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The *a* Sequence Is Dispensable for Isomerization of the Herpes Simplex Virus Type 1 Genome

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The herpes simplex virus type 1 (HSV-1) genome consists of two components, L (long) and S (short), that invert relative to each other during productive infection to generate four equimolar isomeric forms of viral DNA. Recent studies have indicated that this genome isomerization is the result of DNA replication-mediated homologous recombination between the large inverted repeat sequences that exist in the genome, rather than site-specific recombination through the terminal repeat *a* sequences present at the L-S junctions. However, there has never been an unequivocal demonstration of the dispensability of the latter element for this process using a recombinant virus whose genome lacks *a* sequences at its L-S junctions. This is because the genetic manipulations required to generate such a viral mutant are not possible using simple marker transfer, since the cleavage and encapsidation signals of the *a* sequence represent essential *cis*-acting elements which cannot be deleted outright from the viral DNA. To circumvent this problem, a simple two-step strategy was devised by which essential *cis*-acting sites like the *a* sequence can be readily deleted from their natural loci in large viral DNA genomes. This method involved initial duplication of the element at a neutral site in the viral DNA and subsequent deletion of the element from its native site. By using this approach, the *a* sequence at the L-S junction was rendered dispensable for virus replication through the insertion of a second copy into the thymidine kinase (TK) gene of the viral DNA; the original copies at the L-S junctions were then successfully deleted from this virus by conventional marker transfer. The final recombinant virus, HSV-1::L-SΔ*a*, was found to be capable of undergoing normal levels of genome isomerization on the basis of the presence of equimolar concentrations of restriction fragments unique to each of the four isomeric forms of the viral DNA. Interestingly, only two of these genomic isomers could be packaged into virions. This restriction was the result of inversion of the L component during isomerization, which prevented two of the four isomers from having the cleavage and encapsidation signals of the *a* sequence in the TK gene in a packageable orientation. This phenomenon was exploited as a means of directly measuring the kinetics of HSV-1::L-SΔ*a* genome isomerization. Following infection with virions containing just the two packaged genomic isomers, all four isomers were readily detected at a stage in infection coincident with the onset of DNA replication, indicating that the loss of the *a* sequence at the L-S junction had no adverse effect on the frequency of isomerization events in this virus. These results therefore validate the homologous recombination model of HSV-1 genome isomerization by directly demonstrating that the *a* sequence at the L-S junction is dispensable for this process. The strategy used to remove the *a* sequence from the HSV-1 genome in this work should be broadly applicable to studies of essential *cis*-acting elements in other large viral DNA molecules.

Recent advances in the elucidation of the complex molecular biology of large DNA viruses have been driven largely by the development of technologies through which the genomes of these pathogens can be readily manipulated. Numerous methods have been devised by which protein-coding sequences can be selectively deleted from large viral genomes (3, 14, 16, 28, 32). Even if the protein encoded by a targeted sequence is required for virus replication, the desired deletion can still be engineered through the use of *trans*-complementing cell lines which express the essential polypeptide (8, 15). However, such approaches cannot be used to manipulate essential *cis*-acting sites in a genome, since the deletion of such elements necessarily creates a nonviable virus incapable of self-propagation.

One experimental system that would benefit greatly from the development of methods for manipulating essential *cis*-acting sites is the study of genome isomerization in herpes simplex virus type 1 (HSV-1). The HSV-1 genome is a 152-kb linear double-stranded DNA molecule that can be divided into L (long) and S (short) segments on the basis of the presence of the intervening inverted repeat sequences *a*, *b*, and *c* (see Fig. 1). The L and S components of the genome are able to invert relative to each other at high frequency, resulting in the appearance of four equimolar isomeric populations of DNA (7, 11, 31). This phenomenon of genome isomerization is one of the most unique yet enigmatic aspects of the life cycle of herpesviruses. Although initial investigations into this process argued that a site-specific recombination system which utilized the *a* sequence as its *cis*-acting element was responsible for HSV-1 genome isomerization events (4, 21–23), more recent studies have indicated that homologous recombination through any of the inverted repeats of the genome (that is, the *b* and *c* sequences, as well as the *a* sequence) will promote inversion of the L and S components (30, 33, 35, 38, 39, 41). Thus, the controversy surrounding the mechanism of genome isomerization and the role of the *a* sequence in this process has remained unresolved.

A definitive experiment that would settle the debate over the role of the *a* sequence in herpesvirus genome isomerization

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would be to create a recombinant HSV-1 in which the *a* sequences have been deleted from the junctions between the L and S components. The site-specific recombination model predicts that isomerization would not occur in this virus since it lacks the *a* sequence, whereas the homologous recombination model predicts that all four isomers would still be generated in this virus because it retains other regions of significant sequence homology (that is, the *b* and *c* sequences). Unfortunately, the *a* sequence cannot be deleted outright from the viral DNA by conventional marker transfer experiments because it contains sequence motifs that are required for cleavage and packaging of the replicated genome into capsids (6, 38). A novel two-step strategy was therefore devised by which the *a* sequence or any essential *cis*-acting element of any large DNA virus can be selectively deleted from its native site in the genome. This approach was used to successfully construct a recombinant virus that lacked *a* sequences at its L-S junctions. The existence of four equimolar isomeric forms of genomic DNA in cells infected with this virus unequivocally demonstrated that the *a* sequence is dispensable for HSV-1 genome inversion events and is unlikely to play any significant role in this process. These results therefore validate the homologous recombination model of herpesvirus genome isomerization.

MATERIALS AND METHODS

Cells and viruses. Vero cells were maintained in Dulbecco's modified essential medium supplemented with 10% fetal bovine serum. HSV-1 (strain KOS) was the wild-type strain used in this work. The HSV-1 ICP4 deletion mutant *d*202 and the ICP4-expressing cell line E5 (8) were generously provided by Neal DeLuca, University of Pittsburgh, Pittsburgh, Pa.

Plasmid constructions. pHSV-106::GAL/*a* was the plasmid used in marker transfer experiments to construct the recombinant virus *d*202::GAL/*a*. This plasmid was generated as follows: a fragment containing the *a* sequence was removed from pCW101 (41) by *Bst*EII-*Bsp*MI digestion, blunt ended, and cloned into the *Sma*I sites of pSG424 (29). A fragment containing the *a* sequence and the adjacent GAL4 gene was removed from this plasmid by *Xba*I-*Hin*dIII digestion, blunt ended, and cloned into a blunt-ended *Kpn*I site within the TK gene of pHSV-106 (20) to yield pHSV-106::GAL/*a*. This procedure disrupted the TK open reading frame, so that incorporation of pHSV-106::GAL/*a* sequences into the *d*202 genome would allow for the selection of TK-deficient recombinant viruses.

pUCSG1-EK1::Tn*5*/Δ*a* was the marker transfer plasmid used to construct the recombinant virus HSV-1::L-SΔ*a*. This plasmid was generated as follows: the L-S junction- and ICP4 gene-containing *Eco*RI-*Kpn*I fragment of pSG1-EK1 (10) was cloned into the *Eco*RI and *Kpn*I sites of pUC19 to generate pUCSG1-EK1. The *a* sequence was then deleted from this plasmid by replacing its *Dra*III-*Bsa*AI fragment with that of the *a* sequence deletion construct pSG424*b*Δ*ac* (30) to generate pUCSG1-EK1Δ*a*. The sequences of the L-S junction fragment that were deleted in this construct extended to the ends of the L-S junction that had been inserted into the TK gene of pHSV-106::GAL/*a*, so that no overlapping flanking sequences existed between the two which could be used to repair the deletion once it was incorporated into the *d*202::GAL/*a* virus. A 1.4-kb *Hin*dIII-*Sma*I fragment from the bacterial transposon Tn*5* was then cloned into the blunt-ended *Dra*III site of pUCSG1-EK1Δ*a* to yield the plasmid pUCSG1-EK1::Tn*5*/Δ*a*.

Generation of recombinant viruses. The recombinant virus *d*202::GAL/*a* was constructed by insertion of the *a* sequence into the TK gene of the *d*202 genome (8), using the marker transfer construct pHSV-106::GAL/*a*. Ten micrograms of pHSV-106::GAL/*a* DNA was cotransfected with 10 μg of infectious *d*202 viral DNA into ICP4-expressing E5 cells (8) by using calcium phosphate precipitation as described previously (39). Cell-free supernatants of the resulting transfection progeny were used to infect fresh E5 cells in the presence of 100 μg of 5′-bromodeoxycytidine per ml. DNAs from these plaques were screened for the presence of GAL4 sequences in dot blot hybridizations, since it was probable that any genome which had incorporated the GAL4 DNA would also have incorporated the adjacent *a* sequence.

