JOURNAL OF VIROLOGY, Feb. 1991, p. 938–944
0022-538X/91/020938-07$02.00/0

Vol. 65, No. 2

The Open Reading Frames U_L3, U_L4, U_L10, and U_L16 Are Dispensable for the Replication of Herpes Simplex Virus 1 in Cell Culture

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Received 14 August 1990/Accepted 31 October 1990

By means of insertion and deletion mutagenesis, we have constructed four herpes simplex virus 1 recombinants, each lacking most sequences encoding a different open reading frame. The deleted genes are located in the unique sequences of the long component and include those designated U_L3, U_L4, U_L10, and U_L16. The recombinant virus R7211 lacks 579 of the 696 bp of U_L3. The recombinant virus R7217 lacks 307 of the 597 bp of the U_L4 open reading frame. R7216 contains a 972-bp deletion within the 1,419-bp open reading frame of U_L10, whereas R7210 lacks 988 bp of the 1,119-bp U_L16 open reading frame. Growth curves indicated that the yields of these viruses in Vero and BHK cell cultures were only slightly reduced from or in some instances equivalent to that of the parent virus. The function of the gene products is not known. It is of interest to note that (i) the U_L16 open reading frame maps entirely within the single intron of U_L15 and (ii) on the basis of the extent and size of hydrophobic domains, the U_L3 and U_L10 gene products were predicted to be membrane proteins.

The genome of herpes simplex virus 1 (HSV-1) consists of two covalently linked components, L and S, which can invert relative to each other. Each component consists of unique sequences (U_L and U_S) flanked by inverted repeats (7, 25, 29). The virus specifies an excess of 70 polypeptides, but the function of most of these gene products is not known (8, 9, 16). Studies done in the last few years have revealed that 11 of 12 genes in U_S are dispensable for growth in cells in culture, although most appear to be required for viral replication and pathogenesis in experimental animal systems (13–15, 17, 24). The U_L sequence was reported to encode 56 open reading frames (16). While many of the genes encoded in this region have been shown to be essential for replication, the function of many genes remains unknown. As part of an effort to determine the roles of viral genes in viral replication and in the biology of the virus in its multicellular hosts, this laboratory and others have systematically probed individual genes to determine whether they are required or dispensable for growth in cells in culture (2, 6, 11, 13–15, 17, 20, 23). In this paper we report that four genes mapping in the U_L sequence are dispensable for growth in cells in culture. The open reading frames of the genes deleted in this study were designated U_L3, U_L4, U_L10, and U_L16 (16). Of particular interest is the deletion of U_L16, totally contained within the intron of another gene, that of U_L15.

The procedures we have employed for deletion of HSV-1 genes involve the use of the thymidine kinase (*tk*) gene as a selectable marker since it can be selected both for and against (23). When these studies began, the sequence of HSV-1 was not known, and both the sites for insertion of the *tk* gene for subsequent deletion and the target gene were dependent on mapping the cloned DNA fragment for suitable restriction endonuclease cleavage sites. Since most convenient sites turned out to be within coding sequences of target genes, failure to obtain mutants containing the *tk* insert could be attributed to technical failure or to indispensibility of the gene. With the availability of the nucleotide sequence of the viral genome (16), it became possible to target the insertion of the *tk* gene specifically to the nucleotide sequence between two open reading frames. Since the selection against the inserted *tk* gene is more powerful than the selection for the *tk* insert, failure to obtain deletions of both the *tk* and target gene more accurately define the requirement of the target gene for viral replication in cell culture.

MATERIALS AND METHODS

Cells and viruses. HSV-1 strain F [HSV-1(F)] is the prototype HSV-1 strain used in this laboratory (4). Recombinant viruses in this report were derived from HSV-1(F)Δ305, a thymidine kinase deletion mutant (*tk* deletion mutant) of HSV-1(F) described elsewhere (22). Titrations and preparation of viral stocks and viral DNA were done in Vero cells. Transfections were done in rabbit skin cells originally obtained from J. McClaren. Selection of tk^+ viruses was done in human 143TK$^-$ cells originally obtained from Carlo Croce. The cell lines were maintained in Dulbecco's modified Eagle medium supplemented with either 5% newborn calf serum (Vero and rabbit skin cells) or 5% fetal calf serum and 40 μg of bromodeoxyuridine (BUdR) per ml (143TK$^-$ cells).

