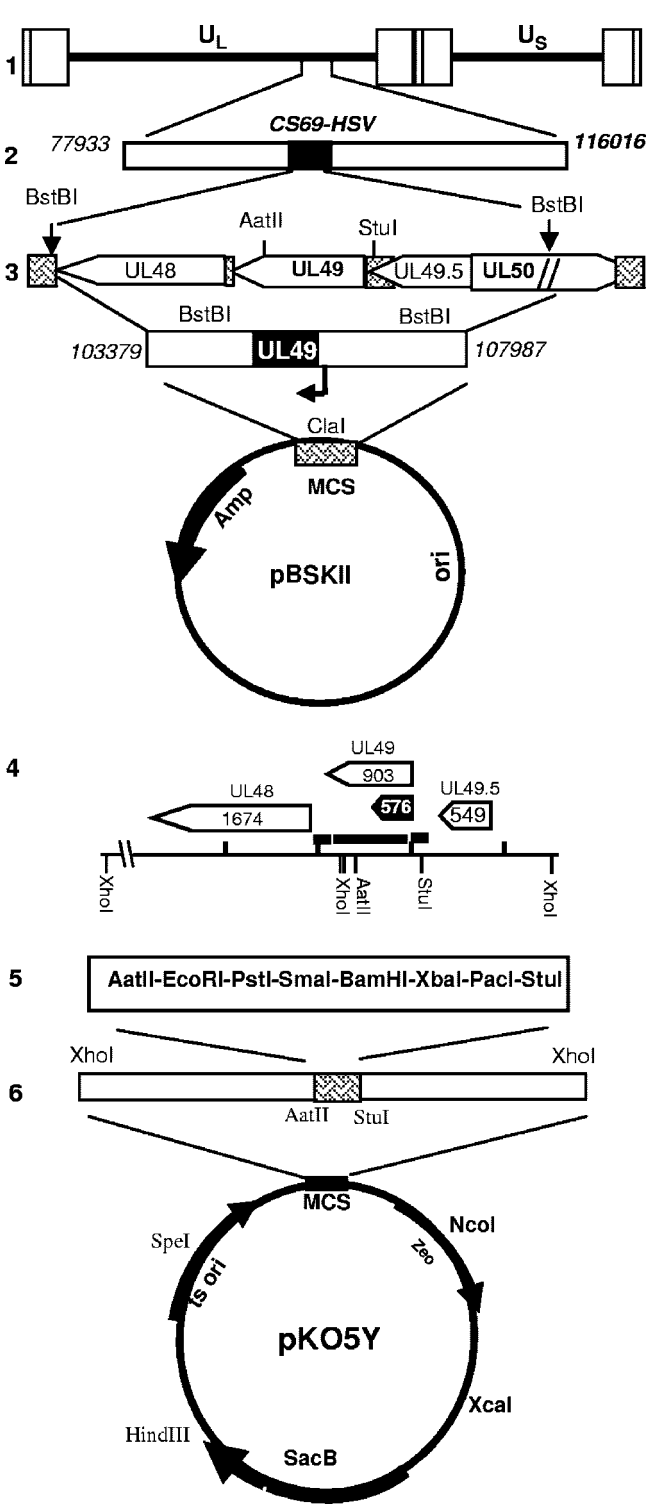

FIG. 1. Schematic representation of cosmid and plasmid DNAs. Line 1, linear representation of the HSV-1 genome. Rectangles represent the inverted repeats flanking the unique sequences (U_L and U_S, represented by thin lines). Line 2, linear representation of cosmid CS69 DNA containing the insertion of HSV-1 sequence spanning nucleotides 77933 to 116016. Line 3, pMTS2, a 4.5-kb BstBI fragment of cosmid CS69 containing the U_L48, U_L49, and $U_L49.5$ genes and a portion of the U_L50 gene. Arrowheads indicate the direction of transcription; hatched boxes, noncoding regions. Line 4, details of the ORFs present in the BstBI fragment and the relevant restriction endonuclease sites used for construction of the deletion mutants. Black arrow indicates the position of the probe used for this study. Line 5, pMTS2 was cleaved with AatII/StuI to remove an 862-bp fragment containing the coding sequence for amino acids 1 to 266 of U_L49. The U_L49 sequence was replaced with a polylinker containing the following restriction sites: StuI, PacI, XbaI, BamHI, SmaI, PstI, EcoRI, and AatII (pMTS3). Line 6, the XhoI fragment derived from partially digested pMTS3, resulting in a 3,653-bp fragment, was transferred to shuttle plasmid pKo5Y at the XhoI site (pMTS4).

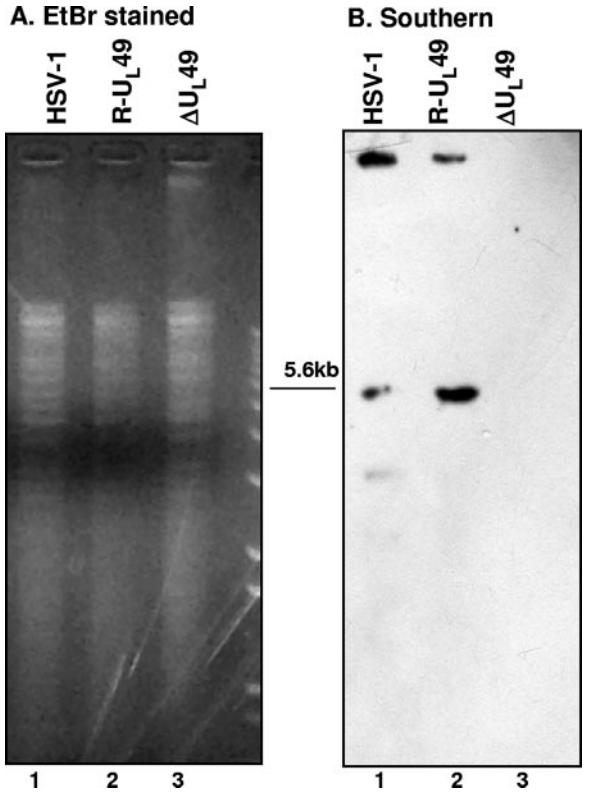

FIG. 2. Verification of the structure of BAC-ΔUL49 DNA. DNAs (10 μg) from BAC-HSV-1, BAC-UL49R, and BAC-ΔUL49, purified from RR1 bacterial cells, were separately digested with EcoRV. DNA was separated by agarose gel electrophoresis, and the blots were analyzed by hybridization with a biotinylated probe encompassing the amino-terminal region of U_L49. (A) Ethidium bromide (EtBr) staining; (B) Southern blotting.

reacted with the appropriate primary antibody overnight at 4°C. The monoclonal antibody to U_S11 and polyclonal antibodies to *vhs* and VP22 were diluted 1:500 in phosphate-buffered saline containing 1% bovine serum albumin and 0.05% Tween 20. The blots were then washed and incubated with alkaline phosphatase (AP)-conjugated anti-mouse immunoglobulin G (Calbiochem) and AP-conjugated anti-rabbit immunoglobulin G (Santa Cruz Biotechnology), respectively. To develop AP-conjugated secondary antibodies, the immunoblots were reacted with the AP buffer 5-bromo-4-chloro-3-indolylphosphate–tetranitroblue tetrazolium (BCIP/TNBT; Calbiochem).

