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The Unique Sequence of the Herpes Simplex Virus 1 L Component Contains an Additional Translated Open Reading Frame Designated U_L49.5

DAVID E. BARKER AND BERNARD ROIZMAN*

Marjorie B. Kovler Viral Oncology Laboratories, University of Chicago, 910 East 58th Street, Chicago, Illinois 60637

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We present evidence for the existence of an additional herpes simplex virus 1 gene designated U_L49.5. The sequence, located between genes U_L49 and U_L50, predicts a hydrophobic protein with 91 amino acids. Attempts to delete U_L49.5 were not successful. To demonstrate that U_L49.5 is expressed, we made two recombinant viruses. First, we inserted in frame an oligonucleotide encoding a 15-amino-acid epitope known to react with a monoclonal antibody. This gene, consisting of the authentic promoter and chimeric coding domain, was inserted into the thymidine kinase gene of wild-type virus and in infected cells expressed a protein which reacted with the monoclonal antibody. The second recombinant virus contained a 5′ U_L49.5-thymidine kinase fusion gene. The protein expressed by this virus confirmed that the first methionine codon of U_L49.5 served as the initiating codon. The predicted amino acid sequence of U_L49.5 is consistent with the known properties of NC-7, a small capsid protein whose gene has not been previously mapped. A homolog of U_L49.5 is present in the genome of varicella-zoster virus, located between homologs of U_L49 and U_L50.

The genome of herpes simplex virus 1 (HSV-1) consists of two unique sequences, U_L and U_S, each flanked by inverted repeats. The inverted repeats of U_L have been designated *ab* and *a′b′*, whereas those of U_S have been designated *a′c′* and *ca* (15). Earlier studies have shown that the genome encodes a total of 75 open reading frames (ORFs), i.e., 12 in U_S, 57 in U_L, and 6 in the inverted repeat sequences (3, 9, 11). In this communication, we report that U_L encodes an additional gene which we have designated U_L49.5.

The circumstances which led us to this conclusion were as follows. We have been systematically probing the HSV-1 genome to determine the role of the viral genes in viral replication and in recent studies demonstrated that the ORFs U_L46, U_L47, U_L U_L51, U_L55, and U_L56 are dispensable with respect to replication in vitro (1, 12). To determine the role of U_L49, we took advantage of recombinant virus R7101, in which the *Bam*HI D′ fragment had been replaced by α27-*tk*, a chimeric gene consisting of the promoter of the α27 gene fused to a portion of the transcribed noncoding sequence and the coding sequence of the viral thymidine kinase (*tk*) gene (1). The objective of these experiments was to delete the inserted *tk* gene and the adjacent sequences in the *Bam*HI F fragment situated in the R7101 virus immediately adjacent to the site of insertion of the chimeric α27-*tk* gene. One plasmid constructed for this purpose was derived from pRB3965 (1), into which we had previously cloned the HSV-1(F) *Eco*RV K fragment mapping at map units 0.690 to 0.727. The HSV-1 sequences in this plasmid were digested with *Bam*HI and *Bgl*II to delete *Bam*HI-D′ and the adjacent terminal 272 bp of *Bam*HI-F and religated to yield pRB4063. In accordance with previously described procedures for gene deletion (1, 13), rabbit skin cells were cotransfected with pRB4063 and intact R7101 DNA and the progeny virus was selected for the Tk^- phenotype. These experiments were repeated several times; none of the 60+ bromodeoxyuridine-resistant isolates obtained by picking isolated plaques contained deletions in the *Bam*HI F DNA fragment.