Reagents and plasmids. Restriction enzymes were obtained from New England BioLabs, Beverly, Md. T4 DNA ligase was obtained from Boehringer Mannheim Biochemicals, Indianapolis, Ind. pRB1028 (23a) contains the *Bam*HI Q fragment of HSV-1(F) in the *Bam*HI site of pGEM3Z (Promega, Madison, Wis.). pRB3367 contains the entire *tk* gene under the control of the α27 promoter (α27-*tk*) and has been described elsewhere (14). The ends of a *Hin*dIII-*Eco*RI fragment carrying the chimeric α27-*tk* gene from pRB3367 were blunted with T4 DNA polymerase (International Biotechnologies Inc., New Haven, Conn.), and the fragment was cloned into the *Sma*I site of pUC18 to yield pRB3621. A

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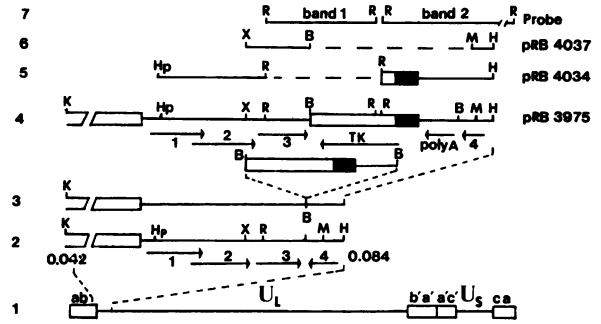

FIG. 1. Sequence arrangements of HSV DNA and of plasmid DNAs used in the construction of U_L3 and U_L4 deletion mutants. (Line 1) Sequence arrangement of HSV-1 DNA. The open rectangular boxes represent the internal repeats flanking U_L and U_S. (Line 2) Organization and direction of transcription of the U_L1 to U_L4 open reading frames. (Lines 3 and 4) Chimeric α27-*tk* gene and upstream polyadenylation signal derived from hepatitis B viral DNA placed between the U_L3 and U_L4 open reading frames of HSV-1(F). The open and filled rectangular boxes represent the *tk* coding sequences and the α27 promoter, respectively. (Line 5) Sequence arrangement of the region of U_L1 to U_L4 after deletion of the U_L3 open reading frame with accompanying α27-*tk* sequences. (Line 6) Cloned HSV-1 sequences used for the deletion of U_L4. (Line 7) Positions of the probe used in hybridizations illustrated in Fig. 3 and of the two unique bands that appear in an *Eco*RV digestion of R7205 DNA (see Fig. 3, lane 3). Dashed lines spanning a region indicate that it is deleted in that particular construct. B, *Bam*HI; H, *Hin*dIII; Hp, *Hpa*I; K, *Kpn*I; M, *Mlu*I; R, *Eco*RV; and X, *Xba*I.

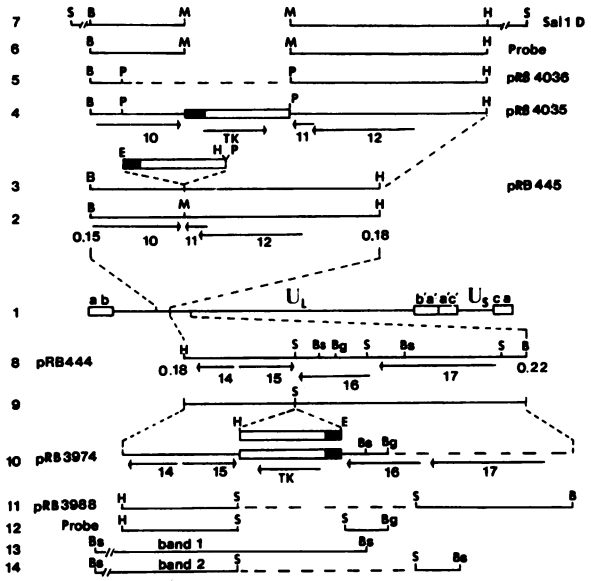

FIG. 2. Sequence arrangements of HSV DNA and of plasmid DNAs used in the construction of U_L10 and U_L16 deletion mutants. (Line 1) Same as line 1 in Fig. 1. (Line 2) Organization and direction of transcription of U_L10 to U_L12. (Lines 3 and 4) Schematic representation of the insertion of the α27 (filled rectangle)-*tk* (open rectangle) gene between U_L10 and U_L11. (Line 5) Representation of the region shown in line 4 after deletion of α27-*tk* and adjacent U_L10 sequences. (Line 6) Location of the DNA probe. (Line 7) The relevant region of DNA that contained U_L10 was contained entirely within the *Sal*I D fragment which hybridized with the probe diagrammed in line 6. (Line 8) Organization and direction of transcription of U_L14 to U_L17. (Lines 9 and 10) Schematic representation of the insertion of the α27-*tk* gene between exon 1 of U_L15 and U_L16. (Line 11) Representation of the region shown in line 8 after deletion of α27-*tk* and adjacent U_L16 sequences. (Line 12) The probe (designated U_L16 in Fig. 4) that was used to document the U_L16^- viruses is shown. The bands labeled 1 and 2 in Fig. 4 are diagrammed in lines 13 and 14. Dashed lines flanked by solid lines represent deleted sequences. B, *Bam*HI; Bs, *Bst*EII; Bg, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; M, *Mlu*I; P, *Pst*I; and S, *Sal*I.