Analysis of shutoff of protein synthesis in infected cells. Replicate 25-cm^2 cultures of Vero cells were either mock infected or infected with 20 PFU of HSV-1(F), ΔU_L41, or ΔU_L49 isolates per cell in the presence of actinomycin D (10 μg/ml) and incubated at 37°C. At 3 h after exposure to virus, the cells were labeled for 1 h with [^{35}S]methionine as previously described (35); then they were harvested, solubilized, resolved in a 12% denaturing polyacrylamide gel, dried, and subjected to autoradiography.

TABLE 1. Virus yields[a] in different cell lines infected with wild-type HSV-1 or ΔU_L49-V1

Cell line	Virus	Yield at the following time (h) after infection:				
		3	12	18	24	48
Vero	HSV-1(F)	2.0×10^3	1.2×10^7	2.0×10^8	2.2×10^7	2.0×10^7
	ΔU_L49-V1	6.0×10^2	5.7×10^5	5.6×10^7	1.4×10^7	1.2×10^7
HEp-2	HSV-1	8.4×10^4	7.3×10^6	4.7×10^7	1.0×10^8	2.0×10^8
	ΔU_L49-V1	1.2×10^4	3.6×10^5	1.4×10^6	3.1×10^6	1.0×10^7
HeLa	HSV-1	2.0×10^3	8.5×10^5	7.5×10^6	1.3×10^7	1.2×10^7
	ΔU_L49-V1	2.0×10^3	8.7×10^4	5.6×10^5	1.3×10^6	1.7×10^6

[a] PFUs were determined by a plaque assay on Vero cell monolayers as described in Materials and Methods.

VHS sequence analysis of PCR products derived from viral RNA and DNA extracted from cells infected with HSV-1(F), ΔU_L49-V0, ΔU_L49-V1, or ΔU_L49-V3 virus. Vero cells were infected with HSV-1(F) or with ΔU_L49-V0, ΔU_L49-V1, or ΔU_L49-V3 mutant virus and were harvested 22 h after infection. Total RNA was extracted with TRIzol reagent according to the manufacturer's instructions (Life Technologies). DNase I treatment, phenol-chloroform extraction, and ethanol precipitation (Fisher Scientific, Houston, TX) were carried out to remove possible DNA contamination. Total RNA (2.5 μg) was then reverse transcribed with 60 U of avian myeloblastosis virus (Promega, Madison, WI) in a total reaction volume of 30 μl. The reverse transcription was primed with an oligo$(dT)_{15}$ primer and performed using a pool of nucleotides consisting of dGTP, dATP, dTTP, and dCTP (Promega) at 1 mM each. Forty units of RNasin (Promega) was added to each reaction mixture. The mixture containing only the RNA template and the oligo$(dT)_{15}$ was first heated at 70°C for 10 min, then chilled on ice, and, after the addition of the other components, incubated at 42°C for 45 min, shifted to 52°C for 45 min, and then heat inactivated at 95°C for 5 min.

Viral DNAs from HSV-1(F), ΔU_L49-V0, ΔU_L49-V1, and ΔU_L49-V3 viruses were extracted as described elsewhere (30) and used as templates for PCR amplification.

cDNAs obtained from reverse-transcribed viral RNAs and viral DNAs extracted from infected cells were amplified by PCR under the following conditions: 1 min at 95°C, 45 s at 60°C, and 2 min at 72°C. The primers used were VHS forward (5′-ATGGGTTTGTTCGGGATGATGAAG-3′) and VHS reverse (5′-CTACTCGCTCCAGAATTTGGCCAG-3′). The PCR products were then cloned into the pGEM-T Easy vector according to the manufacturer's instructions (Promega) and sequenced.

RESULTS

Isolation of ΔU_L49 mutant viruses. The construction of the HSV-1-BAC ΔU_L49 mutant virus is illustrated in Fig. 1 and described in detail in Materials and Methods. The ΔU_L49 mutant virus was isolated by transfection of Vero cells with plasmids containing the BAC-ΔU_L49 DNA. Of the numerous independent transfections, several yielded foci of degenerating cells, but virus could not be passaged to uninfected cells. In three instances, transfections in Vero cells yielded viruses capable of transmission from cell to cell. These were designated V0, V1, and V3. One isolate, designated H-3, was obtained from transfected HEp-2 cells. In the initial studies, V1 was designated as the prototype virus. This virus was plaque purified, grown in Vero cells, and extensively characterized (data not shown).

During the course of these studies, it became apparent that the ΔU_L49-V1 virus carries an additional mutation in the U_L41 ORF. At that point we analyzed all viruses isolated from transfectants with respect to the U_L41 ORF sequence. As described below, viruses V0 and V1 carry an identical mutation. V3 carries a different mutation that blocks the expression of the U_L41 ORF. Finally, the H-3 mutant virus, less extensively characterized, was found to be phenotypically similar to V3.

Verification of the structure of BAC-ΔU_L49 DNA. Equal amounts of BAC-HSV-1, BAC-U_L49R, and BAC-ΔU_L49 plasmid DNAs extracted from RR1 bacteria were separately digested with EcoRV, electrophoretically separated on an agarose gel, and hybridized with a biotinylated DNA probe containing the amino-terminal sequence of U_L49. As expected, and as shown in Fig. 2, the probe hybridized with a 5.6-kb fragment derived from digested BAC-HSV-1 or BAC-R-U_L49 DNA but not with BAC-ΔU_L49 DNA. Identical results were obtained by hybridizing viral DNA extracted from cells infected by wild-type virus or the ΔU_L49 mutant (data not shown).

