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The Herpes Simplex Virus Type 1 (HSV-1) *a* Sequence Serves as a Cleavage/Packaging Signal but Does Not Drive Recombinational Genome Isomerization When It Is Inserted into the HSV-2 Genome

JAMES R. SMILEY,* CAROL LAVERY, AND MARGARET HOWES

Molecular Virology and Immunology Program, Pathology Department, McMaster University, Hamilton, Ontario, Canada L8N 3Z5

Received 15 May 1992/Accepted 24 August 1992

We inserted the terminal repeat (*a* sequence) of herpes simplex virus type 1 (HSV-1) strain KOS into the *tk* gene of HSV-2 strain HG52 in order to assess the ability of the HSV-1 *a* sequence to provoke genome isomerization events in an HSV-2 background. We found that the HSV-1 *a* sequence was cleaved by the HSV-2 cleavage/packaging machinery to give rise to novel genomic termini. However, the HSV-1 *a* sequence did not detectably recombine with the HSV-2 *a* sequence. These results demonstrate that the viral DNA cleavage/packaging system contributes to a subset of genome isomerization events and indicate that the additional recombinational inversion events that occur during infection require sequence homology between the recombination partners.

The genome of herpes simplex virus (HSV) is a ca. 150-kb linear DNA duplex that consists of a long (L) and a short (S) segment flanked by large inverted repeats, termed *b* and *c*, respectively. In addition, the genome is terminally redundant, and an inverted copy of the terminal repeat (or *a* sequence) is present at the L-S junction (Fig. 1) (reviewed in reference 23). The L and S segments invert freely, giving rise to four isomeric forms of viral DNA (10, 25, 26, 39). Although genome segment inversion is a common feature of herpesvirus genomes (24), the mechanism and functional significance of this process remain unclear.

The *a* sequence serves as the target of at least two types of DNA transactions that could contribute to genome isomerization. First, it bears the recognition sequences for the viral DNA cleavage/packaging system (6, 29, 33, 35). HSV DNA circularizes by end-to-end ligation early during infection and gives rise to large "endless" replicative intermediates (perhaps head-to-tail concatemers); these intermediates are then processed to mature linear unit-length genomes by site-specific cleavage at *a* sequences (11–13, 20, 30, 32, 33, 35, 36). The cleavage reaction involves two site-specific breaks that are made at defined distances from the *pac1* and *pac2* signals located within the *a* sequence (27, 35). In addition, the maturation process duplicates the DNA sequences that lie between these breaks so that L-S junctions bearing a single *a* sequence give rise to two termini, each bearing an *a* sequence (7, 35). The *pac1* and *pac2* sequences are the only regions of strong homology among the *a* sequences of a variety of herpesvirus genomes (1, 3, 4, 9, 15, 16, 18, 34); therefore, the finding that several herpesvirus cleavage/packaging systems can process heterologous *a* sequences (30, 31) implies that the *pac* homologies are sufficient for the reaction.

The potential for genome isomerization mediated by the cleavage/packaging system stems from the presence of an inverted copy of the *a* sequence at the L-S junction; as diagrammed in Fig. 1, cleavage of concatemers or circles derived from one isomeric form of HSV DNA can produce one other isomer in which both segments are inverted. This hypothetical pathway is therefore potentially capable of directly generating half of the observed genome segment inversion events. If one further proposes that the two resulting isomers can undergo end-to-end ligation in subsequent rounds of infection, this pathway might also contribute to the remaining isomerization events. However, the extent to which the use of alternative cleavage frames and end-to-end ligation contribute to genome isomerization has not yet been directly assessed.

The *a* sequence also drives recombination events that are potentially capable of generating all four genome isomers. Thus, HSV recombinants bearing an additional copy of the *a* sequence inserted into the thymidine kinase (*tk*) gene display all of the additional genome isomers that are predicted to arise from recombination between the novel *a* insert and the resident *a* sequences located at the L and S termini and the L-S junction (2, 19, 27, 28, 35), and at least some of these products have been shown to arise through bona fide recombination events (27). Mocarski et al. (19) initially proposed that these recombination events are site specific and uniquely targeted to *a* sequences. However, it has since become apparent that a variety of other DNA fragments are also able to induce analogous rearrangements when they are duplicated in the HSV genome (14, 21, 22, 34, 37). Nevertheless, the *a* sequence appears to be considerably more recombinogenic than other DNA fragments of equivalent length, implying that its activity is boosted by specialized processes (8, 27, 37, 38).

