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The Null Mutant of the U_L31 Gene of Herpes Simplex Virus 1: Construction and Phenotype in Infected Cells

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Earlier studies have shown that the U_L31 protein is homogeneously distributed throughout the nucleus and cofractionates with nuclear matrix. We report the construction from an appropriate cosmid library a deletion mutant which replicates in rabbit skin cells carrying the U_L31 gene under a late (γ_1) viral promoter. The mutant virus exhibits cytopathic effects and yields 0.01 to 0.1% of the yield of wild-type parent virus in noncomplementing cells but amounts of virus 10- to 1,000-fold higher than those recovered from the same cells 3 h after infection. Electron microscopic studies indicate the presence of small numbers of full capsids but a lack of enveloped virions. Viral DNA extracted from the cytoplasm of infected cells exhibits free termini indicating cleavage/packaging of viral DNA from concatemers for packaging into virions, but analyses of viral DNAs by pulsed-field electrophoresis indicate that at 16 h after infection, both the yields of viral DNA and cleavage of viral DNA for packaging are decreased. The repaired virus cannot be differentiated from the wild-type parent. These results suggest the possibility that U_L31 protein forms a network to enable the anchorage of viral products for the synthesis and/or packaging of viral DNA into virions.

The U_L31 gene is predicted to encode a basic protein of 306 amino acids (25). The gene appears to be regulated as a γ_2 gene inasmuch as transcription was inhibited in cells infected and maintained in the presence of a DNA synthesis inhibitor (1-β-D-arabinofuranosylthymine) (18). The U_L31 amino acid sequence predicts a relatively hydrophobic protein with a hydrophilic domain at the amino terminus, a nuclear localization signal, and several consensus phosphorylation sites for casein kinase II, cyclic AMP-dependent kinase, and protein kinase C (21). Earlier studies from this laboratory demonstrated that the U_L31 protein is phosphorylated and nucleotidylylated, localizes diffusely throughout the nucleus, and cofractionates with the nuclear matrix (6, 9). The compartment occupied by the U_L31 protein, the entire nucleus except for the nucleoli, exceeds the nuclear domain involved in the synthesis and transcription of viral DNA or that associated with capsid and capsid-tegument structures late in infection. Numerous attempts to delete the gene in the absence of a cell line expressing the U_L31 gene were not successful.

In this report, we describe the construction of a recombinant virus from which the U_L31 gene was deleted. To facilitate the construction of the mutant, we made a set of overlapping cosmids including two from which the entire U_L31 open reading frame was deleted. We also constructed a cell line which is readily transfected with high efficiency and which expressed a U_L31 gene designed to preclude its recombination with the recombinant virus from which the U_L31 open reading frame had been deleted. The cell line, rabbit skin cells, has been used for high-efficiency recombination for production of a large number of recombinant viruses in the past 17 years (29). The advantage of the system used in the construction of the recombinant virus is that it obviates the need to select or screen the progeny of transfection for the recombinant viruses with the desired genotype (11). To ensure that only the absence of U_L31 is responsible for the phenotype of the null mutant, the deleted sequences were restored. In this report, we show that the deletion mutant was highly deficient in its ability to multiply and that in noncomplementing cells infected with the deletion mutant, viral DNA synthesis was reduced as was the cleavage/packaging of DNA; consistent with these observations, we observed a decrease in the envelopment and export of virions from infected cells.

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MATERIALS AND METHODS

Cells and viruses. Rabbit skin cells and human 143TK$^-$ cells were obtained from John McLaren and Carlo Croce, respectively; Vero cells were obtained from American Type Culture Collection. Human lung fibroblast (HLF) cells were obtained from George Kemble (Aviron, Mountain View, Calif.). Cells were maintained in Dulbecco's modified Eagle medium supplemented with either 5% newborn calf serum (rabbit skin cells and Vero cells), 5% fetal bovine serum (143TK$^-$ cells), or 10% fetal bovine serum (HLF cells). Infected cells were maintained in medium consisting of mixture 199 supplemented with 1% calf serum (199V). Virus titer was measured on infected cells maintained in 199V plus 0.2% human immunoglobulin. RD14 and RD17 U_L31-expressing cell lines were two independent clones derived from rabbit skin cells and maintained in 10% fetal bovine serum. Herpes simplex virus 1 strain F [HSV-1(F)], a limited-passage virus, serves as the prototype HSV-1 strain in this laboratory (16).

Generation of U_L31 polyclonal antibody. A DNA fragment encoding U_L31 codons 1 to 163 was cloned into an *Escherichia coli* anthranilate synthetase (TrpE) expression vector (22). The fusion protein was induced after 2-h log-phase growth of transformed *E. coli* with 20 μg of indoleacrylic acid per ml. After 4 h of additional incubation, the bacteria were resuspended in lysis buffer (50 mM Tris-HCl [pH 7.5], 5 mM EDTA, 3 mg of lysozyme per ml) and stored on ice for 2 h. The lysate was then adjusted to 0.3 M NaCl and 0.65% Nonidet P-40 and centrifuged in a Sorvall SS34 rotor at 10,000 rpm for 10 min. The pellet was solubilized in disruption buffer (50 mM Tris-HCl [pH 7.0], 2% sodium dodecyl sulfate [SDS], 0.7 M β-mercaptoethanol, 2.75% sucrose) and subjected to electrophoresis in a denaturing polyacrylamide gel. The gel was stained with cold 0.1 M KCl (7). The fusion protein was excised from the gel and electroeluted into a buffer containing 50 mM NH_4HCO_3 and 0.1% SDS. The eluted protein was vacuum dried, resuspended in water, and precipitated with acetone three times to remove excess SDS. The final suspension was sent to Josman Laboratories (Napa, Calif.) for production of rabbit polyclonal antiserum.

Construction of recombinant plasmids, cosmids, and viruses. All plasmids were constructed by standard techniques as described elsewhere (34). Viral DNAs for construction of recombinant viruses were purified on a 5 to 20% potassium acetate gradient (19). pRB4953 contained the *Hin*cII/*Bam*HI DNA

segment of the *Bam*HI W fragment cloned from the R4606 viral genome (9). This clone contained the U_L31 coding sequence tagged in frame at the 5′ terminus with a sequence encoding the human cytomegalovirus (CMV) epitope, downstream of the HSV-1 gD promoter. pRB5106 contained a 3.4-kb *Pst*I/*Xba*I DNA fragment encoding a portion of the U_L30, the entire U_L31 and U_L32, and a portion of the U_L33 open reading frames. pRB182 carrying the *Bam*HI W DNA fragment served as a probe (probe 2) of nucleotide sequence arrangement in this domain of the HSV-1 genome. The construction of R4603 was described elsewhere (9).