Growth properties of the ΔU_L49-V1 mutant virus. Two series of experiments were done to characterize the growth properties of the ΔU_L49-V1 mutant virus. In the first, replicate cultures of Vero, HEp-2, and HeLa cells were exposed to 1

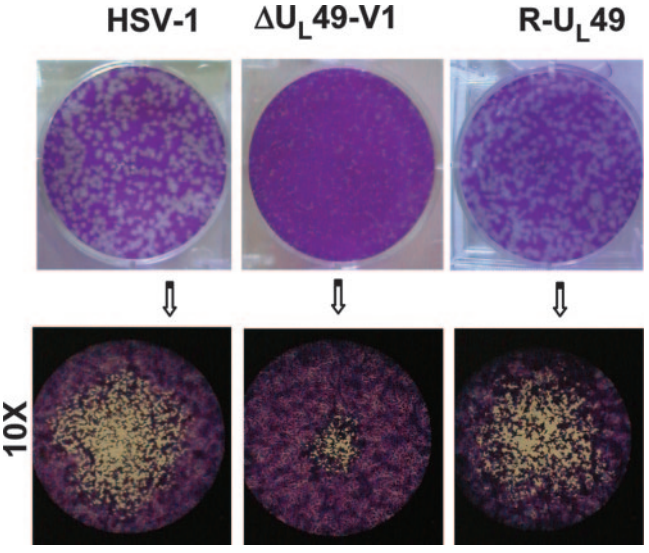

FIG. 3. Growth properties of wild-type HSV-1 and ΔU_L49 mutant viruses. Plaque formation in Vero cells infected with wild-type HSV-1, R-U_L49, or the ΔU_L49 deletion mutant is shown. Following infection, cells were overlaid with a methylcellulose-containing medium for 3 days in duplicate plates for each dilution. Cells were then fixed, stained with crystal violet, and visualized with an inverted microscope (higher magnification, ×10).

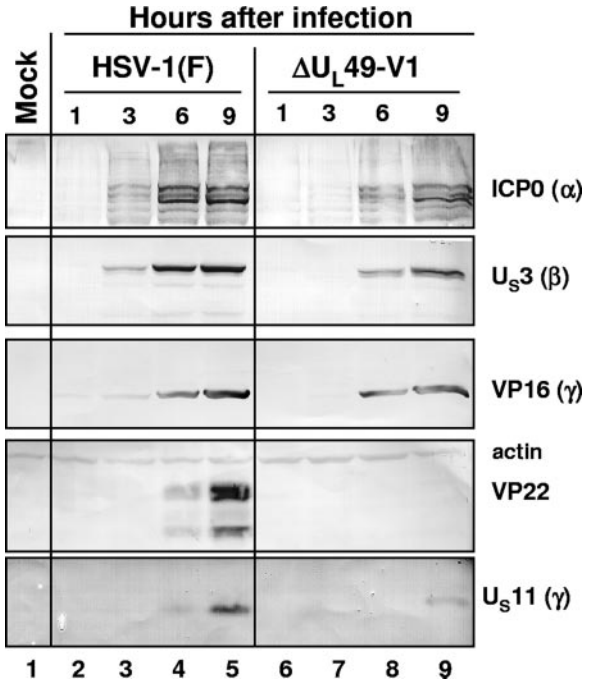

FIG. 4. Viral protein synthesis in Vero cells infected with the ΔU_L49 mutant virus. Confluent cell monolayers were either mock infected (lane 1) or infected with 10 PFU/cell of either HSV-1(F) (lanes 2 to 5) or the ΔU_L49 mutant virus (isolate V1) (lanes 6 to 9). The cells were harvested at the indicated times and processed as described in Materials and Methods. Equal amounts of proteins were electrophoretically separated on a 10% denaturing polyacrylamide gel, transferred to a nitrocellulose sheet, and reacted with antibodies against representative α (ICP0), β (U_S3), and γ (γ_1, VP16 and VP22; γ_2, U_S11) proteins. An anti-actin antibody was used as a control.

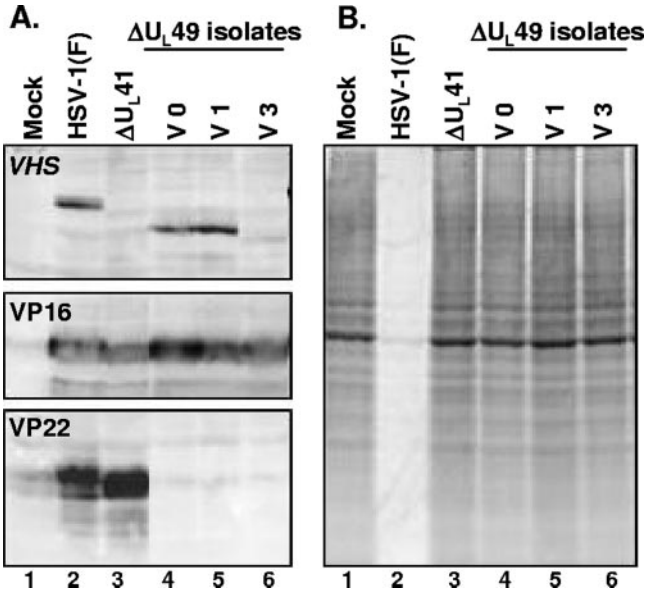

FIG. 5. *vhs* accumulation and shutoff of protein synthesis in Vero cells infected with ΔU_L49 mutant isolates. (A) Confluent cell monolayers were either mock infected (lane 1) or infected with 10 PFU per cell of HSV-1(F) (lane 2), ΔU_L41 (lane 3), or ΔU_L49 mutant virus isolates (lanes 4 to 6). The cells were harvested 18 h after infection and processed as described in Materials and Methods. Equal amounts of proteins were electrophoretically separated on a 10% denaturing polyacrylamide gel, transferred to a nitrocellulose sheet, and reacted with antibodies against *vhs*, VP16, and VP22. (B) Virion host shutoff activity. Replicate 25-cm^2 cultures of Vero cells were either mock infected (lane 1) or infected with 20 PFU per cell of HSV-1(F) (lane 2), ΔU_L41 (lane 3), or ΔU_L49 mutant virus isolates (lanes 4 to 6) in the presence of actinomycin D (10 μg/ml) and then incubated at 37°C. At 3 h after exposure to the virus, the cells were labeled for 1 h with [^{35}S]methionine; then they were harvested, solubilized, resolved on a 12% denaturing polyacrylamide gel, dried, and subjected to autoradiography.

PFU/cell of ΔU_L49-V1 virus. At different times after infection, as indicated in Table 1, the cells were harvested and the virus yield was titered on Vero cells. The fundamental conclusion of these titrations was that the yields of the mutant virus differ significantly from those of the wild-type virus. In particular, we noted that in both Vero and HeLa cells and, to a lesser extent, in HEp-2 cells, the amounts of mutant virus detected at 12 h after infection were lower than those of wild-type virus. This difference was still evident with the passing of time, i.e., at 24 h and 48 h after infection, in HeLa and HEp-2 cells. In contrast, in Vero cells, the yields of the mutant viruses at the later time points were similar to those of wild-type HSV-1 (Table 1).

Another noteworthy observation was that the plaque size of the mutant virus was significantly smaller than that of the wild-type virus or the repaired virus (Fig. 3).