The recombinant virus HSV-1::L-SΔ*a* was constructed by deletion of the *a* sequences from the L-S junctions of *d*202::GAL/*a* with the concomitant repair of the ICP4 gene defect of this virus using the marker transfer construct pUCSG1-EK1::Tn*5*/Δ*a*. Like any removal of reiterated sequences in HSV-1 DNA, this procedure involved the initial incorporation of the deletion by marker transfer into one of the genome repeats, followed by duplication of the deletion in the other repeats through virus-mediated gene conversion events (5, 8, 17, 27, 37). Marker transfer was carried out by cotransfection of 10 μg of pUCSG1-EK1::Tn*5*/Δ*a* and 15 μg of *d*202::GAL/*a* DNA into E5 cells by using calcium phosphate precipitation as described previously (39). The resulting progeny virus was plated onto noncomplementing Vero cells to select for ICP4$^+$ recombinants. DNAs from these plaques were screened for the presence of Tn*5* sequences in dot blot hybridizations, since it was probable that marker transfer which had both repaired the ICP4 deletion and introduced the Tn*5* sequence was likely to have incorporated the intervening *a* sequence deletion at the L-S junction as well.

Isolation and manipulation of viral DNA. The genomic structures of *d*202::GAL/*a* and HSV-1::L-SΔ*a* were confirmed by extensive Southern blot analysis of viral DNA. Viral DNA was prepared from plaques that were positive in dot blot hybridizations by infecting E5 or Vero cells at a multiplicity of infection of 0.1. Upon attaining complete cytopathic effect, the cells were treated with lysis buffer (100 mM Tris-HCl [pH 8.0], 10 mM EDTA [pH 8.0], 1% sodium dodecyl sulfate, 1% laurylsarcosine, 100 μg of proteinase K per ml) for 12 h at 37°C. Viral DNA was purified from this lysate by cesium chloride centrifugation, dialyzed against TE buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA), ethanol precipitated, resuspended in TE buffer, and subjected to restriction enzyme digestion. Alternatively, the viral DNA in the lysate was subjected to repeated phenol-chloroform extraction and ethanol precipitation prior to restriction enzyme digestion. Viral DNA prepared by either of these methods contained a mixture of concatameric replication intermediate DNA as well as unit-length DNA that had been packaged into capsids. The latter DNA form was itself purified from extracellular virions present in the medium of infected cells. Purification was accomplished by first removing cellular debris from the medium by centrifugation at 2,000 × *g* for 15 min and then pelleting the virion particles in the resulting supernatant by centrifugation at 37,500 × *g* for 1 h. The pelleted virions were treated with lysis buffer for 12 h at 37°C; the virion DNA in this lysate was then subjected to repeated phenol-chloroform extraction and ethanol precipitation prior to restriction enzyme digestion.

*Sst*I-generated viral DNA fragments were separated on standard 0.8% agarose gels prior to Southern blot analysis. *Hin*dIII- or *Xba*I-generated viral DNA fragments were separated by using the Bio-Rad contour-clamped homogeneous electric field gel electrophoresis unit. Gels (0.8% agarose in 0.5× TBE buffer [50 mM Tris, 90 mM boric acid, 0.5 mM EDTA]) were run at 145 V for 19 h at 4°C with switch times ramped from 1.5 to 4.0 s. DNA was transferred to GeneScreen Plus nylon membranes (NEN-DuPont) and hybridized with probes radiolabeled by using a random-primed labeling kit (Boehringer Mannheim). Restriction fragments used as probes included the 193-bp *Eco*RI fragment of pLΔR-193 (30) (the *a* sequence-specific *a* probe), the 3.3-kb *Sst*I fragment of pCW101 (41) (the L-S junction-specific *bac* probe), *Hin*dIII-linearized pKST (40) (the *b* sequence-specific *b* probe), the 3.4-kb *Hin*dIII fragment of pTn5Δ1 (39) (the Tn*5*-specific Tn*5* probe), and the 500-bp *Hin*dIII-*Xba*I fragment of pSG424 (29) (the GAL4-specific GAL probe). The resulting blots were visualized by autoradiography and quantitated by using a Betagen PhosphorImager.

RESULTS

Construction of HSV-1::L-SΔ*a*, a recombinant HSV-1 which lacks *a* sequences at its L-S junctions. The obvious complication in deleting the *a* sequence outright from the L-S junctions of the HSV-1 genome is that it is a required *cis*-acting element which is necessary for cleavage and encapsidation of viral DNA (6, 38). It was therefore necessary to devise a novel strategy by which the removal of this element could be accommodated. The rationale behind the two-step scheme that was employed (illustrated in Fig. 1) is that the introduction of a second copy of the *a* sequence into a neutral site in the genome should make the native copies of this element at the L-S junctions expendable. Thus, the first step in constructing a recombinant HSV-1 genome which lacks *a* sequences at its L-S junctions involved the insertion of a second copy of this element into the TK gene of the genome (Fig. 1). This site was chosen for three reasons. (i) It maps in the L component at a considerable distance from the L-S junctions. (ii) Disruption of the TK gene in this manner does not impair virus replication in cell culture. (iii) TK-deficient recombinant viruses can be readily selected by using 5′-bromodeoxycytidine (28, 32, 34). Once this second copy of the *a* sequence has been inserted into the TK gene, it should be able to provide *cis* complementation to a recombinant virus lacking *a* sequences at the L-S junction by acting as the cleavage and packaging signals for newly replicated viral DNA.

The parental virus in this first step was *d*202, which possesses a deletion in the viral ICP4 gene and must therefore be grown in a cell line that complements this defect (8); the ICP4$^-$