572-bp *Kpn*I fragment containing a hepatitis B polyadenylation signal originally contained in a plasmid obtained from P. Tiollais (26) was cloned from pRB3879 (12a) into the *Kpn*I site of pRB3621 to yield pRB3973. pRB442 contains a 6.3-kbp *Kpn*I-*Hin*dIII fragment which encompasses the left end of the U_L region of HSV-1(F), including the region between 0.0415 and 0.084 map units. A *Bam*HI fragment from pRB3973 containing the α27-*tk* and the hepatitis B poly(A) sequences was cloned into the unique *Bam*HI site of pRB442 which is located between the *Bam*HI E and C fragments of the HSV-1(F) genome to yield pRB3975 (Fig. 1, line 4). pRB447 contains a *Xba*I-*Hin*dIII fragment from pRB442, spans HSV-1 DNA from 0.072 to 0.084 map units, and contains sequences corresponding to the *Eco*RV N and *Eco*RV O fragments of HSV-1(F) (1).

To delete U_L3, pRB3975 was cleaved with *Eco*RV and ligated. This plasmid was designated pRB4034 (Fig. 1, line 5).

pRB447 contains the U_L3 and U_L4 open reading frames. To delete U_L4, pRB447 was cleaved with *Mlu*I and *Bam*HI; the ends were then blunted with T4 DNA polymerase and religated. This plasmid was designated pRB4037 (Fig. 1, line 6).

In order to delete U_L10, the 4.85-kbp *Bam*HI-*Hin*dIII fragment was subcloned from the *Hin*dIII J fragment of HSV-1(F) into pGEM3Z (Promega) to yield pRB445. This plasmid contained the open reading frames of U_L10, U_L11, U_L12, and most of U_L13 and spanned the region between 0.15 and 0.182 map units of HSV-1(F) DNA. All of the sequences of pRB445 were contained within the *Sal*I D fragment of HSV-1(F). A unique *Mlu*I site in pRB445 between the U_L10 and U_L11 open reading frames was cut and was filled in with T4 DNA polymerase. A *Hin*dIII-*Eco*RI fragment from pRB3367 which contained the α27-*tk* construct was cloned into the destroyed *Mlu*I site of pRB445 to yield pRB4035 (Fig. 2, lines 3 and 4).

To delete the U_L10 sequences, pRB4035 was cut with *Pst*I and was ligated to yield pRB4036. This collapse removed sequences between the *Pst*I site within the U_L10 open reading frame and the *Pst*I site immediately downstream of the α27-*tk* gene (Fig. 2, line 5).

To delete U_L16, a *Hin*dIII-*Bam*HI fragment of HSV-1(F) DNA was subcloned from *Hin*dIII-J into pGEM3Z as pRB444. The HSV DNA contained in this fragment spanned the region between the left end of *Hin*dIII-J to the *Bam*HI site between the *Bam*HI A and M fragments and corresponded to the region between 0.182 and 0.220 map units of HSV-1(F) DNA. A *Bgl*II-*Hin*dIII fragment of pRB444 subcloned into the *Bam*HI and *Hin*dIII sites of pGEM3Z and designated pRB443 was made so that the *Sal*I site between the first exon of U_L15 and the U_L16 open reading frames was unique. pRB443, which was also used as a DNA probe, contained sequences that spanned the region between 0.182 and 0.199 map units of HSV-1(F) DNA. The *Bgl*II site that was used to clone this fragment defines the division of the *Bgl*II O and P fragments. The unique *Sal*I site of pRB443 was cut and was blunted with T4 polymerase, and a similarly

blunt-ended α27-*tk* construct (obtained as a 1.8-kbp *Eco*RI-*Hin*dIII fragment from pRB3367) was inserted. This plasmid was designated pRB3974 (Fig. 2, line 10). To construct a plasmid in which U_L16 sequences were deleted, pRB444 was cut partially with *Sal*I and was ligated to yield pRB3988 (Fig. 2, line 11).

Selection of recombinant viruses. tk^- progeny viruses were selected on 143TK$^-$ cells in the presence of BUdR. tk^+ progeny were selected on 143TK$^-$ cells overlayed with HAT medium, which is Dulbecco's modified Eagle medium supplemented with hypoxanthine, aminopterin, thymidine, and 5% fetal bovine serum.

Purification and analysis of viral DNA. Viral DNA was prepared from NaI gradients as previously described (30). Nick translations were done according to the manufacturer of a kit designed for this purpose (Du Pont, Wilmington, Del.). The separation of DNA fragments and transfer to nitrocellulose were done according to the method of Southern (27) as modified by Longnecker and Roizman (14). Hybridization conditions were as previously described (19, 21). Some fragments for nick translation and cloning were prepared by using glass powder in saturated sodium iodide (28). All DNA fragments were purified after separation in low-gelling-temperature agarose (FMC Bioproducts, Rockland, Maine).

