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Generation of an Inverting Herpes Simplex Virus 1 Mutant Lacking the L-S Junction *a* Sequences, an Origin of DNA Synthesis, and Several Genes Including Those Specifying Glycoprotein E and the α47 Gene

RICHARD LONGNECKER AND BERNARD ROIZMAN*

The Marjorie B. Kovler Viral Oncology Laboratories, The University of Chicago, Chicago, Illinois 60637

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The herpes simplex virus genome consists of two components, L and S, that invert relative to each other to yield four isomeric arrangements, prototype (P), inversion of the S component (I_s), inversion of the L component (I_l), and inversion of both components (I_{sl}). Previous studies have shown that the 500-base-pair *a* sequences flanking the two components contain a *cis*-acting site for inversion. In an attempt to insert a third copy of the α4 gene, the major regulatory gene mapping in the repeats flanking the S component, a fragment containing the α4 gene and an origin of DNA synthesis, was recombined into the thymidine kinase gene mapping in the unique sequences of the L component. The resulting recombinants showed massive rearrangements and deletions mapping in the S component and in the junction between the L and S components. One recombinant (R7023) yielded two isomeric DNA arrangements, a major component consisting of I_s and a minor component consisting of I_{sl}. In these arrangements, the genome lacked the gene specifying glycoprotein E and all contiguous genes located between it and the α0 gene in the inverted repeats of the L component. Among the deleted sequences were those encoding an origin of viral DNA synthesis, the α47 gene, and the *a* sequences located at the junction between the L and S components. The recombinant grew well in rabbit skin, 143TK$^-$, and Vero cell lines. We conclude that the four unique genes deleted in R7023 are not essential for the growth of herpes simplex virus, at least in the cell lines tested, and that the *b* sequence of the inverted repeats of the L component also contains *cis*-acting sites for the inversion of herpes simplex virus DNA sequences.

In this paper we report the deletion of several contiguous viral genes and an origin of viral DNA replication from the herpes simplex virus 1 (HSV-1) genome coincident with the duplication of a gene in a distant portion of the viral genome. Relevant to this report are the following.

(i) The HSV-1 genome contains a minimum of 50 genes (9). The density of open reading frames deduced from the nucleotide sequence of the S component (20) suggests the existence of approximately 70 genes. These genes form several groups (i.e., α, β_1, β_2, γ_1, and γ_2) that are coordinately regulated and sequentially ordered in a cascade fashion (10, 11). The five α genes are the first genes to be expressed after infection (10, 11). A functional α4 gene product is required for transition from α to β gene expression (5, 14, 15, 33). α27 and α22 play a role in late gene expression (36, 37). The functions of α0 and α47 are unknown.

(ii) The 150-kilobase-pair (kbp) HSV-1 genome consists of two components, L and S, that invert relative to each other such that viral DNA extracted from infected cells or virions consists of four isomers differing in orientation relative to each other (8, 34, 38). Each component consists of unique sequences (U_l or U_s) flanked by inverted repeats (38, 45). The inverted repeats of the L component were designated as *ab* and *b'a'* (45) and contain in their entirety the α0 and $\gamma_1$34.5 genes (3), whereas those of the S component designated as *a'c'* and *ca* (45) each contain a copy of the α4 gene (25) and an origin of viral DNA synthesis (23, 41, 43). A third origin of DNA synthesis has been mapped in the U_l sequence (40, 48). The shared *a* sequence, 500 base pairs in length for HSV-1 strain F [HSV-1(F)] (22), is contained in one to several copies at the L component terminus and at the L-S junction, but only one copy of the *a* sequence is present at the S component terminus (18, 46). The *a* sequence has been shown to contain a *cis*-acting site for the inversion of L and S components relative to each other (2, 21, 22) for the cleavage of concatemeric DNA and packaging of unit-length genomes into capsids (42, 44).

(iii) Earlier reports from this laboratory described two generalized techniques for gene inactivation and for deletion of DNA sequences from the HSV-1 genome (12, 13, 32, 35). The first involved insertion by recombination through homologous flanking sequences of the thymidine kinase (TK) gene into a target sequence followed by deletion of the TK gene and of target sequences at the site of insertion (12, 32). The second generalized technique involves random transposition of a mini-Mu phage containing the TK gene into a target fragment, transfection of competent cells with intact DNA from TK$^-$ virus and the fragment containing the mini-Mu inserts, and selection of TK$^+$ virus progeny (13). The function of the TK gene is to act as a marker for the selection of TK$^+$ recombinants carrying the inserts and of TK$^-$ recombinants from which the TK gene and target sequences had been deleted by recombination. The insertional mutagenesis studies demonstrated that at least 9.7 kbp, i.e., the DNA sequences the size of the mini-Mu carrying the TK gene, can be inserted with the HSV-1 genome without affecting the ability of the viral DNA to replicate and become packaged into virions. The stability of the genomes carrying inserts varies depending on the nature and location of the insert. Specifically, insertions into the internal inverted repeats can result in isolation of viruses

* Corresponding author.

carrying large deletions encompassing both the internal inverted repeats and adjacent unique sequences (13, 27, 28).

In this report we show that duplication of a fragment carrying the α4 gene and an origin of DNA replication resulted in massive rearrangements of the S component. One of these rearrangements analyzed in detail involved the deletion of contiguous sequences containing an origin of DNA synthesis and the genes for several proteins including those specifying glycoprotein E (gE) and the α47 gene. Equally significant, although the *a* sequences at the junction between the L and the S components were deleted, the L component continued to invert, indicating the existence of additional *cis*-acting sites for inversion within the *b* sequence of the terminal repeats of the L component.