We recently conducted an extensive analysis of the *cis*-acting sequences required for the recombinational activity of the HSV type 1 (HSV-1) *a* sequence (27). Our results indicated that completely nonoverlapping segments of the *a* sequence had detectable activity, implying that recombination does not invariably occur by cleavage and ligation reactions at a single specific site. An important finding to emerge from this analysis was that the tandemly reiterated sequences present within the *a* sequence, previously pro-

* Corresponding author.

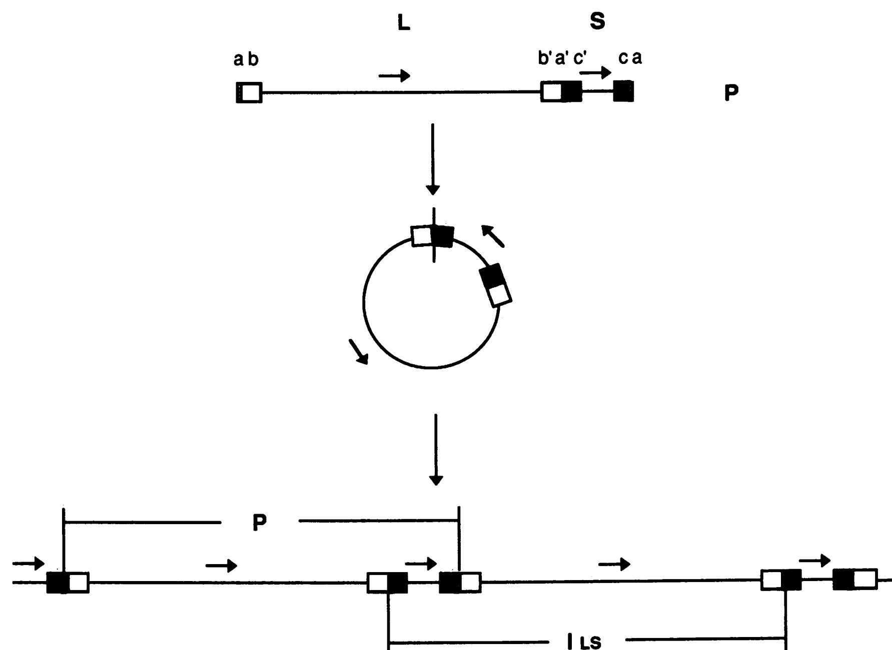

FIG. 1. Structure and replication of the HSV genome. The figure shows the structure of the HSV genome and illustrates the potential for genome isomerization driven by the viral DNA cleavage/packaging system. In the case depicted here, concatemers or circles derived from the prototype (P) isomer can be processed to produce either the P or the inverted L-inverted S (I_{LS}) arrangement.

posed to serve as the target of a site-specific recombination system (2), could be deleted without eliminating recombinational activity. Although this analysis did not identify specific sequences within the *a* sequence that are essential for recombination, we found that recombination was strongly dependent on the integrity of the sequences immediately adjacent to the positions of *pac*-dependent DNA cleavage. Taken in combination, these data led us to propose that the site-specific DNA cleavages induced by the cleavage/packaging system stimulate the initiation of generalized recombination by providing recombinogenic free DNA ends. This model predicts that inter-*a* recombination requires extended DNA sequence homology between the recombining partners.

The *a* sequences of HSV-1 and HSV-2 display essentially no sequence homology, aside from short regions spanning the *pac*1 and *pac*2 signals (3). A previous report has shown that the HSV-2 cleavage/packaging system can cleave transfected HSV-1 *a* sequences (30), but it is not clear from the published literature whether the HSV-1 and HSV-2 *a* sequences are capable of recombining with each other. In one report that bears on this issue, Davison and Wilkie (5) found that HSV-1 × HSV-2 recombinants bearing heterotypic *ba* or *ca* repeats displayed reduced but detectable levels of genome isomerization. However, the interpretation of these observations was complex, for two reasons. First, the heterotypic *a* sequences were flanked by large regions of partial homology contributed by the heterotypic *b* and/or *c* repeats. Second, these recombinants were unstable and gave rise to freely inverting derivatives in which partial identity between the repeats was restored by recombination or conversion events. Although some of these recombination events involved crossovers between HSV-1 and HSV-2 sequences within or close to the heterotypic *a* sequences, crossover events also occurred within the heterotypic *b* and *c* regions. Therefore, this study did not indicate whether the HSV-1 and HSV-2 *a* sequences are able to recombine in the absence of extended flanking-sequence homology.

