JOURNAL OF VIROLOGY, Jan. 1994, p. 34–47
0022-538X/94/$04.00+0

Vol. 68, No. 1

Requirement for Double-Strand Breaks but Not for Specific DNA Sequences in Herpes Simplex Virus Type 1 Genome Isomerization Events

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Received 16 June 1993/Accepted 22 September 1993

Herpes simplex virus type 1 (HSV-1) genome isomerization occurs as a result of DNA replication-mediated homologous recombination between several sets of inverted repeat sequences present in the viral DNA. The frequency with which this recombination occurs has been demonstrated to be dependent upon DNA homology length rather than specific sequences. However, the smallest of the viral inverted repeats, the *a* sequence, has been shown to function as a recombinational hot spot, leading to speculation that this sequence may represent a specific element through which genome isomerization is mediated. To investigate this apparent paradox, a quantitative transient recombination assay system was developed and used to examine the recombinogenic properties of a panel of *a* sequence mutants. This analysis revealed that the presence of both the pac1 and pac2 elements was both necessary and sufficient for the induction of high-frequency recombination events by the *a* sequence. However, it was the double-strand break promoted by pac1 and pac2 during cleavage and packaging at the *a* sequence, and not the DNA sequences of the elements themselves, which appeared to be critical for recombination. This was illustrated (i) by the inability of the same pac1 and pac2 sequences to mediate inversion events in cells infected with an HSV-1 mutant which was competent for DNA replication-dependent recombination but defective for the cleavage and packaging process and (ii) by the ability of double-strand breaks generated in non-HSV-1 DNA by an in vivo-expressed restriction endonuclease to significantly stimulate the initiation of recombination events in virus-infected cells. Thus, the *a* sequence appears to act as a hot spot for homologous recombination simply because it happens to coincide with the site of the double-strand break which is generated during the cleavage and packaging process, not because it contains discrete sequences which are required for this activity. However, it was found that this enhanced recombinogenicity disappeared when the element was flanked by regions of extensive sequence homology, particularly that of the large inverted repeats which flank the *a* sequence at its natural site in the HSV-1 genome. These findings are consistent with a model for HSV-1 genome isomerization in which recombination is initiated primarily by multiple random double-strand breaks which arise during DNA replication across the inverted repeats of the genome, rather than by a single specific break which occurs at the *a* sequence during the cleavage and packaging process.

The herpes simplex virus type 1 (HSV-1) genome is a 152-kb linear double-stranded DNA molecule divided into two segments, a 108-kb long (L) component and a 13-kb short (S) component. The L and S segments are flanked by the inverted repeat sequences *b* (9 kb) and *c* (6.5 kb), respectively. The genome itself is bracketed at either end by one or more copies of a direct repeat, the *a* sequence (250 to 500 bp), and contains one to several inverted copies of the *a* sequence separating the L and S components. The HSV-1 genome can therefore be represented by the following arrangement (9, 26, 38):

ab ---------- L ---------- *b'a'c'* ------- S ------- *ca*

Four equimolar isomers of viral DNA exist during productive HSV-1 infection, since the L and S components invert relative to each other at a high frequency (7, 9). These inversions were originally postulated to be the result of a site-specific recombination event occurring at the *a* sequence, on the basis of two lines of evidence. First, deletion of the entire internal L-S junction, which includes the *a* sequence, from the viral genome resulted in the appearance of noninverting or "frozen" genomes which represented only a single isomeric form (10, 11, 21). Second, the duplication of genomic segments containing the *a* sequence, but not other unique HSV-1 sequences within the viral DNA, resulted in novel inversion events in the genome (4, 14, 15, 17). Since these initial studies suggested that only the *a* sequence was capable of mediating inversion events in the HSV-1 genome, it was concluded that the *a* sequence contained a signal for site-specific recombination which initiated this isomerization process. However, later evidence has indicated that HSV-1 genome isomerization can result from generalized recombination between any of the inverted repeat sequences flanking the L and S components. For example, a mutant virus which had lost two-thirds of the *b* sequence and the entire *a* and *c* sequences of the L-S junction was still capable of undergoing L component inversion during productive infection (12). It was also found that other HSV-1 sequences which lack homology to the *a* sequence promoted similar recombination events when duplicated in the viral genome (10, 11, 20–22, 37). Finally, when sequences of the prokaryotic transposable element Tn5 were incorporated into the viral genome, they were found to undergo inversion in a manner which was entirely analogous to L and S component inversion (39). This novel rearrangement event, which is typically not observed in the transposon's