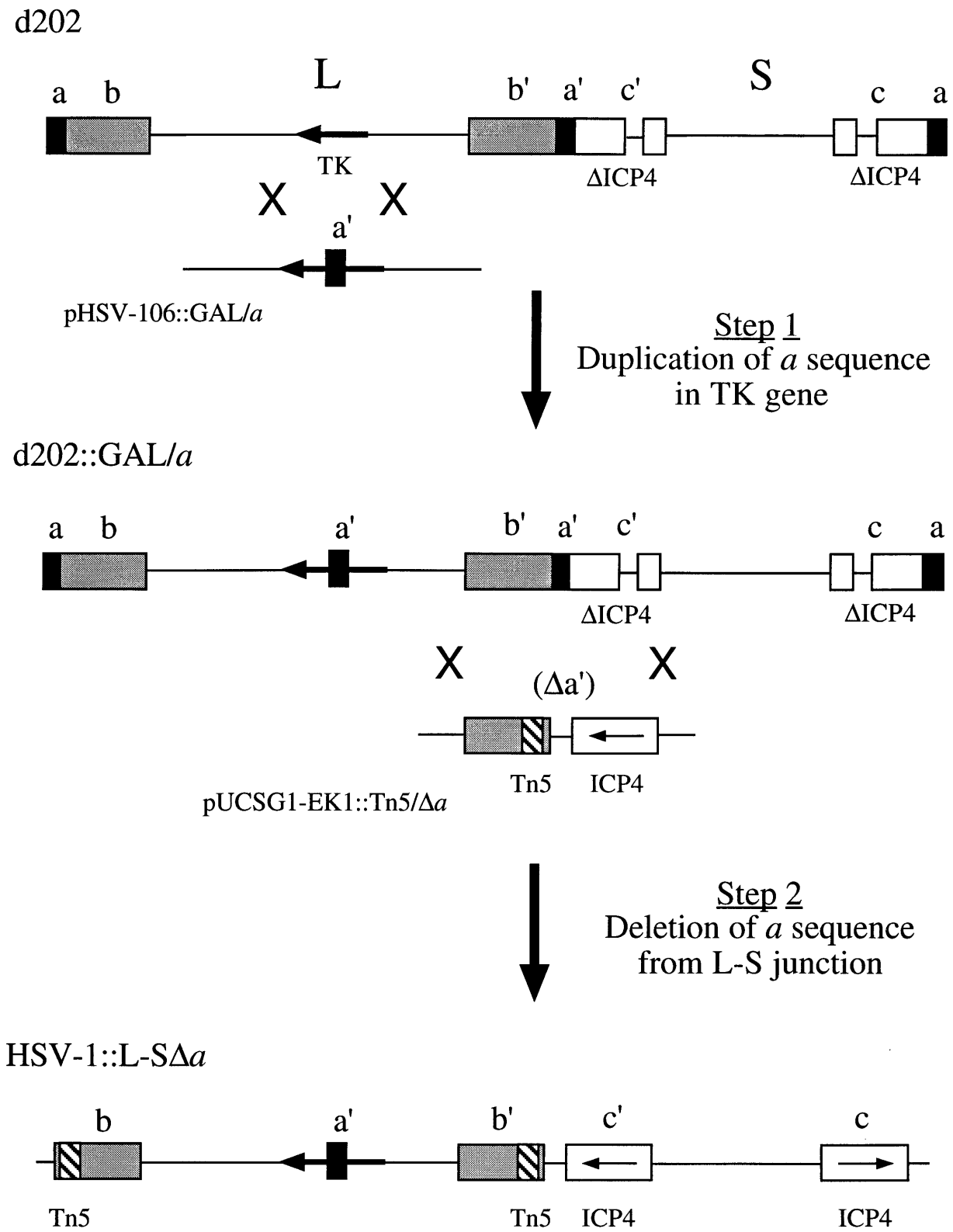

FIG. 1. Two-step scheme for deletion of the *a* sequence from its native locus in the L-S junctions of the HSV-1 genome. A new copy of the *a* sequence was first inserted into a neutral locus, the TK gene, and then the native copies of the *a* sequence were removed from the L-S junctions. Note that the second step is driven by the restoration of a gene essential for HSV-1 replication, ICP4, and involves an initial marker transfer event at one L-S junction followed by duplication of the deletion at the other L-S junctions by conversion events. The inverted repeat sequences *a*, *b*, and *c* are shown as black, grey, and white boxes, respectively.

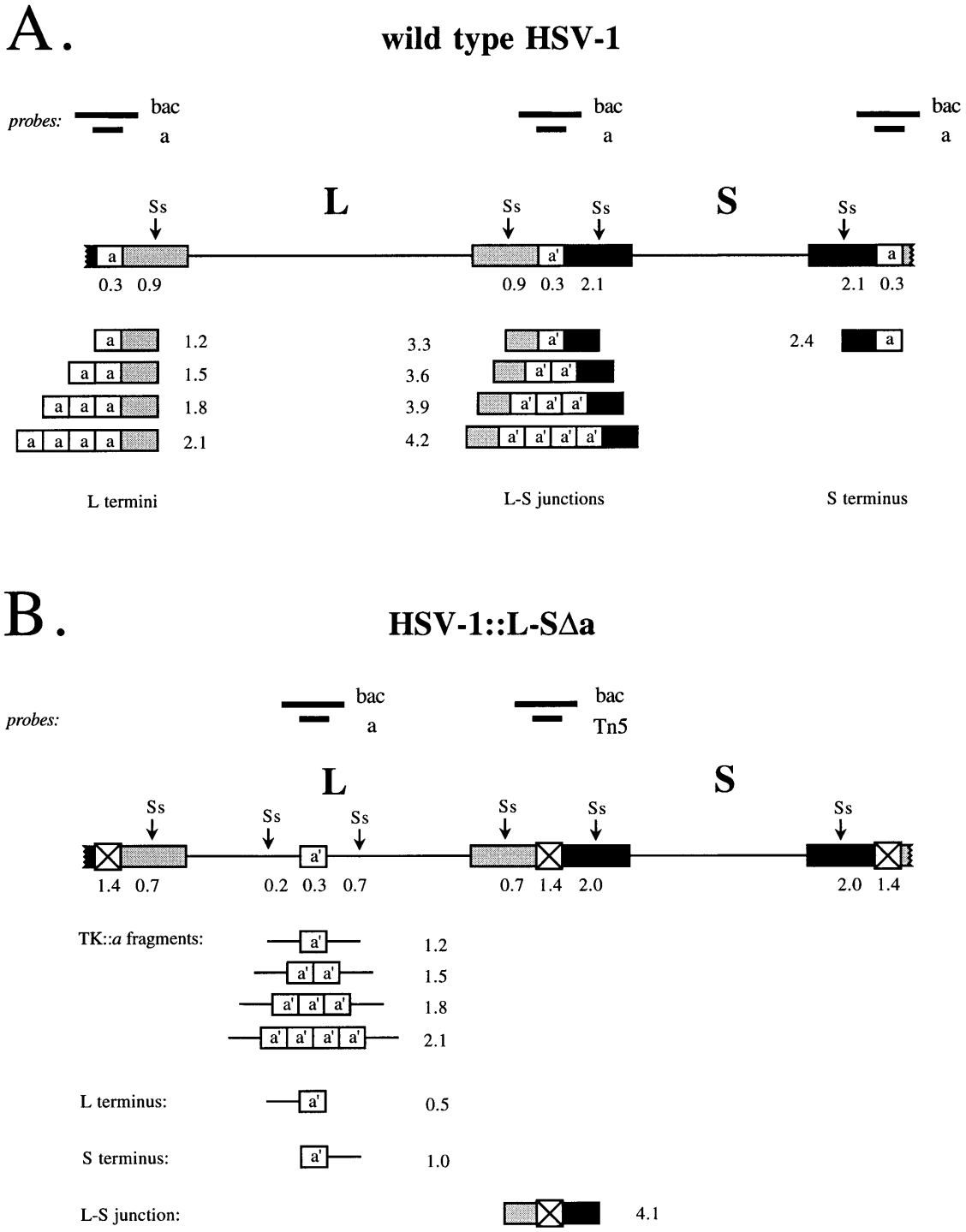

FIG. 2. *Sst*I fragment maps of wild-type HSV-1 (strain KOS) and the *a* sequence deletion mutant HSV-1::L-SΔ*a*. The locations of relevant *Sst*I (Ss) sites in the genomes of HSV-1 (strain KOS) (A) and HSV-1::L-SΔ*a* (B) are denoted by arrows. The *Sst*I site 0.7 kb to the right of the *a* sequence in HSV-1::L-SΔ*a* was derived from the viral TK gene, while the *Sst*I site 0.2 kb to the left of the *a* sequence was fortuitously acquired during the cloning steps used to insert this element into a cloned TK gene. The structures and sizes of those *Sst*I fragments recognized by the indicated *a* sequence-specific, L-S junction-specific, and Tn*5*-specific probes (*a*, *bac*, and Tn*5*, respectively) are shown below the genomes. The inverted repeat sequences *a*, *b*, and *c*, are shown as white, grey, and black boxes, respectively; the locations of the coupled *a* sequence deletion and Tn*5* fragment insertion in the HSV-1::L-SΔ*a* genome are denoted by white boxes containing an X.

phenotype of *d*202 was exploited as a selection strategy in the second step of the virus construction. Insertion of the *a* sequence into the TK gene of the HSV-1 genome was accomplished by marker transfer experiments involving cotransfection of infectious *d*202 DNA and the plasmid pHSV-106::GAL/*a*, which contains a cloned TK gene into which an *a* sequence has been inserted (Fig. 1). The structure of the resulting recombinant virus, *d*202::GAL/*a* (Fig. 1), and the ability of its *a* se-

