

Herpes Simplex Virus 1 Recombinants with Noninverting Genomes Frozen in Different Isomeric Arrangements Are Capable of Independent Replication

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Received 18 February 1986/Accepted 24 April 1986

Herpes simplex virus 1 genome consists of two covalently linked components, L and S, that invert relative to each other to yield four equimolar isomeric populations designated P (prototype), I_s (inversion of S component), I_L (inversion of L component), and I_{LS} (inversion of L and S components) differing in the orientation of the two components. Previous studies have yielded recombinant genomes frozen in the P isomeric arrangement, reinforcing suggestions that the four isomers may not be functionally equivalent. We report the isolation of recombinants produced by insertional mutagenesis with α TK mini-Mu that are frozen in I_s and I_{LS} arrangements. Thus, all isomeric forms of herpes simplex virus DNA appear to be capable of independent replication and must be considered as functionally equivalent.

The herpes simplex virus 1 (HSV-1) DNA genome is approximately 144 kilobase pairs (kbp) in length (6) and consists of two covalently linked components designated L and S. Each component consists of largely unique sequences (U_L and U_S) flanked by inverted repeats (18, 19). The inverted repeat sequences of the L component have been designated as *ab* and *b'a'*, whereas those of the S component have been designated as *c'a'* and *ca* (Fig. 1A) (19). A remarkable property of the HSV-1 genome is the ability of the L and S components to invert relative to each other. As a consequence, viral DNA extracted from wild-type virions or infected cells consists of four equimolar populations differing in the relative orientation of the two components (3). Early studies in this (9) and another (16) laboratory, which were reviewed by Roizman (17), on the structure of HSV-1 \times HSV-2 recombinants suggested that most if not all recombinants with an odd number of crossovers could be shown in a single isomeric arrangement which minimized the number of crossover events. This arrangement was given the designation prototype (P). The other three arrangements were then designated as I_s (inversion of S component), I_L (inversion of L component) and I_{LS} (inversion of both components) (3, 10).

The observation that HSV-1 \times HSV-2 recombinants could be represented in a single arrangement which minimized the number of recombinational events suggested the possibility that only the P arrangement of HSV DNA is actually capable of initiating replication and that other arrangements were by-products of viral replication. This interpretation of the results was reinforced by the observation that two series of independently derived noninverting HSV-1 recombinants from which the internal inverted repeats had been deleted were all arrested in the P arrangement (5, 11, 12). In this study we report the isolation and characterization of two viable HSV-1 viral recombinant viruses frozen in I_s and I_{LS} arrangements, respectively. The two recombinants were generated by the application of a rapid procedure for target-specific insertional mutagenesis of the HSV genome utilizing the α TK mini-Mu derivative of the transducing bacteriophage Mu (5).

As described elsewhere in detail (5), the procedure consists of three steps. In the first step, an *Escherichia coli* strain carrying both a helper Mu prophage and a 9.6-kbp mini-Mu prophage that has a functional thymidine kinase (TK) gene under the control of the HSV α 4 gene promoter and regulatory region (designated as α TK mini-Mu or α TK-mM) is transfected with plasmid DNA containing the target HSV DNA sequences. Induction by growth of the bacterial cells at an elevated temperature results in replication, transposition, and packaging of both the helper Mu and α TK-mM. As a result of transposition, the α TK-mM integrates into many different sites within the plasmid DNA, producing cointegrate plasmid-*E. coli* chromosome structures. In the cointegrate structure the plasmid DNA is flanked by single copies of the α TK-mM. Packaging of these structures begins at the leftmost copy of the α TK-mM and proceeds toward the second copy of the α TK-mM until a headful of DNA (approximately 38 kbp) has been packaged. The α TK-mM prophage is 9.6 kbp in size. Recombinant plasmids up to 18 kbp in size yield phage DNA consisting of two copies of the α TK-mM flanking the plasmid DNA, whereas plasmids greater than 18 kbp in size yield phages whose DNA lack portions or all of the second α TK-mM copy. Because at least part of the second α TK-mM is required for the second step of this procedure, plasmids greater than 28 kbp cannot be utilized.

In the second step, the lysates produced by induction and consisting of packaged cointegrate structures and helper Mu genomes, are used to infect an *E. coli* RecA⁺ strain lysogenized by a helper Mu prophage. The presence of the helper Mu prophage prevents the infecting cointegrate structure from transposing into the *E. coli* DNA. Homologous recombination between the flanking copies of the α TK-mM results in the formation of plasmid molecules containing single copies of the α TK-mM inserted at different sites. *E. coli* carrying these plasmids can be selected by growth in kanamycin, since the α TK-mM contains a kanamycin resistance gene (Fig. 1D). Isolated plasmid DNA consists of a pool of plasmid molecules each containing a single α TK-mM inserted at different sites within the HSV DNA sequences and nonessential vector sequences.

