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Construction and Properties of a Recombinant Herpes Simplex Virus 1 Lacking Both S-Component Origins of DNA Synthesis

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The herpes simplex virus 1 (HSV-1) genome contains three origins of DNA synthesis (Ori) utilized by viral DNA synthesis proteins. One sequence (Ori_L) maps in the L component, whereas two sequences (Ori_S) map in the S component. We report the construction of a recombinant virus, R7711, from which both Ori_S sequences have been deleted, and show that the Ori_S sequences are not essential for the replication of HSV-1 in cultured cells. In addition to the deletions of Ori_S in R7711, the α47 gene and the 5′ untranscribed and transcribed noncoding regions of the U_S11 gene were deleted, one of the α4 promoter-regulatory regions was replaced with the simian virus 40 promoter, and the α22 promoter was substituted with the α27 promoter. The total amount of viral DNA synthesized in Vero cells infected with the Ori_S-negative (Ori_S^-) virus was approximately that seen in cells infected with the Ori_S-positive virus. However, cells infected with the Ori_S^- virus accumulated viral DNA more slowly than those infected with the wild-type virus during the first few hours after the onset of DNA synthesis. In single-step growth experiments, the yield of Ori_S^- progeny virus was reduced at most fourfold. Although a single Ori_S (R. Longnecker and B. Roizman, J. Virol. 58:583–591, 1986) and the single Ori_L (M. Polvino-Bodnar, P. K. Orberg, and P. A. Schaffer, J. Virol. 61:3528–3535, 1987) have been shown to be dispensable, this is the first indication that both copies of Ori_S are dispensable and that one copy of an Ori sequence may suffice for the replication of HSV-1.

The herpes simplex virus 1 (HSV-1) genome consists of two covalently linked components, L and S, that are inverted relative to each other such that DNA extracted from virions consists of four populations differing solely in the relative orientations of the components (7, 11). Each component consists of a unique sequence (U_L or U_S) flanked by inverted repeats (31, 38). In addition, each component contains either one (Ori_L; L component) or two (Ori_S; S component) sequences that confer upon DNA fragments the capacity to be amplified by proteins specified by the virus (15, 21, 33, 34, 35, 37, 41). The Ori_L sequence maps approximately in the middle of the unique sequence of the L component (U_L), whereas the two Ori_S sequences map within the repeats flanking the unique sequence (U_S) of the S component. Both Ori_L and Ori_S are located between divergently transcribed genes; Ori_L is located between the U_L29 and U_L30 genes, whereas Ori_S maps between the α4 gene and either the α22 or the α47 gene. The two Ori_S sequences are identical but inverted relative to each other. Moreover, they share homologous sequences with the Ori_L sequence (15, 41). The evidence that both Ori_S sequences and the Ori_L sequence can function as a DNA replication origin utilized by HSV DNA replication proteins has been obtained in a transient plasmid replication assay (15, 35, 37, 41). Also, a protein specified by the U_L9 open reading frame has been shown to bind to specific sites within each origin (9, 23, 40), and the U_L9 gene is essential for the replication of HSV-1 (4). Nevertheless, mysteries regarding the functions of the origins of DNA synthesis during viral replication abound.

A key problem stems from the nature of the replication of HSV DNA. This DNA circularizes within a very short time after infection in the absence of de novo protein synthesis (24). In principle, circular DNA could replicate by a theta model, which depends heavily on each initiation event in the origins of DNA synthesis, or by a rolling-circle model, in which the dependence on the origins is minimal and occurs at most only once at the initiation of DNA synthesis. Attempts to uncover forms consistent with theta replication have not been successful. The evidence is consistent with the conclusion that both defective genomes, amplified in *trans* by viral enzymes furnished by the expression of nondefective genomes, and nondefective genomes themselves accumulate as head-to-tail concatemers, in accordance with a rolling-circle model of DNA replication (3, 13, 14, 37). If this model is correct, it is not clear why the HSV genome contains and presumably conserves three origins of DNA replication.

In previous studies, workers in our laboratory and subsequently others (16, 25) reported that at least one origin, either one Ori_S or Ori_L, can be deleted without affecting the ability of the virus to replicate in cells in culture. Furthermore, Ori_L is not required for the establishment and reactivation of the virus in trigeminal neurons harboring latent virus in mice inoculated by the corneal route (25). Attempts to delete both Ori_S sequences from the HSV-2 genome were unsuccessful, and it has been suggested that at least one Ori_S sequence is essential for viral DNA replication (32). In this paper, we report the properties of a genetically engineered virus from which both Ori_S sequence have been deleted. The phenotypic properties of the recombinant cannot be differentiated from those of the wild-type parent with respect to DNA synthesis in cells in culture.

MATERIALS AND METHODS

Cells and viruses. The properties and propagation of HSV-1 strain F [HSV-1(F)] and the thymidine kinase (*tk*) deletion mutant HSV-1(F)Δ305 were previously described (8, 26). Vero cells (American Type Culture Collection), rabbit skin cells (obtained from J. McClaren), and 143TK$^-$

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cells (obtained from Carlo Croce) were propagated as described elsewhere (2).

Plasmids. Standard procedures were used in all constructions described in this report (29). Plasmid pRB124 carries the *Bam*HI X fragment of HSV-1(F) in pBR322 (16). pRB179 carries the *Bam*HI T fragment of HSV-1(F) in pGEM-3Z (1). The sequence arrangement of plasmids carrying the α4 gene fused to the promoter-enhancer sequence of simian virus 40 (SV40) is shown in Fig. 1. Plasmid pRB4325, in which the HSV-1 *tk* gene fused to the α27 promoter-regulatory sequences is inserted into the α22 promoter-regulatory region, was constructed in several steps. In the first step, a *Hin*fI fragment of pRB257 containing nucleotides −968 to +53 of the α27 gene was cloned into the *Nru*I site located downstream of the α47 promoter in pRB421 to generate plasmid pRB4012. The orientation of the α27 promoter inserted in pRB4012 was the same as that of the α47 promoter. In the second step, plasmid pRB4012 was partially digested with *Tth*111I, filled in with the Klenow fragment of *Escherichia coli* DNA polymerase, and ligated with a 1.5-kb *Bgl*II-*Nco*I fragment encoding the *tk* gene, which had been excised from pRB3351 and filled in with the Klenow fragment. The resulting plasmid, pRB4276, carries the *tk* gene cloned in the *Tth*111I site of the α27 promoter-regulatory domain, in a direction such that the *tk* gene is transcribed from the α47 promoter. In the final step, pRB138, which carries the *Bam*HI N fragment of HSV-1(F) DNA in the *Bam*HI site of pBR322, was digested with *Rsr*II, and the resulting larger fragment, which contains the α22 coding sequences, was ligated with the 2.7-kb *Rsr*II fragment of pRB4276 to generate pRB4325. The direction of ligation was such that the *tk* gene was under the control of the α22 promoter, whereas the α22 gene was under the control of the α27 promoter. For generation of pRB4397, in which the functional *tk* gene and Ori_S sequences are deleted, pRB4325 was digested with *Bss*HII, and the larger fragment was recircularized with T4 DNA ligase. Two DNA fragments with deletions across Ori_S in the R7711 virus (see below) were cloned from the viral DNA as *Bam*HI fragments into pGEM-3Zf(+) (Promega, Madison, Wis.). pRB4488 carries the 1.3-kb *Bam*HI fragment extending from the α4 5′ transcribed nontranslated leader region to the *Bam*HI site present in the α27 promoter-regulatory sequence. pRB4489 carries the 2.7-kb *Bam*HI fragment, which includes the SV40 promoter-α4 fusion junction.