MATERIALS AND METHODS

Viruses and cells. The properties of HSV-1(F) were reported elsewhere (6). Recombinant R314 carries the *Bam*HI Z fragment inserted into the *Bgl*II site of the *Bam*HI Q fragment of HSV-1(F), linking the promoter regulatory domain and transcription initiation site of α4 gene to the 5′ transcribed noncoding and coding sequences of the TK gene (31). The recombinant viruses described in this report were derived from R314. Infected cell lysates for gel electrophoresis and immunoprecipitations were made in Vero cells. Cotransfections were done in rabbit skin cells originally obtained from J. McLaren. Vero cells were used for the preparation of viral stocks, virus titrations, biotin-avidin-enhanced surface immunoassays, and the preparation of viral DNA from infected cell lysates. Growth medium for cells consisted of Dulbecco modified Eagle minimal essential medium supplemented with 10% calf serum. The maintenance medium, 199-V, consisted of mixture 199 supplemented with 1% calf serum.

Media, buffers, and solutions. Disruption buffer consisted of 0.05 M Tris (pH 7.0), 8.5% (wt/vol) sucrose, 5% (vol/vol) β-mercaptoethanol, and 2% (wt/vol) sodium dodecyl sulfate. For immunoprecipitations, phosphate-buffered saline (8.2 mM Na_2HPO_4, 1.5 mM KH_2PO_2, 0.14 M NaCl, and 2.5 mM KCl) was supplemented with 1% (vol/vol) Nonidet P-40, 1% sodium deoxycholate, 10^{-5} M sodium-α-ptosyl-L-lysine chloromethyl ketone, and 10^{-5} M L-1-tosylamide-2-phenylethyl chloromethyl ketone.

Plasmids. The DNA cloning procedures were as previously described (30). The sources, derivations, and sequence contents of cloned HSV-1(F) DNA fragments used as probes in hybridization tests are listed in Table 1. pRB103 carries the *Bam*HI Q fragment in the *Bam*HI site of pBR322 (30). pRB3471 contains the entire α4 promoter-regulatory region from the *Sac*I site in *Bam*HI-Z and the α4 structural gene as present in pRB3094 (12) inserted into *Bam*HI-Q with a portion of the TK gene deleted.

Preparation of viral DNAs. Viral DNAs were prepared by preparative centrifugation of the cytoplasmic fraction of infected cell lysates in NaI density gradients (47).

DNA cotransfections. The cotransfection procedures were as described by Graham and Van der Eb (7) and modified by Kousoulas et al. (16). Rabbit skin cells were treated with 200 μg of DEAE-dextran per ml in phosphate-buffered saline for 5 min and washed with 10 mM Tris (pH 7.5)–1 mM EDTA–150 mM NaCl immediately before adding the precipitated DNAs. DNAs in 0.5 ml of *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid buffer were precipitated onto rabbit skin cells by the addition of 30 μl of 2.2 M $CaCl_2$ and incubation at room temperature for 30 min. Growth medium was added 10 min after the addition of the $CaCl_2$-precipitated DNAs to the rabbit skin cells. After incubation for 6 to 10 h, the medium was replaced and the cells were further incubated.

Selection of recombinant viruses. TK^- progeny was selected on $143TK^-$ cells maintained in medium containing a mixture of 199-V supplemented with 3% fetal bovine serum and bromouracil deoxyribose (40 μg/ml). TK^+ progeny was selected on $143TK^-$ cells maintained in HAT medium consisting of a mixture of Dulbecco modified Eagle medium supplemented with 10% fetal calf serum, hypoxanthine, aminopterin, and thymidine.

Analysis of DNA by restriction endonuclease digestion and hybridization. Restriction endonucleases were purchased from New England BioLabs, Beverly, Mass. T4 DNA ligase was obtained from Boehringer Mannheim Biochemicals (Indianapolis, Ind.). Restriction endonuclease fragments were separated on SeaKem Me agarose (FMC Corp., Marine Colloids Div., Rockland, Maine) and transferred to nitrocellulose paper (Schleicher & Schuell, Inc., Keene, N.H.) by a modification of the procedure of Southern (39). In this modification the agarose gel was sandwiched between two sheets of nitrocellulose, and absorbent paper towels were placed on both sides of the gel. DNA probes were labeled with [^{32}P]dCTP by nick translation with a kit from New England Nuclear Corp., Boston, Mass. The conditions for the hybridization were as previously described (21, 30). Autoradiograms were made with Kodak X-Omat X-ray film (Eastman Kodak Co., Rochester, N.Y.).

Antibodies. Hybridomas producing monoclonal antibodies

TABLE 1. DNA probes used in analysis of R7023 DNA

Sequence probed	Source or derivation and sequence of probe	Plasmid designation	Reference if previously published
*Bam*HI-Y	*Bam*HI-Y in *Bam*HI site of pBR322	pRB113	30
*Bam*HI-N and -Z	*Bam*HI-N in *Bam*HI site of pBR322	pRB114	30
*Bam*HI-X	*Bam*HI-X in *Bam*HI site of pBR322	pRB124	30
*Bam*HI-B	Left *Bam*HI-*Sac*I subfragment of *Bam*HI-B, contains unique sequences only; in *Bam*HI-*Eco*RI site of pBR322	pRB404	27, 28
*Bam*HI-E	Right *Bam*HI-*Xba*I subfragment of *Bam*HI-E, contains unique sequences only; in *Bam*HI-*Xba*I site of pUC18	pRB422	
a	1.1-kbp *Hae*II fragment spanning the L-S junction and containing an *a* sequence in *Bam*HI site of pACYC177	pRB601	21, 22
*Bam*HI-P	*Bam*HI-P without *a* sequences; in *Eco*RI-*Bam*HI site of pUC9	pRB3133	J. Hubenthal-Voss, unpublished data
gD gene	2.7-kbp *Sac*I subfragment of *Bam*HI-J in *Sac*I site of pUC19	pRB3445	
gE gene	1.9-kbp SacI subfragment of *Bam*HI-J in *Sac*I site of pUC19	pRB3450	

H600 (reactive with gE-1), H1301 (reactive with gE-1+2), H1379 (reactive with gG-1), and H1380 (reactive with gD-1) were derived from BALB/c mice immunized with HSV-1(F) and described in a previous report (1a) and were kindly provided by Lenore Pereira. The rabbit polyclonal antibody made against gE was a gift from Patricia G. Spear and is described elsewhere (26).