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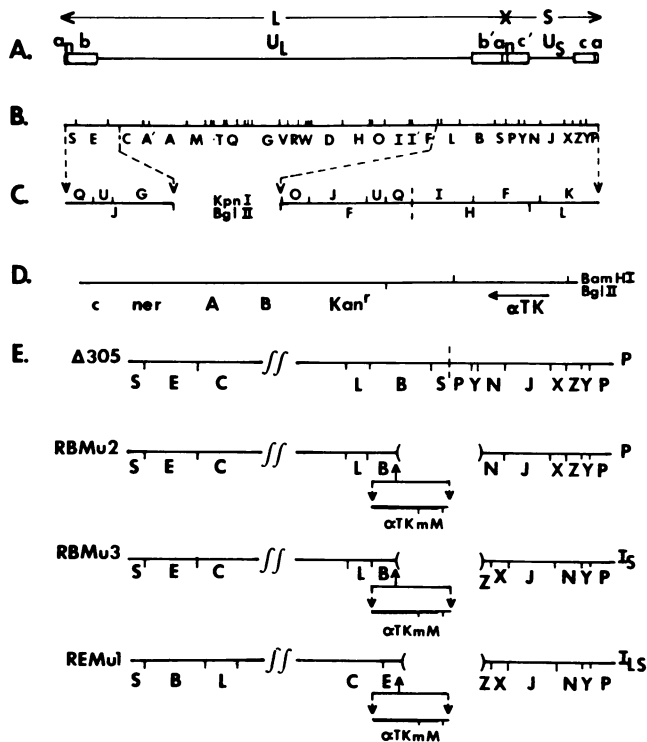


FIG. 1. Restriction endonuclease maps and sequence arrangement of α TK-mM and of the wild-type and recombinant HSV-1 virus genomes. (A) Sequence arrangement of HSV-1 DNA. The open boxes represent terminal sequences (*ab* and *ca*) of the L and S components, respectively, that are reiterated internally in an inverted orientation (*b'a'c'*). (B) *Bam*HI restriction endonuclease map of wild-type HSV-1 DNA. The designations of the small fragments are not shown. (C) Expanded portion of the termini of the L and S components of the HSV-1 DNA genome in prototype (P) arrangement showing the *Kpn*I and *Bgl*II cleavage sites. The dashed vertical line represents the junction of the L and S components. (D) Sequence arrangement of the α TK-mM genome showing *Bam*HI and *Bgl*II cleavage sites. A, B, *ner*, and *c* are mini-Mu genes; *Kan*^r refers to the gene for kanamycin resistance, and α TK refers to the TK structural gene fused to the regulatory and promoter domains of the α 4 gene. (E) Sequence arrangement of the termini of the L and S components of HSV-1 Δ 305 DNA and of three recombinant HSV-1 virus genomes showing the *Bam*HI cleavage sites and the site of insertion of the α TK-mM. HSV-1 Δ 305 is shown in the P arrangement. The dashed vertical line represents the junction of the L and S components. The parentheses identify the domains of the internal inverted repeat sequences deleted in the recombinant viruses. The thin line of the α TK-mM represents the α 4 promoter and regulatory domains and the right terminus of the mini-Mu sequences that have been deleted as a result of homologous recombination as described in the text.

In the third step, the α TK-mM plasmids are cotransfected into rabbit skin cells with intact DNA of a virus, HSV-1 Δ 305 from which 700 bp of the TK gene had been deleted (14). The progeny of the transfection are plated on TK⁻ cells under HAT (hypoxanthine, aminopterin, thymidine) medium for isolation of TK⁺ viral progeny carrying the α TK-mM insert. The procedures for induction, infection, and isolation of recombinant plasmids using the mini-Mu system have been described elsewhere (5).