In the second series of experiments, we measured the accumulation of proteins belonging to different kinetic classes. Replicate cultures of Vero cells were exposed to 1 PFU of wild-type or mutant virus per cell. The cells were harvested at 1, 3, 6, or 9 h after infection, solubilized, subjected to electrophoresis in denaturing gels, and reacted with antibodies to ICP0, U_S3 protein kinase, VP16, VP22, U_S11, and actin as described in Materials and Methods. The results shown in Fig. 4 indicated the following: (i) as expected, the cells infected with the mutant failed to accumulate VP22 protein (lanes 6 to 9); (ii) the accumulation of ICP0, U_S3, VP16, and U_S11 in lysates of mutant-virus-infected cells lagged by at least 3 h the accumulation of the corresponding protein in wild-type-virus-infected cells (compare lane 7 with lane 3).

All independently isolated ΔU_L49 mutants fail to express functional *vhs* protein. The impetus for the next series of experiments stemmed from two observations. First, the studies described above showed a delay in the accumulation of viral protein in infected cells—a phenotype reminiscent of that of *vhs* minus mutants. Second, in parallel experiments reported elsewhere, we noted that the accumulation of *vhs* protein required the presence of VP22 and VP16 (52). The failure of accumulation of *vhs* protein in the absence of VP22 or VP16 was most likely the result of the high toxicity of *vhs* protein, inasmuch as a mutated protein lacking RNase activity accumulated even in the absence of VP16 or VP22. To test the hypothesis that the ΔU_L49 mutants are also defective in the U_L41 gene, four series of experiment were conducted.

In the first, we tested whether the ΔU_L49-V1, -V0, and -V3 mutant viruses expressed *vhs* protein and whether it was active. Specifically, confluent cell monolayers were either mock infected or infected with 10 PFU of HSV-1(F), ΔU_L41, or ΔU_L49 mutant virus isolates per cell. The cells were harvested 18 h after infection and processed as described in Materials and Methods. Equal amounts of proteins were electrophoreti-

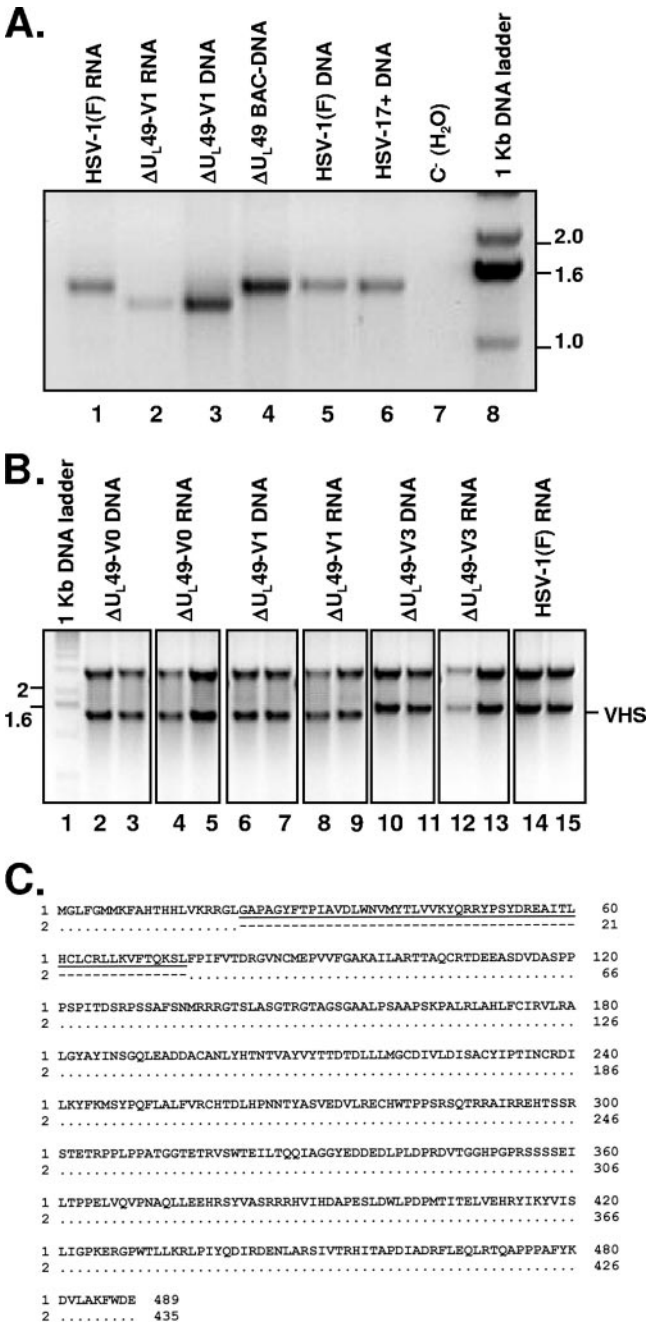

FIG. 6. Expression of the *vhs* ORF in Vero cells infected with ΔU_L49 deletion mutant viruses. (A) The U_L41 ORF was amplified from either RNA (lane 2) or DNA (lane 3) purified from ΔU_L49-V1-infected cells and was compared to the U_L41 ORF obtained from RNA (lane 1) or DNA (lane 5) extracted from cells infected by HSV-1(F) or from DNA from HSV-17+-infected cells (lane 6). Lane 4, ΔU_L49 BAC DNA used as a template; lane 8, 1-kb DNA ladder. PCR products were resolved on a 2% agarose gel and stained with ethidium bromide. The image was acquired with an Eagle Eye II still-video system (Stratagene, La Jolla, CA) and is shown inverted for clarity. (B) The U_L41 ORFs of HSV-1(F), ΔU_L49-V0, ΔU_L49-V1, and ΔU_L49-V3 viruses were amplified from DNA and RNA, and the PCR products were cloned into a T-vector. Two different clones for each sample were analyzed by EcoRI restriction. The image of the ethidium bromide-stained agarose gel was acquired and shown as for panel A. The upper band in panel B represents the T vector, and the lower band is the VHS ORF (VHS). DNA was used as a template for lanes 2, 3, 6, 7, 10, and 11; RNA was used as a template for lanes 4, 5, 8, 9, and 12 to 15. Lane 1, 1-kb DNA ladder; lanes 2 to 13, RNA and DNA obtained from Vero cells infected with either ΔU_L49-V0 (lanes 2 to 5), ΔU_L49-V1 (lanes 6 to 9), or ΔU_L49-V3 (lanes 10 to 13); lanes 14 and 15, RNA obtained from Vero cells infected with HSV-1(F). (C) Predicted *vhs* protein sequence from the consensus nucleotide sequence generated from the ΔU_L49-V1 virus (sequence 2) compared to that amplified from BAC-ΔU_L49 DNA (sequence 1). A deletion of a 162-bp segment close to the N terminus results in an in-frame deletion of 54 amino acids (amino acids 22 to 75).

cally separated on a 10% denaturing polyacrylamide gel, transferred to a nitrocellulose sheet, and reacted with antibodies against *vhs*, VP16, and VP22. The key finding shown in Fig. 5A is that the *vhs* protein did not appear to accumulate in cells infected with the V3 mutant (lane 6), whereas in lysates of both the V1 and V0 mutants, the accumulated *vhs* protein migrated faster than the wild-type protein (compare lanes 4 and 5 with lane 2).

