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Genetics

Characterization of a viable, noninverting herpes simplex virus 1 genome derived by insertion and deletion of sequences at the junction of components L and S

(chimeric genes/recombination/*a* sequences/genetic engineering)

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ABSTRACT **Earlier studies have shown that the DNA of herpes simplex virus 1 consists of two covalently linked components, L and S, each flanked by inverted repeats. The two components can invert, and viral DNA extracted from infected cells or virions consists of equimolar concentrations of four populations differing solely in the orientation of L and S components relative to each other. This paper describes a recombinant virus (I358) generated by an insertion of a chimeric thymidine kinase gene within the reiterated sequences of the S component and deletions that eliminated most of the internal inverted repeats at the junction between the L and S components. A characteristic of I358 is that the L and S components are frozen in one (prototype) orientation. Inversion of L and S components is therefore not required for the replication of viral DNA.**

The DNA of human herpes virus 1 (herpes simplex virus 1, HSV-1) consists of two covalently linked components, L and S, making up 82% and 18% of total DNA, respectively (1, 2). Each component consists of unique sequences bracketed by inverted repeats (1). The inverted repeats of the L component, designated as *ab* and *b'a'*, each contain 6% of viral DNA, whereas the inverted repeats of the S component, *a'c'* and *ca*, each make up 4.3% of total DNA (2). Whereas the terminal of the S component usually contains one, the junction between the L and S components and the L terminus of the DNA may contain several *a* sequences (3, 4). An intriguing property of HSV DNA is that the L and S components can invert relative to each other. Thus, DNA extracted from virions or from cells infected with wild-type virus consists of four equimolar populations differing in the orientation of the L and S components relative to each other (5, 6). These four populations have been designated as P (prototype), I_L (inversion of L component), I_S (inversion of S component), and I_{LS} (inversion of both L and S components) (5). The prototype was selected on the basis of experiments suggesting that only recombinants derived from crosses between DNAs in the prototype arrangement were amplified (7). Subsequent studies have shown that inversions in HSV-1 DNA are the consequence of site specific recombinations through the *a* sequences (8, 9) mediated by *trans*-acting viral gene products (10).

The physiologic function of the inversions is not known (11, 12). If, in fact, there is a physiologic requirement for the inversions, it is satisfied in other ways in many herpesvirus DNAs inasmuch as of the five known sequence arrangements for herpesvirus DNAs (13), only two—i.e., group D, containing pseudorabies (14) and equine herpesvirus 1 (15), and group E, containing HSV-1, HSV-2, bovine mammillitis virus (16), and human cytomegalovirus (17–19)—exhibit inversions of sequences flanked by inverted repeats.

In this paper, we report on the properties of a viable recombinant virus designated as [HSV-1F(I358)] from which most of the internal inverted repeats were deleted and which no longer inverts.

MATERIALS AND METHODS

Viruses. HSV-1 strain F [HSV-1(F)] and the thymidine kinase-negative (TK^-) virus HSV-1(F)Δ305 carrying a 700-base-pair (bp) deletion in the *TK* gene were described elsewhere (10, 20, 21).

Plasmids. The cloned HSV-1(F) *Bam*HI DNA fragments were described elsewhere (22). pRB602 carries the *Hae* II fragment spanning the L–S junction and containing two *a* sequences and portions of adjacent *b* and *c* sequences (9, 10, 23). pRB408 carries the *Bgl* II/*Sac* I fragment from the HSV-1 *TK* gene (10, 11); this sequence was deleted from the Δ305 virus genome. pRB316 carries the *Bgl* II/*Bam*HI fragment of *Bam*HI fragment Q fused to *Bam*HI fragment N in the orientation that juxtaposes the promoter–regulator region of *α 4* gene to the *TK* gene (20). To construct the *Hin*dIII fragment O–*TK* chimeric fragment (pRB358), the *Pvu* II fragment of *α–TK* from pRB316 was blunt end ligated with the *Hin*dIII O fragment contained in pRB209 that had been cleaved with *Bgl* II and digested with phage T4 DNA polymerase (24). The orientation of the O–*TK* chimera in pRB358 was determined from restriction enzyme mapping (data not shown). The *Eco*RI clone (pRB9002) of the junction-spanning region of I358 was cloned as described (22, 25), except that *Eco*RI-digested I358 DNA was electrophoretically separated on an agarose gel and the desired fragment was purified from a gel before ligating with *Eco*RI-digested pACYC184 (26). The recombinant plasmids were selected by colony hybridization with a ^{32}P-labeled *Bam*HI Q fragment containing the *TK* gene.