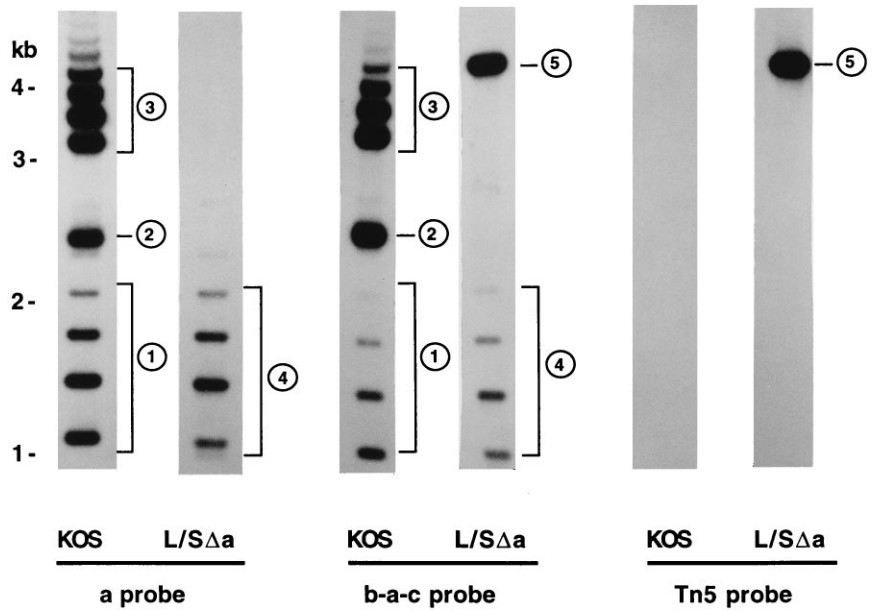

FIG. 3. Southern blot analysis of *Sst*I-digested wild-type HSV-1 (strain KOS) and *a* sequence deletion mutant HSV-1::L-SΔ*a* DNAs. Viral DNA was digested with *Sst*I, and the resulting fragments were separated by agarose gel electrophoresis. Following alkaline transfer, the membranes were hybridized with the *a* sequence-specific (left lanes), L-S junction-specific (middle lanes), and Tn*5*-specific (right lanes) probes shown in Fig. 2 (*a*, *bac*, and Tn*5*, respectively). A molecular size scale is indicated to the left; specific fragments or fragment families are identified to the right of each lane by circled numbers (1, 1.2-kb L terminal fragment family of wild-type HSV-1; 2, 2.4-kb S terminal fragment of wild-type HSV-1; 3, 3.3-kb L-S junction fragment family of wild-type HSV-1; 4, 1.2-kb TK::*a* insertion fragment family of HSV-1::L-SΔ*a*; and 5, 4.1-kb L-S junction fragment of HSV-1::L-SΔ*a*). Note that HSV-1::L-SΔ*a* is also predicted to contain a 1.0-kb S terminal fragment and a 0.5-kb L terminal fragment family (Fig. 2); however, these fragments were present at very low concentrations and were not efficiently resolved under the electrophoresis conditions used in this experiment.

quence duplication in the TK gene to promote cleavage and encapsidation events were confirmed by extensive Southern blot analysis of viral DNA (19).

The second step of the construction strategy involved the actual deletion of the *a* sequences from the L-S junctions of the *d*202::GAL/*a* genome. Since it was anticipated that the loss of these elements would confer a significant replication disadvantage on this virus and make it difficult to purify from its *d*202::GAL/*a* parent, a selection scheme involving restoration of the mutated ICP4 gene was utilized to increase the likelihood of obtaining the desired recombinant. This procedure involved marker transfer of the plasmid construct pUCSG1-EK1::Tn*5*/Δ*a*, which contained an *a* sequence deletion that was flanked by an intact ICP4 gene on one side and a fragment of the bacterial transposon Tn*5* and part of the *b* sequence on the other (Fig. 1). Recombinant viruses in which the ICP4 deletion had been repaired using this plasmid were isolated by direct selection on noncomplementing Vero cells. These isolates were subsequently screened for the presence of Tn*5* sequences, since ICP4$^+$ Tn*5*$^+$ recombinants were predicted to acquire the *a* sequence deletion mapping between these two markers (Fig. 1). Extensive Southern blot analyses of several of these ICP4$^+$ Tn*5*$^+$ recombinants confirmed that their genomes had indeed lost all copies of the *a* sequence at their L-S junctions. Representative results for one of these isolates, HSV-1::L-SΔ*a*, are presented in this work.

Characterization of the genome structure of HSV-1::L-SΔa. The successful removal of the *a* sequences from the L-S junctions of the HSV-1::L-SΔ*a* genome was confirmed by Southern blot analysis of *Sst*I-digested viral DNA. Wild-type HSV-1 (strain KOS) DNA is predicted to contain three *Sst*I fragments that hybridize to either L-S junction-specific or *a* sequence-specific probes: a 3.3-kb fragment corresponding to the L-S junction, a 2.4-kb fragment corresponding to the S terminus of the genome, and a 1.2-kb fragment corresponding to the L terminus of the genome (Fig. 2). Since the L-S junction and L terminus typically contain one to several *a* sequence reiterations of 0.3 kb each, they both form families of fragments (i.e., the 3.3-, 3.6-, 3.9-, and 4.2-kb fragments in the 3.3-kb fragment family and the 1.2-, 1.5-, 1.8-, and 2.1-kb fragments in the 1.2-kb fragment family [Fig. 2]). Each of these predicted fragments was detected in hybridizations of Southern blots of *Sst*I-digested HSV-1 (strain KOS) DNA with either L-S junction-specific or *a* sequence-specific probes (Fig. 3).

The removal of the *a* sequences from the L-S junctions of the HSV-1::L-SΔ*a* genome was predicted to result in the loss of all three of these *Sst*I fragment families. The 3.3-kb *Sst*I fragment family originating in the L-S junction should be replaced by a single 4.1-kb *Sst*I fragment containing the Tn*5*-derived sequence tag and lacking the viral *a* sequence (Fig. 2). The results of Southern blot analysis of *Sst*I-digested HSV-1::L-SΔ*a* DNA confirmed that the 3.3-kb fragment family was indeed missing and that a novel 4.1-kb fragment could be detected which hybridized to L-S junction-specific and Tn*5*-specific probes but not to an *a* sequence-specific probe (Fig. 3). This demonstrated that the *a* sequence had been successfully deleted from the L-S junctions of this virus. As a result of this loss, terminal fragments resulting from cleavage and encapsidation at the L-S junction should be absent in HSV-1::L-SΔ*a* DNA. This was confirmed by the absence of both the 2.4-kb S terminal fragment and the 1.2-kb L terminal fragment family in *Sst*I digestions of viral DNA (Fig. 3). A 1.2-kb *Sst*I fragment family was detected by Southern blot analysis of HSV-1::L-SΔ*a* DNA which comigrated with the 1.2-kb L terminal fragment family of the wild-type virus (Fig. 3). However, this was in fact derived from the copy of the *a* sequence (and its amplified derivatives) that had been inserted into the TK gene of HSV-1::L-SΔ*a* (Fig. 2), as confirmed by its hybridization to