Infection and radiolabeling of cells. Monolayer cultures containing 4×10^6 cells were exposed to 10 PFU of virus per cell. After 1 h of adsorption, the inoculum was replaced with maintenance medium containing only 10% of the usual amount of methionine and supplemented with [^{35}S]methionine (New England Nuclear Corp., Boston, Mass.) to a final concentration of 25 μCi/ml.

Preparation of infected cell extracts for immunoprecipitation. Infected Vero monolayers were washed one time with phosphate-buffered saline, harvested, and pelleted by centrifugation at $2,000 \times g$ for 5 min before being solubilized in immunoprecipitation buffer. Cell lysates were sonicated and then centrifuged for 1 h at 25,000 rpm in a Beckman SW 50.1 rotor. Protein A-Sepharose (Sigma Chemical Co., St. Louis, Mo.) was swelled in immunoprecipitation buffer on ice and washed twice with cold immunoprecipitation buffer. The secondary antibody was adsorbed to the protein A-Sepharose on ice for 1 h and then washed three times. The primary antibody was adsorbed to the secondary antibody-protein A-Sepharose complex for 1 h on ice and then washed three times with cold immunoprecipitation buffer. The cell lysates were added to antibody-protein A-Sepharose complexes so that one-third of a plaque dish was precipitated with 5 mg of protein A-Sepharose. This mixture was incubated for 2 h with occasional shaking. The complexes were washed three times with immunoprecipitation buffer. Proteins were removed from the antigen-antibody-protein A-Sepharose complex by the addition of disruption buffer. The solubilized material was boiled for 2 min before electrophoresis.

Polyacrylamide gel electrophoresis. The solubilized immunoprecipitate was separated on 9.25% (vol/vol) polyacrylamide gels containing sodium dodecyl sulfate and was cross-linked with diallytartardiamide as described by Morse et al. (25).

Biotin-avidin-enhanced surface immunoassay. The procedure was carried out, as described by Kousoulas et al. (16) and subsequently modified by Ackerman et al. (1), by using the Vecta stain kit (Vector Laboratories, Inc., Burlingame, Calif.).

RESULTS

Construction of recombinants exhibiting novel deletions. The initial purpose of the studies described below was to insert a copy of the α4 gene into the TK gene. For this purpose the plasmid pRB3471, containing the α4 gene fused to the *Eco*RI-*Pst*I fragment carrying the 3′ terminus of the TK gene from the *Bam*HI Q fragment, was cotransfected with intact DNA of recombinant R314. TK$^-$ progeny were selected in 143TK$^-$ cells in the presence of bromouracil deoxyribose (Fig. 1A). The reason for using the R314 recombinant rather than the wild-type HSV-1(F) virus is that R314 DNA contained the promoter-regulatory domain of the α4 gene inserted into the *Bgl*II cleavage site and therefore provided flanking sequences for homologous recombination necessary for the insertion of the α4 gene into that site. Analyses of the recombinants (data not shown) indicated that all contained the α4 gene inserted into the TK gene. However, without exception, all clones tested exhibited extensive rearrangements of the S component and of the junction between the L and S components. One of the recombinant virus clones, described in this report, was of particular interest because of extensive deletions not previously described. To facilitate the analyses of the recombinant, we deleted the inserted copy of the α4 gene by cotransfecting intact viral DNA with the *Bam*HI Q DNA fragment from pRB103 into rabbit skin cells and selecting TK$^+$ viruses in 143TK$^-$ cells maintained in HAT medium. The resulting recombinant designated as R7023 was TK$^+$ and contained a *Bam*HI Q fragment indistinguishable with respect to restriction endonuclease cleavage sites and mobility from the wild-type HSV-1(F) fragment. The deletions and rearrangements that differentiate it from the wild-type HSV-1(F) virus described below were in the S component and in the reiterated sequences at the junction between the L and S components.

Structure of recombinant R7023. The analyses described below indicate that R7023 DNA differs from HSV-1(F) DNA with respect to the following. (i) The S component is frozen in an inverted orientation relative to the P arrangement. (ii) The DNA sequences deleted from R7023 extend from approximately the left third of the L component inverted repeat sequence *b* to approximately, but not into, the gene specifying gD. (iii) The L component inverts notwithstanding the absence of the *a* sequence at the junction between the L and S components. Therefore, R7023 DNA is present in two isomers. The major population is in I_s arrangement, whereas a minority is in I_{sl}. These conclusions are supported by the following data.

(i) *Bam*HI digests of the R7023 DNA indicate the presence of two novel fragments of 15.6 and 14.0 kbp (Fig. 1C; Fig. 2, lanes 1 and 2). The ^{32}P-labeled unique sequences contained in the left end of the *Bam*HI B fragment (Fig. 1B) hybridized with the wild-type *Bam*HI-B and band 1 (Fig. 2, lanes 3 and 4), whereas the ^{32}P-labeled unique sequences from the right end of *Bam*HI-E (Fig. 1B) hybridized with the homologous *Bam*HI-E and band 2 (Fig. 2, lanes 5 and 6). In addition, band 1 and band 2 hybridized (Fig. 2, lanes 7 through 10) with the *Sac*I fragments from *Bam*HI-J (Fig. 1B). These analyses indicate that *Bam*HI-B and *Bam*HI-E located near the left and right termini of the L component in the P orientation (Fig. 1C) are present in digests in intact form and that at least portions of *Bam*HI-E and *Bam*HI-B are linked to sequences mapping in the S component. Therefore, bands 1 and 2 are fragments spanning novel junctions between the L and S components.