The significance of our results stems from two considerations. (i) Deletion of the *Bam*HI D′ fragment from HSV-1 did not affect the ability of the resulting deletion mutants, R7101 and R7108, to replicate, and therefore *Bam*HI-D′ does not contain sequences essential for replication in cells in culture (1). (ii) Hall et al. (7) and McGeoch et al. (11) concluded that U_L49 initiated at map unit 0.696, i.e., downstream from the 272 bp of the *Bam*HI F fragment we had attempted to delete (Fig. 1A, lines 2, 4, and 5). One implication of our results was that the sequence we attempted to delete was contained within an element or gene that was essential for viral replication. This hypothesis was supported by studies of Hall et al. (7), who identified a small ORF and a late γ_2 transcript which initiates in *Bam*HI-D′ at approximately map unit 0.701 (Fig. 1A, line 5). Since there was no evidence that the ORF was expressed, it was subsequently ignored. The transcript has been of particular interest to us inasmuch as its promoter was used as a prototype of a γ_2 gene (10). Since the putative ORF was located between U_L49 and U_L50, we designated it U_L49.5. Because of discrepancies in the nucleotide sequences reported for the HSV-1(KOS) (7) and HSV-1(17) (11) U_L49.5s, we sequenced the HSV-1(F) U_L49.5 ORF cloned in plasmid pRB3965. The HSV-1(F) sequence of U_L49.5 is similar to that of HSV-1(17); it differs from the latter in three nucleotide replacements which result in two conservative amino acid substitutions (Table 1). HSV-1(KOS) shares these two amino acids with HSV-1(F).

The U_L49.5 ORF is expressed from a γ_2 transcript which originates at map units 0.701, 110 bp to the right of the *Bam*HI site, and is coterminal with the U_L49 transcripts at map unit 0.677 or 0.689. Since the ORF was not previously shown to be expressed, we attempted to label the U_L49.5 gene product by introducing the coding sequence for a known epitope into the coding sequence of the U_L49.5 gene. We inserted into the *Bgl*II site of pRB3965 a synthetic

* Corresponding author.

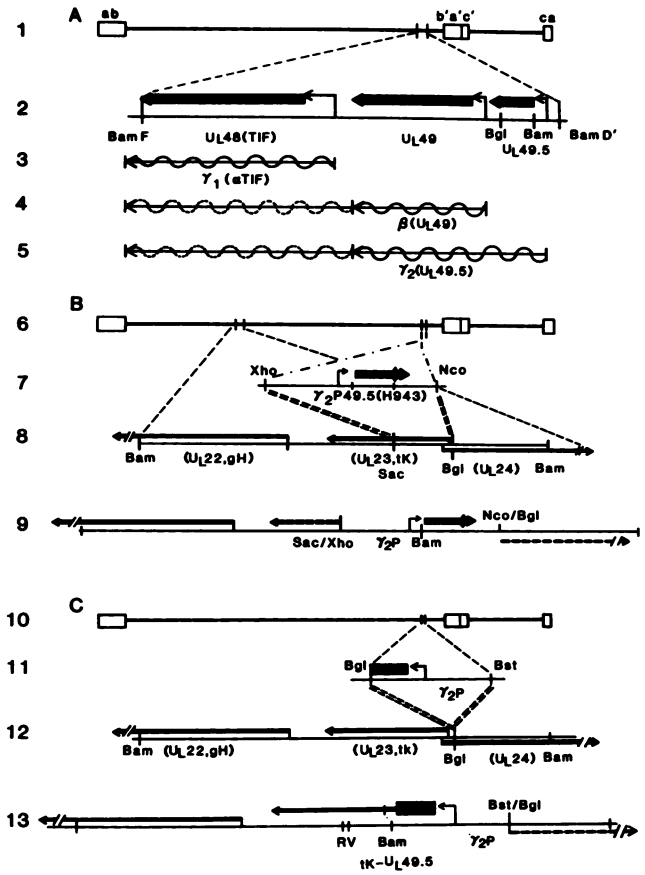

FIG. 1. Schematic representation of the genome of HSV and HSV-1(F) (6) plasmid constructs. In panel A, line 1, the HSV-1 genome, a, b, and c are the terminal repetitive sequences reiterated internally. In line 2, the positions of ORFs U_L48, U_L49, and $U_L49.5$ (determined in this study) are shown. The heavy lines show the coding sequences; the thin lines to the small arrowheads represent 5′ transcribed nontranslated RNA sequences. Lines 3, 4, and 5 show the mRNAs of this partially 3′-coterminal family of genes (7). The γ_2 transcript (line 5), which encodes $U_L49.5$, initiates in *Bam*HI-D′, upstream of the U_L49 gene, but coterminates with β transcripts of U_L49 3′ of either U_L49 or U_L48 (7). Panel B shows the schematics of the construction of recombinant R7126. Line 7 shows the 1,300-bp *Xho*I-*Nco*I fragment of pRB4257, which contains the late promoter and mRNA transcriptional initiation signals from *Bam*HI-D′ and the entire $U_L49.5$ gene with the ICP4 epitope (diamond). Line 8 shows the insertion of chimeric $U_L49.5$ into the *tk* (U_L23) gene. Line 9 shows the structure of pRB4304 and the corresponding region of the R7126 recombinant. The epitopically labeled $U_L49.5$ gene was inserted in place of the *Sac*I-*Bgl*II sequences of the *tk* gene. Panel C is a schematic representation of the construction of R7127. Line 11 shows (i) the 943-bp *Bgl*II-*Bst*EII fragment from pRB3965, which contains the γ_2 promoter and the first 80 5′ codons of $U_L49.5$ inserted into *Bam*HI-Q (line 12) to yield the sequence arrangement of pRB4305 and (ii) the corresponding domain of the R7127 recombinant. In the chimeric gene inserted in this recombinant, the promoter is that of $U_L49.5$, translation initiates from the $U_L49.5$ AUG, and the sequences translated are those of $U_L49.5$ (80 codons), the remaining 50 bases of the *tk* leader, and the *tk* structural gene.