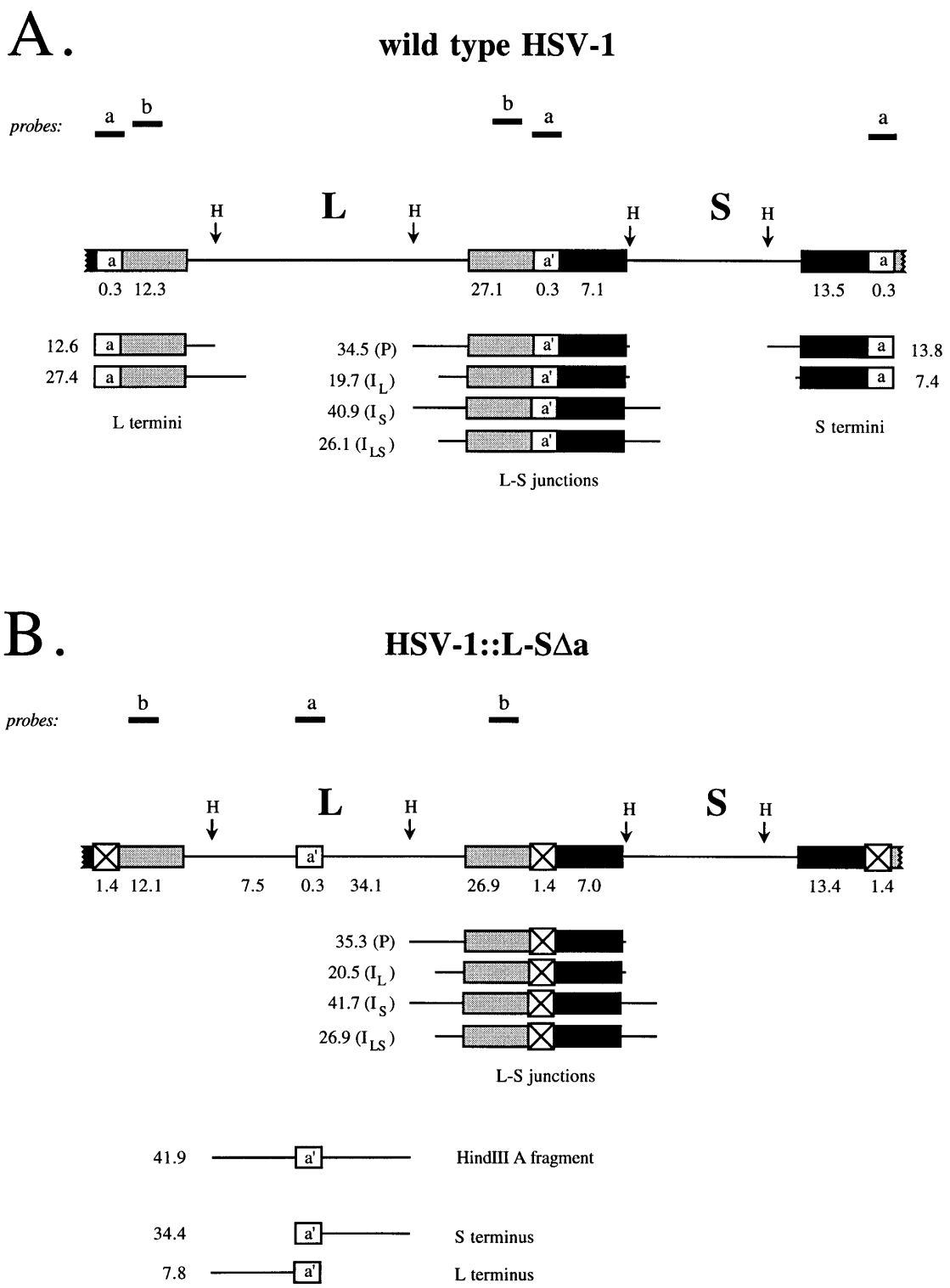

FIG. 4. *Hin*dIII fragment maps of wild-type HSV-1 (strain KOS) and the *a* sequence deletion mutant HSV-1::L-SΔ*a*. The locations of relevant *Hin*dIII (H) sites in the genomes of HSV-1 (strain KOS) (A) and HSV-1::L-SΔ*a* (B) are denoted by arrows. The structures and sizes of those *Hin*dIII fragments recognized by the indicated *a* and *b* sequence-specific probes (*a* and *b*, respectively) are shown below the genomes. The isomeric source of each of the four L-S junction fragments is identified by the standard convention of prototype (P), inverted L (I_L), inverted S (I_S), and inverted L and S (I_{LS}). The inverted repeat sequences *a*, *b*, and *c*, are shown as white, grey, and black boxes, respectively; the locations of the coupled *a* sequence deletion and Tn*5* fragment insertion in the HSV-1::L-SΔ*a* genome are denoted by white boxes containing an X.

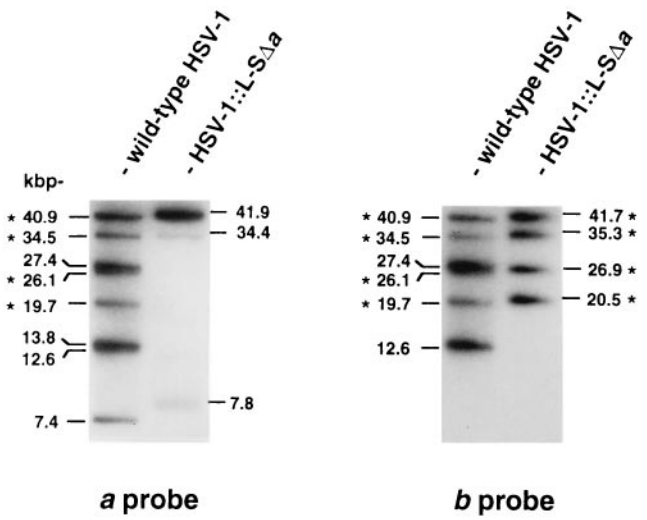

FIG. 5. Southern blot analysis of *Hin*dIII-digested wild-type HSV-1 (strain KOS) and *a* sequence deletion mutant HSV-1::L-SΔ*a* DNAs. Viral DNA was digested with *Hin*dIII, and the resulting fragments were separated by pulsed-field gel electrophoresis. Following alkaline transfer, the membranes were hybridized with the *a* sequence-specific and *b* sequence-specific probes shown in Fig. 4. The molecular sizes of the fragments are indicated beside each lane; asterisks indicate fragments containing L-S junctions.

TK-specific and GAL4-specific probes (19). This 1.2-kb fragment family represented the only *Sst*I fragments in HSV-1::L-SΔ*a* DNA that hybridized to the *a* sequence-specific probe, verifying that the *a* sequence which had been inserted into the TK gene represented the only copy of this element that remained in this virus.

The successful removal of the *a* sequences from the HSV-1::L-SΔ*a* genome was also apparent in the failure of an *a* sequence-specific probe to hybridize to those viral *Hin*dIII fragments which contained L-S junction sequences. In wild-type HSV-1, there are eight *Hin*dIII fragments containing *a* sequences which should be recognized by this probe: four fragments mapping to L-S junctions that are unique to each of the four isomers of viral DNA, two fragments mapping to the L terminal end of the genome, and two fragments mapping to the S terminal end of the genome (Fig. 4). Positive hybridization to an *a* sequence-specific probe was observed for all eight of these fragments (Fig. 5). The four fragments which correspond to the genomic termini of wild-type HSV-1 DNA are absent in HSV-1::L-SΔ*a* (Fig. 5), since the latter virus generates novel L and S terminal ends as a result of cleaving and packaging at the *a* sequence inserted into its TK gene (Fig. 4). However, as demonstrated with a *b* sequence-specific probe below, the four L-S junction fragments present in wild-type HSV-1 DNA were also found in HSV-1::L-SΔ*a*. Nevertheless, these fragments did not hybridize to the *a* sequence-specific probe (Fig. 5), since each had undergone deletion of this element (Fig. 4).

Thus, none of the eight *a* sequence-containing fragments in *Hin*dIII-digested wild-type HSV-1 were detected in HSV-1::L-SΔ*a*; however, three novel fragments were detected by the *a* sequence-specific probe in this virus (Fig. 5). The largest of these fragments was the 41.9-kb TK gene-containing *Hin*dIII A fragment into which the *a* sequence had been inserted in the first step of the construction of HSV-1::L-SΔ*a*. This fragment is cleaved into 34.4- and 7.5-kb fragments during encapsidation of viral DNA, both of which contain an *a* sequence (Fig. 4) and which were therefore capable of hybridizing to an *a* sequence-specific probe (Fig. 5). These two fragments, but not their 41.9-kb parent, were detected in analyses of DNA purified from virions (19), confirming this precursor-product relationship and the identity of the 34.4- and 7.5-kb fragments as the new termini of the HSV-1::L-SΔ*a* genome.

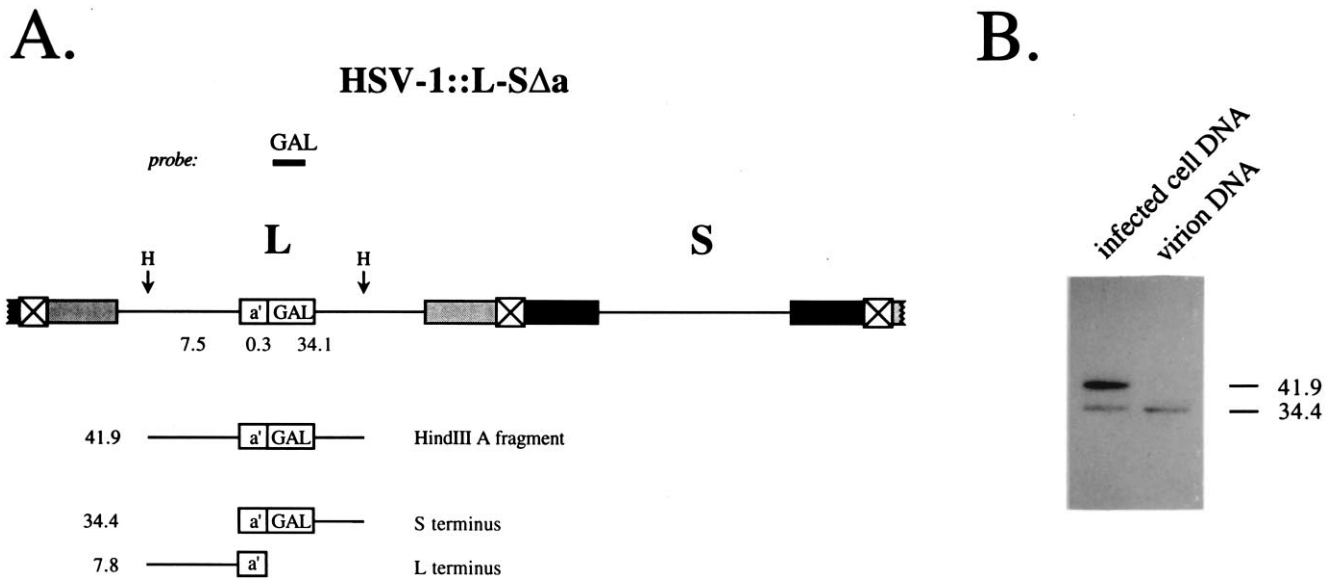

FIG. 6. Southern blot analysis of *Hin*dIII-digested HSV-1::L-SΔ*a* virion DNA. (A) *Hin*dIII fragment map of HSV-1::L-SΔ*a*. The locations of relevant *Hin*dIII (H) sites are marked by arrows. The structures and sizes of those *Hin*dIII fragments recognized by the indicated GAL4-specific probe (GAL) are shown below the genome; note that the 7.8-kb L terminal fragment will not be recognized by this probe. The inverted repeat sequences *a*, *b*, and *c*, are shown as white, grey, and black boxes, respectively; the locations of the coupled *a* sequence deletion and Tn*5* fragment insertion in the HSV-1::L-SΔ*a* genome are denoted by white boxes containing an X. (B) Southern blot analysis of *Hin*dIII-digested HSV-1::L-SΔ*a* DNA. DNA isolated from either infected cells or purified virions was digested with *Hin*dIII, and the resulting fragments were separated by pulsed-field gel electrophoresis. Following alkaline transfer, the membranes were hybridized with the GAL4-specific probe shown in panel A. The molecular sizes (in kilobases) of the fragments are indicated to the right of the blot.