Viral DNA. Vero cells in 850-cm^2 roller bottles were infected with virus at a multiplicity of infection of 0.01 PFU per cell and incubated for 3 to 4 days at 34°C. For harvest, the cultures were washed once with phosphate-buffered saline (PBS) (140 mM NaCl, 3 mM KCl, 10 mM Na_2HPO_4, 1.5 mM KH_2PO_4), scraped into PBS, and recovered by centrifugation at 3,000 rpm in a Beckman JS-13 rotor for 5 min at 4°C. The cell pellet was resuspended in 150 mM NaCl–1.5 mM $MgCl_2$–0.1% Nonidet P-40–10 mM Tris-HCl (pH 7.4), the suspension was vortexed briefly, and then nuclei were removed by centrifugation as described above. The supernatant fluid was removed to a fresh tube, adjusted to 0.2% sodium dodecyl sulfate–5 mM EDTA–50 mM 2-mercaptoethanol, and extracted twice by gentle shaking with 1:1 phenol-chloroform. Nucleic acids were precipitated with 2 volumes of ethanol and recovered by centrifugation at 10,000 rpm in a Sorval SS-34 rotor for 15 min at 4°C. The nucleic acid pellet was suspended in 1 ml of TE (1 mM EDTA, 10 mM Tris-HCl [pH 8.0]) and treated with RNase A (20 μg/ml) for 15 min at 37°C. Viral DNA was separated from oligoribonucleotides and other small contaminants by sedimentation through a gradient of 5 to 20% potassium acetate in 10 mM Tris-HCl (pH 8.0)–5 mM EDTA at 27,000 rpm in a Beckman SW41 rotor for 17 h at 4°C. The supernatant fluid was discarded, and the viral DNA pellet was resuspended in TE. Excess salt was removed by precipitation of the viral DNA with ethanol. The precipitate was resuspended in TE and stored at 4°C.

Recombinant viruses. Procedures for the construction of recombinant HSVs by insertion and deletion of the *tk* selectable marker were previously described (27, 28). The structure and purity of recombinant viruses were evaluated by hybridization of diagnostic probes with restriction enzyme-digested DNAs from the recombinant viruses. Samples of DNA were digested with restriction enzymes, electrophoretically separated on 0.8% agarose gels, and transferred to ZetaProbe membranes (Bio-Rad Laboratories, Richmond, Calif.). Blots were probed with ^{32}P-labeled DNA by use of protocols recommended by the manufacturer. Double-stranded DNAs to be used as probes were labeled with [α-^{32}P]deoxynucleotide triphosphates by use of a nick translation kit (DuPont, Wilmington, Del.). A synthetic DNA oligonucleotide probe was labeled at its 5′ terminus with [γ-^{32}P]ATP and T4 polynucleotide kinase.

Transient replication assay. Test plasmids (0.5 μg) were transfected into Vero cells grown in 25-cm^2 flasks by the calcium precipitation method (29) with 4.5 μg of salmon sperm DNA as a carrier. Four hours later, the cells were incubated with 15% glycerol in PBS for 2 min, washed with PBS without glycerol, and reincubated at 37°C. At 19 h posttransfection, the cells were exposed to 10 PFU of HSV-1(F) per cell for 1 h. At 32 h posttransfection, total cell DNA was extracted from the cells as described elsewhere (5). Portions of the resulting DNA samples equivalent to 10^6 cells were digested with 40 U of *Bam*HI and 100 U of *Dpn*I overnight. The digests were separated on 0.8% agarose gels, transferred to ZetaProbe membranes, and hybridized with nick-translated pGEM-3Zf(+) DNA.

Time course of viral DNA synthesis. Vero cells in 25-cm^2 culture flasks were infected with 5 PFU of test virus per cell. At various times after infection, total cell DNA was isolated as described elsewhere (5), and one-fifth of each sample was digested with *Bam*HI. Digests were electrophoretically separated on agarose gels, transferred to ZetaProbe membranes, and hybridized successively with excess ^{32}P-labeled pRB179 and pRB124 DNAs carrying *Bam*HI T and *Bam*HI X fragments of HSV-1(F) DNA, respectively. The amount of radioactive DNA hybridizing to each band was quantified with a Betascope 603 blot analyzer (Betagen).

RESULTS

Experimental design. To determine whether HSV-1 required at least one Ori_S sequence, we systematically deleted both Ori_S sequences. The strategy used in these experiments was as described earlier (27, 28) and was based on a technique in which the *tk* gene selectable marker, sandwiched between flanking sequences homologous to the target sequence, was first recombined at a site at or near the target sequence and then deleted along with the target sequence in a second recombinational event. The procedures were straightforward and relied on the observation that although double recombinational events through flanking sequences are relatively infrequent, procedures for the selection of either *tk*-positive or *tk*-negative virus progeny resulting from such events are available (27, 28).

