JOURNAL OF VIROLOGY, Apr. 1997, p. 2666–2673 Vol. 71, No. 4
0022-538X/97/$04.00+0

The U_L15 Gene of Herpes Simplex Virus Type 1 Contains within Its Second Exon a Novel Open Reading Frame That Is Translated in Frame with the U_L15 Gene Product

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Received 6 November 1996/Accepted 20 December 1996

The U_L15 gene of herpes simplex virus type 1 is composed of two exons. A mutation previously shown to preclude viral DNA cleavage and packaging at the nonpermissive temperature was identified as a change from a highly conserved serine to proline at codon 653. Separate viral mutants that contained stop codons inserted into exon I of U_L15 (designated S648) or an insertion of the *Escherichia coli lacZ* gene into a truncated U_L15 exon II [designated HSV-1(ΔU_L15ExII)] were constructed. Recombinant viruses derived from S648 and HSV-1(ΔU_L15ExII) and containing restored U_L15 genes were constructed and designated S648R and HSV-1(ΔU_L15ExIIR), respectively. Unlike HSV-1(ΔU_L15ExIIR) and S648R, the viruses containing mutant U_L15 genes failed to cleave and package viral DNA when propagated on noncomplementing cells. As revealed by electron microscopy, large numbers of enveloped capsids lacking viral DNA accumulated within the cytoplasm of cells infected with either S648 or HSV-1(ΔU_L15ExII) but not in cells infected with HSV-1(ΔU_L15ExIIR) or S648R. Thus, one function of the U_L15 gene is to effectively prevent immature particles lacking DNA from exiting the nucleus by envelopment at the inner lamella of the nuclear membrane. Cells infected with HSV-1(ΔU_L15ExII) did not express the 75,000- or 35,000-apparent-M_r proteins previously shown to be products of the U_L15 open reading frame, whereas the 35,000-apparent-M_r protein was readily detectable in cells infected with S648. We conclude that at least the 75,000-M_r protein is required for viral DNA cleavage and packaging and hypothesize that the 35,000-M_r protein is derived from translation of a novel mRNA located partially or completely within the second exon of U_L15.

At least three types of capsids that differ in electron microscopic appearance can be detected in the nuclei of cells infected with wild-type herpesviruses (17). Type A capsids consist of an icosahedral shell surrounding an electron-lucent core. Type B capsids resemble type A capsids but contain an internal circular structure or scaffold, whereas type C capsids contain the external shell, lack the internal scaffold, and contain an electron-dense core consisting of tightly packed genomic DNA (7). It is likely that type A and C capsids are derived from type B capsids (18, 22). Type C capsids likely become enveloped at the inner lamella of the nuclear membrane and are transported through the cytoplasm as enveloped virions to the extracellular space (24). Type A capsids are viewed as the consequence of an aborted packaging reaction in which the scaffold is removed but DNA is not inserted.

The herpes simplex virus type 1 (HSV-1) genome contains two components, long (L) and short (S), that are covalently linked and that contain unique sequences (U) flanked by inverted repeats (26, 32). During the cleavage and packaging reaction, concatemeric viral DNA that accumulates in the nuclei of infected cells is cleaved within *a* sequences located within the inverted repeats to yield linear genomes containing one copy of the *a* sequence at the S terminus and one to several copies at the L terminus (33). In cells infected with HSV-1, linear viral genomes have not been detected in the absence of (i) preassembled capsids (14), (ii) the U_L6 gene product that encodes a minor capsid protein dispensable for the production of morphologically normal HSV type B capsids (20, 21, 27, 29, 35), and (iii) at least the U_L15, U_L25, U_L28, U_L32, and U_L33 gene products of unknown function(s) (1, 2, 4, 27, 28, 34). The focus of these studies is on the U_L15 gene products of HSV-1.

The U_L15 gene is highly conserved among all herpesviruses for which sequence data are available (3, 9, 12, 13). Although U_L15 is invariably spliced to join two or more exons in wild-type herpesvirus genomes, HSV-1 replicates to high titers when U_L15 is expressed as a single cDNA copy (6, 10). Of interest is the observation that U_L15 and its counterparts in other herpesviruses have homology with the ATP binding terminase gp17, a protein required for DNA cleavage and packaging of bacteriophage T4 DNA, suggesting the possibility that the proteins perform similar functions (12).

Previous work indicated that antibody directed against sequences encoded by the second exon of U_L15 recognized two proteins with apparent M_rs of 75,000 and 35,000 and that these proteins shared sequences derived from translation of the 3′ end of U_L15 exon II (4). The goals of the current study were to determine the origin of the 35,000-apparent-M_r protein and to begin to determine the respective functions of the individual U_L15-encoded proteins.

MATERIALS AND METHODS

Cells and viruses. HEp-2, Vero, and rabbit skin cells were obtained from Bernard Roizman, University of Chicago, and were maintained as previously described (5). The HSV-1(F) isolate has been described previously (15). The temperature-sensitive mutant HSV-1(mP)*ts*66.4, bearing a temperature-sensitive mutation within the second exon of the U_L15 gene, has also been described elsewhere (23).

Purification and analysis of viral DNA. Cytoplasmic viral DNA was purified for analytical purposes as previously described (30). Transfection of viral DNA, agarose gel electrophoresis, the production of radiolabeled DNA probes, transfer of viral DNA to nitrocellulose, and hybridization conditions were as previ-

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ously described (5). DNA sequencing was performed by using a modification of the method of Sanger et al. (25) on an ABI automated sequencer, using dye-conjugated dideoxynucleotides or by using [^{35}S]dATP-radiolabeled DNA and dideoxynucleotides.

DNase protection assay. The assay was performed essentially as described by Vlazny et al. (31) and modified by Poon and Roizman (23). Vero cells were infected with various viruses at 5.0 PFU per cell. Twenty-two hours after infection, the cells were washed twice in phosphate-buffered saline, resuspended in 0.5% Triton X-100–10 mM NaCl–10 mM Tris-HCl (pH 7.5)–1.5 mM $MgCl_2$, and sonicated for 10 s. Under these conditions, cytoplasmic capsids containing viral DNA and some unpackaged nuclear viral DNA escape into the lysis buffer (23). Lysates were clarified at 14,000 × *g* for 2 min, and the supernatants were incubated for 15 min in the presence or absence of 50 μg of DNase I (Boehringer Mannheim) per ml at 37°C. To stop the DNase I digestion, proteinase K (200 μg/ml; Boehringer Mannheim) and sodium dodecyl sulfate (0.2%) were added, and the mixture was incubated for an additional 30 min at 37°C. The samples were then extracted with phenol-chloroform-isoamyl alcohol (24:24:1), ethanol precipitated, and resuspended in 10 mM Tris-HCl–1 mM sodium EDTA (pH 8.0) prior to digestion with *Bam*HI.