In the second series of experiments, replicate 25-cm^2 flask cultures of Vero cells were either mock infected or exposed to 20 PFU of HSV-1(F), ΔU_L41, or ΔU_L49 mutant virus isolates per cell in the presence of actinomycin D (10 μg/ml) and then incubated at 37°C. At 3 h after exposure to virus, the cells were labeled for 1 h with [^{35}S]methionine; then they were harvested, solubilized, and resolved on a 12.5% denaturing polyacrylamide gel. The gel was then dried and subjected to autoradiography. This is a classic test of *vhs* activity, and as shown in Fig. 5B, *vhs* was active in cells infected with the wild-type virus (lane 2) but not in cells infected with any of the three ΔU_L49 mutants tested (lanes 4 to 6) or in cells infected with the ΔU_L41 mutant virus (lane 3).

The objective of the third series of experiments was to test the integrity of the U_L41 ORF in the BAC-ΔU_L49 DNA clone and in the viruses isolated by transfection of BAC DNAs. In this series of experiments, the U_L41 ORF was first amplified from either RNA or DNA purified from ΔU_L49-V1-infected cells and then compared to those obtained from RNA and DNA extracted from cells infected by wild-type virus (either strain F or strain 17+) as well as to BAC-ΔU_L49 plasmid DNA. The results shown in Fig. 6A were as follows. The U_L41 ORF amplified from the BAC-ΔU_L49 plasmid DNA (lane 4) was identical to those recovered from cells infected with wild-type virus (lanes 1, 5, and 6), and sequence analysis later confirmed that indeed the BAC-ΔU_L49 DNA contained a wild-type U_L41 ORF. However, the U_L41 ORF amplified from either DNA or RNA purified from Vero cells infected with the ΔU_L49-V1 isolate appeared to be shorter. The U_L41 ORFs of isolates V0 and V3 were also amplified in parallel, and the PCR products were cloned into a T-vector (Fig. 6B, lanes 2 to 5 and 10 to 13, respectively). At least four clones from each sample were sequenced. As shown in Fig. 6C, the U_L41 ORF in isolate V1, compared to that amplified from BAC-ΔU_L49 plasmid DNA, lacked a 162-bp segment close to the N terminus, resulting in a protein product with an in-frame deletion of 54 codons. The V0 U_L41 ORF contained an identical deletion (data not shown). In contrast, the U_L41 ORF from isolate V3, even though it appeared to be full length (Fig. 6B, lanes 10 to 13), contained a deletion of 2 nucleotides toward the 3′ end

that resulted in a frame shift and early stop codon formation. However, based on the predicted amino acid sequence, the 414-amino-acid protein product should share the first 286 amino acids with wild-type *vhs* protein. We conclude that all of the independently derived viable ΔU_L49 mutant viruses isolated in the course of this study failed to express an intact, functional *vhs* protein. Furthermore, analyses of the U_L41 ORF indicate that the isolates exhibited at least two different mutations.

Virions derived from transfected BAC-U_L49R DNA contain a full-length *vhs* protein. In the studies described above, we noted that all of the progeny derived from independent transfections of BAC-ΔU_L49 DNA either failed to express the *vhs* protein or encoded a truncated form. Even though the BAC-ΔU_L49 DNA encoded an intact U_L41 ORF (Fig. 6A, lane 4, and C), the question arose whether the U_L41 ORF contained in the transfected DNA was defective or whether the only virus that could replicate was that which spontaneously mutated in the course of transfection. The experimental design of our studies included the rescue of the U_L49 gene in the BAC-ΔU_L49 DNA. The rationale was that if the defect occurred in the course of the manipulation of the BAC-HSV-1 DNA, it would be conserved in the repaired virus. Hence the expression of an intact U_L41 ORF by the repaired virus would signify that the defects in the U_L41 genes in viral progeny of BAC-ΔU_L49 DNA resulted after transfection of the viral DNAs into mammalian cells. In this experiment, purified virions prepared from Vero cells that had been infected with either wild-type virus (HSV-1), the virus derived from transfection of BAC-U_L49R DNA (R-U_L49), or ΔU_L49 isolate V1 or V3 were solubilized, and 10 μg of viral proteins was electrophoretically separated on a denaturing gel, transferred to a nitrocellulose sheet, and probed with polyclonal antibodies to the VP22 and *vhs* proteins or with a monoclonal antibody to the U_S11 protein. As shown in Fig. 7, the *vhs* band contained in the R-U_L49 virions could not be differentiated from that contained in wild-type virions (Fig. 7A, compare lanes 1 and 2). The higher-molecular-weight band present in all the samples (Fig. 7A, top, lanes 1 to 4) is a result of cross-reactivity of the anti-vhs polyclonal antiserum. Furthermore, the *vhs* in R-U_L49 virions was active, inasmuch as the protein synthesis shutoff observed in cells infected by R-U_L49 (Fig. 7B, lane 5) was identical to that caused by wild-type HSV-1 (lane 2). We conclude that the BAC DNAs transfected into cells contained a U_L41 gene capable of being expressed and that the mutant selection occurred after transfection.

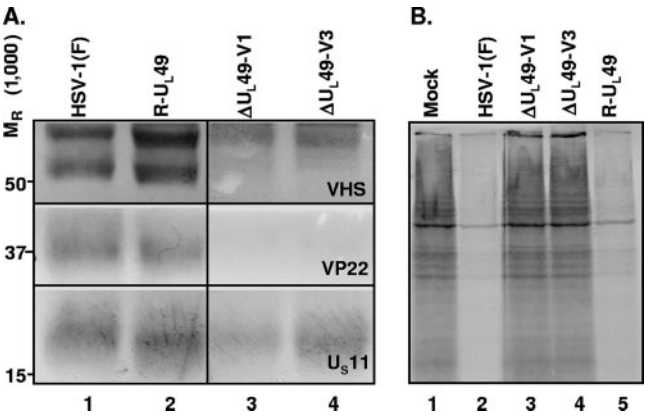

FIG. 7. (A) Immunoblot analysis of virion lysates of wild-type HSV-1 and ΔU_L49 viruses. Purified virions derived from Vero cells infected with wild-type HSV-1(F) (lane 1), R-U_L49 (lane 2), ΔU_L49-V1 (lane 3), or ΔU_L49-V3 (lane 4) virus at a multiplicity of infection of 5 PFU/cell and collected 24 h after infection were solubilized and processed for blotting. Equal amounts (10 μg) of viral proteins were electrophoretically separated, transferred to a nitrocellulose sheet, and probed with polyclonal antibodies to VP22 and *vhs* or with a monoclonal antibody to U_S11, as described in Materials and Methods. (B) Virion host shutoff activity. Replicate 25-cm^2 cultures of Vero cells were either mock infected (lane 1) or infected with 20 PFU per cell of HSV-1(F) (lane 2), ΔU_L49-V1 (lane 3), ΔU_L49-V3 (lane 4), or R-U_L49 (lane 5) virus in the presence of actinomycin D (10 μg/ml) and were incubated at 37°C. At 3 h after exposure to the virus, the cells were labeled for 1 h with [^{35}S]methionine; then they were harvested, solubilized, resolved on a 12% denaturing polyacrylamide gel, dried, and subjected to autoradiography. Lane 1, mock-infected control.