(ii) The electrophoretically separated, immobilized *Bam*HI digest of R7023 DNA lacked the wild type *Bam*HI-J, -X, and -Z and the fragment SP spanning the junction between the L and S components (Fig. 2, lanes 7 through 14, 17, and 18). Thus, although R7023 retained one of the two copies of *Bam*HI Y fragments inasmuch as the ^{32}P-labeled probe hybridized with a fragment comigrating with *Bam*HI-Y (Fig. 2, lanes 15 and 16), it failed to hybridize with the ^{32}P-labeled *Bam*HI-X probe (Fig. 2, lanes 11 and 12). ^{32}P-labeled *Bam*HI-N hybridized to the homologous fragment but not to *Bam*HI-Z, with which it shares inverted repeat *c* sequences (Fig. 2, lanes 13 and 14). Plasmid pRB601 containing the *a* sequence from HSV-1(F) hybridized to the terminal *Bam*HI S and P fragments but not to the DNAs in bands 1 and 2 (Fig. 2, lanes 19 and 20). Similarly pRB3133 containing the terminal *Bam*HI P fragment minus the terminal *a* sequence hybridized to a fragment comigrating with wild-type *Bam*HI-P but not to the DNAs in bands 1 and 2 (Fig. 2, lanes 17 and 18). We conclude that bands 1 and 2,

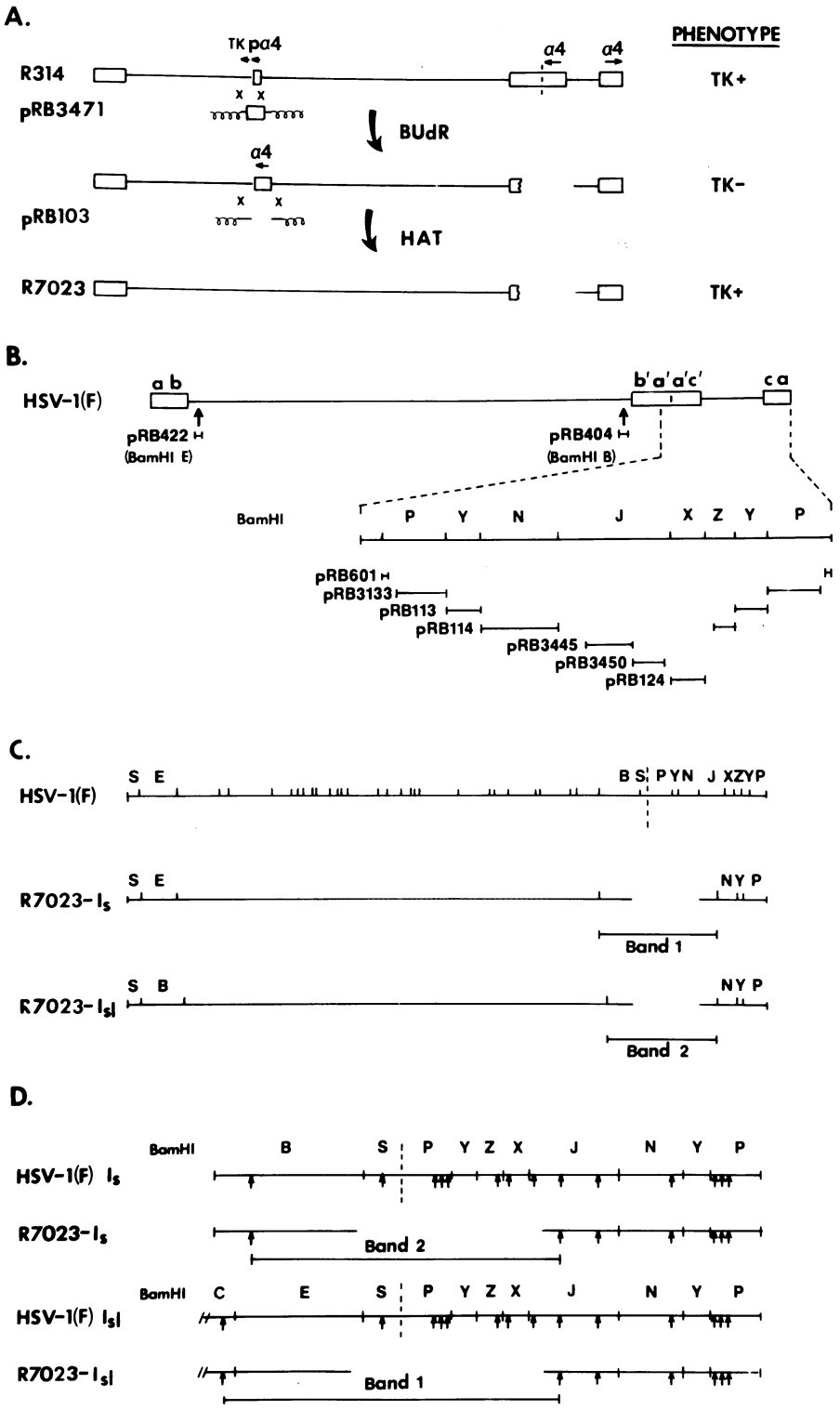

FIG. 1. Viral DNA sequence arrangements in HSV-1(F) and the HSV-1(F) recombinants R314 and R7023. (A) Schematic diagram of the construction of the recombinant R7023. Recombinant R314 (31) contained *Bam*HI-Z inserted into the *Bgl*II site of *Bam*HI-Q such that the promoter of the α4 gene was fused to the 5′ transcribed noncoding and coding sequences of the TK gene. The TK gene was expressed and regulated as an α gene. Cotransfection of intact R314 viral DNA with pRB3471 carrying an intact α4 gene yielded TK^- progeny carrying extensive deletions. Cotransfection of viral DNA which had undergone deletions with pRB103 and selection of TK^+ virus in HAT medium was done to simplify analysis of the deletion recombinants. Recombinant R7023 was selected from among the progeny of this transfection. (B) Schematic diagram of the sequence arrangement of HSV-1(F) virus showing the *Bam*HI restriction endonuclease cleavage sites in the internal inverted repeats and in the S component. The domains of the cloned HSV-1(F) DNA fragments used as probes (Fig. 2 and 3) and listed in Table 1 are shown below the restriction endonuclease map. (C) *Bam*HI cleavage maps of HSV-1(F) DNA and of the two isomers of R7023