We constructed two series of recombinant viruses. The objective of construction of the first set of viruses was to end up with a recombinant virus in which the U_L31 gene was flanked by unique *Pme*I sites. The 5′ end of the U_L31 and 3′ end of the U_L32 open reading frames overlap. The recombinant virus R5127 was designed to insert a unique *Pme*I restriction endonuclease site into the previously constructed recombinant virus R4603 (9), in which an α27-*tk* gene was inserted between U_L31 and U_L32 into a sequence that duplicated the overlap and separated the coding domains of U_L31 and U_L32. To create R5127, intact R4603 DNA was cotransfected with pRB5127 (29). This plasmid was derived by insertion of the oligonucleotide 5′CTGTTTAAACTATACGTAGGTACAGAGG3′ into the *Dra*III site of pRB4601. The consequence of this insertion was to separate U_L31 from U_L32 and insert into the intervening space a *Pme*I site. This oligonucleotide contained sequences 3′ of the *Dra*III site to the stop codon of the U_L32 coding sequence. The duplicated initiator methionine codon of U_L31 within the oligonucleotide was mutated to ACG without changing the amino acid residue of the U_L32 gene. However, the original ATG of U_L31 gene downstream to the *Dra*III site remained unchanged. The progeny of the transfection was plated in 143TK$^-$ cells in the presence of bromodeoxyuridine (40 μg/ml); the recombinant R5127 was selected, and its sequence was verified.

The design of the studies required the genetic engineering of a second *Pme*I site at the 3′ end of U_L31, between U_L31 and U_L30. The U_L31 and U_L30 coding sequences overlap by 51 bp. To insert α27-*tk* between the U_L30 and U_L31 genes, we constructed a plasmid (pRB5130) containing the α27-*tk* gene inserted into an *Nco*I site within a duplication of the overlapping sequences. To recombine this sequence into the virus, it was necessary to add to pRB5130 the appropriate flanking sequences of U_L31 and U_L30 genes to enable double homologous recombination. The flanking 1-kb sequences of U_L31 and U_L30 coding domains were generated by PCR using the primers 5′CGGACGTCTACGGCGG AGGAAACTCGTCGAATGTTG3′ plus 5′CGGACGTCAGGTATTTGTACC AAAGCCCACCGCGC3′ and 5′CACTAGTATTCCATGCGCATCATCTAC G3′ plus 5′CACTAGTCATGCTAGAGTATCAAAGGC3′, respectively. In the plasmid used to recombine the insert between U_L30 and U_L31, the 1 kb of DNA flanked the insert and enabled the selection of R5130 recombinant virus from the progeny of cotransfection of the plasmid DNA with intact R5127 DNA. In the next step, the α27-*tk* gene in R5130 was replaced with the oligonucleotide 5′CATGCGTTTAAACTCCA3′ to generate pRB5131. This oligonucleotide maintained the separation of the coding domains of U_L30 and U_L31 and in addition inserted a *Pme*I site in the intervening sequences. The virus obtained using this plasmid was designated R5131.

The objective of the second set of viruses was to construct a U_L31 null mutant. This step required a set of cosmids containing overlapping sequences of the wild-type genome and cosmids from which the U_L31 gene had been deleted.

All cosmids used in this study were constructed with viral DNA derived from either HSV-1(F) or R5131. To construct the cosmid library, the *Eco*RI fragment containing the multiple cloning site of the SuperCos I cosmid vector (Stratagene, La Jolla, Calif.) was replaced with an *Eco*RI fragment containing *Eco*RI/*Pac*I/*Sse*8387I/*Spe*I/*Bam*HI/*Nde*I/*Eco*RV/*Pac*I/*Eco*RI multiple cloning sites to generate pRB78. Partial *Sau*3AI digests of HSV-1(F) or partial *Mbo*I digests of R5131 DNA were dephosphorylated and ligated to the *Bam*HI-digested pRB78 cosmid vector and packaged into lambda phage with the Gigapack XLII (Stratagene) packaging extract according to the manufacturer's instructions.

Ampicillin-resistant clones were initially analyzed by restriction mapping and, in the case of clones derived from R5131, by hybridization. Three cosmids derived from the HSV-1(F) DNA and used in this study were pBC1004, pBC1006, and pBC1007 (Fig. 1B). Cosmid pBC1005 contained the *Nde*I A fragment, and cosmid pBC1008 contained the *Bgl*II F/H fragment, of HSV-1(F) DNA. Two clones, pBC1000 and pBC1002, derived from R5131 DNA were digested with *Pme*I to excise U_L31 and religated to yield pBC1001 and pBC1003, respectively. All cosmids were end sequenced to verify mapping. Cosmids pBC1001, pBC1003, pBC1004, pBC1005, pBC1006, pCB1007, and pBC1008 were shown to contain nucleotides 44614 to 79398, 62657 to 102527, 133052 to 17029, 12136 to 57747, 2945 to 45035, 77933 to 116016, and 106750 to 142759, respectively.

To construct the recombinant virus R5132, the cosmids were digested with *Pac*I to release the HSV-1 sequences from the cosmid vector and centrifuged in a sucrose gradient (15 to 25% [wt/vol] sucrose in TE [10 mM Tris-HCl {pH 8.0} 1 mM EDTA]), 25,000 rpm for 16 h at room temperature in an SW41 rotor. The fractions containing high-molecular-weight DNA were pooled, dialyzed, and ethanol precipitated before transfection. Approximately 1 μg of each cosmid was used for transfection by the calcium phosphate procedure (14). The deletion in R5132 was repaired by cotransfection of R5132 viral DNA and plasmid pRB 5106 (Fig. 1C, line 4) into rabbit skin cells. The progeny virus was designated R5133.