We inserted a 317-nucleotide (nt) *a* sequence derived from HSV-1 strain KOS into the *tk* gene of HSV-2 strain HG52 in order to determine whether the cleavage/packaging reaction could be uncoupled from recombinational genome isomerization within the context of the viral genome. Plasmid pA23 bears the HSV-1 *a* sequence (lacking any additional flanking HSV sequences) inserted at the *Bam*HI site in pUC18 (27). We first modified pA23 by inserting a *Kpn*I linker into the *Hinc*II site in the polylinker; this manipulation allowed the *a* sequence and flanking polylinker DNA to be excised as a *Kpn*I fragment. The *Kpn*I *a* fragment was then cloned in both orientations into the unique *Kpn*I site in plasmid L3PK1KpnΔ (Fig. 2). L3PK1KpnΔ carries a mutant version of the HSV-2 *tk* gene which lacks a 180-nt internal *Kpn*I fragment; this deletion inactivates TK activity (17). The resulting *tk*-deficient deletion/substitution mutations were then transferred into the genome of HSV-2 HG52 by DNA-mediated marker rescue. Following selection of *tk*-deficient progeny by plaque purification in the presence of 100 μg of thymine arabinoside per ml, recombinants bearing the desired inserts were identified by Southern blot hybridization of *Bam*HI-cleaved viral DNA (data not shown). We chose two recombinants for further analysis. One, Ub5′, bore the HSV-1 *a* sequence oriented with its Ub sequence closest to the 5′ end of the *tk* gene; the other, Uc5′, carried the *a* sequence in the opposite orientation.

We first verified that the recombinant viral genomes bore the predicted insert within the *tk* gene. The *Kpn*I fragment bearing the inserted *a* sequence contains two internal *Bam*HI sites contributed by pUC polylinker sequences (Fig. 2). To test for the acquisition of these sites, packaged viral DNA was extracted from cytoplasmic nucleocapsids, cleaved with *Bam*HI, and then examined by Southern blot hybridization with radiolabeled L3PK1KpnΔ plasmid DNA