Plasmids and cosmids. Plasmid pRB4120 contains the promoter, polyadenylation signal, and coding sequences of the U_L15 gene as a cDNA copy. HSV sequences in this plasmid were sufficient, when incorporated into a recombinant HSV genome, to allow viral replication in the absence of other U_L15 sequences (6). To produce a cell line capable of supporting replication of viruses bearing mutations within the U_L15 gene, a *Hin*dIII fragment from plasmid pRB3464 (kindly supplied by Bernard Roizman) containing a neomycin resistance gene driven by the HSV thymidine kinase promoter was inserted into the *Hin*dIII site of pRB4120. The resultant plasmid, pRB4496, contained the neomycin resistance gene upstream of the U_L15 cDNA such that both genes were transcribed in the same direction.

Schematic representation of DNA sequences relevant to the construction of pJB29, containing a *lacZ* expression cassette in U_L15 exon II, is shown in Fig. 1B, lines 2 and 3, and proceeded as follows. pJB5 contains a 2,063-bp fragment of HSV-1(F) DNA from a *Sal*I site at codon 375 in the second exon of U_L15 to a *Bgl*II site within U_L18 coding sequences. pJB5 was digested with *Mlu*I, and the ends were blunted with T4 polymerase. pJB5 was then digested with *Bam*HI, and a fragment that lacked codons 509 to 735 from U_L15 exon II was isolated. To insert a selectable marker into the U_L15 gene, a *Bgl*II/*Xho*I DNA fragment containing a *lacZ* gene driven by the simian virus 40 (SV40) promoter and terminated by the SV40 polyadenylation signal (a gift from Donald Holzschu, Cornell University) was inserted into the exon II deletion of pJB5 to generate a plasmid designated pJB29.

A bacterial cosmid was constructed such that a DNA oligomer encoding termination codons in all three potential open reading frames and containing an *Xba*I site was inserted into the *Sau*3AI site at position 29639 in HSV DNA located in exon I of the U_L15 gene (19). The insertion of the 16-bp DNA oligomer, TAA TC TAG AT TAG ATC (stop codons underlined), and its complement was confirmed by sequencing cosmid DNA. The remaining cosmids used for reconstitution of the HSV-1(17) viral genome have been described previously (11).

Electron microscopy. Infected Vero cells were fixed in 2.5% glutaraldehyde in 0.07 M sodium cacodylate buffer (pH 7.4). The cells were embedded in Epon and prepared for electron microscopy essentially as previously described (8). Thin sections were viewed in a Philips EM 201 electron microscope with an accelerating voltage of 80 kV and 20-μm objective aperture.

Immunoblotting. Infected HEp-2 cell lysates were separated on polyacrylamide gels, transferred to nitrocellulose, and reacted with U_L15-specific antiserum as previously described (4).

RESULTS

Construction of a cell line capable of rescuing U_L15 mutants. Plasmid pRB4496, containing a cDNA copy of the U_L15 gene and a gene encoding neomycin resistance (see Materials and Methods), was transfected into rabbit skin cells. Cells expressing neomycin resistance were selected by growth in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum and 500 μg of G418 per ml. G418-resistant cells were then cloned by limiting dilution in the same medium. Individual cell lines were then screened for the ability to support the replication of HSV-1(mP)*ts*66.4, containing a temperature-sensitive mutation within exon II of the U_L15 gene, at the nonpermissive temperature of 40°C (23). One cell line capable of supporting replication of HSV-1(mP)*ts*66.4 at 40°C (data not shown) was designated clone 17 and was selected for further studies.