RESULTS

Experimental design. The general strategy for the construction of deletion mutants was to place the α27-*tk* gene between the coding domains of genes targeted for mutagenesis. The *tk* gene was chosen for insertional mutagenesis because it can be selected both for and against (23). *tk* was placed under the control of the α27 gene in order to preclude double recombinational events which would enable the rescue of the *tk* gene in the deletion mutant HSV-1(F)Δ305. Lastly, the α27-*tk* gene was inserted between two genes rather than into the coding domain of the target gene to facilitate the selection of insertion mutants since the function of the target genes was seldom known. To select mutants lacking the target genes, the DNAs of viruses containing the inserted α27-*tk* gene were cotransfected with HSV-1 DNA fragments with deletions in the site of the *tk* insertion and in the flanking sequence containing the target gene. By applying this strategy, we have constructed viruses in which the U_L3, U_L4, U_L10, and U_L16 open reading frames have been deleted. Figures 1 and 2 show the locations of U_L3, U_L4, U_L10, and U_L16 and the sequence arrangements of the plasmids used for the deletion of these genes. Table 1 summarizes the genotypes of the mutant viruses and the plasmids used for their construction.

Construction of U_L3 and U_L4 deletion mutants. To delete U_L3 and U_L4, rabbit skin cells were cotransfected with HSV-1Δ305 DNA and pRB3975. In pRB3975, the α27-*tk* gene was positioned between U_L3 and U_L4 (Fig. 1, lines 3 and 4) and between U_L4 and the sequence AAATTA, which was reported to serve as the natural polyadenylation signal for both U_L4 and U_L5 (16). To allow the transcription of U_L5, an essential gene (31, 32), to terminate properly, a poly(A) sequence which was originally derived from hepatitis B virus was placed in pRB3975. This sequence was in a position that allowed it to function as a polyadenylation signal for the transcription of both U_L4 and U_L5 (Fig. 1, line 4). A tk^+ virus, R7205, was selected in 143TK$^-$ cells overlaid with HAT medium.

The sequence arrangement of R7205 was probed as shown in Fig. 3. The DNAs of R7205, of HSV-1(F)Δ305, and of HSV-1(F) were cleaved with *Eco*RV, and the digest was electrophoretically separated on a 1.0% agarose gel and transferred to two nitrocellulose sheets. One sheet was probed with an *Eco*RV-*Hin*dIII fragment of pRB447 (designated U_L3&4 in Fig. 3). This fragment hybridized to the 4.4-kbp *Eco*RV N fragment of HSV-1(F) and contained sequences corresponding to both U_L3 and U_L4.

TABLE 1. Summary of virus constructions

Virus	Phenotype[a]	Plasmid and virus used for construction
HSV-1(F)	wt	None
HSV-1(F)Δ305	TK$^-$	pRB305 and HSV-1(F)[b]
R7205	TK$^+$	pRB3975 and HSV-1(F)Δ305
R7208	U_L3^-, TK$^-$	pRB4034 and R7205
R7211	U_L3^-, TK$^+$	pRB1028 and R7208
R7213	U_L4^-, TK$^-$	pRB4037 and R7205
R7217	U_L4^-, TK$^+$	pRB1028 and R7213
R7212	TK$^+$	pRB4035 and HSV-1(F)Δ305
R7215	U_L10^-, TK$^-$	pRB4036 and R7212
R7216	U_L10^-, TK$^+$	pRB1028 and R7215
R7206	TK$^+$	pRB 3974 and HSV-1(F)Δ305
R7207	U_L16^-, TK$^-$	pRB3988 and R7206
R7210	U_L16^-, TK$^+$	pRB1028 and R7207

[a] wt, Wild type.
[b] See reference 21.

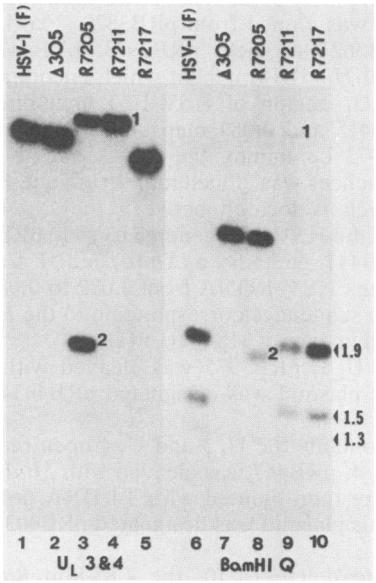

FIG. 3. Autoradiographic image of *Eco*RV digests of U_L3^- and U_L4^- mutant DNAs. Viral DNAs were electrophoretically separated on a 1.0% agarose gel, transferred to two nitrocellulose membranes, and hybridized to a radiolabeled *Eco*RV-*Hin*dIII fragment of pRB447 that contained sequences of U_L3 and U_L4 (lanes 1 to 5) or to labeled *Bam*HI Q fragment containing the *tk* gene (lanes 6 to 10). Lanes 1 and 5, HSV-1(F); lanes 2 and 7, HSV-1(F)Δ305; lanes 3 and 8, R7205; lanes 4 and 9, R7211; lanes 5 and 10, R7216. The numbers to the right indicate the size (in kilobases) of selected bands. The location of the bands numbered 1 and 2 is described in the text and shown in Fig. 1.