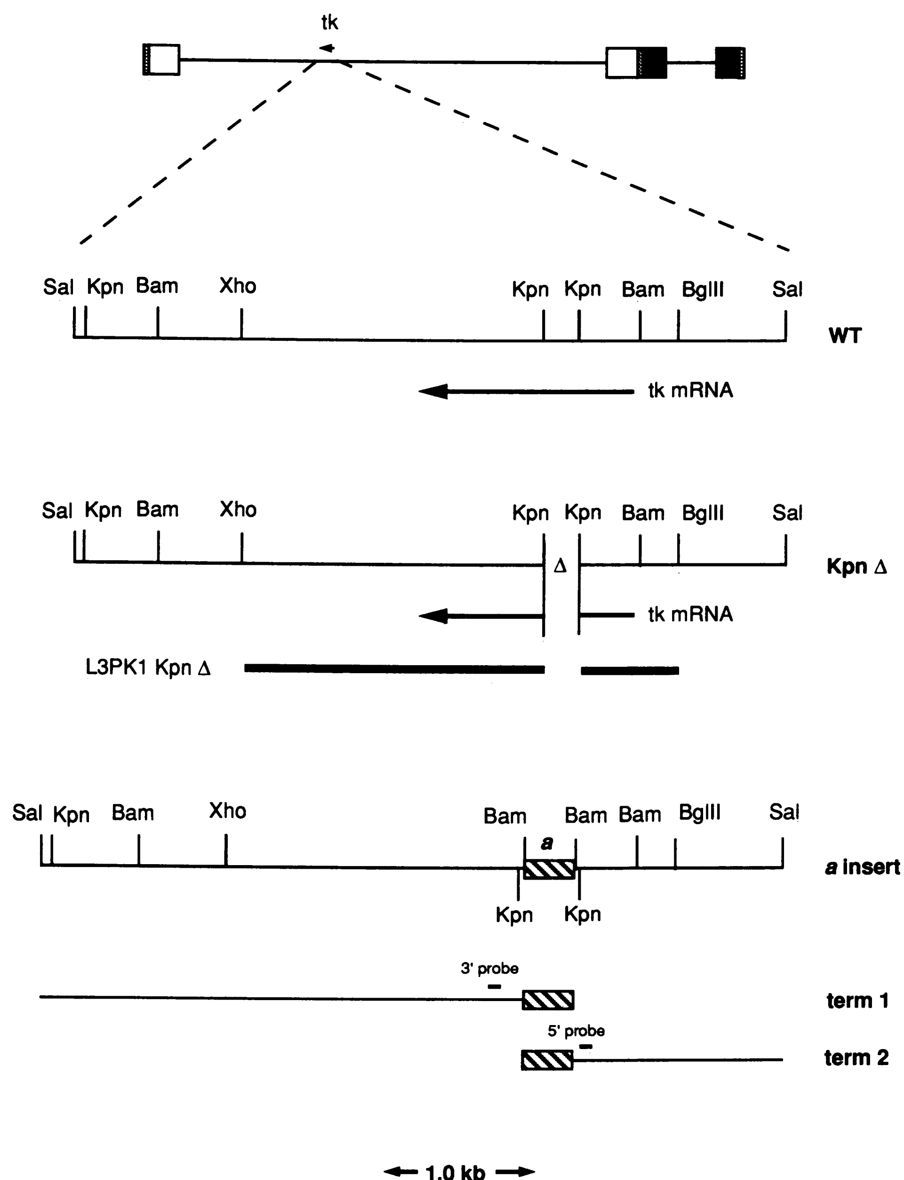

FIG. 2. Structures of recombinant viral genomes. The upper portion of the figure depicts the structure of the *tk* region of the HSV-2 strain HG52 genome (wild type [WT]). Recombinant viral genomes (*a* insert) were produced by inserting a *Kpn*I fragment bearing the HSV-1 *a* sequence into the *Kpn*I site on plasmid L3PK1KpnΔ (KpnΔ). This plasmid bears *tk* sequences extending from the *Xho*I to the *Bgl*II site and lacks a 180-nt *Kpn*I fragment found in wild-type DNA. The lower portion of the figure depicts the predicted structures of novel termini produced by cleavage at the inserted *a* sequence and indicates the oligonucleotide probes used in Fig. 4 (3′ probe and 5′ probe).

as a probe (Fig. 3). The probe detected fragments of 3.9 and 3.2 kb with wild-type HSV-2 DNA; the 3.2-kb fragment spans the site of the *a* insert in recombinants Ub5′ and Uc5′. As predicted, the 3.2-kb fragment was replaced by fragments of 2.8 and 0.4 kb in both recombinants (note that L3PK1KpnΔ does not bear an *a* sequence and therefore does not detect the ca. 320-nt *Bam*HI *a* fragment in recombinants Ub5′ and Uc5′). As another test, we looked for the predicted 120-nt increase in size of fragments that span the site of the insertion. The predicted size increase in the major hybridizing band was observed in both *Sal*I and *Bgl*II plus *Xho*I digests of virion DNA (Fig. 3, most clearly visible in the *Bgl*II plus *Xho*I digest; the origins of the additional minor bands are described below). Similar results were obtained with *Pvu*II (data not shown).

We then examined the recombinants for the presence of the predicted submolar *tk*-related fragments diagnostic of cleavage/packaging and recombinational genome segment inversion. If the inserted *a* sequence serves as a target for the HSV-2 cleavage/packaging system, packaged viral DNA should display two novel submolar termini that end within the *tk* gene (Fig. 2). Fragments of the predicted sizes were readily detected in *Sal*I and *Bgl*II plus *Xho*I digests of viral DNA (Fig. 3, terminus 1 and terminus 2 [ca. 3.4 and 1.5 kb in *Sal*I digests and ca. 2.2 and 1.1 kb in *Bgl*II plus *Xho*I digests, respectively]; note that terminus 1 and terminus 2