Construction and Analysis of the Recombinant Virus. pRB358 DNA was cotransfected with Δ305 viral DNA as described (21). TK^+ viruses were selected by plating the progeny of transfected rabbit skin cells on human strain 143 (TK^-) cells under hypoxanthine/aminopterin/thymidine (HAT) medium (20). The virus was plaque purified in HAT medium three times then transferred to Vero (African green monkey kidney) cells and plaque purified twice more.

To determine whether I358 was free of Δ305 parent virus that could act as a helper, the stock was plated in the presence of thymidine arabinonucleoside (100 μg/ml of medium) (8). The fraction of the virus stock resistant to thymidine arabinonucleo-

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Abbreviations: HSV, herpes simplex virus; TK, thymidine kinase; bp, base pair(s).

side, and therefore TK$^-$, was less than 0.001%.

Hybridization of labeled probes to DNA fragments immobilized on nitrocellulose was done as described (27). The DNA probes were labeled either by the forward exchange reaction with [γ-^{32}P]ATP and phage T4 polynucleotide kinase (28) or by nick-translation (29) with [α-^{32}P]dCTP (New England Nuclear).

RESULTS

Construction of the I358 Recombinant Virus. The initial objective of these studies was to inactivate and then delete putative genes spanning the *Bgl* II cleavage site of the *Hin*dIII DNA fragment O of HSV-1 (Fig. 1*A*). The protocol we chose was similar to that used to inactivate and then delete the α gene 22 (21). Specifically, the HSV-1 TK gene was inserted into the *Bgl* II site of *Hin*dIII O fragment (Fig. 1*B*) and the chimeric fragment was then recombined into HSV-1(F)Δ305 (Fig. 1*C*). The experimental design takes advantage of the observation that TK$^+$ viruses are readily selected in TK$^-$ cells in hypoxanthine/aminopterin/thymidine medium, whereas TK$^-$ viruses can be selected by virtue of their failure to phosphorylate and then incorporate into their DNA analogues of thymidine such as its arabinonucleoside. To ensure that in the selected TK$^+$ recombinants the *TK* gene was carried into the viral genome by homologous recombination of the *Hin*dIII O fragment rather than through recombination of the gene in its natural location, we replaced the sequences upstream from the *Bgl* II site containing the promoter–regulator region of the *TK* gene with the corresponding sequences of the α *4* gene (Fig. 1B). Because the *TK* gene in the chimeric fragment and the partially deleted *TK* gene resident in the viral DNA shared only the sequences downstream from the *Sac* I sites, the probability of recombination of the *TK* gene at its natural position was minimal.