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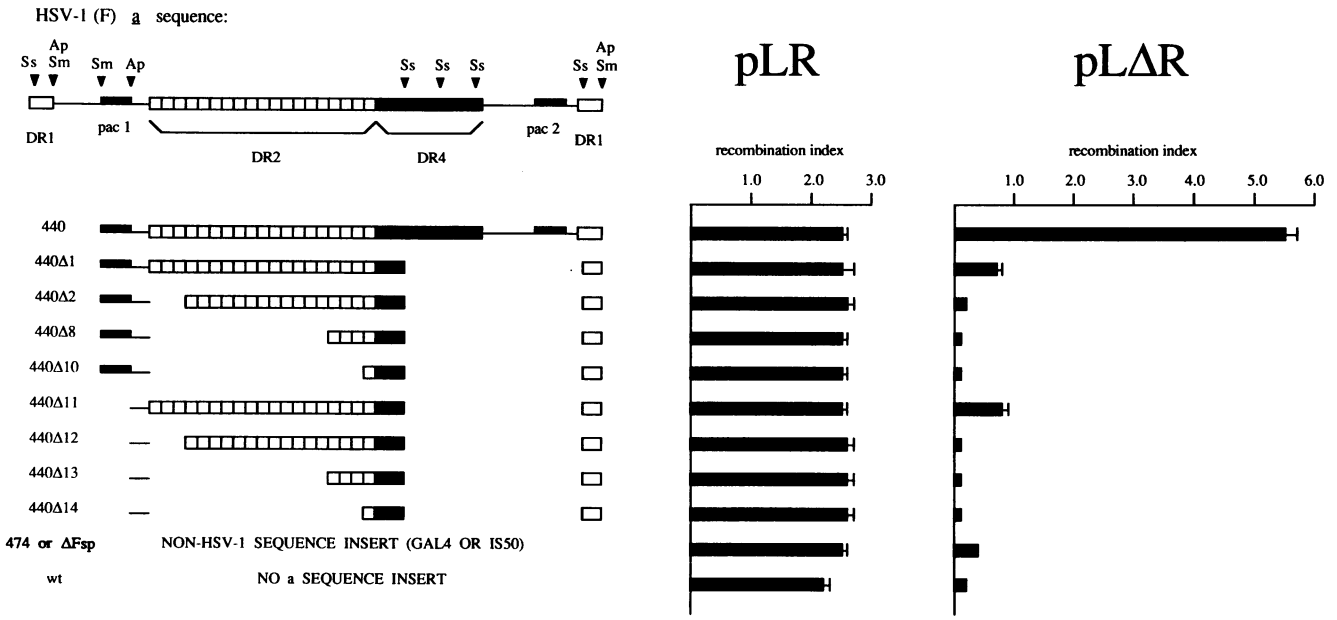

FIG. 1. Construction of HSV-1 *a* sequence derivatives containing deletions in the DR2 repeat array and quantitation of their recombinogenic potential. At left are subfragments of the HSV-1 *a* sequence containing anisomorphic and nonanisomorphic DR2 repeat arrays. 440 represents a wild-type *a* sequence derivative which possesses both pac1 and pac2 elements as well as a complete DR2 array. The eight deletion derivatives of 440 shown have lost one or both pac elements from various wild-type or mutant versions of the DR2 repeat array. 474 and ΔFsp are two non-HSV-1 DNA inserts which are composed of 474 bp of the yeast gene *GAL4* and 600 bp of Tn*5* sequences, respectively. wt, wild-type plasmid without an *a* sequence insert. Restriction sites are shown for *Apa*I (Ap), *Sma*I (Sm), and *Sst*II (Ss). At right the relative contributions of these *a* sequence subfragments to Tn*5* inversion in a plasmid transfection-superinfection assay are summarized. The recombination index (with associated standard deviation) corresponds to the ratio of inversion frequency in the *a* sequence-containing Tn*5* plasmid pLR or pLΔR to the deletion frequency in the internal standard plasmid pGAL-STD. Each plasmid transfection was performed in triplicate.

natural host, *Escherichia coli*, was shown to be caused by high-frequency homologous recombination between the duplicated inverted IS*50* elements of Tn*5*. Furthermore, the seven enzymes which constitute the HSV-1 DNA replication machinery were found to direct these recombination events independent of specific DNA sequence requirements (39).

These results indicate that any inverted repeat homology should be sufficient for recombination to occur in the HSV-1 genome. However, it remains unclear how the *a* sequence, which is apparently sufficient but not required for genome isomerization, can act as a recombinational hot spot by directing inversion events over great distances within the viral genome (4, 27, 37). The *a* sequences of different HSV-1 strains, although highly variable, contain a number of common features (5, 13, 37). These include a complex of short tandem guanosine-cytosine-rich reiterations (the DR2 repeat array), two terminal reiterations (the DR1 repeats), and two sets of unique sequences (U_b and U_c) possessing highly conserved signals (pac1 and pac2, respectively) that direct both the cleavage of concatameric viral DNA into unit-length genomes and its packaging into capsids (6, 37) (Fig. 1). Additional strain-specific reiterations of the *a* sequence which may sometimes be found between the DR2 repeat array and the U_c sequences include the DR4 repeats of HSV-1 strain F (13) (Fig. 1) and the DR3.5 repeats of HSV-1 strain Justin (16).