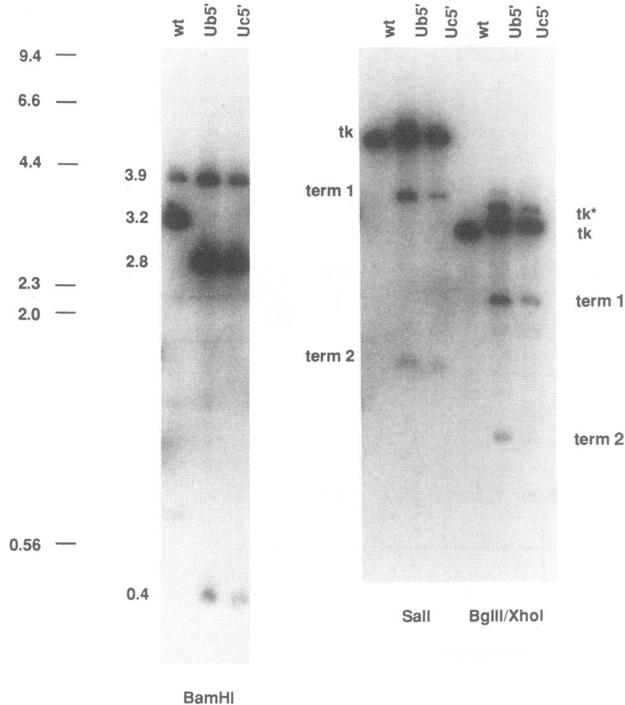

FIG. 3. Southern blot analysis of recombinant viral genomes. Viral DNA extracted from cytoplasmic nucleocapsids was cleaved with the indicated restriction endonucleases and then subjected to electrophoresis through a 0.8% agarose gel. Following transfer to a nylon membrane, the blot was hybridized to ^{32}P-labeled L3PK1KpnΔ DNA. tk, uncleaved *tk* fragment; tk*, *tk* fragment bearing two tandem *a* sequences; term 1, terminus 1; term 2, terminus 2. The sizes (in kilobases) and positions of *Hin*dIII-digested phage λ marker fragments are indicated. wt, wild type.

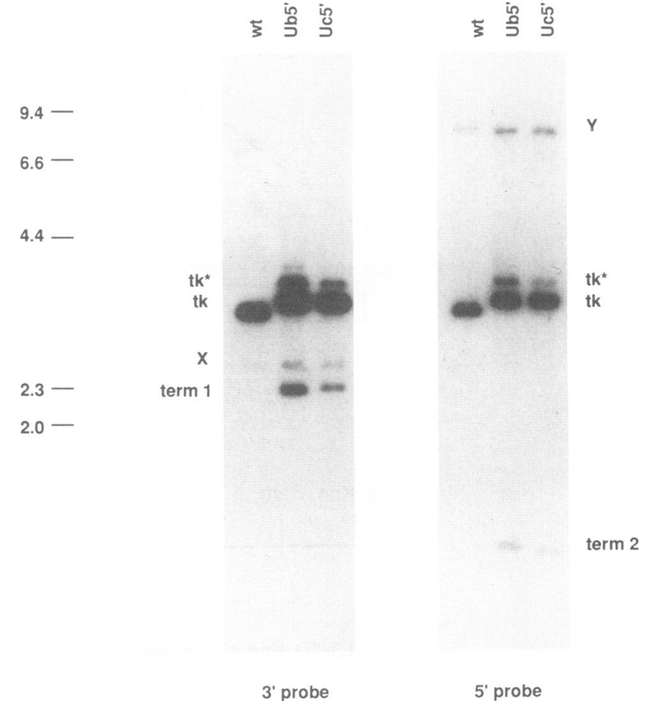

FIG. 4. Hybridization of recombinant viral DNAs to oligonucleotide probes. Viral DNA extracted from cytoplasmic nucleocapsids was cleaved with a mixture of *Bgl*II and *Xho*I and then subjected to electrophoresis through a 0.8% agarose gel. Following transfer to nylon membranes, the blot was hybridized to ^{32}P-labeled 20-mers derived from *tk* sequences 3′ and 5′ to the inserted *a* sequence (ATGTGGTACAAGTCCCCGTT and CTATCGCCTCCCTGCTG TGC, respectively). Fragment nomenclature is described in the legend to Fig. 3 and in the text. X and Y are cross-hybridizing fragments unrelated to *tk* sequences. The sizes (in kilobases) and positions of *Hin*dIII-digested λ fragments are indicated.