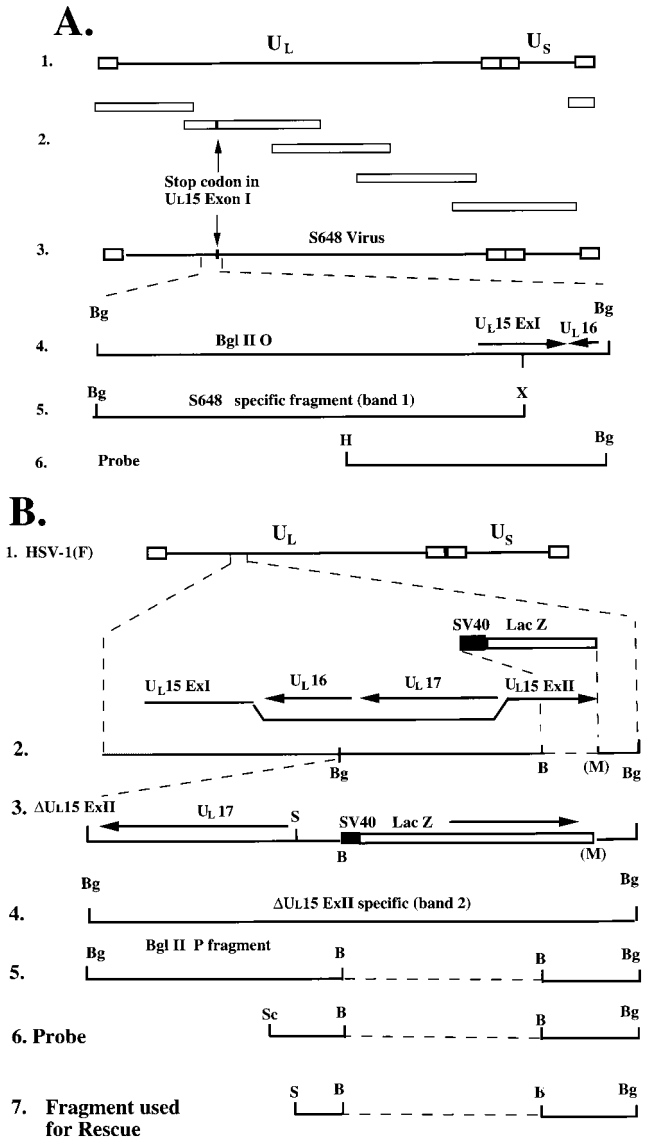

FIG. 1. Schematic representation of colinear HSV sequences relevant to the production and documentation of U_L15 insertion and deletion viruses. (A) Line 1, representation of the HSV-1 genome. Open rectangles represent inverted repeat regions flanking the U_L and U_S components. Lines 2 and 3, colinear representation of a set of cosmid DNAs cotransfected into cells for production of the S648 recombinant virus. The position of an oligomer inserted within U_L15 exon I encoding termination codons in all three open reading frames is indicated. Line 4, schematic representation of sequences in the *Bgl*II O fragment of HSV-1 DNA. The arrows represent the directions and approximate lengths of the indicated open reading frames. The position of a unique *Xba*I site within the DNA oligomer inserted within U_L15 exon I (ExI) is indicated. Line 5, schematic representation of an S648-specific fragment as a consequence of digestion of viral DNA with *Xba*I and *Bgl*II. Line 6, colinear representation of the probe used in the experiment illustrated in Fig. 2. Bg, *Bgl*II; H, *Hin*dIII; X, *Xba*I. (B) Line 1, representation of the HSV-1 genome. Open rectangles represent inverted repeats flanking the unique regions (solid line). Lines 2 and 3, schematic representation of the construction of a plasmid containing a *lacZ* gene driven by an SV40 promoter into a truncated second exon of the U_L15 gene. Filled rectangle, SV40 promoter; open rectangle, *lacZ* sequences. Line 4, schematic representation of the HSV-1(ΔU_L15ExII)-specific *Bgl*II fragment as shown in Fig. 2 (labeled band 2). Line 5, schematic colinear diagram of the normal *Bgl*II P fragment of HSV. The dashed line indicates colinear sequences not contained within the *Bgl*II P fragment of wild-type viruses. Line 6, schematic colinear diagram of the exon II probe used in the experiment shown in Fig. 2. This probe contained U_L15 exon II sequences and hybridized with DNA fragments schematically represented in lines 4 and 5 and shown in Fig. 2, lanes 4 to 6. Line 7, HSV-1 DNA fragment used to restore the deletion/insertion in HSV-1(ΔU_L15ExII) to wild-type sequences. B, *Bam*HI; Bg, *Bgl*II; H, *Hin*dIII; M, *Mlu*I; S, *Sal*I; Sc, *Sac*I; X, *Xba*I.

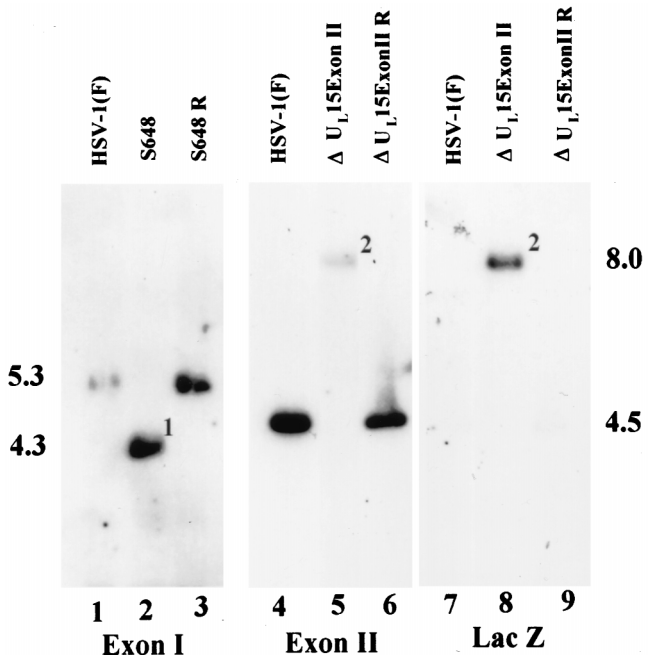

FIG. 2. Digital scanned images of autoradiographs of electrophoretically separated viral DNAs digested with *Bgl*II and *Xba*I and probed with radiolabeled U_L15 and *lacZ* sequences. Lanes contain viral DNA purified from cells infected with the indicated viruses. The probes used are indicated below the numbered lane markers. Bands labeled 1 and 2 are specific for S648 and HSV-1(ΔU_L15ExII), respectively. The sequences contained within bands 1 and 2 are schematically represented in Fig. 1A, line 5, and Fig. 1B, line 4, respectively. The sizes (in kilobase pairs) of the DNA fragments are indicated as the left and right.

Construction of viruses bearing mutations in the first and second exons of the U_L15 gene and viruses bearing restored U_L15 genes. To construct a virus with a mutation within the first exon of U_L15, a DNA oligomer containing stop codons in all three potential open reading frames was inserted into the first exon of the U_L15 gene within cosmid DNA (see Materials and Methods). This DNA and cosmid DNAs (schematically represented in Fig. 1A, line 2) comprising the rest of the HSV-1(17) genome were cloned as overlapping DNA fragments flanked by *Pac*I restriction sites. The cosmids were digested with *Pac*I, releasing the HSV-1 DNA from vector sequences, and the DNA fragments were cotransfected into clone 17 cells. It was expected that by double recombination of homologous sequences, complete viral genomes bearing the inserted mutation would give rise to infectious progeny in cotransfected clone 17 cells (Fig. 1A, lines 2 and 3) (11). Viral progeny of the cotransfection were plaque purified twice, and one virus, designated S648, was selected for further study. A spontaneously arising revertant within the S648 viral stock was designated S648R. The presence of the stop codons inserted into U_L15 exon I of S648 viral DNA and the restoration of wild-type exon I sequences in S648R DNA were confirmed by DNA sequencing (not shown).

Viral DNAs from HSV-1(F)-, S648-, and S648R-infected cells were digested with *Bgl*II and *Xba*I, transferred to nitrocellulose, and probed with radiolabeled DNA delimited by a *Hin*dIII site downstream of U_L14 coding sequences and a *Bgl*II site within U_L16. The probe is designated exon I in Fig. 2, lanes 1 to 3, and schematically represented in Fig. 1A, line 6. As shown in Fig. 2, the introduction of the *Xba*I site into the first exon of U_L15 of S648 genomic DNA caused a truncation of the *Bgl*II O fragment in *Bgl*II/*Xba*I-digested DNA from approximately 5.3 kbp, as seen in HSV-1(F) DNA, to 4.3 kbp (Fig. 2, lane 2, band 1). As expected, the elimination of the *Xba*I site in S648R restored the *Bgl*II O fragment to a size electrophoretically indistinguishable from that of the wild-type *Bgl*II O fragment (Fig. 2; compare lanes 1 and 3). These data indicate that S648 viral DNA contains a novel *Xba*I site within the first exon of U_L15; we therefore deduce that stop codons were inserted into U_L15 exon I as designed.