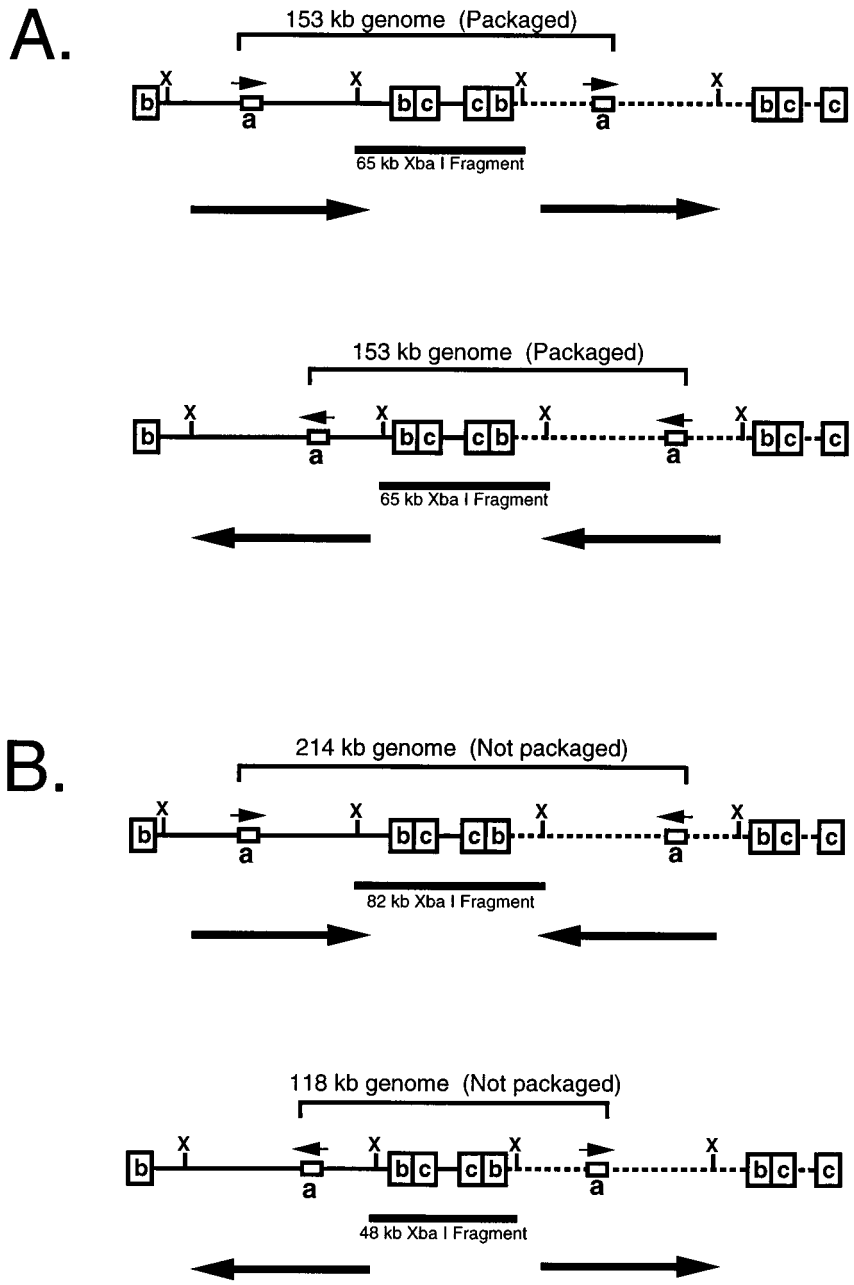

FIG. 7. *Xba*I fragments resulting from inversion of the L component of the HSV-1::L-SΔ*a* genome. Two adjacent genomes in a replicating concatamer are depicted (solid line and dashed line); their *a*, *b*, and *c* sequences are denoted by white boxes, and the orientations of their *a* sequences and L components are indicated by small and large arrows, respectively. For the sake of simplicity, only those *Xba*I sites (X) which flank the L-S junctions of the two genomes are shown. The distances between the *a* sequences for each possible arrangement are indicated above the genomes, and the sizes of the predicted L-S junction-containing *Xba*I fragments are included below the genomes. Of the four possible outcomes illustrated, two are predicted to created genomic DNA that can be packaged into capsids (A) while the remaining two are not (B).

One such analysis is shown in Fig. 6, where viral DNA was hybridized with a probe specific for the GAL4 fragment that had been linked to the *a* sequence before it was inserted into the TK gene of HSV-1::L-SΔ*a*. *Hin*dIII-digested DNA isolated from infected cells, which represents a mixture of concatameric replication intermediate DNA as well as unit-length DNA that has been packaged into capsids, contained both the 41.9-kb precursor fragment and the processed 34.4-kb S terminal fragment. However, DNA that had been isolated directly from purified virions contained only the 34.4-kb S terminal fragment (Fig. 6); the 7.8-kb L terminal fragment was not detected in this analysis because it lacks the GAL4 sequences recognized by the probe used. The appearance of these novel terminal fragments and the absence of the four terminal fragments present in wild-type HSV-1 were consistent with cleavage and packaging of HSV-1::L-SΔ*a* DNA initiating within the TK gene and not at the L-S junctions. This observation further confirmed the deletion of the *a* sequence from the latter regions of the HSV-1::L-SΔ*a* genome.

If normal genome isomerization were occurring in

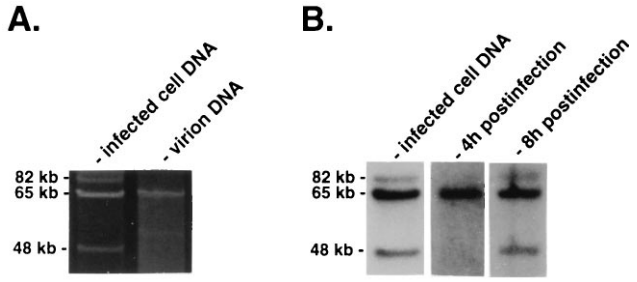

FIG. 8. Analysis of *Xba*I-digested HSV-1::L-SΔ*a* DNA. (A) Analysis of *Xba*I-digested HSV-1::L-SΔ*a* virion DNA. DNA isolated from either infected cells or purified virions was digested with *Xba*I; the resulting fragments were separated by pulsed-field gel electrophoresis and stained with ethidium bromide. The molecular sizes of the fragments are indicated at the left. (B) Southern blot analysis of *Xba*I-digested HSV-1::L-SΔ*a* DNA. DNA was isolated from either infected cells that had reached complete CPE or cells that had been infected at high multiplicity for either 4 or 8 h. Each DNA was digested with *Xba*I, and the resulting fragments were separated by pulsed-field gel electrophoresis. Following alkaline transfer, the membranes were hybridized with the Tn*5*-specific probe shown in Fig. 2. Although the autoradiographic images shown are of equal intensity, the actual signal of the bands in the DNA harvested at 8 h was over an order of magnitude greater than that harvested at 4 h as a result of amplification following the onset of DNA replication during infection. The molecular sizes of the fragments are indicated at the left. The map locations of the fragments identified in either panel are illustrated in Fig. 7.