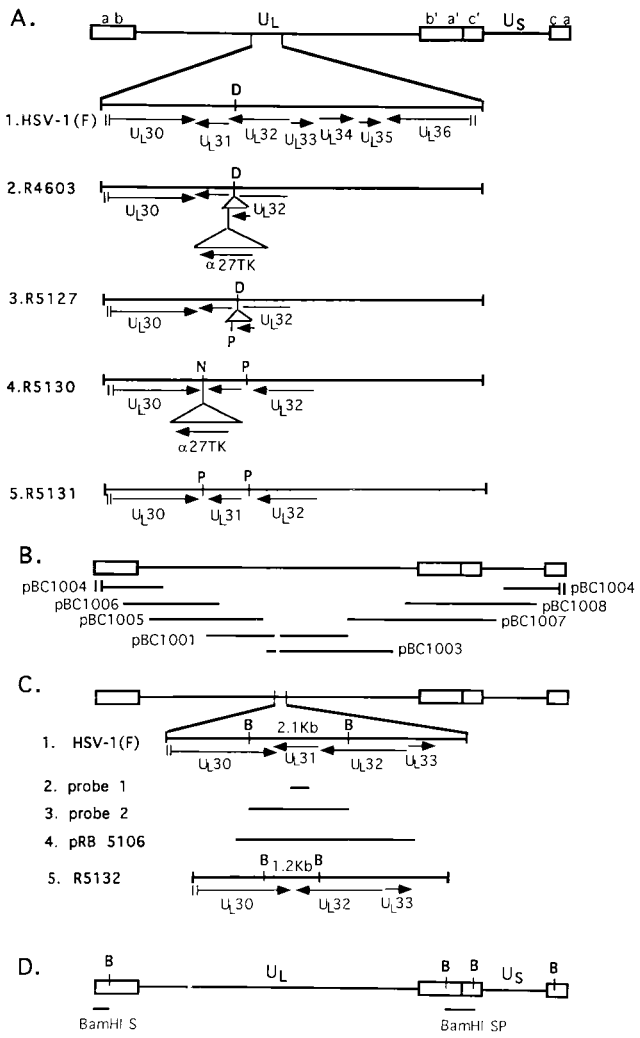

FIG. 1. Schematic diagram of recombinant viruses, cosmids, and plasmids. (A) Line 1, genome organization of HSV-1 and position of the U_L31 gene. The open boxes represented terminal sequences *ab* and *ca* repeated internally in an inverted orientation. U_L and U_S are the unique long and unique short sequences, flanked by the inverted repeats. The overlapping region of U_L31 and U_L32 was duplicated in an oligonucleotide cloned into the *Dra*III site (D), and an α27-*tk* chimeric gene was then recombined into the engineered intergenic region (9). In a subsequent step, the chimeric α27-*tk* gene was replaced by an oligonucleotide containing a *Pme*I (P) restriction endonuclease cleavage site within the duplicated region separating the U_L31 and U_L32 genes to yield the recombinant virus R5127 (line 3). In the next step, the α27-*tk* gene was first recombined into the *Nco*I (N) site of the engineered intergenic region of U_L30 and U_L31 to create R5130 (line 4) and then replaced with an oligonucleotide containing a second *Pme*I site to generate R5131 (line 5). (B) The cosmid set used to generate U_L31 deletion virus R5132. All cosmids except for pBC1001 and pBC1003, which were derived from R5131, were derived from HSV-1(F). (C) Genome organization of U_L31 deletion virus R5132. Line 1, the position and predicted size of the *Bam*HI (B) W fragment in the wild-type HSV-1(F) genome. Line 2, U_L31-specific PCR product used for detection of U_L31 DNA sequences (probe 1), which encompassed nucleotides 177 to 708 of the U_L31 coding sequence. Line 3, location of the *Bam*HI W fragment of HSV-1(F) used as probe 2. Line 5, genome organization of U_L31 deletion mutant R5132 and predicted size of the truncated *Bam*HI W fragment resulting from the deletion. Line 4, plasmid used to generate the repaired virus R5133, containing a 3.4-kb *Pst*I/*Xba*I fragment. (D) Positions of *Bam*HI S and BamHI SP fragments in the viral genome.

Construction of a cell line expressing U_L31 protein. Subconfluent rabbit skin cells were transfected with plasmids pRB4953 and pSV2neo mixed in a ratio of 10:1 by the calcium phosphate method (14). Approximately 48 h after transfection, the cells were subcultured 1:10 and maintained in medium supplemented

with 10% fetal bovine serum and 400 μg of G418 per ml of medium. Neomycin-resistant colonies were picked, amplified, and tested for expression of the U_L31 protein tagged with the CMV epitope as described below. Two positive clones, designated RD17 and RD14, were used in this study.

Analyses of viral DNA by hybridization. Cytoplasmic DNAs of infected cells were harvested, purified by phenol and chloroform extraction, digested with *Bam*HI, electrophoretically separated on an agarose gel, and transferred to a nylon membrane (Bio-Rad Laboratories, Hercules, Calif.). The hybridization and membrane-stripping procedures were as recommended by the manufacturer. Either plasmid pRB182 labeled with a Nick Translation kit (DuPont NEN) (probe 2) or a PCR product representing the U_L31-specific region end labeled with polynucleotide kinase (U.S. Biochemical) (probe 1) was used as the probe for the presence or absence of specific HSV-1 sequences as detailed in Results. The junction and terminal *Bam*HI fragment were detected in the same manner by a nick-translated probe representing the *Bam*HI S region (pRB4789) (23).

Antibodies. The U_L32 and U_L38 rabbit polyclonal antibodies and U_S11 mouse monoclonal antibody used in this study were described previously (8, 33, 35). The U_L13 rabbit polyclonal antibody was described elsewhere (27). The ICP4 monoclonal antibody G1114 and ICP8 monoclonal antibody G1115 were obtained from Goodwin Institute (Plantation, Fla.). Goat anti-rabbit or anti-mouse alkaline phosphatase-conjugated secondary antibody was purchased from Bio-Rad. Goat anti-rabbit peroxidase-conjugated secondary antibody was purchased from Sigma Chemical Co. (St. Louis, Mo.). The goat anti-rabbit Texas red-conjugated secondary antibody was purchased from Molecular Probes Inc. (Eugene, Oreg.), and the goat anti-mouse fluorescein isothiocyanate (FITC)-conjugated secondary antibody was purchased from Sigma.

Detection of viral proteins in electrophoretically separated, denatured lysates of infected cells. Mock-infected or HSV-1-infected rabbit skin or RD14 cells were harvested in disruption buffer, electrophoretically separated in a denaturing polyacrylamide gel cross-linked with *N,N′*-diallyltartardiamide, electrically transferred to a nitrocellulose membrane (Schleicher & Schuell, Keene, N.H.), blocked with 5% nonfat dry milk in phosphate-buffered saline, reacted with primary antibody, extensively rinsed, reacted with goat anti-rabbit secondary antibody conjugated with alkaline phosphatase (Bio-Rad), rinsed again, and then reacted with color-developing reagent purchased from Sigma. The ECL (enhanced chemiluminescence) immunoblotting procedure was as recommended by the manufacturer (Amersham International plc, Buckinghamshire, England).

Confocal fluorescence microscopy. Subconfluent 24-h cultures of RD14 or rabbit skin cells in four-well slides were exposed to 20 PFU of HSV-1(F) or R5132 per cell and incubated at 37°C. At 18 h after infection, the slides were fixed in methanol at −20°C for 20 min, air dried, blocked with 1% bovine serum albumin and 20% human serum in phosphate-buffered saline at room temperature for 1 h, and then reacted for an additional hour with primary antibodies diluted in phosphate-buffered saline containing 1% bovine serum albumin and 10% human serum. After extensive rinsing, the cells were reacted with a mixture of goat anti-mouse immunoglobulin G (IgG) conjugated to FITC (Sigma) and anti-rabbit IgG conjugated to Texas red (Molecular Probes), rinsed again, then mounted in 90% glycerol in phosphate-buffered saline containing 1 mg of *p*-phenylenediamine per ml (20), and examined with the aide of Zeiss confocal microscope. Images were captured and recorded with software provided by Zeiss.