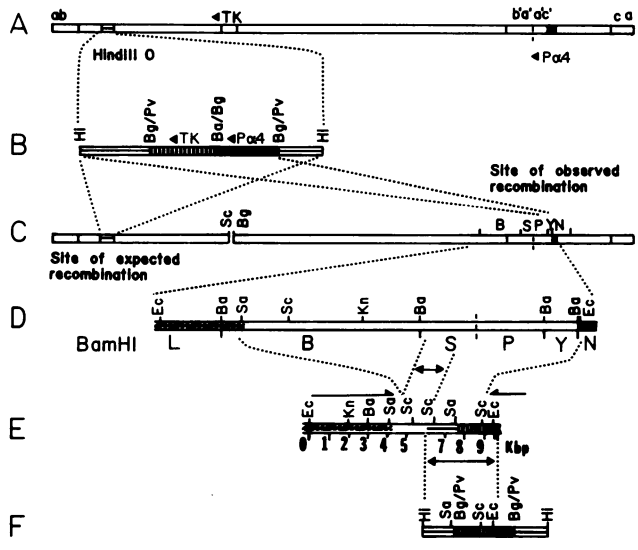

FIG. 1. Sequence arrangements in HSV-1(F)Δ305 and I358 DNAs, in the *Hin*dIII O–α *4 TK* chimeric fragment, and in the fragment spanning the L–S junction of the parental and recombinant viruses. (*A*) Representation of the HSV-1 genome (prototype arrangement), showing the reiterated sequences (*a*, *b*, *c*), the *TK* gene, the *Hin*dIII O fragment, and the α *4* promoter (*P*α*4*) and regulator sequences. (*B*) The *Hin*dIII O–*P*α*4 TK* chimeric fragment from pRB358. (*C*) Diagram of recipient virus Δ305, which has undergone a 700-bp deletion across the *Bgl* II/*Sac* I sites within the *TK* gene. The pRB358 DNA was used in cotransfection with Δ305 DNA and I358 was selected from this progeny. Restriction enzyme cleavage sites were abbreviated as follows: Ba, *Bam*HI; Bg, *Bgl* II; Ec, *Eco*RI; Hi, *Hin*dIII; Kn, *Kpn* I; Pv, *Pvu* II; Sa, *Sal* I; Sc, *Sac* I; Bg/Pv, fusion of fragments at *Bgl* II and *Pvu* II cleavage sites; Ba/Bg, fusion of fragments at *Bam*HI and *Bgl* II cleavage sites. (*D* and *E*) Enlarged scale diagrams of the prototype arrangements of the internal reiterated and surrounding sequences of the parental Δ305 virus (*D*) and of the I358 virus (*E*). (*F*) Diagram of the *Hin*dIII O–*TK* chimera to the same scale as *D* and *E*. The dotted vertical line indicates the L–S junction. The letters L, B, S, P, and Y indicate *Bam*HI fragments. The deleted portions of the Δ305 genome are enclosed in the two pairs of dotted lines and include from right to left all of *Bam*HI Y and P, a portion of *Bam*HI S, and most of *Bam*HI B. The solid arrows show the portions of the chimeric fragment and of the Δ305 virus conserved in I358 recombinant DNA.

To our surprise, none of the recombinants isolated in this study carried the *TK* gene either in the *Hin*dIII O fragment or at its natural location. Recombinant I358, which multiplied as well as the parent virus, was shown as described below to contain the chimeric *TK* gene and portions of the *Hin*dIII O fragment in place of sequences normally located at the L–S component junction as described schematically in Fig. 1. The DNA of this recombinant no longer inverts.

Sequence Arrangement in the DNA of the HSV-1 Recombinant I358. I358 DNA was probed in two series of experiments. Fig. 2 shows a comparison of the electrophoretically separated *Kpn* I, *Bam*HI, and *Bgl* II restriction enzyme digests of I358 and parental DNAs. The figure shows two striking findings. First, the *Kpn* I and *Bgl* II digests indicate that I358 DNA is present in one—i.e., P—arrangement only; the I_L, I_S, and I_{LS} isomers are absent. This conclusion is based on observations (5) that in the case of restriction enzymes that cleave outside the inverted repeated sequences *ab*, *b'a'*, *a'c'*, and *ca*, the wild-type DNA digests contain four different terminal fragments and four fragments spanning the L–S junction, and these are present in concentrations of 0.5 and 0.25 M, respectively, relative to those of fragments generated entirely from unique regions (3). Thus, the 0.25 M junction fragments of Δ305 that were cleaved with *Bgl* II are FH, JH, FL, and JL, whereas the 0.5 M terminal fragments are F, J, H, and L (Fig. 3). As shown in Fig. 2, the molarities of I358 DNA fragments J and L increased relative to those in Δ305 DNA, whereas fragments F, H, JH,