double-stranded DNA fragment made by annealing two complementary single-stranded oligonucleotides as previously described (2). This sequence encodes a 15-amino-acid epitope which occurs in infected cell protein 4 (ICP4), the product of the α4 gene, and is recognized by monoclonal

TABLE 1. Differences in the amino acids sequences of $U_L49.5$s of three strains of HSV-1

Amino acid residue no.	Amino acid in:		
	HSV-1(F)	HSV-1(17)	HSV-1(KOS)[a]
28	His	Arg	His
43	Gly	Gly	Asp
47	Glu	Glu	Gly
51	Ala	Thr	Ala
90	His	His	Gln

[a] Comparison of the HSV-1(KOS) $U_L49.5$ sequence (7) with those of HSV-1(F) and HSV-1(17) suggested that the former contains two frameshifts. To enable comparison of the corresponding sequences of the gene, the sequence was amended by addition of two nucleotides present in both HSV-1(F) and HSV-1(17) to keep it in frame throughout the $U_L49.5$ gene.

antibody H943 (8). The HSV-1 sequences of the resulting plasmid (pRB4257) were sequenced to ensure that the inserted epitope was in frame with the putative $U_L49.5$ gene. Cotransfection of pRB4257 and R7101 DNA, followed by selection for the Tk^- phenotype (13), failed to yield a recombinant virus containing the insert. Given its small size and our failure to delete the $U_L49.5$ gene in our earlier attempts, we hypothesized that the inserted epitope interfered with the function of the $U_L49.5$ gene and that if we altered a second copy of the gene, leaving the native copy intact to provide its essential function, we would more likely succeed in expressing an epitopically labeled product.

Plasmid pRB4257, described above, was digested with *Xho*I (map unit 0.706) and *Nco*I (map unit 0.697) to release a DNA fragment approximately 1,300 bp long which contained the γ_2 promoter and the coding sequences of the putative $U_L49.5$ gene, which was epitopically tagged at the *Bgl*II site (Fig. 1B, line 7) as described above. This fragment was inserted into the *Bgl*II site of pRB3982 after filling in of the ends with T4 DNA polymerase. pRB3982 contains the *Bam*HI Q fragment, from which the 501-bp *Sac*I-*Bgl*II fragment containing portions of the transcribed noncoding sequence and the coding sequence of the *tk* gene had been replaced with a polylinker (Fig. 1B, line 8) to generate pRB4304. Recombinant virus R7126 was generated by cotransfection of rabbit skin cells with pRB4304 and intact HSV-1(F) DNA, and the progeny of the transfection was selected for the Tk^- phenotype. Figure 2 shows photographs of ethidium bromide-stained, electrophoretically separated *Bam*HI (panel A) and *Eco*RV (panel B) digests of R7126 and autoradiograms of these digests transferred to nitrocellulose and probed with *Eco*RV-K and *Bam*HI-Q labeled with ^{32}P by nick translation. As shown in Fig. 2A, lanes 2 and 5, R7126 DNA contained the wild-type *Bam*HI F, L, and D′ fragments, which hybridized with the *Eco*RV-K probe. However, the *Bam*HI Q fragment was altered by inclusion of additional sequences which contained an internal *Bam*HI site. As illustrated in Fig. 1, line 9, the sequences inserted into the *Bam*HI Q fragment of HSV-1(F) would be expected to contain the *Bam*HI site at map position 0.700, that is, the site located immediately upstream of $U_L49.5$. As predicted, the DNA fragments generated by cleavage of the chimeric *Bam*HI Q fragment contained approximately 1,207 and 3,170 bp and migrated just below *Bam*HI-C′ and -R, respectively (Fig. 2, lanes 2 and 5). Similar conclusions were reached from analyses of an *Eco*RV digest of R7126 DNA. In this instance (lanes 10 to 12), the *Bgl*II-*Sac*I deletion in the *tk* gene removed the native *Eco*RV sites within the *tk* gene. The insert lacked *Eco*RV sites, and therefore the chimeric frag-