Pulsed-field gel electrophoresis. Replicate monolayer cultures of Vero cells in 25-cm^2 flasks were exposed to 5 PFU of HSV-1(F), R5132, or R5133 per cell. The infected cells harvested at 16 h after infection were rinsed once with phosphate-buffered saline and resuspended in 0.25 ml of buffer (10 mM Tris-HCl [pH 7.2], 20 mM NaCl, 100 mM EDTA). Each cell suspension was warmed to 42°C, mixed with 0.25 ml of 1% low-melting-point agarose in H_2O, and immediately poured into a 1-ml syringe. After chilling, the agarose plug was extruded into 2 ml of buffer consisting of 1 mg of proteinase K per ml, 100 mM EDTA, 10 mM Tris-HCl (pH 8.0), 50 mM NaCl, 1% *N*-laurylsarcosine (sodium salt), and 0.2% sodium deoxycholate. After digestion for 18 to 24 h at 42°C, the plug was washed 15 times for 20 min each in buffer consisting of 20 mM Tris-HCl (pH 8.0) and 50 mM EDTA, washed two times for 15 min each in 2 mM Tris-HCl (pH 8.0)–5 mM EDTA, and stored at 4°C in the same buffer. Slices 30 μl in size were cut from the agarose plugs, equilibrated with 0.5× Tris-borate-EDTA running buffer, and loaded into wells of a 1.2% agarose gel for electrophoresis.

The 1.2% agarose gels were prepared in 0.5× Tris-borate-EDTA buffer. The gels and buffer were maintained at 11°C during electrophoresis in a Hoefer HG 1000 gel rig (Hoefer Scientific Instruments, San Francisco, Calif.) at 180 V for 40 h, with a pulse time of 300 s and a pulse angle of 120°. After electrophoresis, the DNA was transferred to a nylon membrane (Bio-Rad), hybridized with a ^{32}P-labeled probe generated by nick translation of gradient-purified total HSV-(F) DNA, and subjected to autoradiography. Quantification of DNA was done with computer-aided densitometry of images obtained with an ImageQuant PhosphorImager (Molecular Dynamics).

RESULTS

Detection of native U_L31 protein by polyclonal antibody. Earlier this laboratory reported the construction of recombinant virus R4606, in which the U_L31 gene was tagged in frame at its 5′ terminus with a sequence encoding a CMV epitope to which a monoclonal antibody was available. As a consequence, the tagged U_L31 protein could be detected by a monoclonal antibody specific to the epitope (9). For the purposes of this report, a polyclonal antibody was generated as described in Materials and Methods to detect the native, unaltered form of the U_L31 protein. As shown in Fig. 2A, the antiserum reacted specifically with a viral protein with an apparent M_r of 34,000 in lysates of HSV-1(F)-infected cells (lane 2) and with a protein with an apparent M_r of 37,000 from cells infected with the recombinant virus R4606 carrying a tagged U_L31 gene (lane 3) but not with lysates of mock-infected cells (lane 1). The cellular distribution of the native U_L31 protein in HSV-1(F)-infected cells as determined by immunofluorescence was identical to that of the tagged protein in cells infected with the R4606 recombinant virus (data not shown) (9).

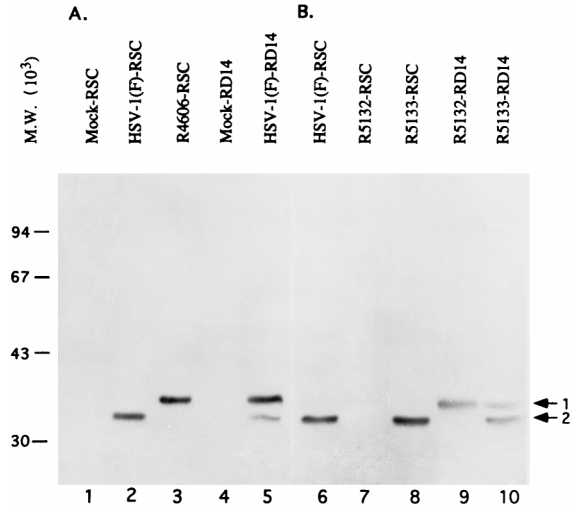

FIG. 2. Photograph of electrophoretically separated proteins of infected cell lysates probed with a U_L31-specific polyclonal antibody. Cells were harvested 18 h after mock infection or infection with the indicated viruses. The proteins were electrophoretically separated on an SDS–12% polyacrylamide gel, electrically transferred to a nitrocellulose membrane, and reacted with the U_L31 polyclonal antibody. (A) The RD14 cell line expressed CMV-tagged U_L31 protein induced by infection with HSV-1(F). Lane 1, mock-infected cells; lane 2, rabbit skin cells infected with HSV-1(F); lane 3, rabbit skin cells infected with CMV epitope-tagged U_L31 virus R4606; lane 4, mock-infected RD14 cells; lane 5, RD14 cells infected with HSV-1(F). (B) U_L31 deletion virus R5132 did not express U_L31 protein. Lane 6, rabbit skin cells infected with HSV-1(F); lane 7, rabbit skin cells infected with R5132; lane 8, rabbit skin cells infected with R5133; lane 9, RD14 cells infected with the R5132; lane 10, RD14 cells infected with R5133. Arrows 1 and 2 indicated the migration of the epitope-tagged and native U_L31 protein, respectively.

Construction of a cell line carrying a U_L31 gene tagged with the CMV epitope and inducible by infection with HSV-1. We have previously shown that the recombinant virus R4606 carrying a U_L31 gene tagged with a CMV epitope replicated to a level comparable to that of the wild-type virus (9), suggesting that the epitope-tagged U_L31 protein is fully functional. To facilitate selection of cells expressing U_L31 protein, we constructed a rabbit skin cell line expressing the epitope-tagged U_L31 protein driven by an HSV-1(F) sequence extending from nucleotides −372 to +92 relative to the transcription initiation site of the gD gene. The objective was to select a cell line in which expression of the U_L31 protein would be inducible by superinfection and the induced protein could be readily differentiated from the wild-type protein encoded by the infecting virus. In addition, the use of a heterologous promoter would be expected to preclude the rescue of a U_L31 null mutant by

recombination. As described in Materials and Methods, two clones, RD14 and RD17, of rabbit skin cells cotransfected with the plasmid encoding U_L31 and neomycin resistance gene and selected for resistance to G418 were found to express the epitope-tagged U_L31 protein upon infection with wild-type virus. To test these clones, the cells were mock infected or infected with 10 PFU of HSV-1(F) per cell. As shown in Fig. 2, the epitope-tagged U_L31 protein was expressed by infected but not by mock-infected RD14 cells (Fig. 2A, lanes 4 and 5). The RD17 cell line showed the same pattern (data not shown). Except for the initial cosmid transfection, most of the work presented in this report was done on the RD14 cell line.

The U_L31 null mutant virus R5132 generated by a cosmid-based system showed the predicted genotype. We have chosen the cosmid system for the construction of the U_L31 null mutant. As described in Materials and Methods, the protocol involved the following steps.

(i) The recombinant virus R4603 described earlier (9) contained the α27-*tk* gene inserted between the U_L31 and U_L32 genes (Fig. 1A, line 2). The α27-*tk* gene was replaced by recombination with an oligonucleotide which regenerated separate termini for the U_L31 and U_L32 genes and inserted between them a *Pme*I site (line 3).

(ii) A DNA fragment that provided authentic but separate termini for the U_L30 and U_L31 genes and also the α27-*tk* gene was recombined into the recombinant virus and designated R5130 (Fig. 1A, line 4). In the next step, the α27-*tk* gene was replaced with an oligonucleotide which maintained the separation of the U_L30 and U_L31 genes and inserted between them the *Pme*I site to yield R5131 (line 5).