As a result of the α27-*tk* insertion, the *Eco*RV N fragment was increased in size from 4.4 kbp in the parent virus to 4.8 kbp (Fig. 3, band 1). In addition, a unique 1.8-kbp fragment (Fig. 3, band 2) appeared in the *Eco*RV digest of R7205 as a result of two closely spaced *Eco*RV sites within the body of the inserted α27-*tk* gene. Evidence in support of this conclusion emerged from analyses of the duplicate nitrocellulose sheets probed with ^{32}P-labeled *Bam*HI Q fragment which contains the wild-type *tk* gene. As expected, both band 1 and band 2 hybridized with the *tk* probe (Fig. 3, lane 8).

In order to delete U_L3, pRB4034 was cotransfected with R7205 viral DNA and TK^- progeny were selected in $143TK^-$ cells in the presence of BUdR. pRB4034 lacks 579 bp of the 696-bp U_L3 open reading frame and 895 bp of the body of the α27-*tk* gene, but the α27 promoter and the hepatitis B virus poly(A) were left intact (Fig. 1, line 5). The resulting virus was designated R7208.

In order to repair the natural *tk* gene of R7208, rabbit skin cells were transfected with the *Bam*HI Q fragment in pRB1028. Six hours later the cells were infected with 1 PFU of R7208 per cell. Progeny tk^+ viruses were selected in $143TK^-$ cells overlaid in HAT medium. To confirm the predicted structure, the *Eco*RV digest of the DNA of one plaque-purified isolate, designated R7211, was electrophoretically separated on a 1.0% agarose gel, transferred to a nitrocellulose sheet, and hybridized with the $U_L3\&4$ probe. As expected, the R7211 DNA was missing the unique 1.8-kbp *Eco*RV fragment of R7205 (Fig. 3, lane 8, band 2) which contained part of the α27-*tk* gene and the bulk of the U_L3 open reading frame. Three *Eco*RV fragments of 1.3, 1.5, and 1.9 kbp hybridized with *Bam*HI-Q in *Eco*RV digests of HSV-1(F) and R7211 DNAs. It should be noted that the deletion in the natural *tk* gene of HSV-1(F)Δ305 extended across a region between a *Bgl*II and *Sac*I site and included portions of the 5′ noncoding and coding sequences of the *tk* gene. Within this 501-bp fragment were two *Eco*RV sites 104 bases apart. The removal of the *Eco*RV sites in the *Bgl*II-*Sac*I deletions of HSV-1(F)Δ305 and R7205 caused a fusion of the truncated 1.5- and 1.9-kbp *Eco*RV fragments to yield a unique 2.9-kbp fragment (fig. 3, lanes 7 and 8).

To delete U_L4, R7205 DNA was cotransfected with pRB4037 into rabbit skin cells, and the progeny of the transfection were plated in $143TK^-$ cells. RB4037 lacked the α27-*tk* sequences, the inserted polyadenylation signal between U_L3 and U_L4, and 307 of the 597 bp of the U_L4 open reading frame. The tk^- and presumed U_L4^- strain selected for further studies was designated R7213. The *tk* gene was repaired as described above, and the mutant containing a deletion solely in U_L4 was designated R7217.

The sequence arrangement of R7217 was verified as follows. The electrophoretically separated *Eco*RV and *Bam*HI fragments of R7217 were transferred to two nitrocellulose sheets and were hybridized with the $U_L3\&4$ and *Bam*HI-Q probes. As expected, R7217 lacked the unique 1.8-kbp fragment (Fig. 3, band 2) of R7205, its tk^+ parent. In addition, the size of the *Eco*RV N fragment decreased in size by approximately 300 bp (Fig. 3, compare lanes 1 and 2 with lane 5). Finally, the *Bam*HI-Q probe revealed that the 2.9-kbp fusion fragment characteristic of the deletion in the natural *tk* gene of HSV-1Δ305 and R7205 (Fig. 3, lanes 7 and 8) was not evident in R7217 DNA. Rather, the 1.9- and 1.5-kbp fragments of HSV-1(F), which were truncated and fused in the DNA of HSV-1Δ305 and R7205, were restored in R7217 DNA to their native sizes (Fig. 3, lanes 6 and 10).