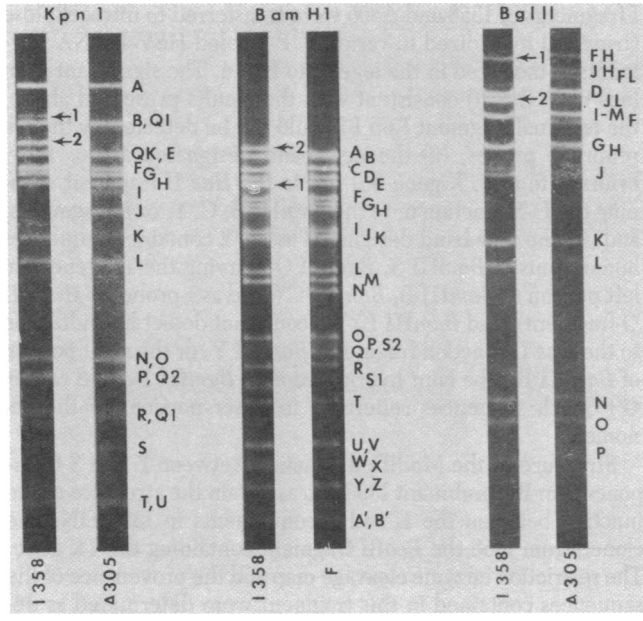

FIG. 2. Photographs of electrophoretically separated *Kpn* I, *Bam*HI, and *Bgl* II digests of viral DNAs. The agarose gels were stained with ethidium bromide and the bands were visualized with UV light. The DNAs identified in the photograph were extracted from the cytoplasm of infected cells and cleaved with the three indicated restriction enzymes. Each pair of digests was subjected to electrophoresis on separate agarose gels.

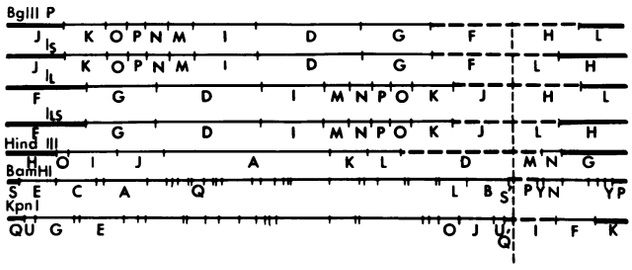

FIG. 3. Diagrams of the HSV-1 genome showing the *Bgl* II restriction endonuclease maps of the four isomeric arrangements (P, I_S, I_L, I_{SL}) and the *Hin*dIII, *Bam*HI, and *Kpn* I maps of the prototype arrangement. The 0.5 M *Bgl* II (terminal) fragments (J, L, F, H) are indicated by heavy solid lines and the 0.25 M (L–S junction) fragments (FH, FL, JH, JL) are designated by broken lines.

and FL of I358 DNA were no longer detectable. Although molar fragments from the unique regions comigrate with FH and JL, the hybridization experiments described below confirm the disappearance of the F and H termini and indicate that the new fragments are aberrant junction fragments that do not correspond to FH and JL. A similar situation is apparent in the case of the *Kpn* I digests. In this instance, the enzyme cleaves within the inverted repeats of the L component but not within the inverted repeats of the S component. Of the two 0.5 M *Kpn* I S component termini, K and I, only K was present.

The second feature of the digests is the presence of two new bands and the absence of DNA bands containing sequences adjacent to the L–S junction in each digest of I358 DNA. The fainter of the new bands was designated as no. 1 and the more abundant was designated as no. 2. The bands were numbered according to sequence homology (data not shown). The missing bands in the *Kpn* I and *Bam*HI digests other than the terminal and junction fragments included *Kpn* I J and *Bam*HI B and N fragments.