HSV-1::L-SΔ*a*, four equimolar L-S junction-derived *Hin*dIII fragments should be discernible in the viral DNA (Fig. 4). As noted above, their existence could not be determined in Southern blot analyses which utilized an *a* sequence-specific probe, since the *a* sequences had been deleted from the L-S junctions of this virus. Thus, a second probe was employed, which hybridized to a portion of the *b* sequence in the L-S junction that mapped just outside of the deletion site (Fig. 4). As predicted, this probe detected the same four L-S junction fragments in wild-type HSV-1 DNA that were recognized by the *a* sequence-specific probe (Fig. 5). More importantly, the *b* sequence-specific probe was able to identify the same four fragments in HSV-1::L-SΔ*a* DNA (Fig. 5), demonstrating the existence of four isomeric forms of genomic DNA in this virus. The relative levels of these four fragments in each virus were determined to be equimolar on the basis of densitometric analysis (19), indicating that HSV-1::L-SΔ*a* underwent genome isomerization as efficiently as wild-type HSV-1.

Exclusion of specific HSV-1::L-SΔ*a* DNA isomers during encapsidation and the kinetics of their reappearance following infection. One of the unique features of the HSV-1::L-SΔ*a* genome is that it is predicted to encapsidate only two of its four isomers. This restriction should arise whenever one of two adjacent genomes in a replicating concatamer undergoes inversion of the L component, resulting in the translocation of one of the two *a* sequences required for cleavage and packaging of the genome. This *a* sequence will no longer have the correct orientation or unit-length distance relative to that of the second *a* sequence to promote normal DNA encapsidation (Fig. 7). To confirm this prediction, viral DNA was digested with *Xba*I, which generates fragments that are unique to each of the four possible L component orientations in two adjacent genomes. The two arrangements which contain L components in direct orientations possess *a* sequences in both the proper spacing (153 kb) and direction for packaging of unit-length DNA genomes; both are predicted to generate a 65-kb *Xba*I fragment (Fig. 7A). In contrast, the two arrangements which contain L components in inverted orientations possess *a* sequences that are both spaced improperly (214 or 118 kb) and juxtaposed incorrectly for normal packaging of viral DNA; these are predicted to generate either an 82- or 48-kb *Xba*I fragment (Fig. 7B).

Analysis of *Xba*I-digested infected-cell DNA on ethidium bromide-stained gels revealed the presence of the 82-, 65-, and 48-kb fragments (Fig. 8A). The 65-kb fragment was detected at twice the molar ratio of either of the other two fragments, indicating that it was present in two of four isomers, and the identities of all of these bands as L-S junction-containing fragments were confirmed by Southern blot analysis (Fig. 8B). Thus, all four of the hypothetical arrangements shown in Fig. 7 were detected at equimolar levels in infected cells, which again confirmed that the HSV-1::L-SΔ*a* genome undergoes normal isomerization. However, when DNA isolated from purified virions was digested with *Xba*I and examined on ethidium bromide-stained gels, only the 65-kb fragment was detected (Fig. 8A). The absence of the 82- and 48-kb fragments in this DNA confirmed that inversion of the L component does prevent two of the four isomers of the replicated HSV-1::L-SΔ*a* genome from being packaged, as predicted in Fig. 7.

Although these results indicated that HSV-1::L-SΔ*a* delivers only two of its four genome isomers into a cell at the onset of infection, the high levels of isomerization observed in this virus indicate that the two missing isomers must be rapidly regenerated during the replication cycle. Thus, the rates of reappearance of the two unpackagable isomers were measured and used as a direct indicator of the kinetics of HSV-1::L-SΔ*a* genome isomerization. DNA isolated from cells infected at a high multiplicity of infection with HSV-1::L-SΔ*a* for various lengths of time were analyzed for the rate of reappearance of the 82- and 48-kb *Xba*I fragments. At 4 h postinfection, only low levels of the 65-kb *Xba*I fragments derived from the unreplicated input genomes could be detected. However, equimolar amounts of all four *Xba*I fragments were apparent by 8 h postinfection, which represented the earliest time point at which replicated viral DNA could be detected (Fig. 8B). This remarkably high recombination rate was comparable to that reported in similar studies with wild-type HSV-1 (1), thereby confirming that the deletion of the *a* sequence from the L-S junction of HSV-1::L-SΔ*a* has no apparent effect on the kinetics of genome isomerization in this virus.

Replicative properties of HSV-1::L-SΔ*a*. HSV-1::L-SΔ*a* typically formed plaques on Vero cells that were difficult to visualize without the aid of a microscope, indicating that this virus possessed a severe replication defect. To investigate this possibility, the replicative abilities of HSV-1::L-SΔ*a* and its HSV-1 (strain KOS) parent were compared in experiments involving determination of virus yields from low-multiplicity infections. Stocks of both viruses were diluted and used to infect 60-mm-diameter dishes. Plates which contained approximately 200 plaques were counted, frozen, thawed, and used in virus titer assays. The final virus titers were then divided by the initial numbers of plaques to determine plaque yield values. HSV-1::L-SΔ*a* had a plaque yield of 46 PFU per plaque, which was more than 4 orders of magnitude lower than the 7.6×10^5 PFU per plaque obtained for wild-type HSV-1 (strain KOS). Possible reasons for this dramatic replication defect are discussed below.

DISCUSSION

A recombinant HSV-1 was constructed in this study which had acquired engineered deletions of all copies of the *a* sequence from its L-S junctions. The genome of this virus, HSV-1::L-SΔ*a*, was found to exist in four equimolar isomeric

forms, and could undergo isomerization at a rate that was indistinguishable from that of wild-type DNA. These results represent the first unequivocal demonstration that the *a* sequence is not required for isomerization of the HSV-1 genome. This finding was consistent with several lines of earlier experimental evidence which together supported the dispensability of this element in HSV-1 genome isomerization; each of these lines of evidence will be briefly summarized below.

First, genome recombination events (both inversion and deletion) can be mediated by HSV-1 sequences which lack any homology to the *a* sequence, including the *Hin*dIII O fragment, the *Bam*HI L fragment, a fragment containing the ICP4 gene promoter, a fragment derived from the *c* sequence, and the inverted repeats of a bacterial transposon, Tn*5* (12, 13, 24–26, 38, 39). In the last instance, inversion events in the Tn*5* transposon were shown through an extensive mutational analysis to occur in the complete absence of sequence specificity (39). Consistent with these results, fine-structure analyses of the *a* sequence have repeatedly proven unable to map discrete elements that are absolutely required for the alleged site-specific recombination event (4, 9, 30, 33, 38).

Second, studies with double jointed genomes bearing *a* sequence duplications have demonstrated that these isolated elements are unable to promote the same high frequency of inversion events as the intact L-S junction, which contains the *b* and *c* sequences in addition to the *a* sequence (4, 33, 38). Moreover, an *a* sequence from HSV-1 that was inserted into the HSV-2 genome was completely unable to mediate any genome isomerization events, even though it retained the ability to promote cleavage and encapsidation of HSV-2 DNA (35). In agreement with these findings, no detectable enhancement in the frequency of intermolecular recombination events was observed between the *a* sequence-containing L-S junction sequences of two different genomes compared with the nonreiterated sequences of the same genomes (36).

Third, inversion events in the HSV-1 genome were shown to be directly mediated by the seven virus-encoded enzymes which make up the HSV-1 DNA replication machinery rather than a single protein (39). In fact, there is currently no evidence for the existence of the single recombinase function that is predicted by the site-specific recombination model in HSV-1. It is unlikely that the cellular nuclease described by Wohlrab et al. (42) which promotes in vitro cleavage of anisomorphic conformations formed by the DR2 repeat arrays of the *a* sequence is responsible for recombination events in vivo, since these conformations can be observed only on supercoiled plasmids (43), and the DR2 repeat arrays themselves are dispensable for high-frequency recombination at the *a* sequence (30, 33). Moreover, the role of the alleged *a* sequence-specific recombinase described by Bruckner et al. (2) in HSV-1 genome isomerization is also questionable, since it is present at high levels in mock-infected cells and functions in the absence of HSV-1 DNA replication, both of which represent situations that fail to support HSV-1 inversion events in all studies carried out to date.

Fourth, although an isolated *a* sequence can behave as a recombinational hot spot when duplicated in the viral genome, this property was shown to be the result of the creation of a recombinogenic double-stranded break within the pac2-DR1-pac1 arrangement of the *a* sequence during cleavage and packaging events (30, 33). The recombinogenic properties of the *a* sequence, but not normal replication-mediated recombination, were shown to disappear in cells that were infected by an HSV-1 cleavage-packaging mutant, and the same properties could be conferred on a non-HSV-1 DNA sequence, provided that it was cleaved in vivo by a restriction endonuclease (30). This last observation directly demonstrated that the viral *cis*- and *trans*-acting components that are responsible for the special properties of the *a* sequence could be functionally replaced by a DNA sequence and endonuclease which were both bacterial in origin. This indicated that only a double-strand break, and not specific viral sequences (such as the pac elements of the *a* sequence), is required for converting a segment of the HSV-1 genome into a hot spot for homologous recombination.