To construct a virus bearing a mutation in U_L15 exon II, clone 17 cells were cotransfected with HSV-1(F) DNA and plasmid pJB29, containing an *Escherichia coli lacZ* gene inserted into U_L15 exon II (see Materials and Methods). It was predicted that double-recombination events between homologous plasmid and viral DNA sequences would give rise to some viral genomes containing a *lacZ* expression cassette inserted into U_L15 exon II. To purify such viruses, clone 17 cells were infected with the progeny of the cotransfection and were overlaid with medium containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) and 1% agarose. Plaques containing recombinant virus were identified by their blue color under the X-Gal overlay and were plaque purified on clone 17 cells five times. One of the plaque-purified recombinant viruses was selected for growth of stock virus and was designated HSV-1(ΔU_L15ExII).

To ascribe any phenotype of HSV-1(ΔU_L15ExII) to the absence of the U_L15 gene, it was necessary to construct a virus that was derived from HSV-1(ΔU_L15ExII) and contained a restored U_L15 gene. To do this, HSV-1(ΔU_L15ExII) viral DNA was purified from infected clone 17 cells and was cotransfected with a plasmid containing HSV-1 DNA delimited by a *Sal*I site at the 5′ end of exon II and a *Bgl*II site within the U_L18 open reading frame (Fig. 1B, line 7). Clear plaques under an X-Gal overlay were selected and screened for the presence of an intact U_L15 gene (not shown). One of these viruses was designated HSV-1(ΔU_L15ExIIR).

Viral DNAs of HSV-1(F), HSV-1(ΔU_L15ExII), and HSV-1(ΔU_L15ExIIR) were purified from infected cells and were digested with *Bgl*II and *Xba*I. The DNA fragments were electrophoretically separated on an agarose gel and transferred to two separate nitrocellulose sheets. One sheet was probed with radiolabeled HSV-1(F) DNA delimited by a *Sac*I site 596 bp upstream of U_L15 exon II and a *Bgl*II site located within U_L18 coding sequences. A schematic representation of the HSV DNA sequences contained in the probe is shown in Fig. 1B, line 6. The other nitrocellulose sheet was hybridized with radiolabeled *lacZ* sequences. The results, shown in Fig. 2, lanes 4 to 9, were as follows.

(i) The exon II probe hybridized with the *Bgl*II P fragment of approximately 4.5 kbp in lanes containing HSV-1(F) and HSV-1(ΔU_L15ExIIR) DNA (Fig. 2, lanes 4 and 6, respectively).

(ii) The *Bgl*II P fragment was not present in HSV-1(ΔU_L15ExII) DNA; rather, a novel *Bgl*II fragment (labeled band 2 in Fig. 2) of approximately 8.0 kbp was recognized by the probe containing U_L15 exon II sequences. Schematic representations of the DNA sequences in band 2 are shown in Fig. 1B, lines 3 and 4.

(iii) A band electrophoretically indistinguishable from band 2 hybridized with the *lacZ* probe (Fig. 2, lane 8).

Because band 2 is of a size expected upon insertion of the *lacZ* expression cassette within the truncated U_L15 exon II as in pJB29 plasmid DNA and contains U_L15 exon II coding sequences and *lacZ* sequences, we deduce that the *lacZ* expression cassette was inserted into U_L15 exon II of HSV-1(ΔU_L15ExII) viral DNA as designed. We also conclude that HSV-1(ΔU_L15ExIIR) contains a restored U_L15 gene.

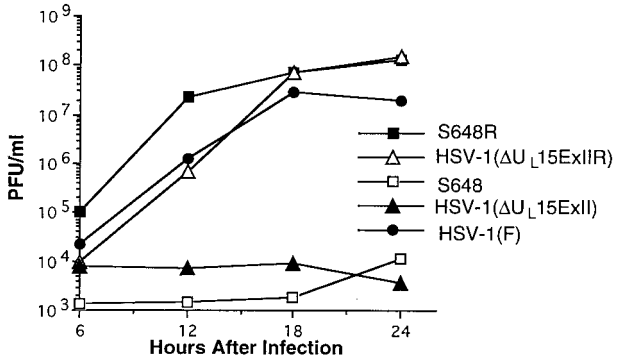

FIG. 3. Vero cells were infected with 5.0 PFU of the indicated viruses per cell. At various times after infection, total infectious virus was measured by titration and plaque assay on clone 17 cells, which support the replication of viruses lacking functional U_L15 genes.

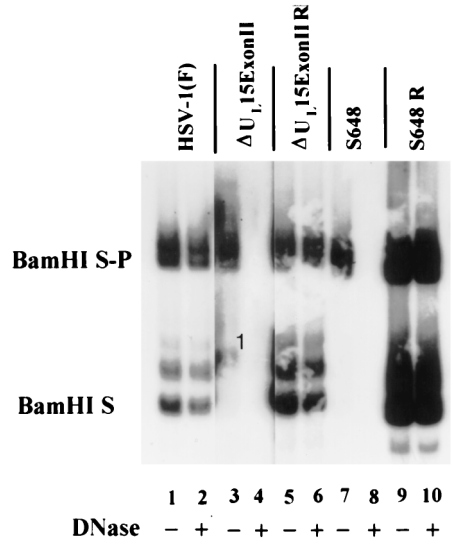

FIG. 4. Digital scanned images of autoradiographs of electrophoretically separated viral DNA probed with end-specific sequences. Lysates of cells infected with various viruses were incubated in the presence (+) or absence (−) of DNase I, and viral DNA was subsequently purified, digested with *Bam*HI, transferred to nitrocellulose, and hybridized with radiolabeled *Bam*HI-S DNA. The positions of the *Bam*HI S fragments representing the terminus of the long component in linear viral genomes and the S-P fragments derived from the junction of the long and short components in linear and concatemeric viral genomes are indicated. The significance of band 1 is discussed in the text.