Two of these features of the *a* sequence, the DR2 repeat array and the pac elements, may enhance the frequency of homologous recombination events in the viral genome by serving as sites for the initiation of recombination. First, when multiple copies of tandem DR2 repeats are present in a negatively supercoiled plasmid, they may adopt a novel secondary structure termed anisomorphic DNA (42). This conformation probably contains single-stranded regions as a consequence of supercoil-induced relaxation, since these sequences have been shown to be highly sensitive to cleavage by single-stranded nucleases. Moreover, a conformation-specific but not sequence-specific nuclease present in both mock- and HSV-infected cells has been shown to preferentially cleave the DR2 repeat array in vitro (41). Although the role that this nuclease plays in recombination in HSV-1 has not yet been determined, these properties of the DR2 array indicate that it may potentially act as a site for the initiation of recombination events. Alternatively, double-strand breaks which are created during cleavage and packaging (cleavage/packaging) events promoted by the pac1 and pac2 elements (6, 19, 37) may serve to stimulate the frequency of homologous recombination events. Smiley et al. (27) have provided compelling evidence in support of this idea by demonstrating a dramatic decrease in the frequency of *a* sequence-mediated recombination events upon the deletion of either pac element.

Thus, nicks within an anisomorphic secondary structure or double-strand breaks resulting from the genome cleavage event may individually or together contribute to the observed enhanced recombination which occurs at the *a* sequence. The potential role that these features play in stimulating recombination events at the *a* sequence was examined in this work. The previously described ability of transfected plasmid-borne Tn*5* sequences to undergo inversion in HSV-1-infected cells (39) was developed into a convenient system for quantitatively analyzing the relative contributions of specific *a* sequence elements to enhanced recombinogenicity. This approach involved inserting wild-type and mutant derivatives of *a* se-

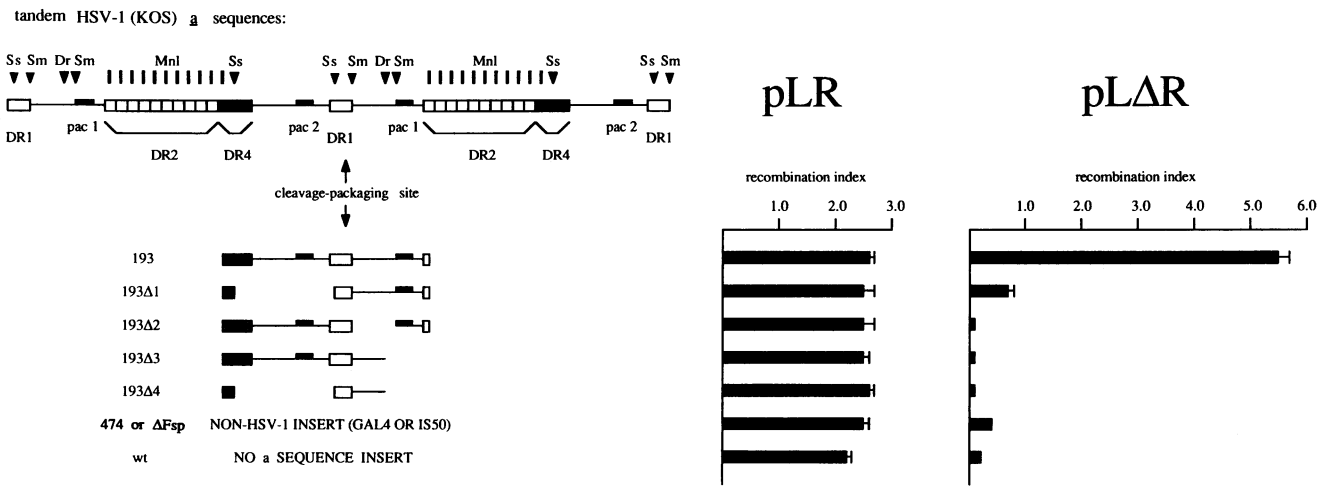

FIG. 2. Construction of HSV-1 *a* sequence derivatives which are defective for cleavage and packaging and quantitation of their recombinogenic potential. At left are cleavage/packaging-proficient and cleavage/packaging-deficient subfragments of a tandem HSV-1 *a* sequence junction. 193 possesses both the pac1 and pac2 elements and represents the minimal signal sufficient for mediating cleavage and packaging of viral DNA. The four deletion derivatives of 193 shown lack one or both of the pac1 or pac2 elements. 474 and ΔFsp are two non-HSV-1 DNA inserts which are composed of 474 bp of the yeast gene *GAL4* and 600 bp of the prokaryotic transposon Tn*5* sequences, respectively. wt, wild-type plasmid without an *a* sequence insert. Restriction sites are shown for *Dra*I (Dr), *Mnl*I (Mnl), *Sma*I (Sm), and *Sst*II (Ss). At right the relative contributions of these *a* sequence subfragments to Tn*5* inversion in a plasmid transfection-superinfection assay are summarized. The recombination index (with associated standard deviation) corresponds to the ratio of inversion frequency in the *a* sequence-containing Tn*5* plasmid pLR or pLΔR to the deletion frequency in the internal standard plasmid pGAL-STD. Each plasmid transfection was performed in triplicate.