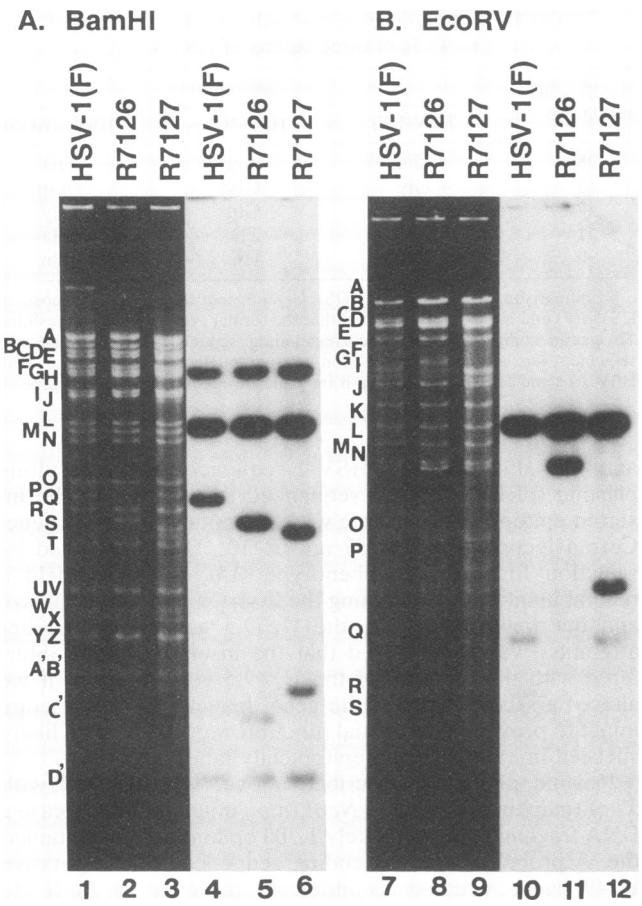

FIG. 2. Photographs of digests of viral DNAs electrophoretically separated in agarose gels and stained with ethidium bromide and autoradiograms of the electrophoretically separated digests obtained after hybridization with selected probes (1). Recombinant viruses R7126 and R7127 were described in Fig. 1. The probes, the *Eco*RV K fragment (map units 0.690 to 0.727), and *Bam*HI-Q were labeled with ^{32}P by nick translation. Letter designations of the restriction fragments appear to the left. For panel A, viral DNAs were digested with *Bam*HI (New England BioLabs). In lanes 4, 5, and 6, hybridization of *Eco*RV-K to native *Bam*HI-F, -L, and -D′ in all three strains indicates that the native copy of $U_L49.5$ is intact. The electrophoretic mobilities of *Bam*HI Q fragments of both R7126 and R7127 (lane 4) were altered by the inserts (see Fig. 1, lines 9 and 13). In panel B, for which viral DNAs were digested with *Eco*RV (1), *Eco*RV-K remained unaltered in all three strains. *Eco*RV-Q and -R were fused with the inserted sequences in R7126 (lane 11) because of deletion of the RV sites in the *Sac*I-*Bgl*II sequence of the *tk* gene. In R7127 (lane 12), the *Sac*I-*Bgl*II sequence was restored, thereby restoring the *Eco*RV restriction sites absent in HSV-1(F)Δ305. However, in this recombinant the additional sequences were inserted into *Eco*RV-R (Fig. 1, line 13).