Deletion of U_L10. The DNAs of HSV-1(F)Δ305 and pRB4035 were cotransfected into rabbit skin cells, and the progeny of the transfection were plated on $143TK^-$ cells overlaid with HAT medium. pRB4035 contained the α27-*tk* gene between the open reading frames of U_L10 and U_L11, with the same direction of transcription as U_L10. The plaque-purified tk^+ progeny virus was designated R7212. To delete U_L10, R7212 DNA was cotransfected with pRB4036 DNA, and the progeny of the transfection of rabbit skin cells were plated on $143TK^-$ cells in medium containing BUdR. In pRB4036, the entire α27-*tk* gene, 972 bp of the 1,419-bp U_L10 open reading frame, the termination codon, and the proposed polyadenylation signal of U_L10 were removed (Fig. 2, line 5). The plaque-purified tk^- virus was designated R7215. In the last step, the *tk* gene was repaired by cotransfection of the R7215 and *Bam*HI Q fragment DNAs and by the selection of tk^+ virus. The plaque-purified tk^+ virus with a deletion solely in U_L10 was designated R7216.

To verify the sequence arrangement of the mutant virus DNAs, the *Sal*I digests of the DNAs of HSV-1(F), HSV-1(F)Δ305, R7212, and R7216 were electrophoretically separated in agarose gels and transferred to nitrocellulose sheets in two directions to obtain two duplicate blots (Fig. 4). One nitrocellulose sheet was probed with nick-translated pRB445 which hybridized with the *Sal*I D fragment of HSV-1(F). As expected, the insertion of the α27-*tk* between U_L10 and U_L11 increased the size of the *Sal*I D fragment in R7212 from approximately 7 kbp as in HSV-1(F) and HSV-1Δ305 (Fig. 4, lanes 1 and 2) to approximately 8.8 kbp (Fig. 4, band 1, lane 3). In addition, this unique 8.8-kbp fragment hybridized with

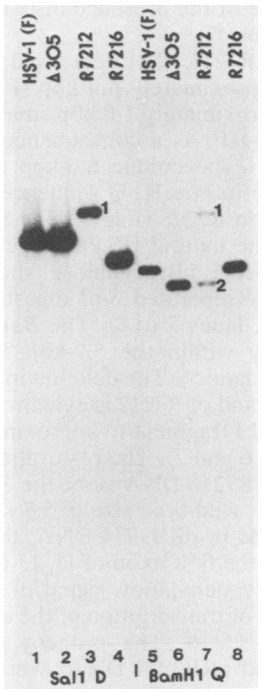

FIG. 4. Autoradiographic image of *Sal*I digests of U_L10^- mutant DNAs. Viral DNA was purified and electrophoretically separated on a 1.0% agarose gel, transferred to two nitrocellulose membranes, and hybridized to either (i) radiolabeled pRB445 which contained a portion of the *Sal*I D fragment and the entire U_L10 open reading frame (lanes 1 to 4) or (ii) the *Bam*HI Q fragment which contains the *tk* gene (lanes 5 to 8). The significance of bands labeled 1 and 2 is described in the text. A diagram of the position of these bands is shown in Fig. 2. Lanes 1 and 5, HSV-1(F); lanes 2 and 6, HSV-1(F)Δ305; lanes 3 and 7, R7212; lanes 4 and 8, R7216.

*Bam*HI-Q because of the presence of *tk* sequences within it (Fig. 4, band 1, lane 7).

The deletion in R7216 DNA decreased the size of the *Sal*I D fragment to approximately 6.0 kbp (Fig. 4, lane 4). This fragment was approximately 1.0 kbp smaller than the *Sal*I D fragment of HSV-1(F) as a consequence of the deletion in U_L10. In addition, the unique 6.0-kbp fragment of R7212 failed to hybridize to *Bam*HI-Q sequences, since the α27-*tk* gene was deleted in R7216 (Fig. 4, lane 8).

To show that the natural HSV-1 *tk* gene was restored in R7216, the duplicate nitrocellulose sheet containing the electrophoretically separated *Sal*I digests was probed with *Bam*HI-Q (Fig. 4, lanes 5 to 8). The *Bam*HI Q fragment is contained entirely within the 5.7-kbp *Sal*I I fragment of HSV-1(F) (Fig. 4, lane 5). The deletion in the natural *tk* gene of HSV-1(F)Δ305 and of R7212 is evidenced by a decrease in the size of the *Sal*I I fragment to approximately 5.2 kbp (Fig. 4, band 2 in lanes 6 and 7). The restoration of the deleted *tk* gene is evident in R7216 DNA since the *Sal*I I fragment was restored to its full, wild-type size of 5.7 kbp (Fig. 4, lane 8).