(iii) A cosmid library was then generated, from which we selected two cosmids containing the U_L31 gene. The U_L31 gene was excised by cleavage with *Pme*I. The religated cosmids were transfected into the RD17 cells with other appropriate overlapping cosmids (Fig. 1B) to regenerate a virus differing from wild type in that it lacked 1 kb of DNA encoding the U_L31 gene (Fig. 1C, line 5). Because the cosmid encoding the natural *tk* gene was derived from HSV-1(F), the resulting recombinant virus, R5132, was also tk^+.

(iv) In the last step, R5132 was repaired by cotransfection of rabbit skin cells with R5132 viral DNA and pRB5106 (Fig. 1C, line 4). The repaired virus was designated R5133.

To analyze the genotypes of these viruses, HSV-1(F), R5132, and R5133 DNAs were individually digested with *Bam*HI, electrophoretically separated in agarose gels, transferred to a nylon membrane, and hybridized to ^{32}P-labeled probe 1 DNA as described in Materials and Methods. Probe 1, derived from the coding domain of the U_L31 gene (Fig. 1C, line 2), hybridized to the 2.1-kb *Bam*HI W fragment of wild-type HSV-1(F) and to the same, restored fragment of R5133 DNA (Fig. 3A, lanes 1 and 3). As expected, probe 1 DNA did not hybridize with the DNA of the deletion virus R5132 (Fig. 3A, lane 2). To delineate the remaining sequences in the deletion mutant, the same blot was stripped and reprobed with a ^{32}P-labeled *Bam*HI W fragment (probe 2 [Fig. 1C, line 3]). This probe hybridized to a fragment with electrophoretic mobility of approximately 1.2 kb (Fig. 1C, line 5; Fig. 3B, lane 2). Because the overlapped region of U_L30 and U_L31 genes was duplicated in the parent viruses R5130 and R5131, this added 80 bp to the size of the *Bam*HI W fragment after excision of the U_L31 gene. These results were consistent with the predicted size of the residual sequences following excision of the U_L31 gene.

U_L31 null mutant virus R5132 did not express U_L31 protein. The purpose of this set of experiments was to verify that R5132 did not express U_L31 protein. Replicate cultures of rabbit skin cells or of RD14 cells were exposed to 10 PFU of HSV-(F), R5132, or R5133 per cell. At 18 h after infection, cells were harvested, solubilized, subjected to electrophoresis on an SDS–12% polyacrylamide gel, transferred to a nitrocellulose sheet, and reacted with antibody to U_L31 as described in Materials and Methods. As shown in Fig. 2B, U_L31 protein was present in lysates of HSV-1(F)- or R5133-infected cells (lanes 6 and 8) but not in lysates of cells infected with R5132 (lane 7).

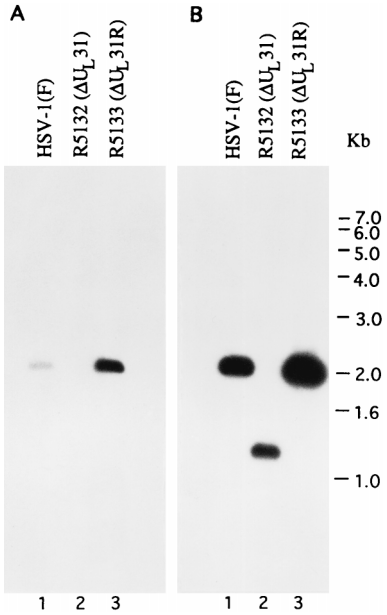

FIG. 3. Photograph of electrophoretically separated digests of viral DNAs reacted with specific probes. HSV-1(F), U_L31 deletion virus R5132, and repaired virus R5133 DNAs were purified from infected Vero cells as described in Materials and Methods, digested with *Bam*HI, separated by agarose gel electrophoresis, and transferred to a nylon membrane. (A) Electrophoretically separated *Bam*HI-digested HSV-1(F) (lane 1), R5132 (lane 2), or R5133 (lane 3) DNA, hybridized with labeled probe 1 DNA (Fig. 1C, line 2). (B) The blot shown in panel A, stripped and hybridized with probe 2 DNA (Fig. 1C, line 3). The 1-kb molecular weight standard ladder (Gibco BRL, Grand Island, N.Y.) is shown at the right.

Both R5132 and R5133 transactivated the expression of the epitope-tagged U_L31 gene resident in RD14 cells (Fig. 2, lanes 9 and 10). In other experiments, we compared the expression of several genes representing different kinetic classes (e.g., ICP4, U_L13, U_L32, and U_S11) (25, 32, 36) in rabbit skin cells infected with R5132 or HSV-1(F), by reacting the respective antibodies with electrophoretically separated, denatured proteins transferred to a nitrocellulose sheet. In all instances (Fig. 4), the expression of these genes was unaffected by the absence of U_L31 protein.

The U_L31 null mutant virus R5132 was defective for growth in nonpermissive cells. Initial characterization of the deletion virus R5132 on rabbit skin cells showed reduced cytopathic effects particularly at low multiplicity of infection. No plaques were also observed in Vero cell cultures infected with 0.1 PFU per cell. In human lung fibroblasts, very small plaques could be seen after prolonged incubation time (data not shown). To assess the ability of R5132 to replicate in cells lacking a resident U_L31 gene, rabbit skin or Vero cells were exposed to 0.1 PFU of HSV-1(F), R5132, or R5133 per cell. The progeny viruses were harvested by freeze-thawing and sonication of the infected cells at 3 or 26 h after infection. Viral yield was measured by titering the viruses on RD14 cells. The 3-h titers measured attached but unpenetrated virus that maintained its

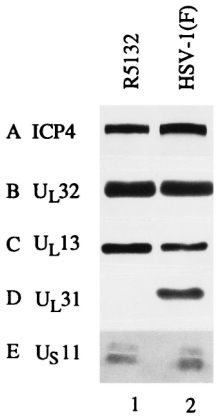

FIG. 4. Photograph of electrophoretically separated proteins from lysates of replicate rabbit skin cultures infected with R5132 or HSV-1(F). The cells were harvested 18 h after infection, electrophoretically separated in denaturing gels, transferred to a nitrocellulose sheet, and reacted with antibodies as shown at the left. (A, D, and E) ICP4, U_L31, and U_S11 proteins were detected with indicated primary antibodies and alkaline phosphatase-conjugated secondary antibodies. (B and C) U_L32 and U_L13 were detected with indicated primary antibodies and peroxidase-conjugated secondary antibodies, followed by the ECL immunoblotting procedure described in Materials and Methods.

infectivity, whereas the bulk of the 26-h titers represent the virus produced after infection. The results of four independent assays shown in Table 1 indicate that R5132 yields were 1,000- to 10,000-fold lower than those of wild-type virus HSV-1(F) or of the repaired virus R5133. RD14 cell line only partially complemented the capacity of the null mutant to replicate inasmuch as the yields of R5132 were 100-fold higher than in the parental, rabbit skin cells infected under the same conditions (data not shown).