quence subfragments into both IS*50* repeats of Tn*5* and then determining the relative increases in Tn*5* inversion frequency that they conferred. The presence of the cleavage/packaging-proficient pac2-DR1-pac1 arrangement was found to be both necessary and sufficient for the enhanced recombinogenic properties of the *a* sequence, while the ability to form anisomorphic DNA in the DR2 repeat array had little influence on recombination frequency. The requirement for a double-strand break created during the cleavage/packaging process in inducing HSV-1 recombination was dramatically confirmed by the demonstration that a restriction endonuclease-generated break in a nonviral DNA sequence had the same stimulatory effect. However, the ability of an isolated *a* sequence to act as a recombinational hot spot was completely abrogated in the presence of extensive flanking sequence homology, particularly that of the *b* and *c* sequences which normally surround the *a* sequence at the L-S junction. These observations serve to resolve the controversy over the role that the *a* sequence plays in HSV-1 genome isomerization by demonstrating that the high frequency of recombination events which arise during DNA replication through the *b* and *c* repeats acts to dilute out the enhanced recombinogenicity exhibited by this element.

MATERIALS AND METHODS

Cells and viruses. HSV-1 (strain KOS) used in superinfections was propagated in Vero cells. Stocks of the UL28 gene deletion virus gCB were prepared by passage in a complementing cell line, C1, which expresses the UL28 gene product (32). Both cell lines were grown in Dulbecco's minimum essential medium supplemented with 5% fetal calf serum.

Plasmid constructions. (i) 440 derivatives. 440 (Fig. 1) was constructed by attaching *Eco*RI linkers onto the 440-bp *Sma*I fragment of the HSV-1 (strain F) *a* sequence of pRB380 (14) and cloning it into the *Eco*RI site of pUC19. 440Δ1 was generated by performing a 160-bp *Sst*II collapse in 440; this resulted in the deletion of the pac2 and DR4 elements of 440 but spared the complete DR2 array. 440Δ2 through 440Δ10 contain spontaneous deletions of integral numbers of DR2 repeats from 440Δ1 and were isolated by passaging this plasmid in the recombination-proficient *E. coli* strain C600. 440Δ11, 440Δ12, 440Δ13, and 440Δ14 represent 440 mutants that possess part or all of the DR2 array but lack both pac elements. They were constructed by transferring the *Apa*I-*Sst*II fragments of 440Δ1, 440Δ2, 440Δ8, and 440Δ10, respectively, into the *Apa*I-*Sst*II sites of the 193Δ4 construct described below.

(ii) 193 derivatives. A *Hae*II fragment containing the two tandem *a* sequences of plasmid pCW103 (40) was isolated by gel purification, digested with *Mnl*I, and shotgun cloned into the *Sma*I site of pSG424 (24). One of the clones which was isolated, 193Δ1, contained a small segment of the *b* sequence, a DR1 repeat, and a complete U_b with pac1 motif. An 80-bp *Sst*II fragment from 440 which contained the pac2 element was then inserted into the *Sst*II site of 193Δ1 to generate 193 (Fig. 2); this derivative therefore contains an intact pac2-DR1-pac1 arrangement, which represents the minimum signal necessary for cleavage and packaging in amplicon assays (19). Finally, a series of *Sma*I- and *Dra*I-generated deletions was used to create the remaining mutant derivatives of 193; these lack pac1 (193Δ3), lack both pac elements (193Δ4), or lack the sequences adjacent to pac1 which are not conserved among herpesviruses yet are essential for the cleavage/packaging process (193Δ2). All 440 and 193 derivatives were sequenced by the dideoxy method to verify their authenticity.

(iii) pL, pLR, and pLΔR. Each of the 440 and 193 derivatives was inserted into the IS*50*-containing plasmid pL to facilitate their incorporation into the Tn*5* shuttle plasmids pLR and pLΔR. pL was generated by first cloning the 2.4-kb *Sma*I fragment containing the left half of the Tn*5* sequences in pMC110::Tn*5* (39) into the *Pvu*II sites of pUC19. An *Eco*RI linker was then inserted into the *Pvu*II site of the $IS50_L$

element of this plasmid. The various *a* sequence derivatives were subcloned via their flanking *Eco*RI sites into pL and screened by restriction site mapping to determine their orientation.