U_L15 mutants do not replicate in noncomplementing cells. To assess the ability of the U_L15 mutants to replicate, Vero cells were infected with 5.0 PFU of either HSV-1(F), S648, S648R, HSV-1(ΔU_L15ExII), or HSV-1(ΔU_L15ExIIR). Cells were harvested at various times after infection and sonicated, and the amount of total infectious virus was determined by plaque assay on clone 17 cells. The result, presented in Fig. 3, shows that wild-type HSV-1(F) and viruses bearing restored U_L15 genes replicated to at least 3×10^7 PFU per ml by 24 h after infection. In contrast, cells infected with HSV-1(ΔU_L15ExII) did not produce significant levels of infectious virus over the 24-h time period. Cells infected with S648 produced a small amount of infectious virus within 24 h: at 6 h after infection, approximately 10^3 PFU per ml were detected, and by 24 h, this number increased approximately 10-fold. This is likely a consequence of the elimination of the inserted stop codons within U_L15 exon I during viral replication because plating the 24-h sample on Vero cells revealed the presence of some virus capable of replicating on Vero cells and containing restored U_L15 genes (data not shown). Nevertheless, the titers produced in S648-infected Vero cells were 3,000- to 10,000-fold lower than those obtained from cells infected with S648R, HSV-1(F), and HSV-1(ΔU_L15ExIIR). We therefore conclude that the mutations in U_L15 exon I (S648) and U_L15 exon II [HSV-1(ΔU_L15ExII)] prevented replication in noncomplementing Vero cells.

U_L15 mutants synthesize but do not cleave or package viral DNA. The observation that a temperature-sensitive mutation in the C terminus of the U_L15 gene prevented DNA cleavage at the nonpermissive temperature (4) led to the prediction that the mutants constructed in this study would fail to cleave viral DNA. To test this possibility, viral DNAs from Vero cells infected with HSV-1(ΔU_L15ExII), HSV-1(ΔU_L15ExIIR), S648, and S648R were purified and then digested with *Bam*HI. DNA fragments were then electrophoretically separated on an agarose gel, transferred to nitrocellulose, and probed with radiolabeled HSV DNA contained within the terminal sequences repeated at the ends of the long component of HSV-1(F) DNA (*Bam*HI S fragment). The results (Fig. 4) were as follows.

(i) As expected, the *Bam*HI-S probe recognized the BamHI S-P fragments of approximately 6.0 kbp that are present at the junctions of the long and short components within cleaved genomes and concatemeric viral DNA (30) (Fig. 4, odd-numbered lanes).

(ii) In cells infected with HSV-1(F), HSV-1(ΔU_L15ExIIR), and S648R, several *Bam*HI S fragments of around 3 to 4 kbp hybridized with the end-specific probe. This was expected since the terminus of the long component contains one or more copies of the approximately 400-bp *a* sequence (33). The presence of these end-specific fragments indicated that wild-type viruses and viruses bearing restored U_L15 genes cleaved genomic DNA normally. In contrast to these results, *Bam*HI S fragments could not be detected in cells infected with HSV-1(ΔU_L15ExII) or S648, indicating that these viruses did not cleave genomic viral DNA into unit-length molecules in noncomplementing Vero cells.

(iii) A surprising observation was the presence of a novel band (band 1 in Fig. 4) that hybridized with the *Bam*HI-S probe in cells infected with HSV-1(ΔU_L15ExII). This band had an apparent length of 3.6 kbp and was reproducibly seen in several experiments, although its intensity on autoradiograms relative to that of the S-P fragment of HSV-1(ΔU_L15ExII) DNA varied. We cannot rule out the possibility that this DNA fragment was derived from the P fragment (located at the terminus of the short component of HSV-1 DNA) as a consequence of *a* sequences present in both *Bam*HI-S and *Bam*HI-P. This DNA fragment might arise as a consequence of several possibilities and is not necessarily the consequence of an aberrant endonucleolytic cleavage event (see Discussion). Characterization of sequences within this 3.6-kbp fragment should provide insight into the mechanism by which it is produced.

To determine the effect of the U_L15 mutations on DNA packaging, we used a previously described DNase protection assay that takes advantage of the fact that viral DNA is rendered resistant to DNase I digestion when packaged into the viral capsid (31). Twenty-two hours after infection, Vero cells were lysed by sonication in the presence of 0.5% Triton X-100, and equivalent samples of clarified supernatant were incubated in the presence or absence of DNase I. The viral DNA was then purified, digested with *Bam*HI, electrophoretically sepa-

rated on an agarose gel, transferred to nitrocellulose, and probed with radiolabeled *Bam*HI-S DNA. Lanes corresponding to DNase-treated lysates of cells infected with HSV-1(F), HSV-1(ΔU_L15ExIIR), and S648R contained readily detectable DNA fragments that hybridized with the *Bam*HI-S probe (Fig. 4, lanes 2, 6, and 10, respectively). In contrast, lanes containing DNA purified from DNase-treated lysates of HSV-1(ΔU_L15ExII) and S648 did not contain detectable *Bam*HI-S DNA. We interpret these data to indicate that in cells infected with either HSV-1(ΔU_L15ExII) or S648, viral DNA is not packaged into capsids and thereby rendered resistant to DNase digestion. Because cells infected with viruses bearing restored U_L15 genes contained DNase-resistant viral DNA, we also conclude that the inability to package viral DNA was a consequence of the respective mutations within the U_L15 gene.

Capsids lacking DNA become enveloped in the absence of functional U_L15. To further investigate the U_L15 null phenotype, Vero cells were infected with S648, S648R, HSV-1(ΔU_L15ExII), and HSV-1(ΔU_L15ExIIR) at 5.0 PFU per cell. At 15 h after infection, the cells were fixed and thin sections were prepared for electron microscopy. The results, presented in Fig. 5, were as follows.

(i) In cells infected with both S648 and HSV-1(ΔU_L15ExII), only type B capsids were present within infected cell nuclei. Cells infected with S648R and HSV-1(ΔU_L15ExIIR) contained B capsids, a few A capsids, and C capsids. These data therefore confirm the conclusion from the DNase protection studies that S648 and HSV-1(ΔU_L15ExII) do not package DNA. Inasmuch as S648R and HSV-1(ΔU_L15ExIIR) form type C capsids and are derived from S648 and HSV-1(ΔU_L15ExII), respectively, these data also indicate that the inability to package viral DNA is a consequence of the respective mutations within the U_L15 gene.