One novel HSV-1 recombinant described in this report (RBMu3) was produced by insertional mutagenesis of the *Bam*HI B fragment with α TK-mM, followed by recombina-

tion of the resulting fragments into the HSV-1 genome. The 10.4-kbp *Bam*HI B DNA fragment of HSV-1(F) was cloned as pRB112 in pBR322 (13). This fragment spans the junction between the unique and the inverted repeat sequences of the L component near the junction between the L and S components in the P arrangement (Fig. 1B). Progeny virus from the rabbit skin cell transfections were passaged four times in human 143 TK⁻ cells (1) under HAT medium. RBMu3 was picked as a single plaque, passaged two times under HAT medium, plaque purified a second time, and passaged two additional times in HAT medium. The virus stock from the last HAT selection was used to infect a confluent roller bottle of Vero cells at a multiplicity of infection of 0.01. The titer obtained from this infection was 10⁹ PFU/ml, which is comparable to yields obtained with HSV-1(F) and HSV-1 Δ 305. Viral DNA used in restriction endonuclease and DNA blot hybridization analyses was isolated from infected Vero roller bottle cultures as previously described (4, 12, 15). RBMu3 DNA contains the α TK-mM inserted 5.2 kbp from the left end of the *Bam*HI B fragment within the internal *b* repeat sequences. In addition, all of the sequences located to the right of the site of the α TK-mM insertion up to and including portions of the *Bam*HI Z fragment were spontaneously deleted. The viral DNA is frozen in the I₁ arrangement. These conclusions are based on the following considerations.

(i) Electrophoretically separated digests of RBMu3 DNA stained with ethidium bromide lacked the DNA fragments *Bam*HI B and SP, *Kpn*I J and K, and *Bgl*II F and L (Fig. 2A). The absence of *Bam*HI B, *Kpn*I J, and *Bgl*II F DNA fragments at their normal position would be expected if they contain the α TK-mM. The absence of *Bam*HI SP, *Kpn*I K, and *Bgl*II L fragments suggests that the internal inverted repeats were deleted. Such a deletion could have occurred if the promoter regulatory region of the α 4 gene contained in the α TK-mM were to recombine with the homologous sequences in their natural location in the *Bam*HI Z fragment such that all of the intervening sequences were deleted.

(ii) Hybridization of electrophoretically separated digests of RBMu3 DNA with ³²P-labeled DNA of a plasmid containing a single *a* sequence confirmed the absence of the *Bam*HI SP fragment spanning the junction between the L and S components and of the terminal *Kpn*I K and *Bgl*II L fragments; the terminal *Kpn*I I and *Bgl*II H fragments were present (Fig. 3B). This observation is consistent with the conclusion that the recombinant lacked at least a portion of the internal inverted repeats and that the S component was frozen in the inverted orientation. The absence of the terminal *Bgl*II F fragment and the presence of the terminal *Bgl*II J fragment is consistent with the conclusion that the L component was frozen in the prototype orientation.

(iii) Digestion of α TK-mM DNA with *Bam*HI produced the 2.1-kbp α TK insert plus two mini-Mu DNA fragments (7.4 and 0.1 kbp) which flank the TK insert (Fig. 1D). Figure 1E shows the predicted orientation of α TK-mM, the novel L-S junction, and termini of RBMu3 DNA. The diagram predicts that the deletion of the junction sequences resulted from recombination of α 4 gene promoter regulatory domains contained in the *Bam*HI Z fragment with the directly repeated corresponding sequences in the α TK-mM inserted in the *Bam*HI B fragment. Insertion of the α TK-mM in the orientation shown at 5.2 kbp from the left end of the *Bam*HI B fragment was deduced by the presence in *Bam*HI digests of a novel 12.6-kbp fragment, designated band 1 in Fig. 2, which hybridized with ³²P-labeled mini-Mu DNA probe and