The initial strategy was to insert one copy of the *tk* gene

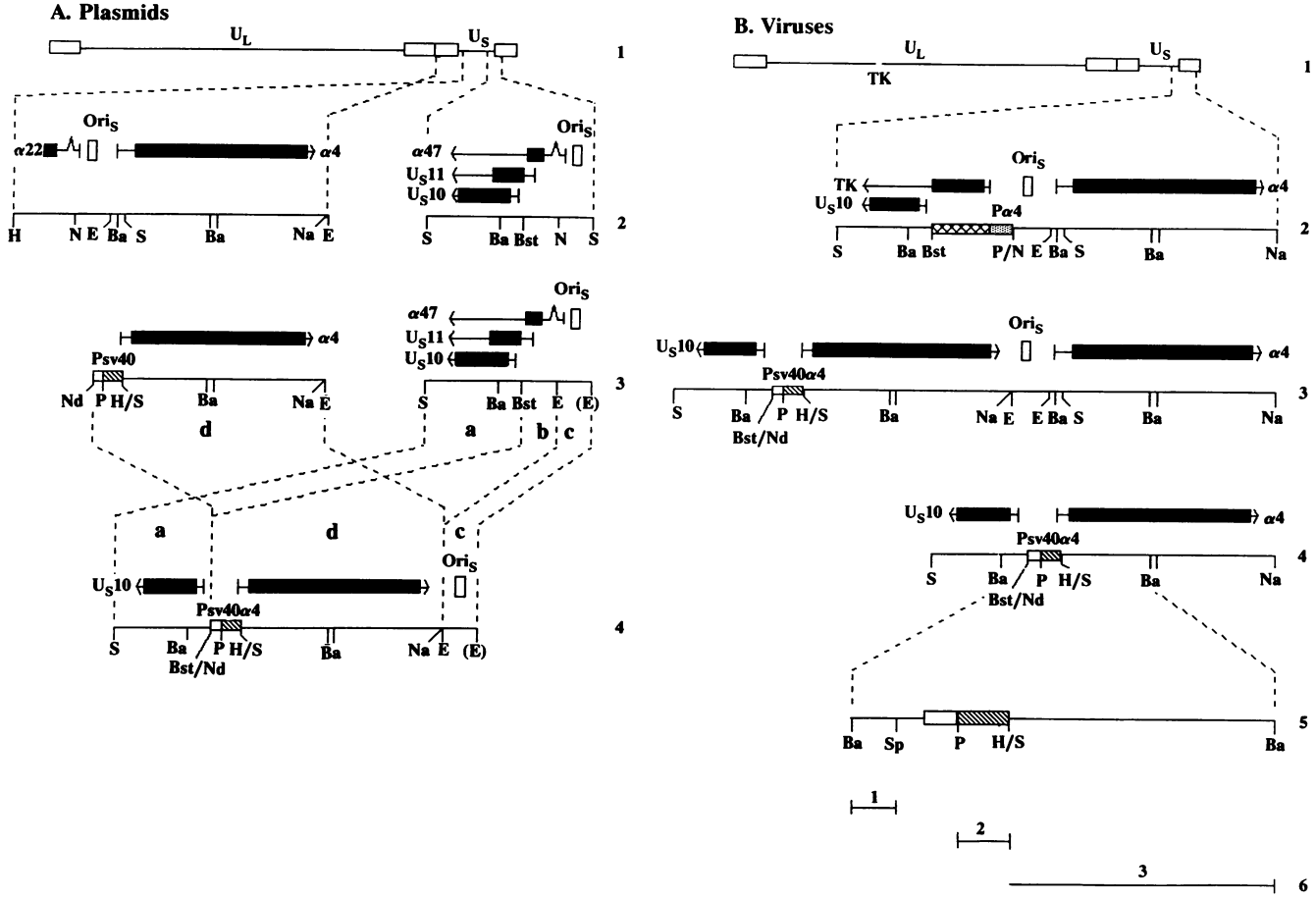

FIG. 1. Schematic construction of recombinant virus R3914. (A) Plasmid construction. Line 1, prototype sequence arrangement of the HSV-1 genome showing unique sequences of the L and S components (thin lines) flanked by inverted repeat sequences (open rectangles). Line 2, expanded representation of the left and right ends of the S component, showing the organization of sequences contained within plasmids pRB3094 and pRB421, respectively. Transcribed sequences are indicated by arrows, protein coding sequences are indicated by filled rectangles, and Ori$_S$ sequences are indicated by open rectangles. Line 3, right, pRB421 was altered by end filling of the unique *Eco*RI site (shown in parentheses) and by insertion of an *Eco*RI linker into the *Nru*I site to create plasmid pRB441. To simplify the presentation of the construction, in this diagram of pRB441, we designated the sequences between the *Sal*I and *Bst*EII sites with the letter a, those between the *Bst*EII site and the *Eco*RI insert with the letter b, and those between the *Eco*RI insert and the end-filled *Eco*RI site with the letter c. Line 3, left, the sequences between the *Sal*I and *Hin*dIII sites of pRB3094 were replaced with the *Hin*dIII-*Nde*I fragment of pSV2CAT (10), which contains the SV40 enhancer-promoter sequences from *Pvu*II to *Hin*dIII (hatched rectangle) and the pBR322 sequences from *Nde*I to *Pvu*II (open rectangle). The resulting plasmid, pRB3906, had the α4 leader coding sequences under the control of the SV40 promoter (Psv40); this chimeric construct could be excised as an *Nde*I-*Eco*RI fragment, designated by the letter d. Line 4, the b sequences of pRB441 were replaced with the d sequences of pRB3906, so that the segments of pRB441 designated a and c would provide flanking sequences for the Psv40-α4 construct (in the order a-d-c) within the resulting plasmid, pRB3915. (B) Virus construction. Line 1, DNA sequence arrangement of HSV-1 recombinant R3630 (19) containing deletions in the natural position of the *tk* gene and in the S component. Line 2, expanded representation of the S component (right end) of recombinant virus R3630, in which the α4-*tk* chimeric gene has replaced the coding sequences between the *Bst*EII and *Nru*I sites of the α47 gene. Transcribed sequences, protein coding sequences, and Ori$_S$ sequences are indicated as in panel A; α4 promoter-regulatory sequences are depicted by a stippled rectangle; and the *tk* gene 5′ transcribed noncoding and coding sequences are indicated by a cross-hatched rectangle. Line 3, diagram showing the intended replacement of the α4-*tk* chimeric gene in R3630 with the chimeric Psv40-α4 construct in plasmid pRB3915 by recombination within the regions of U$_S$10 and Ori$_S$. Line 4, schematic representation of the S component (right end) of R3914, with a deletion of Ori$_S$ resulting from recombination within the regions of U$_S$10 and α4. Line 5, expansion of the mutagenized region in R3914, which is contained within a 2.7-kb novel *Bam*HI fragment. Line 6, three restriction fragments, which together span the mutagenized region in R3914, were used as probes in Fig. 2. Probe 1, sequences between the left *Bam*HI site and the nearest *Sph*I site in the HSV-1 *Bam*HI Z fragment; probe 2, SV40 promoter-enhancer sequences between the *Pvu*II and *Hin*dIII sites excised from pSV2CAT; probe 3, entire HSV-1 *Bam*HI Y fragment. Restriction enzymes: Ba, *Bam*HI; Bst, *Bst*EII; E, *Eco*RI; H, *Hin*dIII; N, *Nru*I; Na, *Nar*I; Nd, *Nde*I; P, *Pvu*II; S, *Sal*I; Sp, *Sph*I. Note that at the junction indicated as H/S, the *Hin*dIII site but not the *Sal*I site was regenerated.

adjacent to each of the two Ori$_S$ sequences within the inverted repeats of the S component, i.e., between Ori$_S$ and the α22 gene in one repeat and between Ori$_S$ and the α47 gene in the other. However, because such a recombinant could not be selected in a single step, we resorted to sequential mutagenesis of the Ori$_S$ sequences.