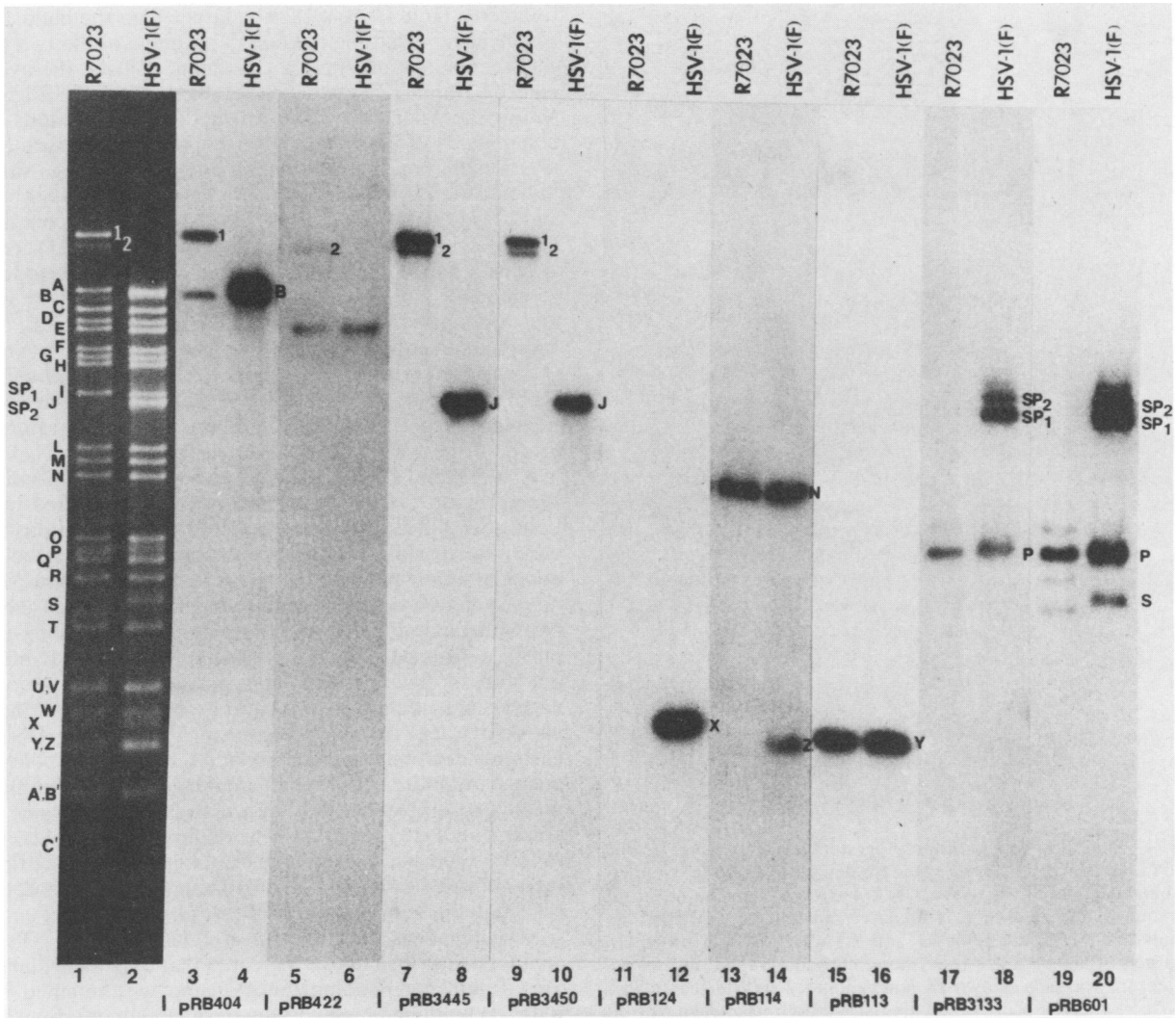

FIG. 2. Photographs and autoradiographs of electrophoretically separated *Bam*HI restriction endonuclease digests of HSV-1(F) and of recombinant R7023 viral DNAs. DNA was transferred to nitrocellulose and hybridized as described in Materials and Methods. Lanes 1 and 2 show ethidium bromide-stained gels. Lanes 3 through 20 show autoradiographic images of duplicates of the gels shown in lanes 1 and 2 hybridized to the following ^{32}P-labeled probes indicated at the bottom of each pair of lanes: pRB404, left terminus of *Bam*HI-B containing unique sequences; pRB422, right terminus of *Bam*HI-E containing unique sequences; pRB3445, *Sac*I fragment from *Bam*HI-J containing the gD-coding region; pRB3450, *Sac*I fragment from *Bam*HI-J containing the gE-coding region; pRB124, *Bam*HI-X; pRB114, *Bam*HI-N; pRB113, *Bam*HI-Y; pRB3133, *Bam*HI-P lacking the terminal *a* sequence; pRB601, *Hae*II fragment spanning the L-S junction and containing an *a* sequence. Bands 1 and 2 refer to the novel bands illustrated in Fig. 1C. The letters to the right identify the *Bam*HI bands hybridizing with the labeled probes. The lower band hybridizing with the labeled probes in lanes 5 and 6 is *Bam*HI-E.

representing novel L-S junction fragments, lack the HSV-1 sequences contained in pRB601 (the *a* sequence), *Bam*HI-Z, and *Bam*HI-X. Because the DNAs contained in bands 1 and 2 contain sequences homologous to *Bam*HI-B and -E, respectively, and *Bam*HI-J, all intervening fragments and at least a portion of the sequences contained in *Bam*HI-B, -E, and -J had to be deleted, since bands 1 and 2 are smaller than the sums of the molecular weights of *Bam*HI-B and -J or *Bam*HI-E and -J.