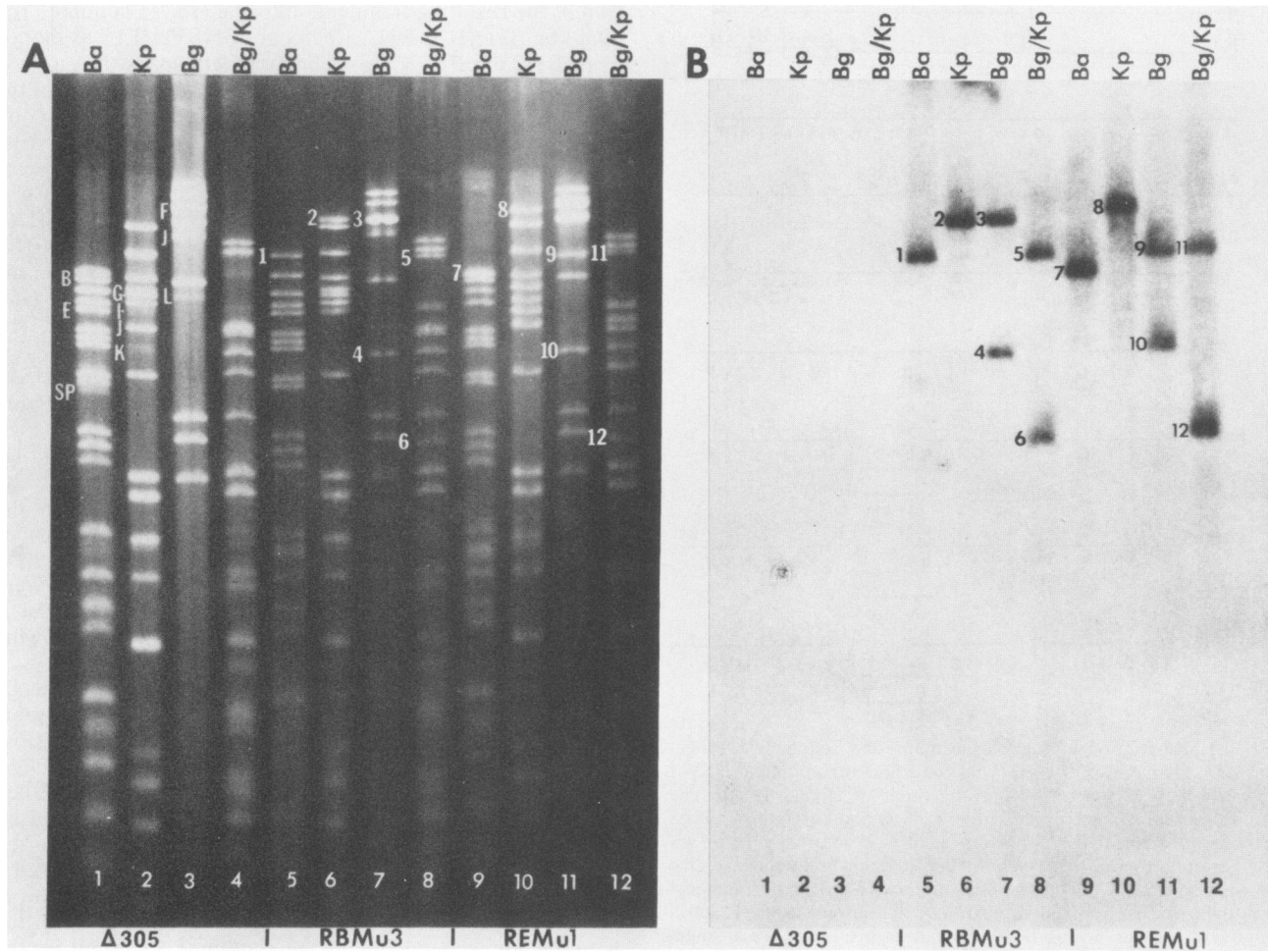


FIG. 2. (A) Photograph of ethidium bromide-stained gel and (B) autoradiogram of electrophoretically separated digests of HSV-1 Δ 305 and recombinant HSV-1 virus DNAs hybridized with 32 P-labeled mini-Mu DNA probe. Isolation, purification, and restriction endonuclease analysis of recombinant virus DNAs were as described (4, 12, 15). DNA probes were labeled with 32 P by a nick-translation kit obtained from New England Nuclear Corp. (Boston, Mass.). Gel electrophoresis, transfer of DNA to nitrocellulose, and hybridization of bound DNA to 32 P-labeled DNA probes were as described (7). The numbers to the left identify novel bands containing α TK-mM sequences. The letters to the left identify the restriction endonuclease fragments *Bam*HI B, E, and SP; *Kpn*I G, J, and K; and *Bgl*III F and L. Ba, *Bam*HI; Kp, *Kpn*I; Bg, *Bgl*III; Bg/Kp, both *Bgl*III and *Kpn*I.

must contain 7.4 kbp of mini-Mu DNA and 5.2 kbp of *Bam*HI-B DNA.

(iv) The *Bam*HI N and Z fragments share homologous sequences contained within the HSV-1 *c* repeat sequences. These contain the α 4 gene promoter and regulatory domains and an origin of DNA replication. Hybridization of electrophoretically separated digests of RBMu3 DNA with 32 P-labeled *Bam*HI-N DNA probe revealed the absence of the *Bam*HI Z fragment and the presence of three novel bands (designated 13, 14, and 15 [Fig. 4B]), which is consistent with the hypothesis that the α 4 promoter and regulatory sequences contained in the α TK-mM recombined with the homologous sequences in *Bam*HI-Z. Digestion of RBMu3 DNA with *Bam*HI yielded novel band 13 (Fig. 4), whose size is 3.6 kbp and which contains the 2.1-kbp α TK insert of α TK-mM and 1.5 kbp of *Bam*HI-Z DNA sequences. Since the *Bam*HI Z fragment is 1.85 kbp long, the deleted internal inverted repeat sequences must contain approximately 250 to 350 bp of *Bam*HI-Z DNA depending on whether or not the terminal 100 bp of α TK-mM DNA was deleted as well. *Kpn*I digestion of RBMu3 DNA yielded novel 18.2-kbp band 14