Construction of HSV-1 lacking one copy of Ori$_S$. During construction of a virus in which a chimeric α4 gene consisting of the coding domain of the gene fused to the SV40 promoter was recombined into the viral genome, a recombinant virus designated R3914 and lacking one copy of the Ori$_S$ sequence was isolated. The parent virus used for this con-

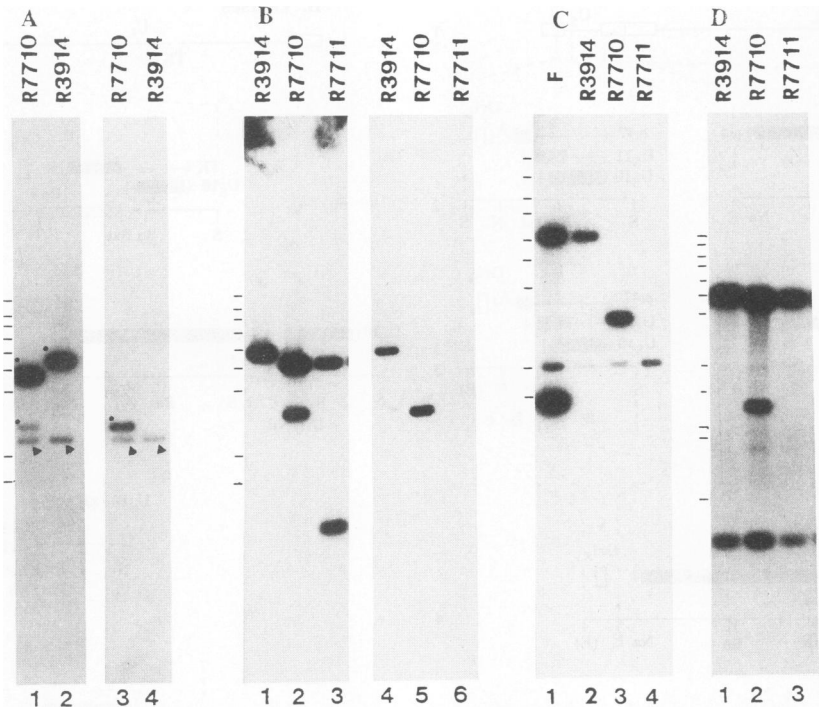

FIG. 2. Autoradiographic images of electrophoretically separated digests of wild-type and recombinant viruses probed for structure. (A) *Bam*HI digests of R7710 (lanes 1 and 3) and R3914 (lanes 2 and 4) DNAs probed first with ^{32}P-labeled pRB138 DNA (lanes 1 and 2), which contains the HSV-1(F) *Bam*HI N DNA fragment in pBR322, stripped, and reprobed with ^{32}P-labeled pRB4540 DNA (lanes 3 and 4), which contains the *Bgl*II-*Nru*I DNA fragment of the HSV-1 *tk* gene (20). Bands hybridizing to the HSV-1 sequences are indicated with dots. The band marked with the arrowhead is the 2.7-kb fragment containing the SV40 promoter-α4 chimeric gene and pBR322 sequences hybridizing with the probes (Fig. 1B, line 4). (B) *Bam*HI digests of R3914 (lanes 1 and 4), R7710 (lanes 2 and 5), and R7711 (lanes 3 and 6) DNAs probed first with the 50-mer oligodeoxynucleotide probe shown in Fig. 3, line 2 (lanes 4 to 6), and then reprobed with the 1.6-kb *Bam*HI-*Sac*I DNA fragment of pRB138, which contains sequences between the left *Bam*HI site and the *Sac*I site of the α22 gene, including Ori$_S$ (lanes 1 to 3). (C) *Bam*HI digests of HSV-1(F) (lane 1), R3914 (lane 2), R7710 (lane 3), and R7711 (lane 4) DNAs probed with the 32-mer oligodeoxynucleotide probe shown in Fig. 3, line 3. (D) *Bam*HI-*Bss*HII digests of R3914 (lane 1), R7710 (lane 2), and R7711 (lane 3) DNAs probed with the same 1.6-kb *Bam*HI-*Sac*I DNA fragment of pRB138 as that used in panel B. The positions of marker DNAs are indicated to the left of each blot; their sizes are 8.5, 7.2, 6.4, 5.7, 4.8, 4.3, 3.7, 2.3, and 1.9 kb. In panel D, the positions of 1.4-, 1.3-, and 0.7-kb bands are also shown.

struction, R3630 (19), carried an α4 promoter-*tk* chimeric gene in place of the α47 gene (Fig. 1B, line 2). DNA of plasmid pRB3915 (Fig. 1A, line 4) was cotransfected with R3630 DNA to isolate a *tk*-negative virus. The possible viral DNA structures within the mutagenized region resulting from the double recombinational event in which the *tk* gene was deleted are depicted in lines 3 and 4 of Fig. 1B. If the right-hand crossover relative to the SV40 promoter occurred within the region near Ori$_S$, the progeny virus would acquire the structure depicted in line 3, in which a total of three copies of the α4 gene would be present in the viral genome and one copy of the α4 gene would be fused to the SV40 promoter. If the right-hand crossover relative to the SV40 promoter occurred within the α4 gene domain, the progeny virus would acquire the structure depicted in line 4, in which one of two copies of the α4 gene would be fused to the SV40 promoter and one of the Ori$_S$ sequences would be deleted. On the basis of tests defined by two criteria (see below), we concluded that isolate R3914 has the structure depicted in Fig. 1B, line 4.

The first criterion was the presence or absence of a 3.5-kb *Bam*HI fragment that contained Ori$_S$ and the 3′ portion of the α4 gene (Fig. 1B, line 3). As shown in Fig. 2C, lane 2, the diagnostic 3.5-kb fragment was not detected by hybridization of the *Bam*HI digest of R3914 DNA with a labeled DNA probe specific for Ori$_S$ and shown in Fig. 3.

The second criterion was the location of an SV40 promoter-α4 chimeric gene in the DNA fragments generated by *Sal*I digestion. If the recombination yielded the construct shown

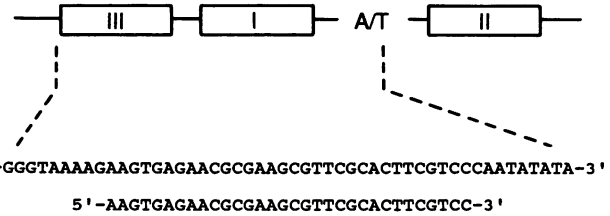

FIG. 3. Diagram of the structure of Ori$_S$ and sequences of the oligodeoxynucleotides that were used as probes for Ori$_S$ sequences. Line 1, schematic structure of Ori$_S$. Rectangles labeled I to III represent U$_L$9 protein binding sites (I and II) or a presumed binding site (III) (9, 22, 39). A/T represents an A/T-rich sequence. Lines 2 and 3 show the synthetic oligodeoxynucleotides that served as probes for Ori$_S$ sequences in viral DNAs in the studies shown in Fig. 2. The 50-mer and 32-mer sequences show 80% (40 of 50) and 97% (31 of 32) identity to Ori$_L$, respectively.