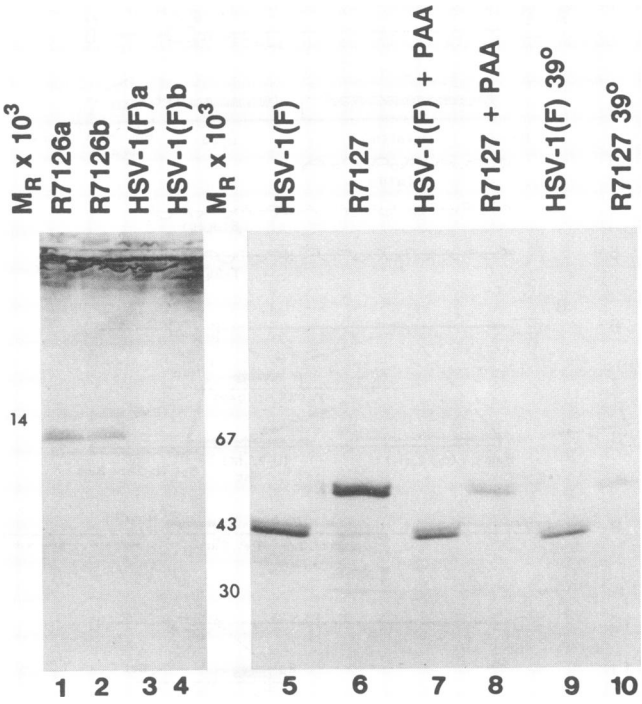

FIG. 3. Photographs of electrophoretically separated proteins from lysates of Vero cells infected with 5 PFU of HSV-1(F) or recombinant virus per cell, electrically transferred to nitrocellulose sheets, and stained with monoclonal antibody H943 to ICP4 (left panel) or rabbit immune serum against thymidine kinase. The right column was as previously described (14). The cells were harvested at 18 h, and electrophoresis was done on 18% (lanes 1 to 4) or 8% (lanes 5 to 10) bisacrylamide gels. The positions of molecular weight (10^3) standard proteins (Pharmacia) are shown to the left of the panels. The contents of lanes 1 to 4 were hybridized with monoclonal antibody H943, which reacts with the α4 epitope introduced into R7126. The detection system was alkaline phosphatase-conjugated goat anti-mouse antibody (Promega). Lanes 1 and 2 show the new 12,000-M_r antigen produced by the epitopically labeled $U_L49.5$ gene, while lanes 3 and 4 contained proteins from isolates of the same transfection that were genetically identical to HSV-1(F), demonstrating that neither transfection nor selection is sufficient to induce the H943-detectable antigen. The bands at the top of the gel contained ICP4, which also reacts with the H934 antibody. Lanes 5 to 10 show the profiles of electrophoretically separated proteins reacted first with polyvalent rabbit anti-thymidine kinase serum and then with alkaline phosphatase-conjugated goat anti-rabbit antibody. The anti-rabbit antibody reacted with the wild-type protein in HSV-1(F) (lanes 5, 7, and 9) or with the chimeric $U_L49.5$-*tk* protein made in cells infected with R7127. The lanes were loaded with equivalent amounts of infected cell lysates. The relative intensity of the 53,000-M_r band versus that of the 41,000-M_r band in lane 6 suggests that the upstream initiation AUG of $U_L49.5$ is preferred over that of the *tk* codon for translational initiation. For lanes 7 and 8, the infected cells were maintained in the presence of phosphonoacetate (PAA; 300 μg/ml; Sigma). For lanes 9 and 10, the infected cells were maintained at 39°C.

ment was predicted to be 4,309 bp long and to comigrate with *Eco*RV-N. In accord with this expectation, the probes hybridized to a DNA fragment of this size (Fig. 2B, lanes 8 and 11). Note that the DNA fragment which hybridized with *Bam*HI-Q and migrated below *Eco*RV-R in lanes 10 to 12 (*Eco*RV-S) mapped at map units 0.291 to 0.299 and therefore shares sequences with a portion of the *Bam*HI Q fragment.

Analyses of lysates of cells infected with two independent isolates of R7126 or with the progeny of transfection which were genotypically identical to HSV-1(F) (designated a and b) were electrophoretically separated on denaturing 18% bisacrylamide gels, electrically transferred to a nitrocellulose sheet, and reacted first with the H943 monoclonal antibody and then with alkaline phosphatase-conjugated goat anti-mouse antibody (Promega) as previously described (14). The antibody reacted with a protein band with an M_r of approximately 12,000 (Fig. 3, lanes 1 and 2), i.e., in good agreement with the M_r of the protein predicted on the basis

of the sequence of U_L49.5 plus the 15 amino acids encoded by the inserted epitope. The dark bands at the top of the gel contained ICP4, which reacted with H943 and entered poorly into the gel.