In the second experiment electrophoretically separated *Kpn* I fragments of I358 and Δ305 were transferred to nitrocellulose strips and hybridized to various ^{32}P-labeled HSV-1 DNA fragments as indicated in the legend to Fig. 4. The significant findings were that (*i*) consistent with the results presented above, the terminal fragment *Kpn* I I could not be detected by the appropriate probes, (*ii*) the new band designated as no. 1 hybridized to the *TK* gene but not to the *Hae* II fragment spanning the L–S junction or to the *Bam*HI B, C, Y, or L fragments, and (*iii*) the new band designated as no. 2 contained sequences homologous to *Bam*HI S, *Bam*HI Q carrying the *TK* gene, the left portion of *Bam*HI B, *Bam*HI C (used as a probe for *Hin*dIII O fragment), and *Bam*HI L. We could not detect hybridization to the *Hae* II junction fragment, *Bam*HI Y, or the right portion of *Bam*HI B. The faint hybridization to *Bam*HI P could reflect G+C-rich sequences reiterated in other portions of the genome.

Structure of the Modified Junction Between L and S Components in Recombinant I358. To ascertain the structure of the junction between the L and S components in I358 DNA we cloned from I358 the *Eco*RI fragment containing the *TK* gene. The restriction enzyme cleavage map and the provenance of the sequences contained in this fragment were determined as described in the legend to Fig. 5 and are depicted in Fig. 1*E*. Because it contains the left end of *Bam*HI B, the cloned *Eco*RI fragment corresponds to the sequences of band no. 2 of Fig. 2. Of the sequences contained between the left end of *Bam*HI B and the *Eco*RI site of *Bam*HI N in the parent virus, only a portion of *Bam*HI S sequences were retained. However, the *Eco*RI fragment contains the *α–TK* chimeric gene and a portion of the

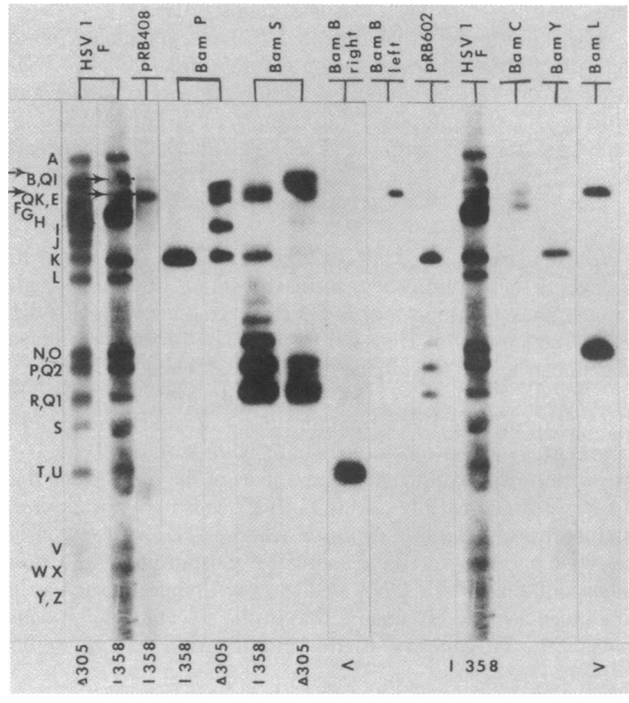

FIG. 4. Autoradiograms of ^{32}P-labeled cloned fragments of HSV-1 DNA hybridized to electrophoretically separated *Kpn* I digests of I358 and Δ305 DNAs transferred to nitrocellulose strips. *Kpn* I digests of parental Δ305 and recombinant I358 DNAs were electrophoretically separated and transferred to nitrocellulose (30). The digests were visualized with ^{32}P-labeled (nick-translated) whole HSV-1(F) DNA. All other probes were cloned HSV-1 fragments purified from agarose after electrophoresis and nick-translated in the presence of [α-^{32}P]dCTP. The genomic locations of *Bam*HI clones are shown in Fig. 3. *Bam*HI B left and *Bam*HI B right represent the left-most and right-most *Bam*HI/*Sac* I fragments of the DNA in the prototype arrangement. Numbers 1 and 2 at left identify the two new DNA fragments in *Kpn* I digests of I358 DNA. pRB408 (*Bgl* II/*Sac* I fragment contained in the *TK* gene) and pRB602 (*Hae* II fragment spanning the L–S junction) were described in *Materials and Methods*.