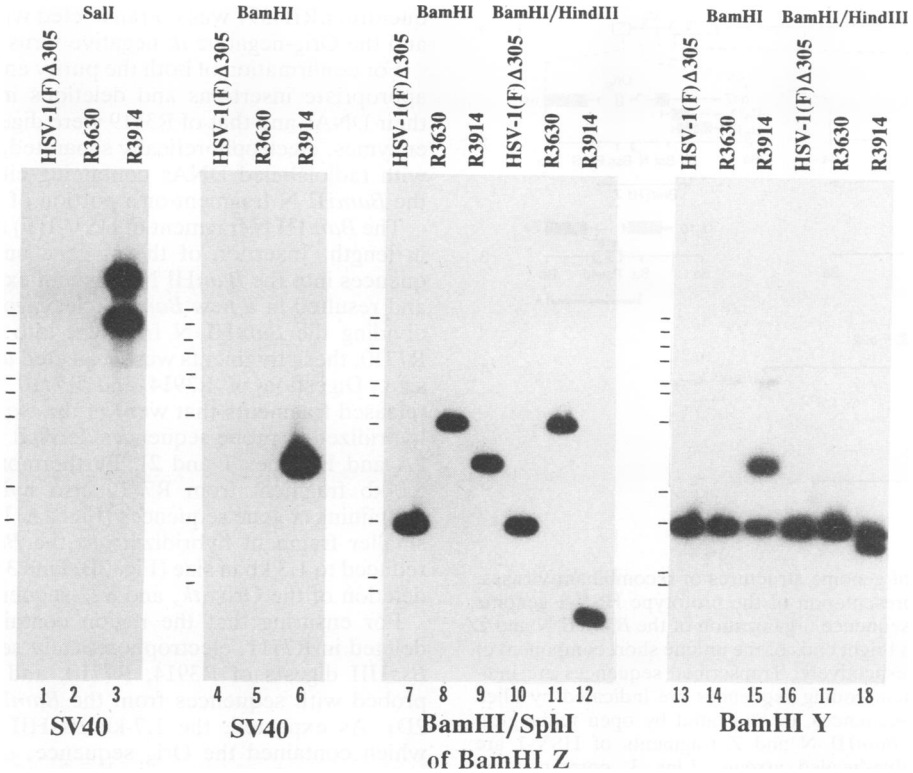

FIG. 4. Autoradiographic images of analyses of the SV40 promoter-α4 chimeric gene in the R3914 genome. Shown are autoradiographic images of *Sal*I digests (lanes 1 to 3), *Bam*HI digests (lanes 4 to 6, 7 to 9, and 13 to 15), or *Bam*HI-*Hin*dIII digests (lanes 10 to 12 and 16 to 18) of HSV-1(F)Δ305 (lanes 1, 4, 7, 10, 13, and 16), R3630 (lanes 2, 5, 8, 11, 14, and 17), or R3914 (lanes 3, 6, 9, 12, 15, and 18) DNAs hybridized with the radiolabeled probes shown in Fig. 1B, line 6. Two of the electrophoretically separated digests (lanes 1 to 3 and 4 to 6) were probed with the ^{32}P-labeled SV40 promoter-enhancer DNA. The electrophoretically separated digest shown in lanes 7 to 12 was first probed with the ^{32}P-labeled 0.29-kb *Bam*HI-*Sph*I fragment containing portions of the U_S10 and U_S11 genes of the *Bam*HI Z fragment. The probe was then stripped off, and the digest was reprobed with the ^{32}P-labeled *Bam*HI Y fragment (lanes 13 to 18). Note that the sizes of the *Bam*HI Y and *Bam*HI Z fragments of HSV-1(F) are roughly equal and that there are two copies of the *Bam*HI Y fragment in HSV-1(F) DNA, inasmuch as the fragment maps in the inverted repeat sequences flanking the S component in HSV-1(F). One *Bam*HI Y sequence was fused to the SV40 promoter-enhancer sequence in R3914. The positions of marker DNAs are indicated to the left of each blot; their sizes are 8.5, 7.2, 6.4, 5.7, 4.8, 4.3, 3.7, 2.3, 1.9, 1.4, and 1.3 kb (the 1.4- and 1.3-kb bands are not shown on the leftmost blot).

in line 3 of Fig. 1B, the SV40 promoter-α4 chimeric gene should be detected by hybridization with the SV40 promoter DNA as a single *Sal*I band 7.4 kb long because there would be two *Sal*I sites surrounding the SV40 promoter-α4 chimeric gene. On the other hand, if the recombination resulted in the construct depicted in line 4 of Fig. 1B, the SV40 promoter-α4 chimeric gene should be detected as *Sal*I bands 13 and 7.5 kb long because of the isomerization of the S component, inasmuch as there would be only one *Sal*I site upstream of the chimeric gene. As shown in Fig. 4, lane 3, SV40 promoter sequences hybridized to two R3914 *Sal*I DNA fragments of the expected sizes. This result is consistent with the recombination event depicted in line 4 of Fig. 1B.

Hybridization studies (Fig. 4) provided additional verification of the structure of the SV40 promoter-α4 chimeric gene in the R3914 DNA. Electrophoretically separated HSV-1(F)Δ305, R3630, and R3914 viral DNAs digested with *Bam*HI or *Bam*HI-*Hin*dIII were hybridized with various radioisotopically labeled DNA probes. The key results were that a 2.7-kb *Bam*HI fragment of R3914 that hybridized with a DNA fragment from the SV40 promoter-enhancer region (lane 6) also hybridized with the unique sequence of the *Bam*HI Z fragment (lane 9) and with the BamHI Y fragment (lane 15). The presence of the *Hin*dIII site between the SV40 sequence and the BamHI Y sequence on this 2.7-kb *Bam*HI fragment (Fig. 1B, line 4) was confirmed because *Hin*dIII digested this 2.7-kb fragment into 1.0- and 1.7-kb fragments that hybridized with *Bam*HI Z (lane 12) and Y (lane 18) probes, respectively. These observations were consistent with a proper fusion between the SV40 promoter and one copy of the α4 gene.

On the basis of these results, we concluded the following: (i) the sequences between the *Bst*EII site of the *Bam*HI Z fragment and the *Sal*I site of the *Bam*HI Y fragment in R3914 were replaced with the SV40 promoter-enhancer sequences and a portion of the pBR322 sequences; (ii) the copy of the α4 promoter-regulatory region nearest the α47 gene was replaced with the SV40 promoter-enhancer sequences; and (iii) the Ori_S sequence discussed in greater detail below, the α47 gene, and the 5′ untranscribed and transcribed noncoding sequences of the U_S11 gene were deleted.

The viability of R3914 was consistent with the previous observations that one copy of Ori_S could be deleted from the virus genome without abolishing the ability of the virus to multiply (16) and that the α47 and U_S11 genes were dispensable for growth in cell culture (17, 19).