(Fig. 4), which contains a small portion of *Bam*HI-Z as discussed below. *Bgl*III digestion of RBMu3 DNA yielded novel band 15 (Fig. 4), 7.3 kbp long, containing 3.7 kbp of α TK-mM and 3.6 kbp of *Bgl*III-L DNA. Deletion of the internal *Bam*HI fragments P and Y plus approximately 350 bp of *Bam*HI-Z would represent a 5.7-kbp deletion in the 9.3-kbp *Bgl*III-L fragment, leaving the 3.6 kbp contained in band 15.

(v) Hybridization of the 32 P-labeled mini-Mu DNA probe to the novel bands designated 2, 3, 4, 5, and 6 in Fig. 2 whose sizes are approximately 18.2, 18.2, 7.3, 12.6, and 5.2 kbp, respectively, yielded results indicating that the orientation of the inserted α TK-mM was as shown diagrammatically in Fig. 1E. *Kpn*I does not cleave the α TK-mM genome. Digestion of RBMu3 DNA with *Kpn*I would produce a novel band (designated 2 in Fig. 2) containing the α TK-mM flanked by *Kpn*I J and K DNA fragment sequences. The right border of α TK-mM must be 6.7 kbp from the *Kpn*I-J/U cleavage site inasmuch as it became inserted 5.2 kbp from the left end of *Bam*HI-B. Deletion of 5.7 kbp from the S component, as described above, would leave 2 kbp of the 7.7-kbp *Kpn*I K