Two comments should be made concerning the results presented in Fig. 2. First, the *Bam*HI S fragments of R7023 are smaller than those of HSV-1(F). This is neither unusual nor entirely unexpected inasmuch as the size of the *a* sequence fluctuates in size depending on the number of reiterations of internal repeats (22). A potentially more interesting observation stems from previous reports that a *Bam*HI fragment (*Bam*HI-P) contains a single *a* sequence and does not form a ladder of bands (18). The additional faint band in lane 17 could indicate that recombinant R7023 contains a small fraction of molecules containing *Bam*HI P

(I_s and I_{sl}) detected in viral DNA digests. Bands 1 and 2 are the novel bands generated in *Bam*HI digests of R7023. (D) Expanded maps of HSV-1(F) DNA in the I_s arrangement and of R7023 DNA in the I_s and I_{sl} arrangements at the junction between the L and S components and in the S component. The letters C, E, B, S, P, Y, Z, X, J, N, and P indicate the *Bam*HI fragments of HSV-1(F). The arrows indicate the location of *Sac*I cleavage sites. Bands 1 and 2 are the two novel *Sac*I fragments generated by the sequence arrangement in the two isomers of R7023 DNA.

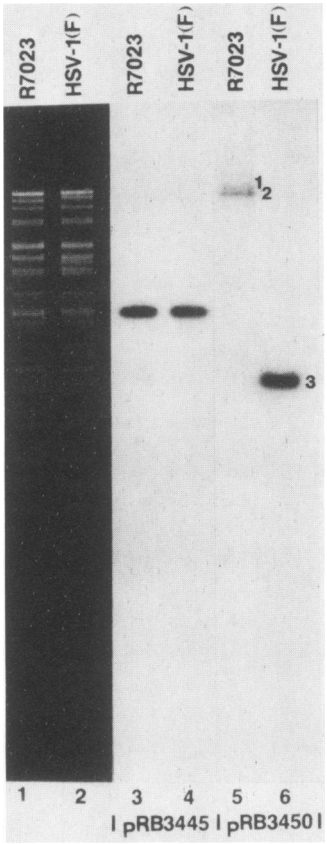

FIG. 3. Photographs and autoradiographs of electrophoretically separated *Sac*I restriction endonuclease digests of HSV-1(F) and of recombinant R7023 viral DNAs. DNA was transferred to nitrocellulose and hybridized with radioactive probes as described in Materials and Methods. Lanes 1 and 2 show ethidium bromide-stained gels. Lanes 3 through 6 show autoradiographic images of duplicates of the gels shown in lanes 1 and 2 hybridized to the following ^{32}P-labeled probes indicated at the bottom of each pair of lanes: pRB3445, *Sac*I fragment from *Bam*HI-J containing the coding region for gD; pRB3450, *Sac*I fragment from *Bam*HI-J containing the coding region for gE. Bands 1 and 2 refer to the novel bands illustrated in Fig. 1D.

fragments with two *a* sequences. This band is absent from HSV-1(F) digests and was not previously seen in recombinants lacking internal inverted repeats of *a* sequences, possibly because of unavailability of a specific *Bam*HI-P probe lacking the shared *a* sequence.

(iii) To map the deletion more precisely, electrophoretically separated *Sac*I digests were hybridized with the two *Sac*I fragments from *Bam*HI-J containing the bulk of the structural sequences of gD and gE, respectively (Fig. 1B). The *Sac*I digest showed two novel fragments, designated as bands 1 and 2 (Fig. 1D and 3), of approximately 9.3 and 9.0 kbp, respectively. The *Sac*I probe from pRB3445 containing the domain of the gD gene hybridized with an R7023 *Sac*I fragment which comigrated with the wild-type HSV-1(F) DNA (Fig. 3, lanes 3 and 4), whereas the *Sac*I probe from pRB3450 containing the domain of the gE gene hybridized to the novel *Sac*I bands 1 and 2 (Fig. 3, lanes 5 and 6), and not to the wild-type HSV-1(F) *Sac*I fragment (Fig. 3, lane 6, band 3), indicating that a portion of these sequences was deleted.

(iv) The sum of the *Bam*HI B (10.5 kbp) and J (6.3 kbp) fragments (16.8 kbp) is 1.2 kbp larger than the band 1 DNA fragment representing the fusion products of the two bands. The recombination site may be deduced from the available data on *Bam*HI-J sequences retained in R7023. Figure 1D shows the *Sac*I cleavage sites at the L-S junctions and S components of HSV-1(F) DNA in the I_s and I_{sl} isomers. At least seven *Sac*I restriction endonuclease cleavage sites had to be deleted from HSV-1(F) DNA to yield the *Sac*I bands 1 and 2 (Fig. 1D). Inasmuch as the DNA of R7023 contains the intact *Sac*I fragment encoding the domain of the gD gene and at least a portion of the 1.8-kbp *Sac*I fragment encoding the gE gene, it may be calculated from the sizes of the band 1 and 2 *Bam*HI fragments that the left terminus of the L component sequences fused to the S component sequences is in the *b* inverted repeat sequences located in *Bam*HI-B or *Bam*HI-E no more than 1.2 kbp from the termini of these fragments closest to the S component in I_s or I_{sl} arrangements of the viral DNAs.

R7023 I_s and I_{sl}: independent recombinants or products of isomerization? Analyses of several plaque-purified populations grown in 25-cm^2 flasks yielded DNA populations that in ethidium bromide-stained preparations contained a single novel junction band (band 1 or 2). However, the second novel junction band invariably appeared on virus passage. Notwithstanding numerous attempts, we failed to plaque purify a virus exhibiting a single novel junction band (data not shown). These results contrast sharply with the observation that identical procedures for plaque purification yielded viruses frozen in one arrangement of the DNA and carrying deletions starting in other domains of the *b* sequence of the *Bam*HI-B and extending into the S component yielded recombinants that appear to be frozen in one DNA arrangement (13, 27, 28). These studies do not lend credence to the hypothesis that failure to obtain pure populations of recombinants frozen in I_s and I_{sl} arrangements, respectively, reflects failures in plaque purification.