*Hin*dIII O fragment. The sequences in band no. 1 have not been cloned. It is apparent from the results presented in Figs. 4 and 5 that we have been unable to detect a large fraction of the inverted repeat sequences including the *a* sequence in both band no. 1 and band no. 2 DNAs. The latter sequence was previously shown to be a site-specific inversion sequence (9, 23).

DISCUSSION

In this paper we report that a recombinant, HSV-1 (I358), is viable—i.e., it multiplies without the parental virus as a helper—even though its DNA no longer inverts. The salient features of the results are as follows:

(*i*) As illustrated schematically in Fig. 6, recombinant I358 appears to have been generated by the recombination between the *α 4* gene promoter–regulator sequences linked to the *TK* gene in the pRB358 plasmid with the homologous sequences contained in the *Bam*HI N fragment of HSV-1(F)Δ305 DNA, followed by two deletions. Although the events leading to the evolution of I358 are likely to occur rarely, we have obtained independently at least two similar recombinants in this study. Another recombinant similar to I358 was obtained by transfection of HSV-1(F)Δ305 DNA with the *α 4–TK* chimera inserted into the HSV-1 *Bam*HI B DNA fragment (unpublished data). These observations reaffirm the conclusions that the sequences replaced by the chimeric *TK* gene are not essential for repli-

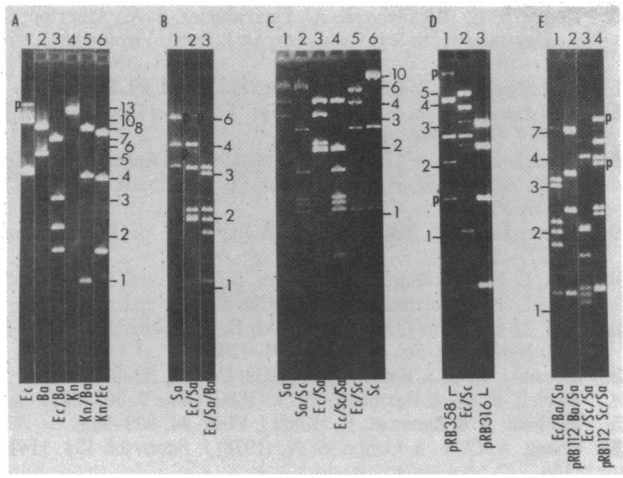

FIG. 5. Photographs of electrophoretically separated HSV-1 DNA fragments prepared for mapping of the sequences spanning the L–S junction in HSV-1 I358. To map the junction the *Eco*RI fragment spanning the sites of the recombinational events in I358 was cloned (pRB9002) and mapped (Fig. 1*E*) as follows:

Lanes A1–6 orient the *Bam*HI and *Kpn* I sites. In lane A1, the 13.5-, 9.98-, and 4.0-kbp bands correspond to incompletely digested pRB9002, the HSV-1 fragment spanning the recombinational sites, and the pACYC184 vector, respectively. Because the vector has a *Bam*HI site (26), the presence of two bands in lane A2 indicates one site in the HSV-1 fragment. This is confirmed by the presence of the 1.71- and 2.3-kbp vector *Eco*RI/*Bam*HI fragments and the 3.0- and 6.78-kbp HSV-1 *Eco*RI/*Bam*HI fragments in lane A3.

Setting the left *Eco*RI site shown in Fig. 1*E* as the 0.0-kbp reference point and knowing that *Bam*HI cleaves pRB9002 into 8.5- and 5.3-kbp fragments (lane A2), we conclude that the *Bam*HI sites are at 3.0 and 11.5 kbp (into the vector sequences). When the pRB9002 linearized with *Kpn* I (lane A4) is cleaved with *Bam*HI, it becomes apparent that the single HSV-1 *Kpn* I site cleaves the 5.3-kbp *Bam*HI fragment into 4.24- and 1.05-kbp fragments (lanes A2, A5). This places the *Kpn* I site 1.95 kbp from the reference point, as shown also by lane A6.