Deletion of U_L16. In pRB3974 DNA, the α27-*tk* gene was inserted between the first exon of U_L15 and the stop codon and proposed polyadenylation signal of U_L16 (Fig. 2, line 10). The direction of transcription of the α27-*tk* gene was the same as that of U_L16. The progeny of transfection of HSV-1(F)Δ305 and pRB3974 DNAs were plated on 143*tk*$^-$ cells under HAT overlay. The plaque-purified *tk*$^+$ virus was designated R7206. The progeny of transfection of R7206 and of pRB3988 DNAs were plated on 143TK$^-$ cells in medium containing BUdR. In pRB3988 DNA, a total of 1,095 bp, including 988 bp of the 1,119-bp U_L16 open reading frame, were deleted. In addition, the termination codon of U_L16 and the sequence AATAAAAA, which may serve as a polyadenylation signal for U_L16 and U_L17 (3), were also removed. The *tk*$^-$ virus selected in this step was designated R7207. In the last step, the *tk* gene of R7207 was repaired to yield a plaque-purified virus with a deletion solely in the U_L16 gene, designated R7210.

To verify the structure of the mutant DNAs, the *Bst*EII digests of R7206 and R7210 DNAs were electrophoretically separated in agarose gels, transferred to nitrocellulose sheets, and hybridized with labeled *Bam*HI-Q and pRB443 probes. The latter plasmid, schematically shown in Fig. 2, line 12, contained the sequences of U_L14, U_L15, and U_L16 and is referred to as U_L16 in Fig. 5, lanes 1 to 4.

pRB443 hybridized with 1.5- and 3.1-kbp *Bst*EII fragments of HSV-1(F)Δ305 and HSV-1(F) DNAs. The α27-*tk* insert in the larger *Bst*EII fragment increased its size to 4.9 kbp, as can be seen in the *Bst*EII digest of R7206 (Fig. 5, band 1, lane 3). A unique 3.5-kbp fragment hybridized with pRB443 in the *Bst*EII digest of R7210 DNA (Fig. 5, band 2, lane 4). This band arose as a consequence of the deletion of a *Bst*EII site within the U_L16 open reading frame; approximately 750 bp was removed from the 1.5-kbp fragment, and 300 bp was deleted from the 3.1-kbp fragment. As a result of the fusion of these truncated fragments, the 1.5- and 3.1-kbp fragments of HSV-1(F) and HSV-1(F)Δ305 DNAs which hybridized to pRB443 were not detected in R7210 DNA. The *Bam*HI-Q probe hybridized to 5.5- and 2.5-kbp *Bst*EII fragments in the HSV-1(F) DNA digest (Fig. 5, lane 5). In the HSV-1(F)Δ305 DNA digest, the 5.5-kbp fragment was reduced in size to approximately 5.1 kbp because of the deletion in the *tk* gene (Fig. 5, lane 6). This fragment comigrated with the unique band 1 of R7206 DNA (Fig. 5, lane 3) which contained the α27-*tk* inserted between exon 1 of U_L15 and U_L16. A 5.0-kbp fragment which hybridized to *Bam*HI-Q also appeared in the

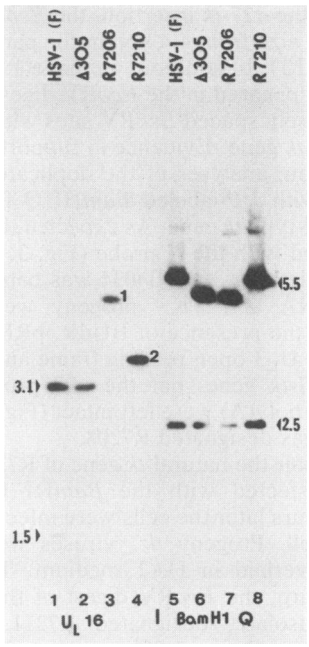

FIG. 5. Autoradiographic image of *Bst*EII digests of U_L16^- mutant DNAs. Viral DNA was electrophoretically separated on a 1.0 agarose gel, transferred to two nitrocellulose sheets, and hybridized to radiolabeled pRB443 which contained some sequences specific for the U_L16 open reading frame (lanes 1 to 4). The other sheet was probed with *Bam*HI-Q sequences (lanes 5 to 8) which contained the *tk* gene. Numbers associated with arrows indicate the size of that band in kilobase pairs. The nature of bands 1 and 2 is described in the text and drawn is Fig. 2.

digest of R7206 DNA, since this virus maintained the 500-bp deletion in the natural *tk* gene of HSV-1(F)Δ305. As a result of the repair of the natural *tk* gene of R7206, R7210 viral DNA contained a 5.5-kbp *Bst*EII fragment which hybridized to the *Bam*HI-Q probe and which could not be differentiated from the corresponding *Bst*EII fragment of HSV-1(F) (compare the 5.5-kbp bands in lanes 5 and 8 of Fig. 5).

Replication of the deletion viruses in Vero and BHK-TK$^-$ cells. Figure 6 shows the growth curves of the deletion viruses in Vero and BHK-TK$^-$ cell lines infected at a multiplicity of 5 PFU per cell. In general, the deletion mutants replicated less well than the wild-type parent in Vero cells. For example, while the yields obtained for R7216 and R7210 at 24 h postinfection were reduced approximately 10-fold relative to those of HSV-1(F), those of R7211 and R7217 grew to titers which were approximately 3-fold lower than those of the wild type.