Construction of HSV-1 lacking both copies of Ori_S. In

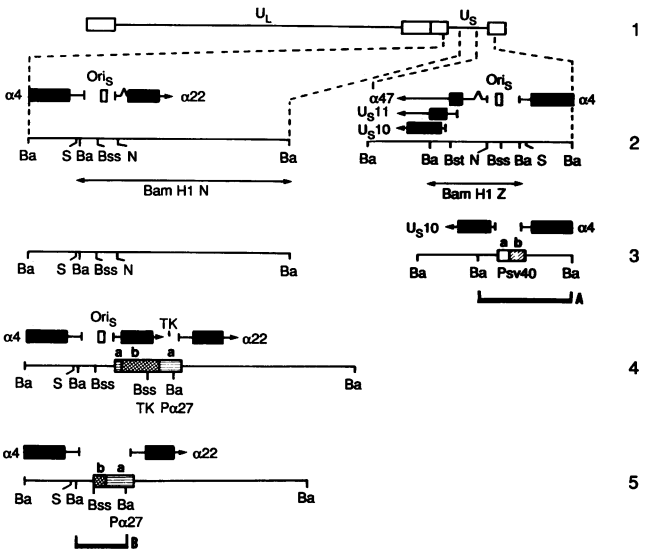

FIG. 5. Summary of genome structures of recombinant viruses. Line 1, schematic representation of the prototype HSV-1 genome arrangement. Line 2, sequence organization of the *Bam*HI N and Z fragments at the left and right ends of the unique short component of the HSV-1 genome, respectively. Transcribed sequences are indicated by arrows, protein coding sequences are indicated by filled rectangles, and Ori_S sequences are indicated by open rectangles. The positions of the *Bam*HI N and Z fragments of HSV-1 are indicated by the double-headed arrows. Line 3, corresponding sequence organization in recombinant R3914. The sequence located between the *Bst*EII and *Sal*I sites of HSV-1(F)Δ305, shown in line 2, which includes most of the *Bam*HI Z fragment, was replaced with the SV40 enhancer-promoter fragment (rectangle b) and *Nde*I-*Pvu*II fragment of pBR322 (rectangle a). The unique 2.7-kb BamHI fragment spanning this mutation (line A) was cloned from the progeny virus R7711, giving rise to pRB4489. Line 4, same as line 2, except that the structure of virus R7710 is shown. Rectangle b, *tk* gene coding sequence; rectangle a, α27 promoter-regulatory sequence. Line 5, same as line 3, except that the structure of recombinant R7711 is shown. The *Bam*HI DNA fragment that contained the junction of the deletion (line B) was cloned into pGEM-3Zf(+) to yield pRB4488. Restriction enzymes: Ba, *Bam*HI; Bss, *Bss*HII; Bst, *Bst*EII; N, *Nru*I; S, *Sal*I.

wild-type HSV-1, Ori_S is located in the promoter-regulatory sequences of both the α22 and the α47 genes (Fig. 5, line 2). In making insertions and deletions around the remaining copy of Ori_S in R3914, we wanted to ensure continued expression of the α22 gene, since this gene is important for growth in primary human and rodent cells (30) and since the properties of a double deletion of α47 and α22 could not be predicted. To accomplish this, we created two plasmids: (i) pRB4325 (Fig. 5, line 4), in which both the *tk* selectable marker and the promoter-regulatory sequences of the α27 gene were inserted near Ori_S in the α22 gene in the *Bam*HI N fragment such that the *tk* gene was under the control of the α22 promoter, whereas the α22 gene was under the control of the α27 promoter, and (ii) pRB4397 (Fig. 5, line 5), in which Ori_S, the α22 promoter-regulatory sequences, and the *tk* selectable marker were deleted. The deletion in pRB4397 did not remove any TAATGARAT motifs, important for α4 gene expression, from the region upstream of the α4 gene promoter. The *tk* insertion plasmid pRB4325 was cotransfected with R3914 viral DNA, and the *tk*-positive virus R7710 was isolated from the transfection progeny. Subsequently, pRB4397 was cotransfected with R7710 viral DNA, and the Ori_S-negative *tk*-negative virus R7711 was isolated.

For confirmation of both the purity and the presence of the appropriate insertions and deletions in R7710 and R7711, their DNAs and that of R3419 were digested with restriction enzymes, electrophoretically separated, blotted, and probed with radiolabeled DNAs containing either sequences from the *Bam*HI N fragment or a portion of the *tk* gene (Fig. 2).

The *Bam*HI N fragment of HSV-1(F) and R3914 is ~4.9 kb in length. Insertion of the *tk* gene and α27 promoter sequences into the *Bam*HI N fragment expanded the fragment and resulted in a new *Bam*HI cleavage site (Fig. 5, line 4), dividing the *Bam*HI N fragment into two fragments. For R7710, these fragments were expected to be 4.4 and 3.0 kb in size. Digestion of R3914 and R7710 DNAs with *Bam*HI released fragments that were of the expected sizes and that hybridized to probe sequences derived from *Bam*HI N (Fig. 2A and B, lanes 1 and 2). Furthermore, as expected, the 3.0-kb fragment from R7710 also hybridized to a probe containing *tk* gene sequences (Fig. 2A, lane 3). In R7711, the smaller fragment hybridizing to the *Bam*HI N probe was reduced to 1.3 kb in size (Fig. 2B, lane 3), consistent with the deletion of the Ori_S, *tk*, and α22 sequences.

For ensuring that the region containing Ori_S had been deleted in R7711, electrophoretically separated *Bam*HI and *Bss*HII digests of R3914, R7710, and R7711 DNAs were probed with sequences from the *Bam*HI N fragment (Fig. 2D). As expected, the 1.7-kb *Bss*HII fragment of R7710, which contained the Ori_S sequence, α22 promoter-regulatory sequences, and part of the *tk* gene, was absent from R7711.

Absence of Ori_S sequences and function in R7711. The minimum sequence required for the Ori_S replication function is quite small (6, 36); the smallest HSV-1 DNA fragment shown to possess replication origin function is 67 bp in length (6). Such a small sequence, if retained in R7711 by some rearrangement, might not be detected by the relatively low resolution analyses described above. For addressing this issue, the same DNA blot as that shown in lanes 1 to 3 in Fig. 2B was probed with an oligodeoxynucleotide probe containing Ori_S sequence (Fig. 2B, lanes 4 to 6). The sequence of the 50-mer oligodeoxynucleotide probe (Fig. 3, line 2) is contained within the minimal HSV-1 DNA segment required for amplification by viral factors, and mutations in this sequence abolish the ability of the DNA to act as an origin (18, 40). The *Bam*HI N fragment (4.9 kb) of R3914 DNA (Fig. 2B, lane 4) and the 3.0-kb fragment derived from the chimeric *Bam*HI N fragment of R7710 DNA (Fig. 2B, lane 5) hybridized with this probe, consistent with the localization of Ori_S on these fragments. The absence of other hybridizing bands is consistent with the absence of Ori_S at the other end of U_S. In contrast, R7711 DNA failed to hybridize with this probe (Fig. 2B, lane 6), suggesting a complete loss of Ori_S sequences from this virus.