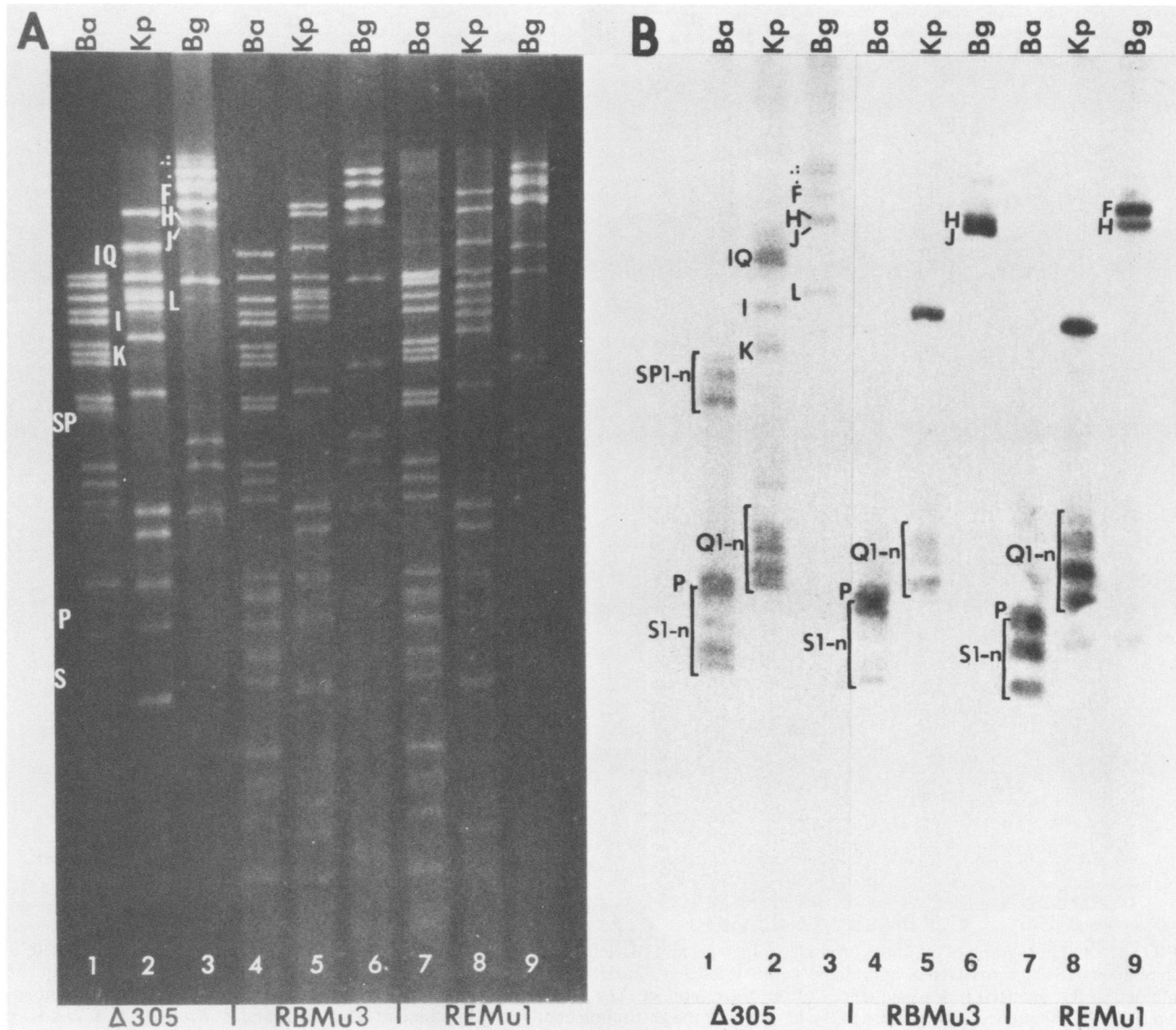


FIG. 3. (A) Photograph of ethidium bromide-stained gel and (B) autoradiogram of electrophoretically separated digests of HSV-1 Δ 305 and recombinant HSV-1 virus DNAs hybridized with 32 P-labeled DNA probe containing a single *a* sequence. The letters to the left identify the restriction endonuclease fragments *Bam*HI SP, P, and S; *Kpn*I IQ, I, K, and Q; and *Bgl*III F, H, J, and L. The terminal *Bam*HI S and *Kpn*I Q and the L-S component junction *Bam*HI SP fragments form ladders of bands differing with respect to the number of *a* sequences (1 to *n*) present (2, 8). The *Bgl*III L-S component junction fragments of Δ 305 (FH, FL, JH, and JL) are indicated by "· · ·". Ba, *Bam*HI; Kp, *Kpn*I; Bg, *Bgl*III.

fragment. Band 2 (Fig. 2) must therefore contain 6.7 kbp from *Kpn*I-J, 2 kbp from *Kpn*I-K, and 9.5 kbp from α TK-mM. *Bgl*III cleaved the α TK-mM once, producing two fragments 5.9 and 3.7 kbp in size (Fig. 1D). Digestion of RBMu3 DNA with *Bgl*III yielded novel bands 3 and 4 (Fig. 2), which contain approximately 11.8 kbp of *Bgl*III F and 5.9 kbp of α TK-mM in band 3 and 3.7 kbp each of *Bgl*III-L and α TK-mM in band 4. Digestion of RBMu3 DNA with both *Bgl*III and *Kpn*I resulted in cleavage of band 2, producing novel bands 5 and 6. The size of the deleted internal inverted repeat sequences is approximately 13.9 kbp.

The second novel HSV-1 recombinant (REMu1) was produced by mutagenesis of the 20-kbp *Hind*III HM fragment containing the L-S junction of the I₁ isomer cloned as pRB201 into pBR322 (4). This fragment contains the entire internal inverted repeat sequences along with some unique

sequences from both the L and S components. With the exception of the unique sequences from the right end of the L component in the P arrangement, all of the *Bam*HI-B HSV DNA sequences contained in pRB112 are also contained in pRB201. However, pRB201 contains unique sequences located at the left end of the L component, including those contained in *Bam*HI E fragment (Fig. 1B). Progeny virus from rabbit skin cell transfections were passaged three times on 143 TK⁻ cells under HAT medium. REMu1 was picked as a single plaque, passaged once under HAT medium, plaque purified twice more, and then passaged an additional three times under HAT medium. The virus stock from the last HAT selection was used to infect a confluent roller bottle of Vero cells at a multiplicity of infection of 0.01. The titer obtained from this infection was 10⁸ PFU/ml, which is comparable to titers obtained with HSV-1(F) and HSV-1