Lanes B1–3 map the *Sal* I sites. The HSV-1 fragment contains two *Sal* I sites (lane B1). Lane B2 indicates that the 3.5-kbp *Sal* I fragment is entirely HSV-1, whereas the 6.14- and 4.12-kbp *Sal* I fragments contain viral and vector sequences. The *Eco*RI/*Sal* I/*Bam*HI digest (lane B3) indicates that the 4.17-kbp (*Eco*RI/*Sal* I) fragment is cleaved by *Bam*HI, placing the *Sal* I cleavage sites at 4.17, 7.68, and 11.8 kbp from the reference site.

Lanes C1–6 map the three *Sac* I sites in HSV-1 DNA (lane C6). The *Sac* I and *Eco*RI/*Sac* I digests (lanes C5–6) identify the 5.37- and 0.6-kbp fragments as *Eco*RI/*Sac* I HSV-1 fragments and the 2.67- and 1.14-kbp bands as *Sac* I/*Sac* I HSV-1 fragments. Because the 4.17-kbp *Eco*RI/*Sal* I fragment is not cleaved by *Sac* I (lanes C3, C4), and because the 1.14-kbp *Sac* I/*Sac* I fragment can fit into only the 3.3-kbp *Eco*RI/*Sal* I fragment, the location of the *Sac* I sites is 5.37, 6.51, and 9.18 kbp from the reference site in the viral fragment.

Lanes D1–3 and E1–4 indicate the provenance of the various cleavage sites. Comparison of *Eco*RI/*Sac* I digests of plasmids pRB358, pRB9002, and pRB316 (lanes D1–3) shows that all three share the 0.6-kbp *Eco*RI/*Sac* I fragment and that pRB358 and pRB9002 share a 2.6-kbp fragment. Therefore the *Sac* I site at 6.51 kbp is derived from the *Hin*dIII O fragment, whereas the *Sac* I site at 8.95 kbp is derived from the chimeric *TK* gene contained in pRB316 (Fig. 1 *E* and *F*).

Comparisons of an *Eco*RI/*Bam*HI/*Sal* I digest of pRB9002 (lane E1) and a *Bam*HI/*Sal* I digest of the *Bam*HI B fragment (lane E2) cloned as pRB112 (see Fig. 1) show that the 1.17-kbp *Bam*HI/*Sal* I fragment is shared. The *Eco*RI/*Sac* I/*Sal* I digest of pRB9002 and the *Sac* I/*Sal* I digest of pRB112 show that a 1.2-kbp fragment is shared. This confirms that the *Sal* I site at 4.17 kbp is derived from *Bam*HI B sequences and suggests that the *Sac* I site at 5.37 kbp may also be derived from the same fragment.

The summary presented in Fig. 1*E* indicates that the sequences from the *Eco*RI reference site through the *Sal* I site at 4.17 kbp are from the *Bam*HI B fragment, whereas the sequences at and to the right of the *Sac* I site at 6.51 kbp are from pRB358. The presence of *Bam*HI S DNA sequences between the *Sac* I sites at 5.37 and 6.51 kbp may be deduced from the data presented in Fig. 4 showing the hybridization of these sequences to band no. 2 DNA transferred to nitrocellulose strips.