DISCUSSION

The initial objective of the studies described in this report was to produce a ΔU_L49 mutant in the genetic background of the viruses used in our studies. The U_L49 gene is not an essential gene for virus replication in cell culture systems in vitro (e.g., Vero or HEp-2 cells), and indeed mutants that either lack the U_L49 gene or express a mutated gene whose products are not packaged into virions have been reported (7, 14, 34). The ΔU_L49 mutant characterized in detail in the studies reported here shares characteristics with other ΔU_L49 mutants. Specifically, viral gene expression is delayed and the yields are lower than those of the wild-type virus. The unexpected finding, however, was the observation that these mutants do not exhibit *vhs* activity. Moreover, analyses of the cell lysates for *vhs* protein revealed that cells infected with one mutant (V3) do not appear to accumulate *vhs* protein, whereas cells infected with either of two other, independently derived virus isolates (V1 and V0) accumulate truncated *vhs* proteins. Whereas V3 encodes a U_L41 ORF with a deletion of 2 nucleotides and consequent frame shifting, the ORFs contained in the V0 and V1 isolates exhibit an in-frame deletion of 54 codons.

One hypothesis that could explain our results is that the U_L41 ORF was spontaneously mutated in the course of the manipulation of the BAC DNA in order to produce the mutant. If this were the case, it would be expected that the mutation would also be present in the BAC DNA in which the U_L49 ORF was restored. The results presented in this report show that the R-U_L49 mutant virus expresses a full-length *vhs* protein, unlike the V0, V1, and V3 mutant viruses derived from the parental BAC-ΔU_L49 DNA, and therefore this hypothesis is not tenable.

A alternative hypothesis is that the viable, replication-competent ΔU_L49 viruses were selected specifically on the basis of a loss of *vhs* activity and therefore that ΔU_L49 mutants exhibiting *vhs* activity are replication incompetent. This hypothesis is supported by three series of observations. (i) As noted in Results, in the course of analyzing the products of transfection, we noted the presence of foci of cells exhibiting cytopathic effects, but these did not yield replication-competent viruses upon passage. (ii) As reported above, the products of indepen-

dent transfections exhibited at least two different mutations that precluded the expression of the *vhs* activity. In addition to the three Vero cell isolates, we also isolated a replication-competent virus from transfected HEp-2 cells, which also failed to express *vhs* activity, although the basis of the failure is not known. The data suggest that the genetic basis of the failure to express *vhs* varies, rendering it highly unlikely that these viruses are derived from a single mutation of the BAC DNA. (iii) In parallel studies carried out in our laboratories and reported elsewhere (52), we noted that VP22 interacts with *vhs*, but only in the presence of VP16. Moreover, expression of *vhs* protein in transfected cells required the expression of both VP16 and VP22. In contrast, a mutated form of *vhs* in which three amino acids were substituted to inactivate the RNase activity was readily expressed in the absence of VP22 or VP16. The necessary conclusion is that both VP16 and VP22 are necessary to neutralize *vhs*. The implication of the present studies is that *vhs* activity in the absence of VP22 is lethal.

vhs minus mutants fail to replicate in animal systems, indicating that in natural infections *vhs* plays an essential role (18, 46, 48). At the same time, the RNase activity—the only activity identified to date for this protein—poses obvious risks to the virus in that it could degrade key viral mRNAs. It is not surprising that the virus has evolved means to constrain the activity of this protein.

ACKNOWLEDGMENTS

We thank Weiran Zhang for excellent technical support.

These studies were aided by grants from the Italian Ministry of University and Research (Research Projects of National Interest) and the University of Messina (PRA) and by National Cancer Institute grants CA87661, CA83939, CA71933, CA78766, and CA88860.