Finally, the highly recombinogenic nature of an isolated *a* sequence was shown to be abrogated when it was flanked by large regions of extended homology that were capable of undergoing generalized recombination, including the *b* and *c* sequences which normally flank the *a* sequence in the HSV-1 genome (30). Conversely, by using a highly quantitative plasmid-based recombination assay, deletion of the *a* sequence was found to have no adverse effect on the recombinogenic potential of an L-S junction (30). The latter result is consistent with the properties of both the HSV-1::L-SΔa virus in this study, whose L-S junctions lacked *a* sequences and yet still promoted normal L and S component inversion, as well as a previously characterized HSV-1 mutant whose L-S junctions had undergone a deletion event that retained only a portion of the *b* sequence and yet still promoted significant L component inversion (18).

The results of this study therefore validate the homologous recombination model of herpesvirus genome isomerization (30, 33, 35, 38, 39, 41) and formally disprove the site-specific recombination model (4, 21–23). It is now clear that while the *a* sequence may be sufficient for promoting recombination events in the viral genome independent of other sequences (4, 33, 38), the behavior of the HSV-1::L-SΔa virus unequivocally demonstrates that this element is not required for this process. In fact, the equimolar levels of isomeric DNA observed in this mutant indicate that the relative contribution of *a* sequence-directed recombination events to genome isomerization is dwarfed by the role of homologous recombination events occurring across the larger *b* and *c* sequences, as predicted in previous studies (30, 38). The potential for homologous recombination to generate isomeric forms of HSV-1 DNA is even more dramatic when it is considered that the L and S components themselves can serve as extended inverted repeats in the recombination reaction. For example, inversion of the L component occurs as a result of recombination between a combined 16 kb of inverted repeat homology created by the *b* and *c* sequences of the L-S junctions of a replicating concatamer (Fig. 9). However, subsequent inversion of the S component will then be able to occur through a combined 140 kb of inverted repeat homology created by an L component and two L-S junctions in the concatamer (Fig. 9). Similarly, an initial S component inversion event will generate 45 kb of inverted repeat homology created by an S component and two L-S junctions through which subsequent L component inversion can take place. With the existence of these extended stretches of inverted repeat homology in replicated viral DNA, it is not difficult to envision why removal of an element as small as the *a* sequence had no apparent effect on the overall frequency of genome isomerization in HSV-1::L-SΔa.

Interestingly, the HSV-1::L-SΔa virus was found to possess a substantial replication defect, as it typically formed very small plaques on Vero cell monolayers which generated yields of infectious virus that were more than 4 orders of magnitude lower than that of wild-type HSV-1. Although the nature of this phenotype is still unclear and is under investigation at the present time, several possible explanations can be imagined. First, a genome equivalent of HSV-1::L-SΔa possesses half the number of *a* sequences that wild-type HSV-1 does for cleavage

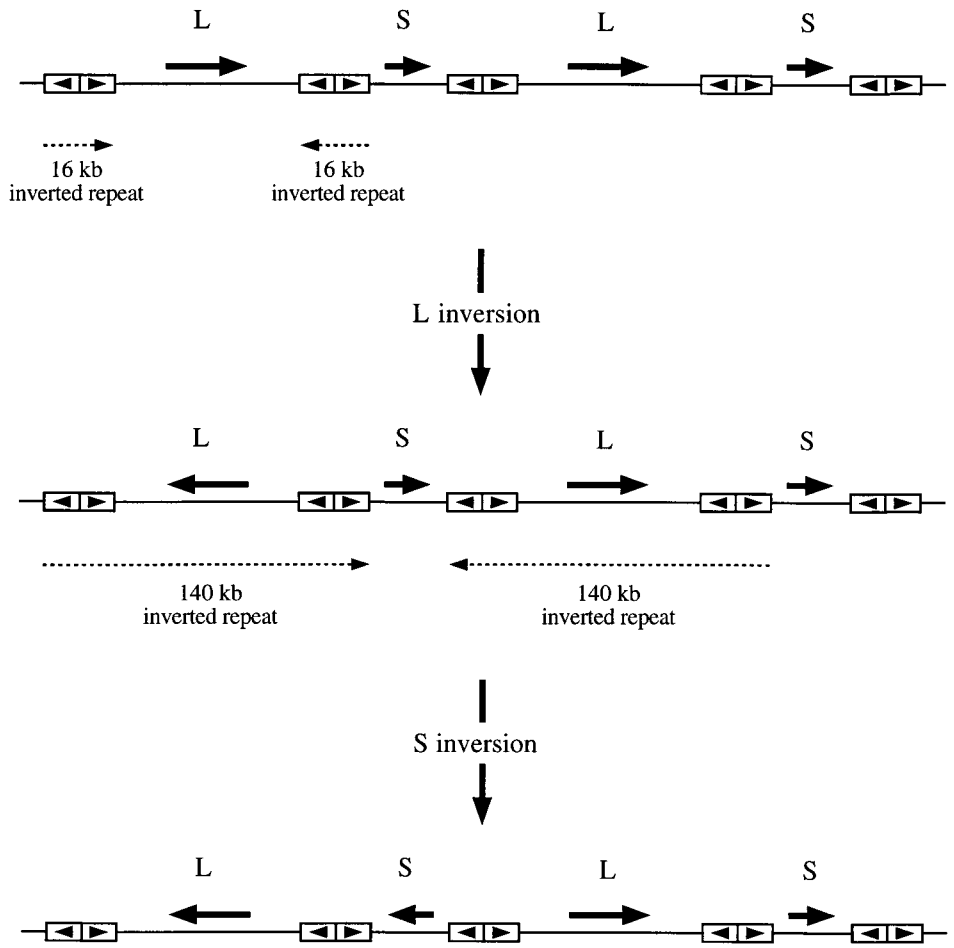

FIG. 9. HSV-1 genome isomerization promoted by large inverted repeats containing the unique segments of the viral DNA. Two adjacent genomes in a replicating concatamer are depicted; the large inverted repeats of their L-S junctions are denoted by white boxes with arrows, and the orientations of their L and S components are indicated by large arrows. Inversion of either component results in the formation of very large templates of inverted repeat homology through which additional recombination events can occur (see text for details).

and packaging, thereby potentially reducing its encapsidation efficiency. Second, previous studies have shown that an *a* sequence duplication at the TK locus generates lower numbers of terminal fragments than those generated by an *a* sequence at its native site in the L-S junction (33); this is presumably because flanking elements of the *b* and *c* sequences of the L-S junction enhance cleavage and encapsidation at the *a* sequence. The absence of these flanking elements at the TK locus in HSV-1::L-SΔ*a* suggests that the *a* sequence at this site may be similarly underutilized, and the significant reduction in the levels of terminal fragments generated in this virus compared with that of wild-type HSV-1 (Fig. 5) would seem to support this hypothesis. Third, only two of the four isomers of HSV-1::L-SΔ*a* were found to be packaged into virions (Fig. 7 and 8). Thus, fully half of the replicated DNA generated by this virus during infection appears to be completely wasted. It is probable that the summed contribution of each of these individual factors almost certainly results in the marked replication defect of HSV-1::L-SΔ*a*.

Finally, the ability to manipulate essential *cis*-acting sites by using the strategy described in this report is a powerful tool that could be applied to any viral genome for which marker transfer technology exists. In addition to targeting DNA maturation signals like the HSV-1 *a* sequence, the effects of removing other *cis*-acting elements such as origins of DNA replication or promoter-enhancer regions from their natural context in a viral genome can now be assessed. This level of genetic analysis has not previously been possible, since deletion of such essential sequences is necessarily lethal to the virus. The mutagenesis scheme employed in this work has very few restrictions that could limit its successful application to other viruses: the *cis*-acting element must be active in its new locus so that it can effectively mediate *cis* complementation, duplication of the element in the genome must have a neutral effect on virus replication, and a selection strategy may be necessary if the final recombinant virus possesses unique properties that provide a barrier to purification. All of these criteria were readily met in the construction of a recombinant HSV-1 that lacks *a* sequences at its L-S junctions and should not be difficult to satisfy in other systems as well.

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