In the BHK-TK$^-$ cell line, peak titers were generally attained by 12 to 18 h after infection at a multiplicity of 5 PFU per cell. The yields of R7217 and R7211 were equivalent to those of the parent HSV-1(F), whereas R7216 and R7210 yields were 10-fold and 3-fold lower than those of HSV-1(F), respectively.

DISCUSSION

In this report we have described the generation of 11 recombinant viruses and the deletion of most of the sequences of the U_L3, U_L4, U_L10, and U_L16 open reading frames. Relevant to these studies are the following.

(i) U_L3 and U_L4 appear to be part of a cluster of dispens-

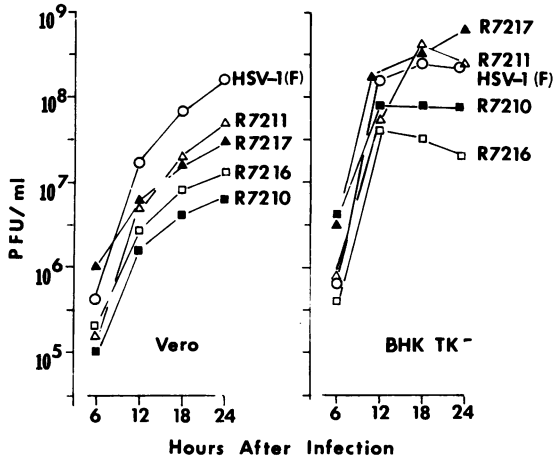

FIG. 6. Growth curves of wild-type and deletion mutants in BHK-TK⁻ and Vero cells. Vero (left panel) and BHK-TK⁻ (right panel) cells were infected at 5.0 PFU per cell with HSV-1(F) (○), R7211 (△), R7217 (▲), R7216 (□), or R7210 (■). Titrations of cell lysates were done on Vero cells.

able genes inasmuch as the inactivation of U_L2 by insertion has been reported (20). We should note that the adjacent, *b*-inverted repeat sequence is also dispensable inasmuch as HSV-1 recombinants lacking the *b* sequence adjacent to U_L1 have been reported (10). The product of the U_L5 gene is required for viral DNA synthesis, and the gene is not dispensable (31, 32).

(ii) The nucleotide sequences of two of the four deleted open reading frames predict that the protein products may have properties of membrane proteins. Thus, McGeoch et al. (16) deduced from the primary sequence that the proteins encoded by U_L10 and U_L3 could be predicted to contain multiple hydrophobic domains which could serve as membrane-spanning regions. Both exhibited potential signal sequences, assuming the second in-frame methionine codons were used to initiate translation. It has been shown that a possible U_L10 homolog is associated with cellular membranes and is present in preparations of human cytomegalovirus virions (12).

(iii) Attempts to delete exon I of U_L15 or both U_L16 and U_L17 together from R7206 containing the α27-*tk* insert between U_L15 exon 1 and U_L16 have not been successful. U_L15 has been shown to consist of two exons, and both the organization of the gene and the amino acid sequence are highly conserved among herpesviruses (3, 16). It is of interest to note that the insertion of the α27-*tk* gene into the intron of U_L15 did not affect the growth of the resulting recombinant R7205. Although we were successful in inserting the α27-*tk* gene between U_L18 and U_L19, attempts to delete U_L18 have not been successful (data not shown). Further studies are required, however, to verify the conclusion that U_L15 and U_L18 are essential.

(iv) The four genes identified in this report join a growing list of genes dispensable for growth in cell culture. In the U_L sequence, the list includes U_L2 through U_L4, U_L10, U_L16, the *tk* gene U_L23 (11), U_L24 (22), the gene U_L44 specifying glycoprotein C (8), U_L39 which encodes ribonucleotide reductase (6), U_L46, U_L47 (1), U_L50 (1, 5), U_L51 (1), U_L55, and U_L56 (18).

We should stress that despite the ability of deletion mutants to grow in cell cultures, they seldom grow in experimental systems or successfully compete in terms of tissue spread and pathogenicity with wild-type viruses. Evidence has been presented that in some cases the deletion mutants grow in some tissues and not others (2, 17, 24). Therefore, the genes dispensable for growth in cell cultures appear to extend the host range of the virus in vivo. The deletion mutants constitute a powerful tool for the identification of the genes' functions and roles in viral pathogenesis.

ACKNOWLEDGMENTS

We thank Susanna Rudofsky for excellent technical assistance.

The studies at the University of Chicago were aided by Public Health Service grants from the National Cancer Institute (CA47451) and the National Institute of Allergy and Infectious Diseases (AI24009 and AI1588).

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