Absence of gE from lysates of R7023-infected cells. The analyses described above indicated that only a portion of the *Sac*I fragment encoding the gE gene was retained in the R7023 recombinant. Several experiments were done to determine whether R7023 specifies in the infected cells an intact or truncated form of the gE. First, R7023-infected Vero cells were tested with monoclonal antibodies to glycoproteins whose genes map in the S component of HSV-1 DNA by biotin-avidin-enhanced surface immunoassays as previously described (1, 16). The infected cells reacted with antibody to gG and gD but not with antibody to gE (Table 2). Furthermore, to verify the failure of anti gE antibody to react with R7023-infected cells, lysates of cells labeled with [^{35}S]methionine were reacted in immune precipitation tests with one monoclonal antibody to gD (H1380), with two independently derived monoclonal antibodies to gE (H600 and H1301), and with a polyclonal rabbit antibody to gE kindly provided by P. G. Spear. The monoclonal antibody to gD yielded a precipitate, but neither H600 nor the rabbit polyclonal antibody precipitated gE from lysates of R7023

TABLE 2. Reactivity of viruses with monoclonal antibodies

Monoclonal antibody	Specificity	Reactivity with:	
		HSV-1(F)	R7023
H600	gE-1	+	−
H1301	gE-1+2	+	−
H1379	gG-1	+	+
H1380	gD-1	+	+

infected cells (Fig. 4). Similar results were obtained with monoclonal antibody H1301 (data not shown).

DISCUSSION

The salient features of the HSV-1(F) recombinant R7023 described in this report are that it lacks one of the three known origins of viral DNA replication and several unique genes, that its L component inverts although the virus lacks an *a* sequence at the site of the inversion, and that its DNA consists of two populations in which I_s forms a majority and I_{sl} forms a minority. It is convenient to discuss each of these features separately.

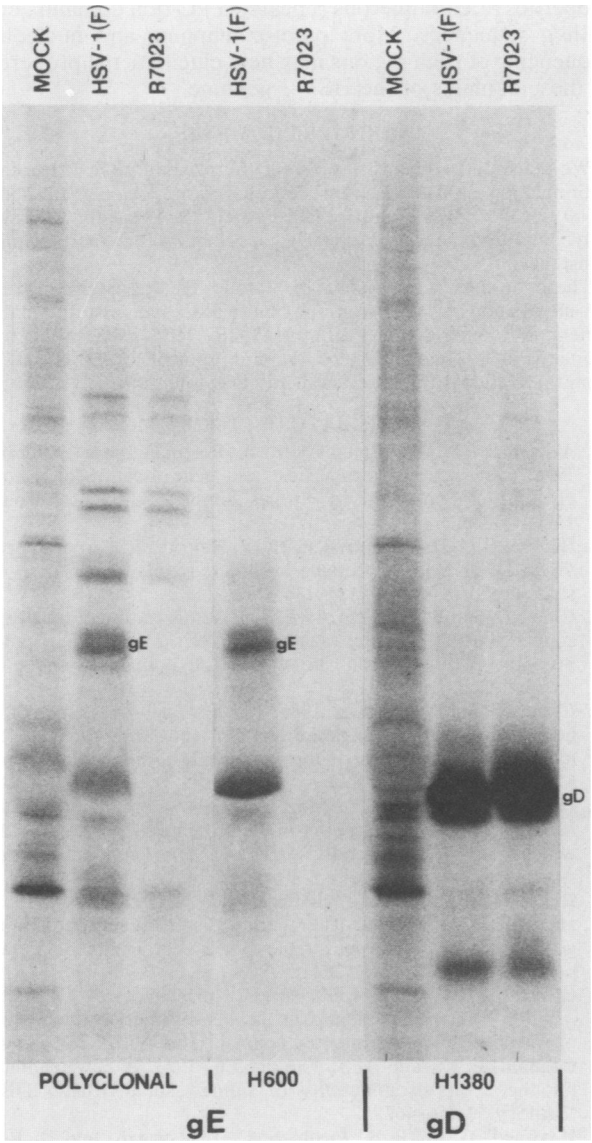

FIG. 4. Autoradiographic images of immunoprecipitations of lysates of Vero cells mock infected or infected with HSV-1(F) or R7023 obtained with monoclonal or polyclonal antibody. Vero cells were labeled 10 to 18 h postinfection with [^{35}S]methionine. The immune precipitation and processing of the precipitates were as described in Materials and Methods. The rabbit polyclonal antibody and monoclonal antibody H600 were made against gE, whereas monoclonal antibody H1380 reacted with gD.

Insertion of a third copy of the α4 gene resulted in extensive deletions and rearrangements at distant sites within the genome. We do not know what property of the inserted sequence caused the deletions and rearrangements, except that it is not the site of the insertion or the α4 gene promoter-regulatory domain or the nearby origin of DNA replication, since insertions of similar size (R3104 [12]) and much larger inserts (R3158 [12]; RBMu1 [13]) and insertion of the α4 promoter-regulatory domain and origin of DNA replication by themselves (e.g., recombinants R314 and R316 [31]) did not cause the arrangements seen in current studies. It is conceivable that a *cis*-acting site or a *trans*-acting gene product repeated in the insert was highly disadvantageous to the virus and that spontaneous mutants reflecting recombinational events across the L-S component junction had a selective advantage over the viral genomes carrying the insert. If this were the case, either the effect of the insertion would be lethal or the recombinational events across the L-S component junction would occur at relatively high frequencies. Since we have seen several instances of rearrangements and deletions at or across the L-S junction (13, 27, 28) caused by insertions at near or distal sites, it is conceivable that the inverted repeat *b'a'c'* sequences at the junction between the L and S components contain recombinational hot spots that destabilize that domain of the genome.