The second recombinant virus was constructed to determine whether the initiation codon was the first ATG of U_L49.5 or, as suggested by Hall et al. (7), the initiating codon was the downstream U_L49 ATG. In this instance, the 943-bp fragment, which contains the γ_2 promoter and the first 80 codons of U_L49.5 (Fig. 1C, line 11), was removed from pRB3965 by cleavage with *Bgl*II and *Bst*EII at map units 0.698 and 0.704, respectively, and inserted into the *Bgl*II site of the *tk* gene as cloned in pRB173 (Fig. 1C, line 12). In this instance, both the fragment derived from U_L49.5 and the pRB173-derived vector were treated with T4 DNA polymerase, to which dGTP was added. As a consequence, the enzyme filled the first base complementary to cytosine in the overhanging ends but removed the other unpaired bases to place the U_L49.5 and *tk* genes in the same translational reading frame. In the resulting plasmid, pRB4305 (Fig. 1C, line 13), initiation of translation at the first ATG of U_L49.5 would yield a chimeric protein consisting of the 80 amino acids of U_L49.5, 17 codons representing the translated product of the 5′ transcribed noncoding sequence of the *tk* gene downstream from the *Bgl*II site, and the coding sequences of the *tk* gene (Fig. 1C, line 13). Since deletions and insertions into the 5′ end of *tk* do not abolish the activity of the enzyme product, we cotransfected rabbit skin cells with pRB4305 and intact *tk* [HSV-1(F)Δ305)] DNA. The tk^+ progeny selected as previously described was plaque purified and designated R7127.

As shown in Fig. 1C, line 13, it could be expected that insertion of the 943-bp fragment into the *tk* gene would introduce the *Bam*HI site from map position 0.700 near the 5′ terminus of the U_L49.5 sequence. The predicted chimeric *Bam*HI Q fragments generated by digestion with that enzyme would contain 1,449 and 3,073 bp, respectively, exactly as found (Fig. 2, lanes 3 and 6). Restoration of the *Bgl*II-*Sac*I sequence from pRB4305 into *Bam*HI-Q restored to the mutated *tk* gene from HSV-1(F)Δ305 the *Eco*RV sites at map unit 0.312. As a consequence, the *Eco*RV Q fragment comigrated with the wild-type fragment (Fig. 2B, lanes 7 and 10). The additional 943 bp from pRB4305 were predicted to increase the size of the *Eco*RV R fragment to 2,436 bp, which would place it slightly below *Eco*RV-P (2,691 bp) in Fig. 2, lanes 9 and 12. The intensity of the hybridization signal of the new band in lane 12 is due to the fact that all of the 943 bp of the U_L49.5 insert were represented in the more highly labeled *Eco*RV-K probe, whereas the restored sequences of *Eco*RV-Q were highlighted by the less highly labeled *Bam*HI-Q probe.

Figure 3, lanes 5 and 6, shows the electrophoretic mobilities of the native HSV-1(F) and chimeric R7127 thymidine kinases as detected by the R161 polyclonal rabbit antiserum made against a peptide predicted by the HSV-1(F) *tk* sequence. The cells were harvested at 18 h postinfection, electrophoretically separated in 8% bispolyacrylamide gels, transferred to a nitrocellulose sheet, and reacted with the polyclonal serum, and then the thymidine kinase polypeptide was visualized by staining with alkaline phosphatase-conjugated goat anti-rabbit antibody (Promega). The electrophoretic mobility of the resulting protein predicted an M_r of 53,000 (Fig. 3, lanes 5 and 6), indicating that translation begins at the first AUG.