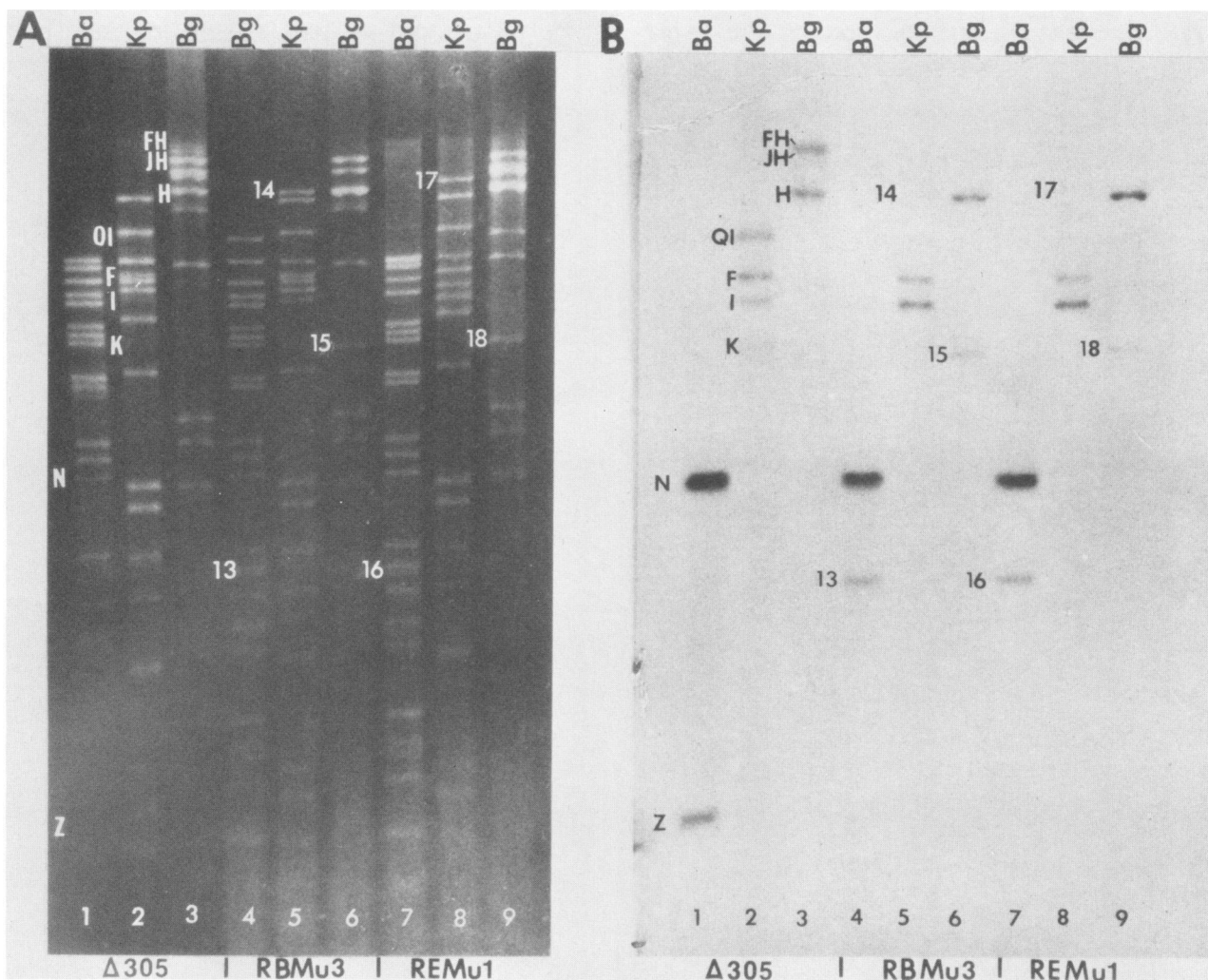


FIG. 4. (A) Photograph of ethidium bromide-stained gel and (B) autoradiogram of electrophoretically separated digests of HSV-1 Δ 305 and recombinant HSV-1 virus DNAs hybridized with 32 P-labeled *Bam*HI-N DNA probe. This fragment and *Bam*HI-Z share DNA sequences contained in the inverted repeat sequences of the S component. The numbers to the left of specific bands refer to novel bands containing *Bam*HI-Z DNA sequences. The letters to the left identify the restriction endonuclease fragments *Bam*HI N and Z; *Kpn*I QI, F, I, and K; and *Bgl*II FH, JH, and H. Ba, *Bam*HI; Kp, *Kpn*I; Bg, *Bgl*II.

Δ 305. Purified virus DNA used in restriction endonuclease and DNA blot hybridization analyses was isolated from infected roller bottles of Vero cells as previously described (4, 12, 15). In recombinant REMu1 DNA the α TK-mM inserted 3.3 kbp from the right terminus of the *Bam*HI E fragment in the P arrangement. The site of insertion is nearly identical to that of RBMu3 within the *b* repeat sequences shared by both *Bam*HI B and E fragments, and the REMu1 genome is frozen in the I_s arrangement. The sequences within *Bam*HI-E from the site of insertion of the α TK-mM up to the α 4 promoter and regulatory sequences contained in *Bam*HI-Z were spontaneously deleted. This conclusion is based on the following.