REFERENCES

1. **Aints, A., M. S. Dilber, and C. I. Smith.** 1999. Intercellular spread of GFP-VP22. J. Gene Med. **1:**275–279.
2. **Beerens, A. M., M. G. Rots, E. F. De Vries, and H. J. Haisma.** 2007. Fusion of herpes simplex virus thymidine kinase to VP22 does not result in intercellular trafficking of the protein. Int. J. Mol. Med. **19:**841–849.
3. **Blaho, J. A., C. Mitchell, and B. Roizman.** 1994. An amino acid sequence shared by the herpes simplex virus 1 alpha regulatory proteins 0, 4, 22, and 27 predicts the nucleotidylylation of the U_L21, U_L31, U_L47, and U_L49 gene products. J. Biol. Chem. **269:**17401–17410.
4. **Brewis, N., A. Phelan, J. Webb, J. Drew, G. Elliott, and P. O'Hare.** 2000. Evaluation of VP22 spread in tissue culture. J. Virol. **74:**1051–1056.
5. **Brignati, M. J., J. S. Loomis, J. W. Wills, and R. J. Courtney.** 2003. Membrane association of VP22, a herpes simplex virus type 1 tegument protein. J. Virol. **77:**4888–4898.
6. **Chi, J. H. I., C. A. Harley, A. Mukhopadhyay, and D. W. Wilson.** 2005. The cytoplasmic tail of herpes simplex virus envelope glycoprotein D binds to the tegument protein VP22 and to capsids. J. Gen. Virol. **86:**253–261.
7. **Duffy, C., J. H. Lavail, A. N. Tauscher, E. G. Wills, J. A. Blaho, and J. D. Baines.** 2006. Characterization of a U_L49-null mutant: VP22 of herpes simplex virus type 1 facilitates viral spread in cultured cells and the mouse cornea. J. Virol. **80:**8664–8675.
8. **Ejercito, P. M., E. D. Kieff, and B. Roizman.** 1968. Characterization of herpes simplex virus strains differing in their effects on social behaviour of infected cells. J. Gen. Virol. **2:**357–364.
9. **Elliott, G., and P. O'Hare.** 1998. Herpes simplex virus type 1 tegument protein VP22 induces the stabilization and hyperacetylation of microtubules. J. Virol. **72:**6448–6455.
10. **Elliott, G., and P. O'Hare.** 1999. Intercellular trafficking of VP22-GFP fusion proteins. Gene Ther. **6:**149–151.
11. **Elliott, G., D. O'Reilly, and P. O'Hare.** 1996. Phosphorylation of the herpes simplex virus type 1 tegument protein VP22. Virology **226:**140–145.
12. **Elliott, G., D. O'Reilly, and P. O'Hare.** 1999. Identification of phosphorylation sites within the herpes simplex virus tegument protein VP22. J. Virol. **73:**6203–6206.
13. **Elliott, G., G. Mouzakitis, and P. O'Hare.** 1995. VP16 interacts via its activation domain with VP22, a tegument protein of herpes simplex virus, and is relocated to a macromolecular assembly in coexpressing cells. J. Virol. **69:**7932–7941.
14. **Elliott, G., W. Hafezi, A. Whiteley, and E. Bernard.** 2005. Deletion of the herpes simplex virus VP22-encoding gene (U_L49) alters the expression, localization, and virion incorporation of ICP0. J. Virol. **79:**9735–9745.
15. **Esclatine, A., B. Taddeo, and B. Roizman.** 2004. The U_L41 protein of herpes simplex virus mediates selective stabilization or degradation of cellular mRNAs. Proc. Natl. Acad. Sci. USA **101:**18165–18170.
16. **Esclatine, A., B. Taddeo, L. Evans, and B. Roizman.** 2004. The herpes simplex virus 1 U_L41 gene-dependent destabilization of cellular RNAs is selective and may be sequence-specific. Proc. Natl. Acad. Sci. USA **101:**3603–3608.
17. **Fang, B., B. Xu, P. Koch, and J. A. Roth.** 1998. Intercellular trafficking of VP22-GFP fusion proteins is not observed in cultured mammalian cells. Gene Ther. **5:**1420–1424.
18. **Geiss, B. J., T. J. Smith, D. A. Leib, and L. A. Morrison.** 2000. Disruption of virion host shutoff activity improves the immunogenicity and protective capacity of a replication-incompetent herpes simplex virus type 1 vaccine strain. J. Virol. **74:**11137–11144.
19. **Geiss, B. J., J. E. Tavis, L. M. Metzger, D. A. Leib, and L. A. Morrison.** 2001. Temporal regulation of herpes simplex virus type 2 VP22 expression and phosphorylation. J. Virol. **75:**10721–10729.
20. **Green, K. L., T. D. Southgate, K. Mulryan, L. J. Fairbairn, P. L. Stern, and K. Gaston.** 2006. Diffusible VP22-E2 protein kills bystander cells and offers a route for cervical cancer gene therapy. Hum. Gene Ther. **17:**147–157.
21. **Hafezi, W., E. Bernard, R. Cook, and G. Elliott.** 2005. Herpes simplex virus tegument protein VP22 contains an internal VP16 interaction domain and a C-terminal domain that are both required for VP22 assembly into the virus particle. J. Virol. **79:**13082–13093.
22. **Hakkarainen, T., T. Wahlfors, O. Merilainen, S. Loimas, A. Hemminki, and J. Wahlfors.** 2005. VP22 does not significantly enhance enzyme prodrug cancer gene therapy as a part of a VP22-HSVTk-GFP triple fusion construct. J. Gene Med. **7:**898–907.
23. **Horsburgh, B. C., M. M. Hubinette, D. Qiang, M. L. MacDonald, and F. Tufaro.** 1999. Allele replacement: an application that permits rapid manipulation of herpes simplex virus type 1 genomes. Gene Ther. **6:**922–930.
24. **Kotsakis, A., L. E. Pomeranz, A. Blouin, and J. A. Blaho.** 2001. Microtubule reorganization during herpes simplex virus type 1 infection facilitates the nuclear localization of VP22, a major virion tegument protein. J. Virol. **75:**8697–8711.
25. **Kwong, A. D., and N. Frenkel.** 1987. Herpes simplex virus-infected cells contain a function(s) that destabilizes both host and viral mRNAs. Proc. Natl. Acad. Sci. USA **84:**1926–1930.
26. **Kwong, A. D., J. A. Kruper, and N. Frenkel.** 1988. Herpes simplex virus virion host shutoff function. J. Virol. **62:**912–921.
27. **Lam, Q., C. A. Smibert, K. E. Koop, C. Lavery, J. P. Capone, S. P. Weinheimer, and J. R. Smiley.** 1996. Herpes simplex virus VP16 rescues viral mRNA from destruction by the virion host shutoff function. EMBO J. **15:**2575–2581.
28. **Lemken, M. L., C. Wolf, W. A. Wybranietz, U. Schmidt, I. Smirnow, H. J. Buhring, A. F. Mack, U. M. Lauer, and M. Bitzer.** 2007. Evidence for intercellular trafficking of VP22 in living cells. Mol. Ther. **15:**310–319.
29. **Martin, A., P. O'Hare, J. McLauchlan, and G. Elliott.** 2002. Herpes simplex virus tegument protein VP22 contains overlapping domains for cytoplasmic localization, microtubule interaction, and chromatin binding. J. Virol. **76:**4961–4970.
30. **Morse, L. S., T. G. Buchman, B. Roizman, and P. A. Schaffer.** 1977. Anatomy of herpes simplex virus DNA. IX. Apparent exclusion of some parental DNA arrangements in the generation of intertypic (HSV-1 × HSV-2) recombinants. J. Virol. **24:**231–248.
31. **Mouzakitis, G., J. McLauchlan, C. Barreca, L. Kueltzo, and P. O'Hare.** 2005. Characterization of VP22 in herpes simplex virus-infected cells. J. Virol. **79:**12185–12198.
32. **Munger, J., A. V. Chee, and B. Roizman.** 2001. The U_S3 protein kinase blocks apoptosis induced by the *d*120 mutant of herpes simplex virus 1 at a premitochondrial stage. J. Virol. **75:**5491–5497.
33. **Murphy, A. L., and S. L. Murphy.** 1999. Catch VP22: the hitch hiker's ride to gene therapy? Gene Ther. **6:**4–5.
34. **Pomeranz, L. E., and J. A. Blaho.** 2000. Assembly of infectious herpes simplex virus type 1 virions in the absence of full-length VP22. J. Virol. **74:**10041–10054.
35. **Poon, A. P. W., and B. Roizman.** 1997. Differentiation of the shutoff of protein synthesis by virion host shutoff and mutant $\gamma_1$34.5 genes of herpes simplex virus 1. Virology **229:**98–105.
36. **Potel, C., and G. Elliott.** 2005. Phosphorylation of the herpes simplex virus tegument protein VP22 has no effect on incorporation of VP22 into the virus but is involved in optimal expression and virion packaging of ICP0. J. Virol. **79:**14057–14068.
37. **Roller, R. J., and B. Roizman.** 1992. The herpes simplex virus 1 RNA binding protein US11 is a virion component and associates with ribosomal 60S subunits. J. Virol. **66:**3624–3632.
38. **Roy, V., J. Qiao, P. de Campos-Lima, and M. Caruso.** 2005. Direct evidence