To create pLR-*a*, pMC110::Tn*5* was first cleaved at its *Sal*I sites to generate two fragments which represented the left and right halves of the Tn*5* transposon. The 6.0-kb *Sal*I fragment containing IS*50*$_R$ was self-ligated to create pR*, and the 2.7-kb *Sal*I fragment containing IS*50*$_L$ was cloned into the unique *Sal*I site of pMC110 (3) to generate pL*. Next, the 1.3-kb *Hinc*II-*Bgl*II fragment from pL, which contains the entire IS*50* element with its 440 or 193 derivative insert, was cloned into the *Hpa*I-*Bgl*II sites of both pL* and pR*. These transposon halves, which now contained identical *a* sequence inserts, were then reassembled to create the intact Tn*5* molecule in plasmid pLR-*a*. This involved ligating the IS*50*$_L$-containing *Bgl*II-*Sst*I fragment of pL* into the *Bam*HI-*Sst*I sites of pR*. pLR and pLR-474 were identical to pLR-*a*, except that the former lacks *a* sequence inserts in its IS*50* elements, while the latter contains a 474-bp insert derived from the *Saccharomyces cerevisiae GAL4* gene as a non-HSV-1 DNA control. This insert was obtained by first cloning the 0.5-kb *GAL4*-containing *Hin*dIII-*Bam*HI fragment of pSG424 into pGEM7 (Promega) and then transferring it into pL as an *Eco*RI fragment for incorporation into pLR.

pLΔR-*a* represents a derivative of pLR-*a* in which all but 236 bp of the IS*50*$_R$ element has been deleted; it was constructed in two steps. First, a 0.2-kb *Hin*dIII-*Bsa*HI fragment of the IS*50* element of pL which contains the *a* sequence insert was transferred into the *Hin*dIII-*Acc*I sites of pUC19. The 2.8-kb IS*50*$_L$-containing *Bgl*II-*Sst*I fragment of pLR-*a* was then transferred into the *Bam*HI-*Sst*I sites of this plasmid, so that the final construct contained identical *a* sequence derivatives in inverted orientations. pLΔR is identical to pLΔR-*a* but lacks *a* sequence inserts in its IS*50* elements. pLRΔFsp, which represents a pLΔR derivative with a non-HSV-1 DNA (i.e., Tn*5*-derived) insert, was constructed by replacing the 1.3-kb *Hpa*I-*Bgl*II fragment of the IS*50*$_R$ element of pLR with the 0.6-kb *Fsp*I-*Bgl*II fragment of IS*50*$_R$.

(iv) pGAL4-STD. The plasmid used as an internal standard in recombination assays, pGAL4-STD, was generated in three steps. First, pSG424-ori$_s$ was constructed by inserting the 0.2-kb ori$_s$-containing *Sma*I fragment of pMC110 into the *Sma*I sites of pSG424. Next, pMC-0.5HK was constructed by inserting the 0.5-kb *Hin*dIII-*Kpn*I fragment of pSG424 into the *Hin*dIII-*Kpn*I sites of pMC110. Finally, pGAL4-STD was assembled by inserting the 0.7-kb *Bgl*II-*Sst*I fragment of pSG424-ori$_s$ into the *Bam*HI-*Sst*I sites of pMC-0.5HK; this resulted in a construct which contains two tandem repeat copies of the yeast gene *GAL4* linked to an HSV-1 origin of replication.

(v) p3.4Hin and p3.4Hin-E. To create p3.4Hin-E, a Tn*5* molecule which contained *Eco*RI sites within both IS*50* elements first had to be constructed. This was accomplished by first cleaving the transposon in pTn5Δ1 (39) into two halves, each of which contained an IS*50* element. The 3.0-kb IS*50*$_R$-containing *Sal*I-*Sst*I fragment of pTn5Δ1 was inserted into the *Sal*I-*Sst*I sites of pMC110 to generate pΔ1R. The 5.8-kb IS*50*$_L$-containing *Bam*HI fragment of pTn5Δ1 was religated to itself to generate pΔ1L. The 1.0-kb *Not*I-*Bgl*II IS*50*-containing fragments of both pΔ1R and pΔ1L were then replaced by the 1.0-kb *Eco*RI linker-containing *Not*I-*Bgl*II fragment of pL to yield pΔ1R-E and pΔ1L-E, respectively. Finally, the 5.8-kb *Sal*I-*Sst*I fragment of pΔ1L-E and the 3.4-kb *Sal*I-*Sst*I fragment of pΔ1R-E were ligated to create pTn5Δ1-E. p3.4Hin-E and p3.4Hin (see Fig. 9) were then constructed by inserting the 3.4-kb *Hin*dIII fragments of pTn5Δ1-E and pTn5Δ1, respectively, into the *Hin*dIII site of a pMC110 derivative whose unique *Eco*RI site had been destroyed. All but a 335-bp segment, which contained the *Eco*RI linkers in p3.4Hin-E, was deleted from the IS*50*$_L$ and IS*50*$_R$ elements during this final step. The construction of the *Eco*RI gene-expressing plasmid pMENs has been described previously (18).