(ii) The electron microscopic appearance of the cytoplasm of cells infected with both S648R and HSV-1(ΔU_L15ExIIR) resembled that of cells infected with wild-type HSV-1(F) (data not shown). Specifically, most of the cytoplasmic particles were enveloped and contained an electron-dense core that closely resembled the appearance of cores found in type C capsids and virions. As noted in studies of wild-type virus infections, particles lacking envelopes but containing electron-dense cores were also present in the cytoplasm of wild-type and restored viruses. In contrast to these results, the cytoplasm of cells infected with S648 and HSV-1(ΔU_L15ExII) contained a large number of enveloped particles containing internal electron lucent cores. Many of these enveloped particles were located within cytoplasmic membranes. Such particles were only very rarely observed in cells infected with viruses bearing restored U_L15 genes or wild-type viruses (24). This unexpected observation strongly suggests that in the absence of functional U_L15, capsids indistinguishable in morphologic appearance from type B capsids are efficiently enveloped at the nuclear membrane of infected cells.

Characterization of a mutation within U_L15 conferring a temperature-sensitive phenotype. Previous studies indicated that a temperature-sensitive virus, HSV-1(mP)*ts*66.4, failed to cleave and package viral DNA at the nonpermissive temperature. Wild-type U_L15 sequences from a *Bam*HI site at codon 509 to the U_L15 C terminus (codon 735) were sufficient to restore the ability of the viral mutant to replicate at 39.5°C (23). To precisely identify the site of the temperature-sensitive mutation, viral DNA encompassing codons 509 to 735 from HSV-1(mP)*ts*66.4 and the parent virus HSV-1(mP) were sequenced, and the data were compared to the previously published DNA sequence of HSV-1(17) U_L15 exon II (19). The differences among the viral sequences of this region are presented in Table 1. Although several silent mutations in HSV-1(mP) and HSV-1(mP)*ts*66.4 that differed from the published sequence of HSV-1(17) were found, only one mutation that was predicted to change the amino acid sequence of HSV-1(mP)*ts*66.4 U_L15 exon II relative to HSV-1(mP) was found. Thus, codon 653 of HSV-1(mP)*ts*66.4 U_L15 encoding serine was altered to a codon encoding proline. Taking into account the facts that (i) cotransfection of wild-type DNA encompassing this region restored viral DNA cleavage and packaging at nonpermissive temperatures and (ii) the mutation at codon 653 was the only mutation predicted to alter the amino acid sequence of this region of the U_L15 gene, we conclude that this mutation is necessary for manifestation of the temperature-sensitive phenotype of HSV-1(mP)*ts*66.4. Alignment of the deduced amino acid sequences with those of other herpesviruses indicate that the serine residue is conserved in all herpesviruses except the Epstein-Barr virus homolog, in which the codon in question is predicted to encode threonine (data not shown).

Origin of the 35,000-apparent-M_r U_L15-encoded protein. Previous studies indicated that antisera directed against the C terminus of the U_L15 protein recognized 75,000- and 35,000-apparent-M_r proteins (4). To determine the origin of the 35,000-apparent-M_r protein, cells were mock infected or were infected with HSV-1(F), S648, or HSV-1(ΔU_L15ExII). Lysates of cells were electrophoretically separated on a denaturing polyacrylamide gel, transferred electrically to nitrocellulose, and probed with the previously described antibody directed against the C terminus of the U_L15 gene. Bound antibody was detected by reaction with alkaline phosphatase-conjugated donkey antirabbit antibody followed by the fixation of colored substrate by alkaline phosphatase. The results (Fig. 6) were as follows.

(i) As described previously, two bands containing proteins with apparent M_rs of 75,000 and 35,000 reacted with the U_L15-specific antiserum in lanes containing HSV-1(F)-infected cell lysates but not in lanes containing mock-infected cell lysates.

(ii) The 75,000- and 35,000-apparent-M_r proteins could not be detected in cells infected with HSV-1(ΔU_L15ExII). This was as expected because virtually all of the sequences predicted to act as antigen in the production of the U_L15-specific antiserum were deleted from the HSV-1(ΔU_L15ExII) genome (4).

(iii) A key observation was the detection of the 35,000-apparent-M_r protein in the absence of detectable 75,000-apparent-M_r protein in the S648-infected cell lysate (Fig. 6, lane 4). We conclude that the 35,000-apparent-M_r protein is not derived from proteolytic cleavage of the 75,000-apparent-M_r protein inasmuch as it can be expressed in the absence of the 75,000-apparent-M_r protein.

DISCUSSION

We have shown that mutations in either exon I or exon II of the U_L15 gene can separately eliminate viral DNA cleavage and packaging, confirming and expanding on previous observations that a temperature-sensitive mutation mapping in the second exon of U_L15 eliminated viral DNA packaging at nonpermissive temperatures (4, 23). We also conclude that at least the 75,000-apparent-M_r U_L15-encoded protein is required for viral DNA cleavage and packaging inasmuch as the S648 virus does not cleave or package viral DNA and does not express the 75,000-apparent-M_r U_L15-encoded protein. The observations that (i) reversion of the exon I mutation afforded the ability to replicate normally and (ii) S648 was able to cleave and package viral DNA when propagated on a cell line containing U_L15