Cells lacking a resident U_L31 gene (nonpermissive cells) infected with the U_L31 null mutant R5132 did not exhibit extracellular virions on cell surfaces. Experiments described above indicated that representative proteins of the various kinetic classes of HSV-1 were made in cells infected with R5132 lacking a resident U_L31 gene. To determine the site of the defect in viral maturation, replicate cultures of RD14 or rabbit skin cells were exposed to 10 PFU of wild-type HSV-1(F), R5132, or R5133 per cell. The infected cells were harvested and processed for electron microscopy at 20 h postinfection. In rabbit skin cells infected with the deletion mutant R5132, large number of empty A and B capsids and occasionally full C capsids were detected in the nucleus (17). No virions, however, were seen in cytoplasm or extracellular space. Large number of L particles could be seen decorating the plasma membrane (Fig. 5A to C) (26, 31), whereas in rabbit skin cells infected with HSV-1(F), both cytoplasmic and extracellular virions were readily seen (Fig. 5E). The defect was rescued in either RD14 cells infected with R5132 (Fig. 5D) or rabbit skin cells infected with the repaired virus R5133 (Fig. 5F and G).

U_L31 deletion virus R5132 showed normal pattern of capsid assembly. The absence of extracellular virions could reflect defects in capsid assembly and/or DNA synthesis and packaging. Earlier studies from this laboratory have shown that late in infection, the capsid protein VP19C encoded by the U_L38 gene colocalized in peripheral nuclear structures designated assemblons containing other capsid proteins, whereas the proteins involved in DNA synthesis (e.g., ICP8) colocalized with proteins involved in transcription of the DNA in a neighboring compartment (12, 30, 35). Immunofluorescence studies using polyclonal antibody to VP19C and monoclonal antibody to ICP8 on RD14 or rabbit skin cell line infected with HSV-1(F) or R5132 showed no substantive difference in the localization of these two proteins. VP19C detected with polyclonal antibody and goat anti-rabbit IgG conjugated with Texas red localized to one or frequently more than one small peripheral nuclear structures, whereas ICP8 detected by the reactivity of anti-mouse IgG conjugated with FITC filled most of the central portion of the nucleus in virtually all infected cells (Fig. 6A and B). The only notable difference is that fewer assemblons were seen in rabbit skin cells infected with R5132 than in RD14 cells infected with R5132 or in either rabbit skin or RD14 cells infected with HSV-1(F).

U_L31 deletion virus R5132 is deficient in accumulation of monomeric DNA in infected Vero cells. Two series of experiments were done. In the first, we tested the presence of junction and terminal fragments to determine whether viral DNA was cleaved and processed for packaging. In principle, monomeric DNA on cleavage with *Bam*HI yields a junction fragment (*Bam*HI SP) connecting the L and S components of viral DNA, a copy of the L-component terminus (*Bam*HI S) (Fig. 1D), and a copy of the S terminus (*Bam*HI P), whereas if cleavage/packaging of concatemeric DNA were blocked, we would have expected to see primarily junction fragments. Cytoplasmic viral DNAs were purified, digested with *Bam*HI, electrophoretically separated on an agarose gel, transferred to a nylon membrane, and hybridized to a ^{32}P-labeled *Bam*HI S fragment to detect both *Bam*HI S and *Bam*HI SP fragments of the viral genome. Although not specifically noted in Fig. 7, because *Bam*HI S also contains an *a* sequence, the probe also detects *Bam*HI P, albeit less efficiently. The results show that cleavage/packaging of viral DNA did occur in R5132-infected cells, as evidenced by the presence of both junction (*Bam*HI SP) and terminal (*Bam*HI S) fragments (Fig. 7, lane 2).

The assay described above and shown in Fig. 7 measures the presence of junction and terminal fragments but not the efficiency of cleavage/packaging of viral DNA. The objective of the second series of experiments was to examine and quantify

TABLE 1. Replication of wild-type and deletion mutants in rabbit skin or Vero cells[a]

Virus	Viral yield (PFU/ml)							
	Rabbit skin cells				Vero cells			
	Expt 1		Expt 2		Expt 3		Expt 4	
	3 h	26 h	3 h	26 h	3 h	26 h	3 h	26 h
HSV1-(F)	1,800	3.7×10^8	27	2.2×10^8	34	6.0×10^7	27	6.3×10^7
R5132	20	3.0×10^4	153	1.2×10^5	172	2.0×10^3	300	3.0×10^3
R5133	25	1.2×10^8	38	2.2×10^8	47	7.0×10^7	17	1.4×10^7

[a] Replicate cultures of rabbit skin cells (experiments 1 and 2) or Vero cells (experiments 3 and 4) were infected with 0.1 PFU of HSV-1(F), R5132, or R5133 per cell. At 3 or 26 h after infection, the cells were harvested and lysed by freeze-thawing and sonication. Viral yield was measured on RD14 cells.