(vi) pBaC and pBΔaC. The first step in generating pBaC was to subclone the 2.7-kb *Eco*RI fragment of pCW101 (40) into the *Eco*RI site of pSG424, yielding pSG424-BaC. The construction of pBΔaC involved first removing the *a* sequence from pCW101 by digesting this plasmid with *Bst*EII-*Bsp*MI, blunting the resulting ends with Klenow fragment, and religating; the 2.2-kb *Eco*RI fragment of this plasmid, pCW101Δa, was then subcloned into the *Eco*RI site of pSG424 to generate pSG424-BΔaC. The *Hin*dIII-*Xba*I fragments of pSG424-BaC and pSG424-BΔaC, which contain 474 bp of *GAL4* DNA sequences in addition to the L-S junction sequences, were then inserted into the *Hin*dIII-*Xba*I sites of pMC110 to create pMC110-BaC and pMC110-BΔaC, respectively. The 2.7- and 2.2-kb *Eco*RI fragments of pCW101 and pCW101Δa, respectively, were also subcloned into the *Eco*RI linker of pL* to generate pL*-BaC and pL*-BΔaC, respectively; the *Bgl*II-partial *Sst*I fragments of these two plasmids were then ligated into the *Bam*HI-*Sst*I sites of pR* to generate pLR-BaC and pLR-BΔaC. Finally, the 5.4- and 4.9-kb *Bgl*II-*Kpn*I fragments of pLR-BaC and pLR-BΔaC, respectively, were ligated with the 6.0- and 5.5-kb *Bam*HI-*Kpn*I fragments of pMC110-BaC and pMC110-BΔaC, respectively, to generate pBaC and pBΔaC, respectively. In both plasmids, the only inverted repeat homology present was derived from the L-S junction of the HSV-1 genome.

Analysis of Tn*5* inversion events in a transfection-superinfection assay. Vero cells were transfected in triplicate with 6 μg of each plasmid by calcium phosphate precipitation and subjected to a 15% glycerol shock after 4 h. The medium was changed after 18 h, and the cells were superinfected with HSV-1 (KOS) or gCB at a multiplicity of infection of 10 to 20. After 24 h, the infected-cell DNA was isolated and treated with RNase, proteinase K, phenol, and chloroform according to the procedure of Weber et al. (39) before being split equally into six Eppendorf tubes for precipitation overnight at −70°C. The DNA from a single tube was then resuspended in 190 μl of water–21 μl of 10× restriction buffer, digested overnight with 30 Units each of the appropriate restriction endonucleases, reprecipitated, electrophoresed on a 0.8% agarose gel, transferred to GeneScreen Plus (NEN DuPont), and hybridized to Tn*5*- or *GAL4*-specific probes which had been radiolabeled with [^{32}P]dCTP with a random-primed labeling kit (Boehringer Mannheim). Southern blots were exposed to Kodak XAR5 film for various lengths of time without an intensifier screen, and the resulting autoradiograms were quantitated with a Hoefer GS 300 densitometer.

S1 nuclease sensitivity assay. Cleavage of *a* sequence subfragments cloned into pL by S1 nuclease was performed by using a modification of the assay described by Wohlrab et al. (42). Briefly, 1.5 μg of each plasmid was treated with 3.5 U of S1 nuclease (Bethesda Research Laboratories) for 30 min at 37°C in 50 μl of reaction buffer (40 mM sodium acetate [pH 4.6], 50 mM NaCl, and 1 mM $ZnSO_4$); the reactions were stopped by the addition of EDTA to a final concentration of 30 mM. The samples were then subjected to phenol and chloroform extraction, ethanol precipitation, digestion with *Pst*I, and electrophoresis on a 0.8% agarose gel.