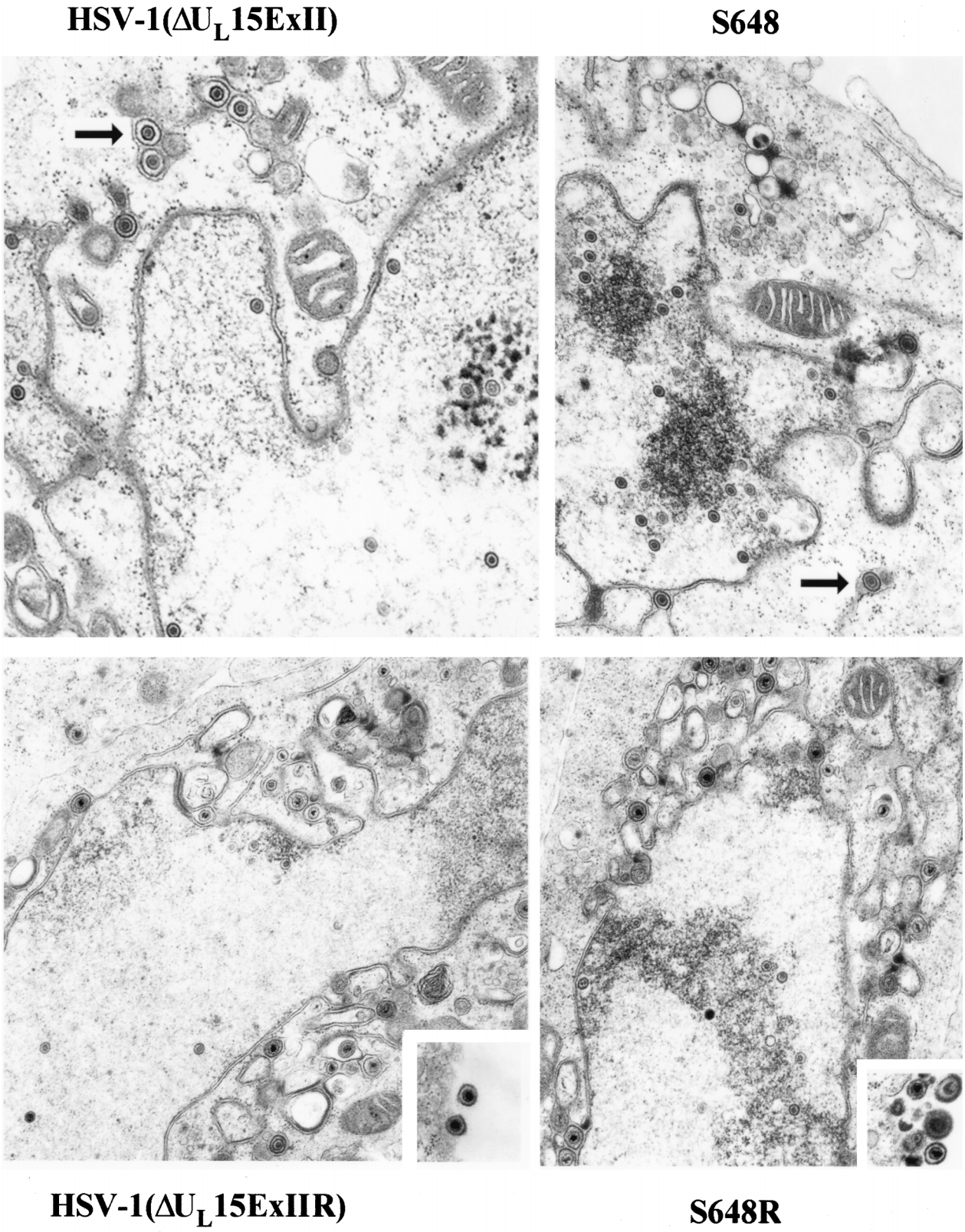

FIG. 5. Scanned digital images of electron photomicrographs of HEp-2 cells fixed at 15 h after infection with the indicated viruses. Thin sections were prepared and viewed in a Philips EM 201 electron microscope. Solid arrows indicate enveloped viral particles lacking DNA in the cytoplasm of cells infected with HSV-1(ΔU_L15ExII) and S648. For size comparisons, capsids are approximately 100 nm in diameter.

TABLE 1. Differences in the DNA and predicted amino acid sequences of relevant portions of U_L15 exon II of HSV-1(17), HSV-1(mP)*ts*66.4 containing a temperature-sensitive mutation that prevents viral DNA cleavage and packaging at the nonpermissive temperatures, and HSV-1(mP), from which HSV-1(mP)*ts*66.4 was derived

Codon[a]	DNA (predicted amino acid) sequence		
	HSV-1(17)[b]	HSV-1(mP)	HSV-1(mP)*ts*66.4
521	GGC (Gly)	GGC (Gly)	GGT (Gly)
536	TTC (Phe)	TTT (Phe)	TTT (Phe)
569	CAT (His)	CAC (His)	CAC (His)
592	CTA (Leu)	CTC (Leu)	CTC (Leu)
607	CTC (Leu)	TTA (Leu)	TTA (Leu)
653	TCC (Ser)	TCC (Ser)	CCC (Pro)
674	GTC (Val)	GTG (Val)	GTG (Val)
681	CTA (Leu)	CTG (Leu)	CTG (Leu)
692	ACT (Thr)	ACG (Thr)	ACG (Thr)
706	GAT (Asp)	GAC (Asp)	GAC (Asp)
730	CCT (Pro)	CCC (Pro)	CCC (Pro)

[a] Codon 1 is the initiation codon of U_L15 exon I.
[b] Data from McGeoch et al. (19).

sequences (data not shown) indicated that the mutation in exon I of U_L15 was responsible for the null phenotype.

An unexpected observation was the presence of large numbers of enveloped capsids lacking DNA that accumulated in the cytoplasm of cells infected with S648 and HSV-1(ΔU_L15ExII). Many of these enveloped particles were incorporated into cytoplasmic vesicles presumably on route to the extracellular space. The morphology of these particles resembled particles seen in the cytoplasm of cells infected with viruses lacking the U_L28 gene (28). Such particles are only rarely observed in cells infected with wild-type HSV-1, suggesting the existence of a mechanism to select type C capsids for envelopment at the inner lamella of the nuclear membrane (24). Such a mechanism may even discriminate between capsids containing DNA of different lengths inasmuch as capsids containing linear genomes significantly shorter than genomic length are retained in the nucleus (31). The studies reported here indicate that in the absence of U_L15, envelopment of capsids occurs independently of viral DNA cleavage or the presence of DNA within the capsid. One possibility consistent with these data is that one of the functions of U_L15, and possibly U_L28, is to exclude particles lacking DNA from becoming enveloped; thus, in the absence of these proteins, envelopment occurs indiscriminately.

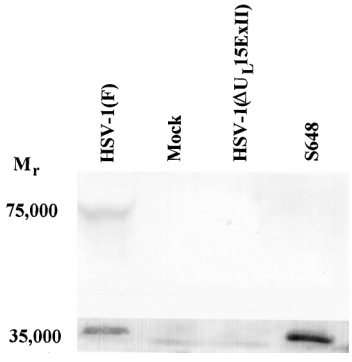

FIG. 6. Digitally scanned image of an immunoblot of electrophoretically separated proteins probed with U_L15-specific polyclonal antibody. HEp-2 cells were mock infected or were infected with the indicated viruses. The proteins were electrophoretically separated, transferred to nitrocellulose, and probed with antibody directed against a fusion protein containing U_L15 exon II-encoded protein. The apparent M_rs of the proteins reacting with the antibody are indicated.