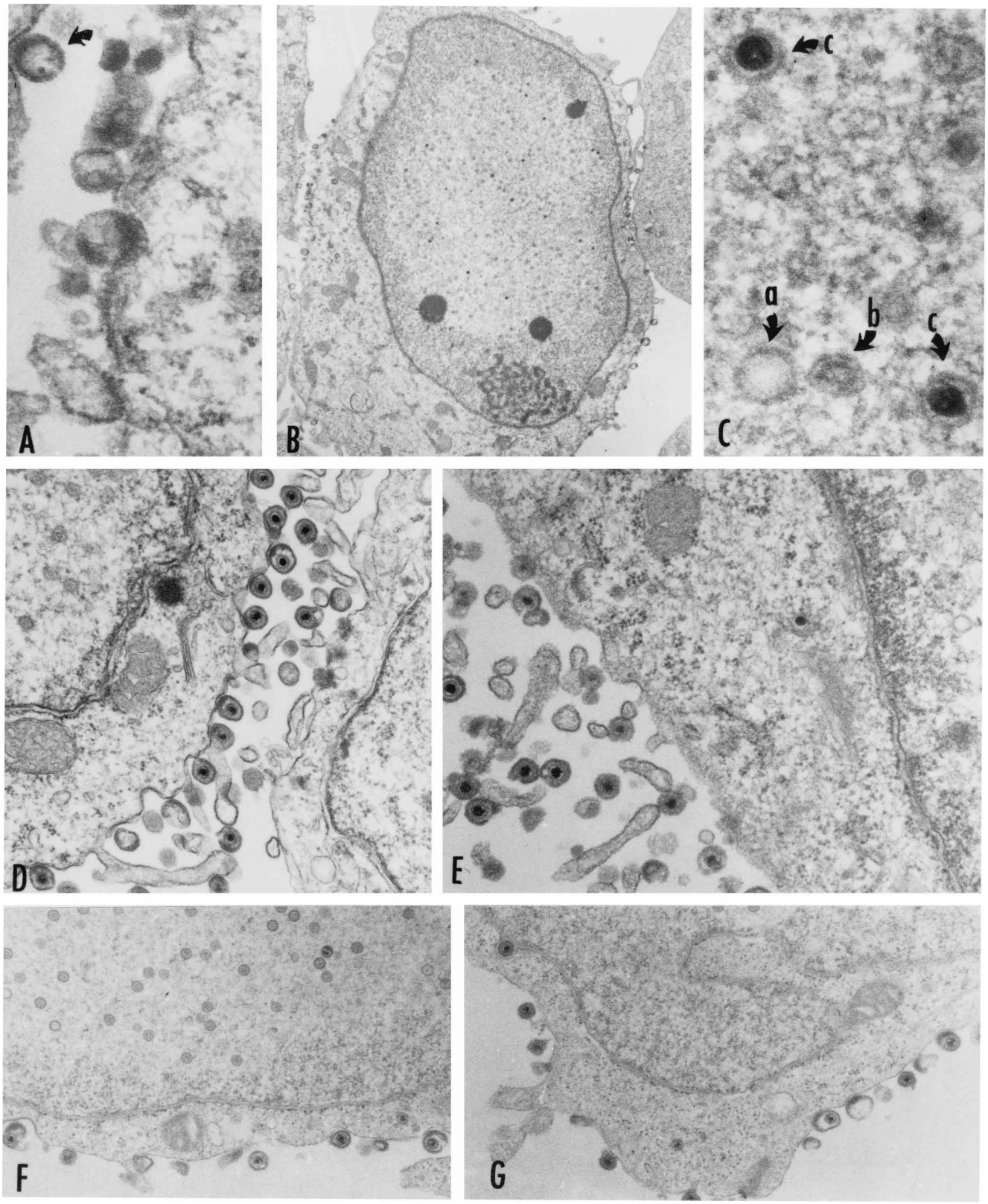

FIG. 5. Electron micrographs of cells infected with wild-type HSV-1(F), U_L31 deletion virus R5132, and repaired virus R5133. Replicate cultures of rabbit skin or RD14 cells were infected with 10 PFU of the indicated viruses per cell. At 20 h postinfection, cells were fixed in glutaraldehyde and prepared for electron microscopy. (A to C) Representative sections of rabbit skin cells infected with U_L31 deletion virus R5132 at lower (B) or higher (A and C) magnification. Note the absence of extracellular virions. Panel C shows the presence of A, B, and C capsids in the nucleus. (D) Representative section of RD14 cells infected with R5132. (E) Representative section of rabbit skin cells infected with HSV-1(F). (F and G) Representative sections of rabbit skin cells infected with R5133. Note that the extracellular virions were readily seen in cells infected with wild-type or repaired virus or in RD14 cells infected with mutant R5132 (D to G).

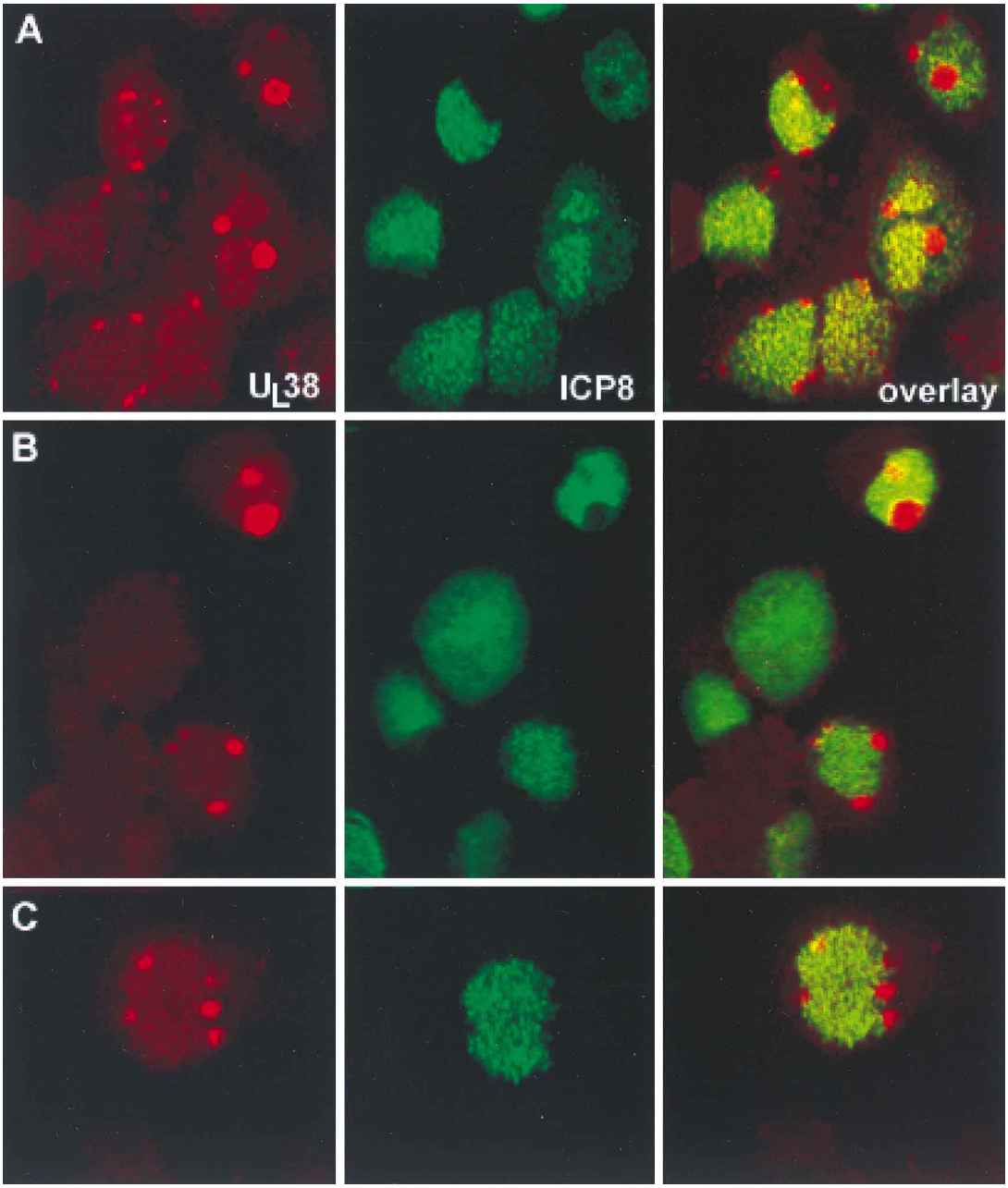

FIG. 6. Confocal micrograph of infected cells staining with antibodies to ICP8 and U_L38. Rabbit skin cells were infected with 20 PFU of HSV-1(F) (A) or R5132 (B) per cell, or RD14 cells were infected with 20 PFU of R5132 per cell (C). The cells were fixed at 18 h after infection and reacted with appropriate antibodies as described in Materials and Methods. U_L38 protein stained with Texas red is shown in the left panels, ICP8 stained with FITC (green) is shown in the middle panels, and the overlay is shown in the right panels.

the relative amounts of monomeric and concatemeric forms of total HSV-1 DNA late in infection. Vero cells infected with HSV-1(F), R5132, or R5133 and harvested at 16 h after infection were processed for pulsed-field electrophoresis. The electrophoretically separated DNA was transferred to a nylon membrane, hybridized to an HSV-1 probe, and quantified as described in Materials and Methods. The results (Table 2) show that in Vero cells infected with the R5132 virus, the amounts of viral DNA made and processed by cleavage to monomeric form were grossly reduced relative to those of HSV-1(F) or R5133.