The chimeric U_L49.5-*tk* protein was expressed from a late promoter (Fig. 1) and therefore could be expected to be

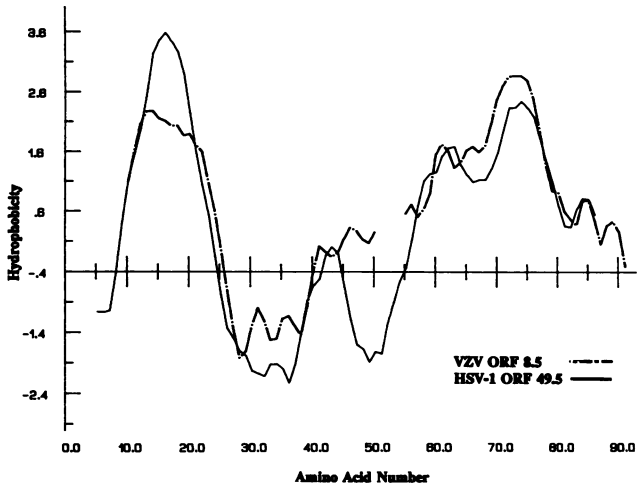

FIG. 4. Kyte and Doolittle hydropathic analysis of the proteins predicted to be encoded by the HSV-1 U_L49.5 gene and VZV ORF 8.5, which is homologous to U_L49.5. The sequences were scanned by using a moving window of seven residues and plotted with a three-residue smoothing function. To align the outputs, five-residue gaps were introduced into the 5′ end near the center of the ORF 8.5 sequence.

dependent on viral DNA synthesis. Addition of phosphonoacetic acid, an inhibitor of viral DNA synthesis, did not significantly inhibit production of native β-*tk* from HSV-1(F)-infected cells but significantly depressed production of U_L49.5-*tk* in R7127-infected cells (Fig. 3, lanes 7 and 8). Similarly, inasmuch as ICP4 of HSV-1(F) contains a *ts* lesion, at the nonpermissive temperature, U_L49.5-*tk* chimeric protein synthesis was virtually eliminated, as would be expected of a late (γ_2) gene (Fig. 3, lanes 9 and 10).

McGeoch et al. (11), in the original publication of the sequence, predicted conservatively, on the basis of available data, a total of 72 ORFs. Since then, it has been shown that the ORF originally designated U_L26 in fact consists of two 3′-coterminal transcriptional units, U_L26 and U_L26.5, each yielding a protein product (9). Another ORF expressing a protein was found in the inverted repeats flanking the U_L sequence, between the terminal *a* sequence and the α0 gene (2, 3). This gene, present in two copies per genome, was designated $\gamma_1$34.5 (3). Discovery of the U_L49.5 gene brings the total number of known ORFs to 76.

The U_L49.5 gene product is expected to be highly hydrophobic (Fig. 4). Its functions are not known. However, the predicted sequence of the U_L49.5 protein is similar to that of a small capsid protein designated NC-7 (4), whose gene has not been mapped. NC-7 was reported to have an apparent molecular weight of approximately 12,000, at least two methionine residues in two different tryptic peptides, and at least five arginine residues. U_L49.5 is predicted to contain four in-frame methionines, the first in a five-residue tryptic peptide, the other three in a large 39-amino-acid peptide derived from the carboxyl half of the protein, and six (or seven) arginines. Trypsin digestion would yield seven peptides, of which the carboxyl terminus (seven amino acids, no arginines) and the single arginine at residue position 6 might not be detected, but the other five peptides, ranging from 3 to 39 amino acids, should have been detected by Cohen et al. (4), who reported four to six arginine-labeled tryptic peptides.

The U_L49.5 ORF is conserved in the genome of varicella-zoster virus (VZV). The homologs of HSV-1 ORFs U_L49 and U_L50 are VZV ORFs 9 and 8. Between ORFs 8 and 9, positioned nearly identically to HSV-1 U_L49.5, there is a small ORF (VZV ORF 8.5) containing 87 codons which overlaps the first 9 codons of ORF 8 (5). In contrast, HSV-1 U_L49.5 contains 91 codons and is separated from U_L50 by 23 bp. The relatedness between HSV-1 U_L49.5 and VZV ORF 8.5 is particularly striking if expressed in the form of a Kyte-and-Doolittle hydropathic plot (Fig. 4). That U_L49.5 is produced from a true late promoter (7, 10; R7127 data in this report) is consistent with its predicted role as a virion (capsid) protein. Were the product of the U_L49.5 gene a capsid protein, it would be predicted to be essential for virus maturation and not dispensable for replication, even in cells in culture, as reported here.

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