An additional hybridization experiment was carried out with a shorter oligodeoxynucleotide probe to detect simultaneously both Ori_S and Ori_L sequences (Fig. 2C). The 32-mer oligodeoxynucleotide (Fig. 3, line 3) is shorter in length and shows greater similarity to Ori_L than that shown in Fig. 3, line 2, and used in the hybridization experiments described above. In *Bam*HI digests of HSV-1(F) DNA, this probe detected the Ori_S sequences as two stronger bands (*Bam*HI N and Z fragments) and the Ori_L sequence as a weaker band (*Bam*HI V fragment). Both R3914 and R7710 yielded only one stronger band in addition to the weaker band, indicating that these viruses possessed only one copy

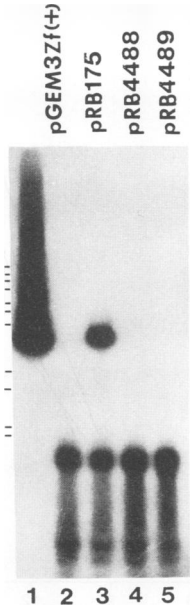

FIG. 6. Transient replication assay. Autoradiographic image of labeled pGEM-3Zf(+) DNA hybridized to electrophoretically separated *Bam*HI and *Dpn*I digests of DNA extracted from Vero cells transfected with test plasmids and infected with HSV-1(F). Test plasmids were pGEM-3Zf(+) (lane 2), pRB175 (lane 3), pRB4488 (lane 4), and pRB4489 (lane 5). Lane 1, linearized pGEM-3Zf(+) size standard. The positions of marker DNAs, the same as those shown in Fig. 2, are shown to the left.

of Ori$_S$ in addition to Ori$_L$. R7711 yielded only the band corresponding to the *Bam*HI V fragment, indicating that R7711 did not retain any Ori$_S$ sequence. Differences in the intensity of the *Bam*HI V band among viruses reflected variations in the amount of viral DNA loaded on each lane.

To test directly for a loss of Ori$_S$ function, we tested DNA fragments spanning the mutagenized regions in a transient replication assay. The *Bam*HI DNA fragments spanning the mutagenized regions from the left and right (with respect to the prototype genome arrangement) ends of U$_S$ (Fig. 5) were cloned from R7711 DNA into vector pGEM-3Zf(+) to yield pRB4488 and pRB4489, respectively. Restriction enzyme mapping of these plasmids further verified the absence of both Ori$_S$ sequences (data not shown). Vero cells were transfected with plasmids and subsequently infected with HSV-1(F). At 13 h postinfection, total cellular DNA was isolated, digested with *Bam*HI and *Dpn*I, electrophoretically separated on an agarose gel, and probed with radiolabeled pGEM-3Zf(+) DNA (Fig. 6). *Bam*HI cleaves each plasmid DNA to release a vector-length fragment, regardless of whether the DNA has replicated, whereas *Dpn*I can cut at multiple sites only in unreplicated DNA. Thus, replicated DNA is detected as a *Dpn*I-resistant, vector-length linear fragment, and unreplicated DNA is detected as small fragments. The positive control plasmid, pRB175, which carries the HSV-1(F) *Bam*HI Z fragment containing one copy of the Ori$_S$ sequence, yielded *Dpn*I-resistant DNA (Fig. 6, lane 3), indicating that it contained an active origin. In contrast, neither of the plasmids carrying the mutagenized fragments cloned from R7711 yielded *Dpn*I-resistant DNA (Fig. 6, lanes 4 and 5), demonstrating that they did not contain sequences with detectable origin activity.

The results of these studies indicated that R7711 DNA lacks both Ori$_S$ sequences and function. The results also demonstrated that the SV40 DNA replication origin contained in the SV40 promoter-α4 fusion could not support transient plasmid replication during HSV-1 infection. This result was consistent with the observations of Heilbronn and Zur Hausen (12), who showed that stimulation of the activity of the SV40 replication origin by infection with HSV-1 was absolutely dependent on the presence of T antigen supplied in *trans*.

Time course of DNA synthesis. The successful isolation of a virus lacking both copies of Ori$_S$ demonstrates that Ori$_S$ is not essential for viral replication, but it seemed likely that the loss of these sequences might alter either the amount of viral DNA synthesized, the time course of synthesis, or the timing of replication of different regions of the genome. For testing these possibilities, Vero cells were infected with 5 PFU of viruses HSV-1(F)Δ305, R3914, and R7711 per cell, and total cellular DNA was harvested from duplicate dishes at 3, 6, 9, 12, and 15 h after infection. The DNAs were digested with *Bam*HI, electrophoretically separated, transferred to a nylon membrane, and hybridized with excess labeled probe (Fig. 7 and 8). The accumulation of viral DNA was assessed with two probe sequences: (i) the *Bam*HI T fragment, located in the L component (0.273 to 0.292 map units) and distant from both Ori$_L$ and Ori$_S$, and (ii) the *Bam*HI X fragment, located in the S component (0.938 to 0.951 map units) and near one of the Ori$_S$ sequences.

The amounts of DNA detected for all viruses at 3 h after infection in this experiment could not be differentiated from those observed at 3 h after infection in the presence of

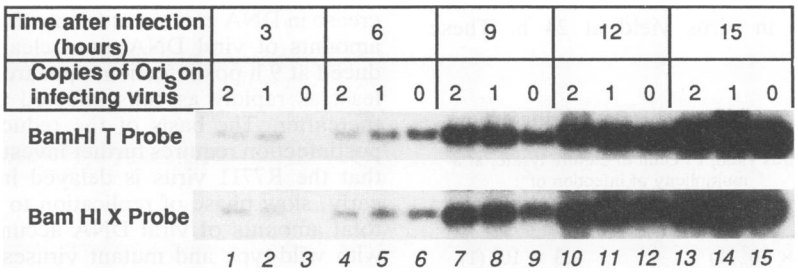

FIG. 7. Autoradiographic images of the time course of viral DNA synthesis. Shown is a representative autoradiographic image of *Bam*HI digests of DNAs extracted from Vero cells infected with HSV-1(F)Δ305 (lanes 1, 4, 7, 10, and 13), R3914 (lanes 2, 5, 8, 11, and 14), and R7711 (lanes 3, 6, 9, 12, and 15) and probed successively with excess labeled plasmids containing the *Bam*HI T (pRB179 [1]) or X (pRB124 [16]) fragment of HSV-1(F). The time after infection at which each sample was harvested is indicated above the lanes. Excess probe was demonstrated by hybridization to serial dilutions of unlabeled probe on the same blot (data not shown).