differ slightly in size between recombinants Ub5′ and Uc5′ because the *a* insert is not precisely centered in the *Kpn*I fragment used to produce these recombinants). The identity of the novel terminal fragments was further confirmed by hybridizing *Bgl*II- and *Xho*I-digested virion DNA to synthetic oligonucleotides derived from the 5′- and 3′-flanking *tk* DNA sequences (Fig. 4). As predicted (Fig. 2), the 3′ *tk* probe hybridized to terminus 1 (and to the uncleaved *tk* fragments), and the 5′ probe hybridized to terminus 2. Unexpectedly, each of these oligonucleotide probes also hybridized to one other fragment of unknown origin (ca. 2.5 and 9 kb for the 3′ and 5′ probes, respectively; labeled X and Y in Fig. 4). Inasmuch as the X and Y signals were also observed with wild-type viral DNA, these bands presumably represent HSV DNA fragments that cross-hybridize with the probes under the conditions used.

Previous work has shown that the cleavage/packaging system duplicates the *a* sequence (7, 35), leading to the accumulation of L-S junctions bearing tandem *a* reiterations. We observed a minor band (tk*) of the size predicted for a *tk* fragment bearing two tandem *a* sequences in the *Sal*I and *Bgl*II plus *Xho*I digests displayed in Fig. 3 and 4 (most clearly seen in the *Bgl*II plus *Xho*I digests). We cloned the tk* *Sal*I fragment from Ub5′ virion DNA and showed, by restriction endonuclease cleavage site mapping, that it bore the predicted tandem duplication of the HSV-1 *a* sequence (data not shown). Taken in combination, these data demonstrate that the HSV-1 *a* sequence was processed by the HSV-2 cleavage/packaging system, confirming previous results of transfection assays (30).

If the inserted HSV-1 *a* sequence was also able to recombine with the HSV-2 *a* sequences located at the L and S termini and L-S junction, additional novel junction fragments representing fusions of *tk* sequences to the *b* and *c* repeats would be formed (for *Sal*I digests, ca. 11.3 and 6.0 kb for recombinant Ub5′ and ca. 9.4 and 7.9 kb for recombinant Uc5′; for *Bgl*II plus *Xho*I digests, ca. 4.3 and 2.2 kb for recombinant Ub5′ and ca. 3.3 and 3.2 kb for recombinant Uc5′). However, we were unable to detect such novel junction fragments even after prolonged exposures of the Southern blots (Fig. 3 and 4). We estimate that novel junctions would have been detected easily if they were present at 5% of the abundance of inversion products observed with an analogous recombinant bearing the HSV-1 *a* sequence inserted into the HSV-1 *tk* gene (27). In addition, we were unable to detect the predicted novel *tk-c* fusion fragments following hybridization of *Pvu*II-, *Sal*I-, *Xho*I-, *Bgl*II-, or *Bgl*II- plus *Xho*I-digested Ub5′ or Uc5′ DNA to an oligonucleotide derived from the *c* region of HSV-2 DNA (data not shown). Thus, although the inserted HSV-1 *a* sequence served as a target for cleavage/packaging, it did not detectably recombine with the HSV-2 *a* sequences located elsewhere in the recombinant viral genomes. These results contrast with the relatively efficient inter-*a* recombination

that is observed following insertion of the same HSV-1 *a* fragment into the *tk* gene in the HSV-1 genome (27).

These data establish that the HSV cleavage/packaging system is capable of generating new genomic termini without provoking the formation of novel junctions. This finding demonstrates that the cleavage/packaging system contributes directly to genome isomerization through the use of alternative cleavage frames on circular or concatemeric precursors (diagrammed in Fig. 1). As noted above, this process can account for half of the genome isomerization events that occur during infection with wild-type HSV-1. The absence of novel junction fragments bearing the inserted *a* sequence further suggests that intermolecular end-to-end ligation does not measurably contribute to the additional genome segment inversions that occur during infection; more likely, these events occur entirely through bona fide crossovers between pairs of homologous inverted sequences. Our data suggest that inter-*a* recombination requires homology between the recombining partners. One interpretation is that HSV-1 and HSV-2 employ distinct specialized recombination systems that are targeted exclusively to their respective *a* sequences. However, we believe that a simpler interpretation of the available evidence is that recombination between *a* sequences occurs through a homology-dependent generalized mechanism that is stimulated by the free ends produced by the cleavage/packaging system (27). These two possibilities could be distinguished by examining additional recombinants bearing two inverted copies of the HSV-1 *a* sequence in an HSV-2 background.

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