A novel 3.6-kbp *Bam*HI fragment containing sequences hybridizing with a probe representing sequences at the terminus of the HSV-1 genome accumulated to detectable levels in cells infected with a virus not expressing either the 75,000- or 35,000-apparent-M_r protein [HSV-1(ΔU_L15ExII)]. This band was sensitive to digestion with DNase, indicating that the majority of this DNA was not packaged into capsids. Determining the nature of this band will require further study, but its variable intensity relative to other DNA fragments from experiment to experiment resembles the behavior of previously characterized defective genomes present in some preparations of HSV DNA (16). We cannot, however, exclude the possibilities that DNA in this band arises from genomes undergoing degradation, recombination, or aberrant endonucleolytic cleavage.

Previous data indicated that antisera directed against the U_L15 C terminus recognized two proteins with apparent M_rs of 75,000 and 35,000 that shared immunoreactive epitopes. Further analysis of these proteins indicated that (i) they were derived in part from the C terminus of the U_L15 open reading frame and (ii) the 35,000-apparent-M_r protein was dependent on viral DNA synthesis for its expression whereas the 75,000-apparent-M_r protein was less so dependent (4). We could not distinguish between the possibilities that (i) the proteins were derived from differentially regulated mRNAs or that (ii) the smaller protein was derived from cleavage of the larger protein and the protease responsible for the cleavage event was dependent on viral DNA synthesis for optimal activity. The following considerations of data presented in this study and previous studies support the hypothesis that the 35,000-apparent-M_r protein is derived from translation of an mRNA located within exon II of U_L15.

(i) The 35,000- and 75,000-apparent-M_r proteins can be expressed independently; thus, in cells infected with the S648 virus bearing termination codons in the U_L15 first exon, the 35,000-apparent-M_r protein, but not the 75,000-apparent-M_r protein, could be detected with the U_L15-specific antibody. This finding rules out the possibility that one protein is derived from proteolytic cleavage of the other.

(ii) Viruses containing a cDNA copy of the U_L15 gene in place of the U_L15 first and second exons express the 35,000-apparent-M_r protein (4). The arrangement of U_L15 sequences in the genomes of these viruses is such that exon II sequences are covalently linked to exon I sequences and are displaced from sequences normally located upstream of U_L15 exon II. Thus, exon II sequences when displaced from their normal position in the viral genome are sufficient for expression of the 35,000-apparent-M_r protein. The promoter and mRNA giving rise to the 35,000-apparent-M_r protein must therefore be located within U_L15 exon II, or the mRNA may be derived from alternative splicing within the U_L15 cDNA.

(iii) Costa et al. (10) previously mapped an mRNA by S1 nuclease analysis initiating approximately 1,000 bases upstream from the U_L15 C terminus and downstream from two potential TATA boxes starting at base positions 33838 and 33901 within exon II (19). Such an mRNA is located at a position appropriate for expression of a 35,000-apparent-M_r protein initiating at codons 418 or 443 within the U_L15 coding sequence (data not shown). An alignment of U_L15 exon II sequences indicates that a methionine codon is present within four codons of position 443 in all mammalian herpesviruses (data not shown). Whether this is indeed an initiation codon or serves as an important residue of the 75,000-apparent-M_r protein will require further studies.

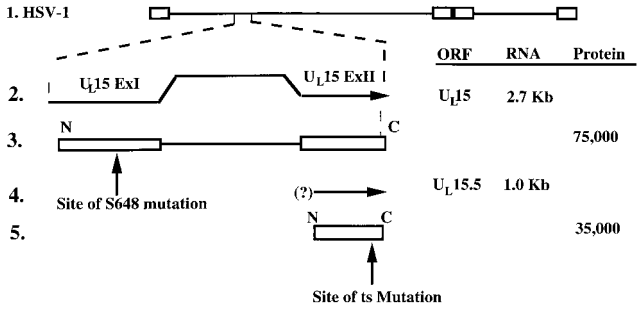

FIG. 7. Schematic collinear representation of U_L15 and $U_L15.5$ mRNAs and proteins. Line 1, representation of HSV-1 genomic DNA. Open rectangles indicate the repeat sequences flanking the unique regions of the long and short components. Line 2, schematic collinear representation of U_L15 mRNA. The positions of exon I (U_L15 ExI) and exon II (U_L15 ExII) are indicated. Line 3, schematic diagram of the U_L15-encoded protein. The positions of the amino terminus (N) and carboxyl terminus (C) and the site of insertion of an oligomer bearing stop codons in the S648 viral genome are indicated. Protein coding sequences are represented by open rectangles. Line 4, schematic representation of the proposed $U_L15.5$ mRNA identified by Costa et al. (10). The 5′ end has not been precisely defined but is located approximately 1.0 kbp upstream of the U_L15 mRNA terminus. Line 5, collinear representation of the $U_L15.5$ gene product with an apparent M_r of 35,000. The site of the temperature-sensitive (ts) mutation in HSV-1(mP)*ts*66.4 is indicated. The apparent M_rs of the U_L15 and $U_L15.5$ gene products are indicated in thousands, and the sizes of the mRNAs encoding the respective proteins are indicated in kilobases. ORF, open reading frame.

These studies therefore support the existence of a novel gene located partially or entirely within exon II of U_L15 that encodes a 35,000-apparent-M_r protein translated in frame with, and coterminal with, the 75,000-apparent-M_r U_L15 gene product. We propose that the putative gene be designated $U_L15.5$. A schematic representation of the proposed mRNAs and proteins relevant to these studies is shown in Fig. 7. Confirmation of this model will require further studies. Parenthetically, this model predicts that of all the mutants described thus far, only the mutation in S648 solely affects the U_L15 gene product.

ACKNOWLEDGMENTS

The studies at Cornell University were supported by Public Health Service grant R01 GM50740 and by a consolidated research grant administered by the New York State College of Veterinary Medicine. D.N. was supported by a World Bank loan to the government of Uganda.

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