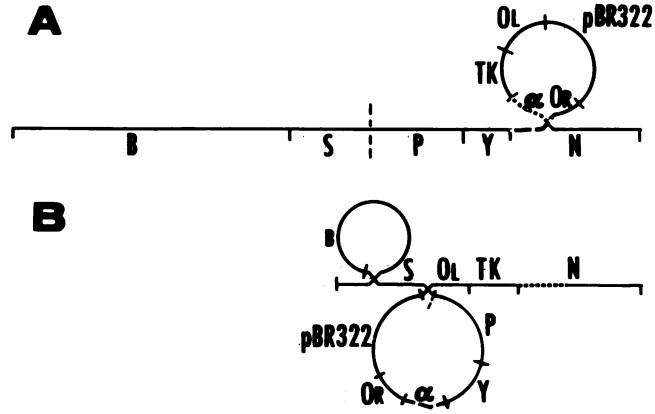

FIG. 6. Diagram representing the hypothetical sequence of events leading to the generation of recombinant I358. The letters B, S, P, Y, and N refer to *Bam*HI fragments. O_L and O_R refer to the portions of the *Hin*dIII O fragment to the left or right of the site of insertion of the *α–TK* chimeric gene when the fragment is in the prototype arrangement of the DNA. *α* identifies the *α4* promoter–regulatory region of *α4* gene derived from the left portion of *Bam*HI N (20). pBR322 is the vector for pRB358. (*A*) Schematic representation of a hypothetical recombination of pRB358 with HSV-1(Δ305). The recombination is shown to occur between the *α* sequences (dotted line) of the plasmid shown as a circle with the homologous sequences (broken line) in *Bam*HI N contained in viral DNA, a portion of which is shown in linear form. (*B*) Formation of two deletions, the first consisting of most of *Bam*HI B and a portion of *Bam*HI S and the second consisting of the distal portion of *Bam*HI S, *Bam*HI P and Y, the *α* promoter–regulator sequence of gene *4*, the left portion of the *Hin*dIII O fragment, and the pBR322 vector.

cation and attest to the usefulness of the *TK* gene as a tool for genetic selection of products of rare recombinational events.

(*ii*) Earlier Preston *et al.* (31) described an HSV-1 × HSV-2 recombinant containing in its virions a preponderance of P and I_S isomers. The presence of the other two isomers indicated that either the inversion or the packaging processes favored two isomers, but that the inversions of L and S components were not abolished. The absence of I_S, I_L, and I_{LS} isomers indicates that I358 DNA does not invert, and that either inversion is not essential for virus multiplication or that some perturbations in the structure of I358 DNA obviated this requirement. Less clear is the significance of the observation that all of the recombinants obtained in this fashion are frozen in the P arrangement of HSV-1 DNA.

The I358 genome is smaller than the wild type by about 10 kbp of DNA. Previous studies have shown that 7.5 kbp of DNA can be inserted into the wild-type genome without affecting the ability of the DNA to be packaged (32). We conclude therefore that I358 could act as a vector for insertion of as much as 7.5

The sizes of the fragments from top to bottom in each lane are given in kbp as follows: In A1, 9.78 and 4.0; in A2, 8.5 and 5.3; in A3, 6.78, 3.0, 2.3, and 1.7; in A4, 13.78; in A5, 8.5, 4.24, and 1.05; in A6, 7.8, 4.0, and 1.95; in B1, 6.14, 4.12, and 3.5; in B2, 4.17, 3.5, 2.1, 2.02, and 1.97; in B3, 3.5, 3.0, 2.1, 1.97, 1.7, and 1.17; in C1, 6.14, 4.12, and 3.5; in C2, 6.14, 2.62, 1.5, 1.2, 1.17, and 1.14; in C3, 4.17, 3.5, 2.1, 2.02, and 1.97; in C4, 4.17, 2.02, 1.97, 1.5, 1.2, 1.17, 1.14, and 0.6; in C5, 5.37, 4.0, 2.67, 1.14, and 0.6; in C6, 9.96, 2.67, and 1.14; in D1, 4.8, 2.67, 2.05, 0.6, and 0.5; in D2, 5.37, 4.0, 2.67, 1.14, and 0.6; in D3, 3.2, 2.5, 1.3, and 0.6; in E1, 3.5, 3.0, 2.1, 1.97, 1.7, and 1.17; in E2, 7.2, 3.5, 2.5, and 1.17; in E3, 4.17, 2.02, 1.97, 1.5, 1.2, 1.17, 1.14, and 0.6; in E4, 5.3, 4.15, 2.5, 2.2, and 1.2. Enzyme abbreviations are as noted in legend of Fig. 1. Approximate scales are indicated in kbp at sides of photographs. DNA in all lanes is pRB3002 except as indicated. The p indicates bands that represent incompletely digested DNA fragments.

kbp of DNA without removing the gene and as much as 20 kbp if the sequences inserted (*TK* and *Hin*dIII O) into I358 were removed.

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