(i) Electrophoretically separated digests of REMu1 DNA stained with ethidium bromide, lacked the fragments *Bam*HI E and SP, *Kpn*I G and K, and *Bgl*II J and L (Fig. 2A). Insertion of the α TK-mM within the *b* repeat sequences of *Bam*HI-E would result in loss of the *Bam*HI E, *Kpn*I K, and *Bgl*II J fragments. The loss of *Bam*HI SP, *Kpn*I K, and *Bgl*II L suggested that the internal inverted repeat sequences had been deleted, as was the case with RBMu3.

(ii) Hybridization of electrophoretically separated digests of REMu1 DNA with a 32 P-labeled *a* sequence probe confirmed the absence of the *Bam*HI SP fragment spanning the L-S junction and of the terminal *Kpn*I K and *Bgl*II L fragments; the *Kpn*I I and *Bgl*II H terminal fragments were present (Fig. 3B), indicating that the S component was frozen in an inverted orientation. The absence of the terminal *Bgl*II J fragment and the presence of the terminal *Bgl*II F fragment indicated that the L component was also frozen in an inverted orientation.

(iii) Hybridization of *Bam*HI digests of REMu1 DNA with 32 P-labeled mini-Mu DNA probe illuminated a 10.7-kbp *Bam*HI fragment designated 7 in Fig. 2, placing the insertion of the α TK-mM 3.3 kbp from the right end of the *Bam*HI E fragment in the P arrangement.

(iv) Hybridization of electrophoretically separated digests of REMu1 DNA with a 32 P-labeled *Bam*HI N DNA probe (Fig. 4) showed the absence of the *Bam*HI Z fragment and the presence of novel bands designated 16, 17, and 18, which is consistent with the hypothesis that the deletion occurred by recombination between the α 4 promoter and regulatory

domains in the α TK-mM and in *Bam*HI-Z. The deleted internal inverted repeat sequences in REMu1 could be expected to be nearly identical to those described for RBMu3 since bands 16 and 18 are the same size as bands 13 and 15 produced by digestion of RBMu3 DNA.

(v) Hybridization of the mini-Mu DNA probe to the novel bands designated 8, 9, 10, 11, and 12 in Fig. 2, whose sizes are 19, 12.6, 7.4, 12.6, and 5.2 kbp, respectively, supports the conclusion that the site of insertion of the α TK-mM is as shown diagrammatically in Fig. 1E. Novel band 8 (Fig. 3) would contain 7.6 kbp of *Kpn*I-G, 9.5 kbp of α TK-mM, and 2 kbp of *Kpn*I-K DNA. Novel band 9 would contain 6.4 kbp of *Bgl*II-J and 5.9 kbp of α TK-mM, whereas band 10 must contain 3.7 kbp of both *Bgl*II-L and α TK-mM DNA. Digestion of REMu1 DNA with both *Bgl*II and *Kpn*I would cleave band 8, producing bands 11 and 12.

Relevant to the conclusions reported in this paper are the following. The observation that HSV-1 \times HSV-2 recombinants could be represented by a single arrangement which minimized the number of recombinational events suggested that only recombinants derived by recombination between P or at most I_s arrangements were amplified. The suggestion carries the caveat that undetected recombinational events occurring near the termini of the inverted repeat sequences (*ab* and *ca*) and which could not be detected would refute the conclusion (9). The results presented in the present study suggest that this was obviously the case. Previously this laboratory reported the isolation of recombinants frozen in the P arrangement (5, 11, 12). The structure of one recombinant designated RBMu2 and obtained by α TK-mM insertional mutagenesis of *Bam*HI-B (5) is illustrated in Fig. 1E. In the present study we report the isolation of recombinants frozen in I_s and I_{is} arrangements. Inasmuch as the recombinants are viable and capable of independent replication, the isomers are functionally equivalent.

We cannot account for the succession of isolations of recombinants frozen in the P arrangement made before the isolation of the recombinants reported here. It is noteworthy, however, that all of the frozen recombinants obtained to date represent viral genomes from which the internal inverted repeat sequences were spontaneously deleted. The propensity of the internal inverted repeat sequences to delete are in retrospect not surprising, given the current evidence that the deleted sequences for the most part contain genes that are duplicated at the termini. Given the facility with which such sequences are deleted in cell culture, their retention in wild-type virus suggests that the inverted repeat sequences do play a role in their natural human hosts that is not essential in the cell culture systems in which they have been isolated.

These studies were aided by Public Health Service grants CA-08494 and CA-19264 from the National Cancer Institute and grant MV-2U from the American Cancer Society. F.J.J. is a Public Health Service Postdoctoral Trainee (PHS-5-T32-CA-09241).

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