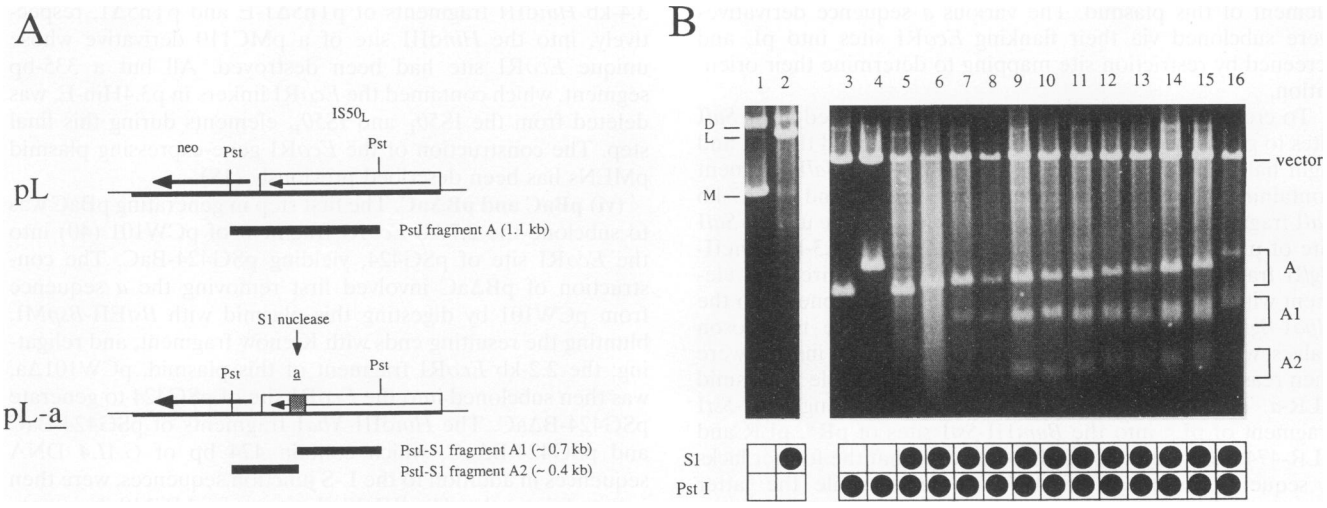

FIG. 3. Ability of *a* sequence subfragments to form anisomorphic DNA. (A) 440 derivatives containing variable numbers of DR2 repeats were inserted into the IS*50* element of plasmid pL to generate a panel of pL-*a* derivatives. Recognition of anisomorphic DNA conformations in pL-*a* plasmids by S1 nuclease will be evident by cleavage of the *a* sequence-containing *Pst*I fragment A to yield two smaller fragments, A1 and A2. (B) Agarose gel electrophoresis of S1 and/or *Pst*I digests of pL and pL-*a* plasmid DNAs. D, L, and M refer to dimer, linear, and monomer species, respectively, present in the first two lanes. In the remaining lanes, A, A1, and A2 refer to the fragments identified in panel A, while the vector band refers to the 3.6-kb vector-containing *Pst*I fragment common to all pL-*a* derivatives. Plasmids were treated with *Pst*I alone, with S1 nuclease alone, or with S1 followed by *Pst*I, as indicated in the grid at bottom. Lanes: 1, 2, 4, and 15, pL-440Δ1; 3 and 5, pL; 6, pL-440Δ10; 7, pL-440Δ9; 8, pL-440Δ8; 9, pL-440Δ7; 10, pL-440Δ6; 11, pL-440Δ5; 12, pL-440Δ4; 13, pL-440Δ3; 14, pL-440Δ2; 16, pL-440.

RESULTS

Construction and characterization of *a* sequence mutants. The enhanced recombinogenic properties of the *a* sequence are thought to be due to a novel anisomorphic DNA conformation occurring at the DR2 repeat elements or to double-strand breaks resulting from cleavage and packaging events mediated by the pac1 and pac2 motifs. In order to examine the possible contribution of these features to recombinogenicity, a series of subfragments of the *a* sequence which contained both wild-type and mutated components was constructed, such that all possible combinations of anisomorphic, nonanisomorphic, cleavage/packaging-proficient, and cleavage/packaging-deficient elements could be prepared for analysis in genetic and biochemical assays.

The first section of the *a* sequence which was examined is found in 440, a *Sma*I fragment of the wild-type *a* sequence which contains the full DR2 array and both pac sequences (Fig. 1). A panel of deletion mutants was constructed which represented both anisomorphic and nonanisomorphic versions of 440 in which one or both pac elements were deleted (Fig. 1). To further explore the role of the pac elements and to establish a possible link between cleavage and packaging with enhanced recombinogenicity, a second subfragment which included only the two pac elements was examined: 193 is an *Mnl*I fragment which contains the pac2-DR1-pac1 arrangement from two tandemly repeated *a* sequences and completely lacks the DR2 array (Fig. 2). An analogous fragment from a closely related HSV-1 strain has previously been shown to function as a minimal cleavage and packaging signal in amplicon assays (19). Mutations in 193 in which either or both pac elements were deleted were then created (Fig. 2).