The domain of the genome deleted in R7023 contains an origin of DNA synthesis and several unique genes. The sequences deleted from R7023 include a portion of *Bam*HI-J and the contiguous fragments *Bam*HI X, Z, Y, P, and S for a total of 13 kbp (Fig. 1). These deleted DNA sequences encode several unique and diploid genes as well as an origin of DNA replication, specifically the following.

(i) The origin of DNA replication deleted from R7023 maps in the *c* sequence of the inverted repeat of the S component, within fragment *Bam*HI Z, and between the genes α4 and α47. As noted in the introduction, HSV DNA contains three known origins of DNA replication, one in the unique sequences of the L component and two in the *c* inverted repeat sequences of the S component. Previous studies have shown that the HSV-1 genome tolerates insertion of a third copy of the S component origin into the unique sequences of the L component (e.g., recombinants R314 and R316 [31], but this is the first instance of a virus carrying only two origins of DNA replication, and the observation that the virus replicates normally raises the question whether this origin and its mirror image in the second, inverted repeat copy of the *c* sequence play a role in viral replication.

(ii) R7023 lacks three known genes mapping entirely in inverted repeat sequences and therefore present in two copies per genome. These are α4 (located in the *c* inverted repeat sequences of the S component), the α0 gene, and a recently discovered $\gamma_1$34.5 gene mapping in the *b* inverted repeat sequences of the L component (3). Earlier studies have demonstrated that single copies of these genes suffice for the replication of HSV-1 (13, 27, 28).

(iii) The unique genes deleted from R7023 include the α47 gene, two open reading frames (no. 10 and 11 [20]) predicted by McGeoch et al. (20) to encode the 33,000-molecular-weight protein of Lee et al. (17) and the 21,000-molecular-weight protein of Dalziel and Marsden (4), respectively, and at least a portion of the gene specifying gE. The absence of the gene specifying the 21,000-molecular-weight protein is of interest because of the suggestion by Dalziel and Marsden (1a, 4) that this protein specifically binds to *a* sequences. We have verified the absence of an intact or truncated gE protein

capable of reacting with rabbit polyclonal antibody and several mouse monoclonal antibodies.

Inasmuch as the recombinant R7023 grows in Vero, 143TK$^-$, and rabbit skin cell lines, these results indicate that the deleted unique genes are not essential for growth in these cell lines. This observation should not be construed to indicate that the genes are not essential for virus growth in its natural host, in experimental animals, or in other cell lines or strains. Previous studies have revealed numerous examples of viral genes that appear to duplicate functions expressed by host cells. The α22 is an example of a gene whose function has not yet been identified, but which has a host counterpart in some cells and not in others. Specifically R325, a recombinant carrying a deletion in the α22 gene (32), grows well in Vero and HEp-2 cell lines but not in mice, rodent cell lines, and human embryonic cell strains (37). Studies now in progress may determine whether this is also the case for the unique genes deleted in R7023.

R7023 L component inverts in the absence of *a* sequences at the novel junction between the L and S components. Two observations with regard to the structure of R7023 should be stressed. First, the R7023 genome consists of two isomers, I_s and I_{sl}, instead of the usual four isomeric arrangements present in wild-type HSV-1 DNA. Previous studies yielded only viruses arrested in the P arrangement (13, 27, 28), and these and other observations (19, 24) raised questions of whether I_s, I_l, and I_{sl} isomers were capable of independent replication and were not merely nonviable rearrangements of the P isomer. This report does not resolve the problem in its entirety, since it could be argued that I_s is the predominant isomer and that the I_{sl} isomer could be a product of inversion incapable of independent multiplication. However, since R7023 was isolated, several other recombinants obtained by other procedures also were found to be arrested in I_s and I_{sl} arrangements (F. Jenkins and B. Roizman, submitted for publication). The significant conclusion to be derived from these observations is that all isomeric forms of HSV-1 DNA are capable of initiating viral replication and yielding infectious progeny.

The second observation regarding the structure of R7023 concerns the presence of two populations representing the I_s and I_{sl} isomers. As noted in Results, we failed to separate the two isomeric forms and believe that they are the products of isomerization rather than independently derived recombinants. In this instance, the rate of isomerization is slower than that of wild-type virus because several passages of plaque-purified stocks were required to produce detectable, but not equimolar, amounts of both isomers.

Previous studies have shown that the *a* sequence contains *cis*-acting and recombination sites for the inversion of both S and L components of HSV-1 DNA. Because *a* sequences are normally present at both termini of the viral genome, it is difficult to imagine a mechanism of inversion of a single component through the inverted *a* sequence of the L-S component junction unless the genomic terminus of the frozen component no longer contains an intact *a* sequence, an unlikely event. The absence of *a* sequences from the novel junction suggests a different *cis*-acting recombination site for inversion. Comparisons of the DNA sequence of R7023 with that of the other frozen recombinants from which sequences near the L-S junction had been deleted suggest that the *cis*-acting site for inversion of the L component in R7023 is within a 5-kbp region near the middle of the *b* sequence located at the right and left termini of the *Bam*HI B and E fragments, respectively, in the P arrangement.

We should note that duplication of HSV-1 sequences in inverted orientation relative to each other led to inversions in one instance (29) but not in others (21, 32). One hypothesis that could explain such observations is that inversions that cause inactivation of essential genes would be nonviable and hence would not accumulate in sufficient amounts to be detected. This explanation may be an oversimplification. For example, one of the fragments which failed to induce additional inversions when duplicated in the viral genome of recombinant R308 (21) was the *Sac*I fragment, which encodes the domain of the gE gene shown here not to be essential for growth in cell culture. Another illustration of a nonessential gene which when duplicated (e.g., recombinant R321 [32]) failed to induce inversions is the TK gene. A more likely explanation is that inverted repeat sequences that cause inversions in the HSV-1 genome share characteristic properties (e.g., numerous repeats, G+C-rich domains, etc.) with *a* sequences. More precise mapping and nucleotide sequencing of such regions may help elucidate the properties of these domains of the HSV-1 genome.

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