for the absence of intercellular trafficking of VP22 fused to GFP or to the herpes simplex virus thymidine kinase. Gene Ther. **12:**169–176.
39. **Schmelter, J., J. Knez, J. R. Smiley, and J. P. Capone.** 1996. Identification and characterization of a small modular domain in the herpes simplex virus host shutoff protein sufficient for interaction with VP16. J. Virol. **70:**2124–2131.
40. **Sciortino, M. T., M. Suzuki, B. Taddeo, and B. Roizman.** 2001. RNAs extracted from herpes simplex virus 1 virions: apparent selectivity of viral but not cellular RNAs packaged in virions. J. Virol. **75:**8105–8116.
41. **Sciortino, M. T., B. Taddeo, A. P. Poon, A. Mastino, and B. Roizman.** 2002. Of the three tegument proteins that package mRNA in herpes simplex virions, one (VP22) transports the mRNA to uninfected cells for expression prior to viral infection. Proc. Natl. Acad. Sci. USA **99:**8318–8323.
42. **Shenk, T.** 2002. Might a vanguard of mRNAs prepare cells for the arrival of herpes simplex virus? Proc. Natl. Acad. Sci. USA **99:**8465–8466.
43. **Smibert, C. A., B. Popova, P. Xiao, J. P. Capone, and J. R. Smiley.** 1994. Herpes simplex virus VP16 forms a complex with the virion host shutoff protein VHS. J. Virol. **68:**2339–2346.
44. **Smiley, J. R.** 2004. Herpes simplex virus virion host shutoff protein: immune evasion mediated by a viral RNase? J. Virol. **78:**1063–1068.
45. **Spear, P. G., and B. Roizman.** 1972. Proteins specified by herpes simplex virus. V. Purification and structural proteins of the herpesvirion. J. Virol. **9:**143–159.
46. **Strelow, L. I., and D. A. Leib.** 1996. Analysis of conserved domains of U_L41 of herpes simplex virus type 1 in virion host shutoff and pathogenesis. J. Virol. **70:**5665–5667.
47. **Strom, T., and N. Frenkel.** 1987. Effects of herpes simplex virus on mRNA stability. J. Virol. **61:**2198–2207.
48. **Suzutani, T., M. Nagamine, T. Shibaki, M. Ogasawara, I. Yoshida, T. Daikoku, Y. Nishiyama, and M. Azuma.** 2000. The role of the U_L41 gene of herpes simplex virus type 1 in evasion of non-specific host defence mechanisms during primary infection. J. Gen. Virol. **81:**1763–1771.
49. **Taddeo, B., A. Esclatine, W. Zhang, and B. Roizman.** 2003. The stress-inducible immediate-early responsive gene IEX-1 is activated in cells infected with herpes simplex virus 1, but several viral mechanisms, including 3′ degradation of its RNA, preclude expression of the gene. J. Virol. **77:**6178–6187.
50. **Taddeo, B., and B. Roizman.** 2006. The virion host shutoff protein U_L41 of herpes simplex virus 1 is an endoribonuclease with a substrate specificity similar to that of RNase A. J. Virol. **80:**9341–9345.
51. **Taddeo, B., W. Zhang, and B. Roizman.** 2006. The U_L41 protein of herpes simplex virus 1 degrades RNA by endonucleolytic cleavage in absence of other cellular or viral proteins. Proc. Natl. Acad. Sci. USA **103:**2827–2832.
52. **Taddeo, B., M. T. Sciortino, W. Zhang, and B. Roizman.** 2007. Interaction of herpes simplex virus RNase with VP16 and VP22 is required for the accumulation of the protein but not for accumulation of mRNA. Proc. Natl. Acad. Sci. USA **104:**12163–12168.
53. **van Leeuwen, H., M. Okuwaki, R. Hong, D. Chakravarti, K. Nagata, and P. O'Hare.** 2003. Herpes simplex virus type 1 tegument protein VP22 interacts with TAF-1 proteins and inhibits nucleosome assembly but not regulation of histone acetylation by INHAT. J. Gen. Virol. **84:**2501–2510.
54. **Vittone, V., E. Diefenbach, D. Triffett, M. W. Douglas, A. L. Cunningham, and R. J. Diefenbach.** 2005. Determination of interactions between tegument proteins of herpes simplex virus type 1. J. Virol. **79:**9566–9571.
55. **Wybranietz, W. A., F. Prinz, M. Spiegel, A. Schenk, M. Bitzer, M. Gregor, and U. M. Lauer.** 1999. Quantification of VP22-GFP spread by direct fluorescence in 15 commonly used cell lines. J. Gene Med. **1:**265–274.
56. **Ye, G. J., and B. Roizman.** 2000. The essential protein encoded by the U_L31 gene of herpes simplex virus 1 depends for its stability on the presence of U_L34 protein. Proc. Natl. Acad. Sci. USA **97:**11002–11007.
57. **Yedowitz, J. C., A. Kotsakis, E. F. M. Schlegel, and J. A. Blaho.** 2005. Nuclear localizations of the herpes simplex virus type 1 tegument proteins VP13/14, vhs, and VP16 precede VP22-dependent microtubule reorganization and VP22 nuclear import. J. Virol. **79:**4730–4743.
58. **Zavaglia, D., M. C. Favrot, B. Eymin, C. Tenaud, and J.-L. Coll.** 2003. Intercellular trafficking and enhanced in vivo antitumour activity of a non-virally delivered P27-VP22 fusion protein. Gene Ther. **10:**314–325.
59. **Zavaglia, D., E. H. Lin, M. Guidetti, O. Pluquet, P. Hainaut, M. C. Favrot, and J. L. Coll.** 2005. Poor intercellular transport and absence of enhanced antiproliferative activity after non-viral gene transfer of VP22-P53 or P53-VP22 fusions into p53 null cell lines in vitro or in vivo. J. Gene Med. **7:**936–944.
60. **Zhou, G., G. J. Ye, W. Debinski, and B. Roizman.** 2002. Genetic engineering of a herpes simplex virus 1 vector dependent on the IL13Rα2 receptor for entry into cells: interaction of glycoprotein D with its receptors is independent of the fusion of the envelope and the plasma membrane. Proc. Natl. Acad. Sci. USA **99:**15124–15129.