DISCUSSION

The salient features of the studies described in this report are as follows.

(i) We took advantage of the capacity of overlapping cosmids to recombine into a full-length genome upon transfection into cells to generate a deletion mutant lacking the U_L31 gene. The design involved construction of cosmids from a virus in which a unique restriction endonuclease cleavage site, *Pme*I, was recombined both between U_L30 and U_L31 and between U_L31 and U_L32. From a cosmid library of the recombinant

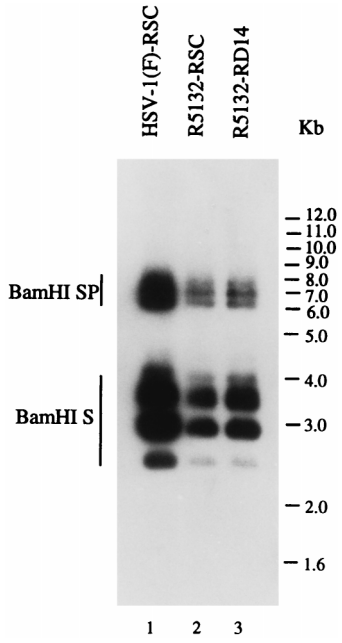

FIG. 7. Photograph of electrophoretically separated digests of viral DNAs hybridized with labeled *Bam*HI S DNA to identify terminal and junction fragments. Cytoplasmic DNAs from rabbit skin cells infected with HSV-1(F) (lane 1), rabbit skin cells infected with R5132 (lane 2), or RD14 cells infected with R5132 (lane 3) were purified and digested with *Bam*HI, separated by agarose gel electrophoresis, transferred to a nylon membrane, and probed with the ^{32}P-labeled *Bam*HI S probe. The positions of the junction fragment *Bam*HI SP and the terminal fragment *Bam*HI S are indicated at the left. The multiple bands reflect the DNA fragments differing in the number of *a* sequence repeats. The 1-kb molecular weigh standard ladder is shown at the right.

virus genome, we identified two cosmids carrying the U_L31 gene flanked by the *Pme*I sites. The U_L31 gene was deleted, and the cosmids lacking the gene supplemented with all other cosmids necessary to generate a full-length viral genome were cotransfected into a cell line stably carrying and expressing only the U_L31 gene.

(ii) The yield of U_L31 deletion mutant in noncomplementing cells was 1,000- to 10,000-fold lower than that of the wild-type virus. Two series of experiments indicated that viral DNA was made but in reduced amounts. Thus, analyses of viral DNA cleaved with *Bam*HI indicated that while infected cells contained unit-length DNA with free termini, the total amount of viral DNA was lower than in wild-type virus-infected cells. Quantification of concatemeric and unit-length DNA separated by pulsed-field electrophoresis also indicated that noncomplementing cells infected with deletion mutant made less viral DNA; furthermore, there was a decrease in the total DNA cleaved to unit-length molecules.

TABLE 2. Monomeric and concatemeric DNA in infected cells at 16 h after infection

Virus	HSV DNA (cpm)[a]		Ratio, concatemer/monomer
	Concatemer	Monomer	
HSV-1(F)	1,403,000	642,100	2.2
R5132	267,700	30,200	8.9
R5133	1,138,000	446,400	2.5

[a] HSV DNA was measured by hybridization of ^{32}P-labeled total viral DNA probe to DNA subjected to pulsed-field electrophoresis. The signal was measured by densitometric analyses in a PhosphoImager.

In addition, although we observed A, B, and C capsids in the nuclei of infected cells, we did not detect enveloped particles in either cytoplasm or extracellular space in R5132-infected rabbit skin cells. The decreased virus yields in complementing cells infected with the deletion mutant were not unexpected. The complementing cells were screened and analyzed over a long period of time. Although the complementing cells infected with the U_L31 mutant produced extracellular virions (Fig. 5D), the ability of these cells to complement U_L31 may be rapidly lost upon serial passage of these cells. The possibility exists that cells which lost or were unable to express the U_L31 gene had a growth advantage and therefore that not all cells in the culture at any one time were able to complement the U_L31 mutant.

(iii) A paradoxical finding became apparent from two observations. First, we noted that cells infected with the deletion mutant exhibited typical cytopathic effects at high multiplicities of infection, and at low multiplicities of infection, small clusters of cells exhibiting cytopathic effects were readily detected. The formation of mini- or microplaques suggested that infectious virus was able to form and be transmitted from cell to cell. A second, reproducible observation was that the yield of infectious virus from cells infected with the deletion mutants was 10- to 1,000-fold greater than would have been expected if the virus was totally incapable of replication (e.g., yield at 3 h after infection [Table 1]). The results shown in Table 1 suggest that the mutant is capable of assembling a very small amount of infectious virus but that the yield estimated in this fashion was of the order of 0.01 to 0.1% of virus made in the same cells by the wild-type virus. Although this result would suggest that the gene is not essential for replication, the efficiency of production of infectious virus in the absence of U_L31 is relatively small. It is likely that the absence of enveloped capsids in our electron microscopy images reflects a paucity rather than complete absence of enveloped virus.

An earlier report from this laboratory indicated that the U_L31 protein is hydrophobic and partitions with the nuclear matrix, that the distribution of the protein is consistent with that of the nuclear matrix rather than with any viral compartment involved with either synthesis and transcription of DNA or assembly, and that capsid proteins were among the nuclear matrix proteins were among the nuclear matrix proteins purified according to standard protocols (9). These observations coupled with the results reported here suggest that the U_L31 protein may form a nuclear network that is possibly associated with the nuclear matrix and which assists the synthesis and packaging of viral DNA but does not per se express a function that defines these processes.

(iv) The nuclear matrix is the center of many nuclear activities such as DNA synthesis, gene expression, and regulation (2, 28). However, the role of nuclear matrix in the viral life cycle remains a largely underexploited area. In the case of cells infected with simian virus 40, there was a good correlation between an increase in encapsidated DNA and an increase in nuclear matrix-associated DNA. Pulse-chase experiments demonstrated that the first newly synthesized encapsidated DNA was present in the nuclear matrix fraction and was slowly released to the chromatin fraction upon chase (15). It has also been speculated that nuclear matrix plays an essential role in HSV virion maturation at the step of capsid assembly, DNA synthesis, cleavage, and packaging (1, 3–5, 13, 24). Herpesviruses appear to have evolved a propensity for duplicating, altering, or complementing cellular functions required for optimal viral replication. It is conceivable that HSV-1 evolved the U_L31 gene to maximize the use of cellular structures so as to provide a site of anchorage for viral DNA synthesis and pack-

aging, a conclusion consistent with a preliminary observation that U_L31 protein binds weakly and nonspecifically to HSV-1 DNA (10). Further studies may help elucidate the role of U_L31 protein in the viral reproductive cycle.

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