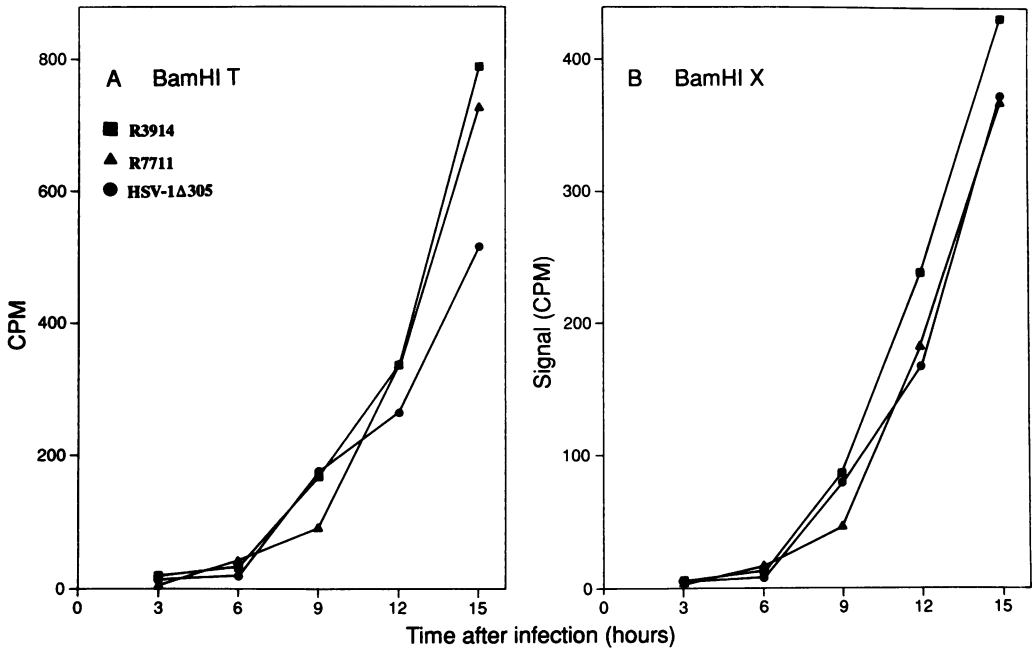

FIG. 8. Graphic representation of the time course of viral DNA synthesis. (A) The amount of labeled *Bam*HI T fragment hybridized (in counts per minute) to HSV-1(F)Δ305, R3914, and R7711 DNAs recovered from infected Vero cells as a function of time postinfection (in hours) is shown. Each time point is the mean of two independent infections. (B) As in panel A, except that the *Bam*HI X fragment was used as the hybridization probe.

cycloheximide (data not shown). Therefore, the amounts of DNA detected at 3 h indicated the levels of unreplicated input DNA. Amplification of DNA could be detected in all infected-cell samples harvested at 6 h postinfection, suggesting that the deletion of one or both copies of Ori_S did not cause a delay in the onset of viral DNA synthesis. Although the amounts of viral DNA made by R7711 were reduced at 9 h, all three viruses showed similar time courses of DNA accumulation and produced nearly equal amounts of progeny DNA. These results indicate that the viruses lacking both Ori_S sequences are not significantly or demonstrably impaired with respect to their capacity to make viral DNA.

Effect of the deletion of both Ori_S sequences on virus multiplication. Vero cells grown in 25-cm^2 dishes were infected at either a low (0.01 PFU per cell) or a high (5 PFU per cell) multiplicity of infection with HSV-1(F)Δ305, R3914, or R7711. At 24 h after infection, cells were lysed by a single cycle of freezing and thawing and sonicated, and the titer of infectious virus in each lysate was determined by a plaque assay with Vero cells (Table 1). Both R3914 and R7711 showed small reductions in virus yield at 24 h. These reductions were more apparent at a low multiplicity of infection but were at most fourfold for R7711. Both mutant viruses also multiplied efficiently in 143TK$^-$, BHK, and HEp-2 cells (data not shown).

TABLE 1. Virus yields from Vero cells at 24 h postinfection

Virus	Virus yield, PFU/ml of culture lysate, at a multiplicity of infection of[a]:	
	5 PFU/cell	0.01 PFU/cell
HSV-1(F)Δ305	4.7×10^7 (1)	9.3×10^6 (1)
R3914	3.3×10^7 (0.70)	5.4×10^6 (0.58)
R7711	2.5×10^7 (0.53)	2.7×10^6 (0.29)

[a] Mean of duplicate infections. Titration was done with Vero cells. The yields of the recombinant viruses relative to those of the parent virus, HSV-1(F)Δ305, are shown in parentheses.

DISCUSSION

We report that we have deleted both Ori_S sequences from HSV-1 recombinant virus R7711. Evidence supporting this conclusion is based on both hybridization analyses of the viral DNA with specific probes and functional assays showing that the DNA sequences that in the wild-type virus contain the Ori_S sequences are not amplified in *trans* by HSV-1 proteins expressed in the transfected cells by superinfecting virus.

The Ori_S-negative virus R7711 yielded approximately fourfold less infectious progeny than the parent virus from which it was derived in single-step growth experiments at a low multiplicity of infection. Cells infected with the virus lacking both Ori_S sequences showed a demonstrable increase in DNA content at 6 h postinfection and, although the amounts of viral DNA were clearly and reproducibly reduced at 9 h postinfection, appeared to accumulate DNA at least as rapidly as cells infected with the wild-type virus thereafter. The basis of the reduced accumulation at 9 h postinfection requires further investigation, but we speculate that the R7711 virus is delayed in the transition from an early, slow phase of replication to a late, rapid phase. The total amounts of viral DNA accumulated in cells infected with wild-type and mutant viruses by 15 h after infection were approximately similar, suggesting that the decreased virus yield of R7711 after single-step replication did not result directly from a defect in DNA synthesis. Indeed, it is possible that the small difference between R7711 and HSV-1(F)Δ305 in virus yield is unrelated to the Ori_S deletion,

since our manipulations affected the structure of at least four viral genes in addition to Ori_S.

We conclude on the basis of our data as well as on the basis of the reports published previously (16, 25) the following. (i) Both Ori_S sequences are dispensable for viral replication in cells in culture. (ii) The evidence indicates that if an origin of viral DNA synthesis is required for the amplification of a wild-type genome, Ori_L is sufficient for this purpose. (iii) Inasmuch as the Ori_L sequence was shown to be dispensable under conditions in which both Ori_S sequences were intact (25) and in this study we showed that both Ori_S sequences are dispensable, we may conclude that none of the three origins is uniquely required for viral replication in cell culture. Rather, if an origin of DNA synthesis is required at all, any one of the three origins will most likely suffice.

In view of the evidence that the absence of two origins of DNA synthesis does not grossly affect the quantity of viral DNA synthesized, the reason for the evolution and conservation of three origins of DNA synthesis remains obscure. Three broad hypotheses can address this point: (i) Ori_S and Ori_L mediate different types of replication initiation (i.e., mediate the assembly of different types of replication complexes, perhaps leading to different mechanisms of replication); (ii) the two origin types are used differently in different cells or at specific stages of the viral life cycle (e.g., lytic versus latent infection); and (iii) these origins are indeed functionally redundant, but the presence of multiple origins in the virus genome increases the probability and/or the rate of successful initiation of DNA replication or decreases the time required to replicate genome DNA. Interestingly, it can be argued that the placement of each of the three origins in the HSV-1 genome could conceivably provide for their conservation independently of selective pressure derived from their function in DNA replication. Ori_L maps between the genes encoding the single-stranded DNA binding protein ICP8 and the viral DNA polymerase. Although the viability of the Ori_L-negative virus (25) demonstrates that the Ori_L sequence is not essential for the expression of these genes, the Ori_L sequence may contribute to proper temporal and quantitative expression. The Ori_S sequences are present in reiterated sequences, and their stability could conceivably be maintained by a sequence conversion mechanism, even in the absence of selective pressure.

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