Prior to examination of the recombinogenic activities of these *a* sequence derivatives in a quantitative sequence inversion assay, the physical and biological properties of the 440 and 193 derivatives were characterized. First, the ability of variable numbers of DR2 repeats to form anisomorphic DNA conformations was examined by an S1 nuclease sensitivity assay (42). 440 and a series of deletion mutants containing variable numbers of DR2 repeats, which potentially represent both anisomorphic and nonanisomorphic conformations (Fig. 1), were subcloned into the IS*50* element of plasmid pL (Fig. 3A) and treated with S1 nuclease. The 440 mutants analyzed included 440Δ1, 440Δ2, 440Δ8, and 440Δ10, which contain 19, 16, 4, and 1 DR2 repeats, respectively (Fig. 1), and 440Δ3, 440Δ4, 440Δ5, 440Δ6, 440Δ7, and 440Δ9, which contain 15, 13, 10, 6, 5, and 3 DR2 repeats, respectively (not illustrated).

An S1-sensitive site was observed in plasmids bearing the 440 insert and its deletion derivatives and could be mapped to the DR2 repeat array by digestion with *Pst*I (Fig. 3B) and other restriction endonucleases (25). Cleavage by S1 was evident in the constructs present in lanes 7 to 16 of Fig. 3B, since their *a* sequence-containing *Pst*I fragments (fragment A in Fig. 3A) had been cleaved by S1 to yield two smaller products (A1 and A2). The breadth of the A1 and A2 bands in those pL-*a* derivatives which contained higher-copy-number inserts (Fig. 3B, lanes 10 to 16) indicated that the S1 cleavage event could occur at variable sites within the DR2 repeat array. However, plasmids with fewer DR2 repeats possessed sharper bands (Fig. 3B, lanes 7 to 9) and thus contained fewer potential nuclease-sensitive sites. In contrast, constructs which lacked an insertion (pL; Fig. 3B, lane 5) or which contained either a single DR2 repeat (pL-440Δ10; lane 6) or a non-HSV-1 DNA insert (25) were resistant to S1 cleavage. Thus, any 440 derivative which contained at least three DR2 elements was found to facilitate the formation of an unwound nuclease-sensitive DNA structure. Moreover, recognition of this structure by S1 nuclease required negative supercoiling of the plasmid template, since cleavage of the DR2 repeat array could be prevented by linearization of the DNA prior to nuclease treatment (25). All of these observations were found to be in complete agreement with the earlier results of Wohlrab et al. (42). This observed S1 sensitivity suggests that

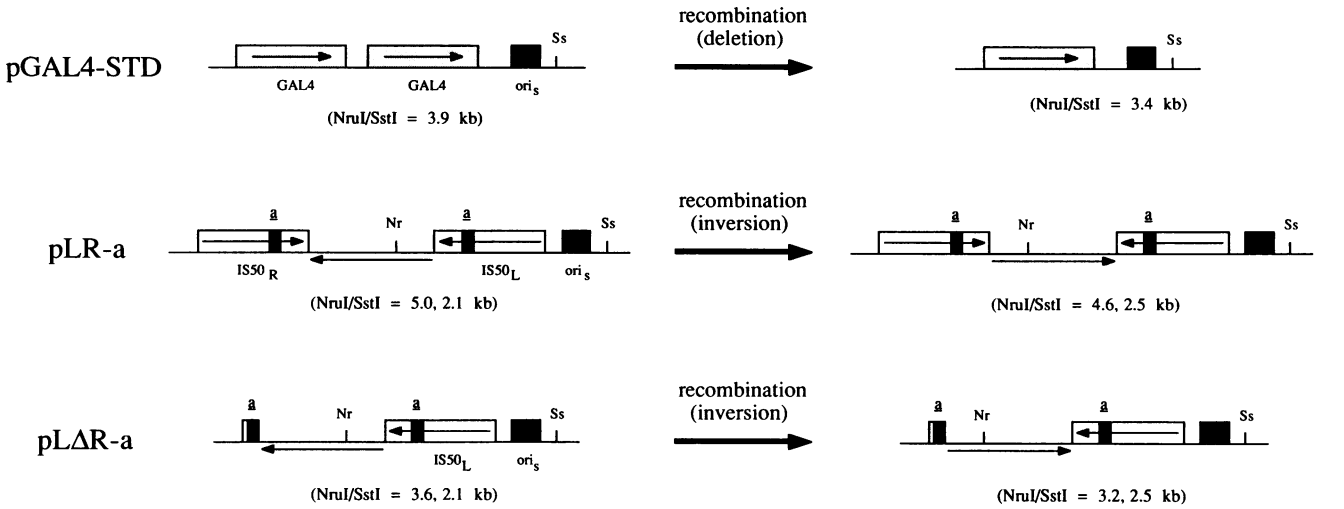

FIG. 4. Plasmids used in the quantitative Tn*5* inversion assay. pGAL4-STD undergoes deletion of one of its two copies of the yeast gene *GAL4* at a constant frequency when replicated in HSV-1-infected cells, making it a useful internal standard for Tn*5* inversion assays. pLR and pLΔR are two Tn*5*-containing plasmids which differ dramatically in their abilities to undergo sequence inversion events in HSV-1-infected cells, since the latter has lost almost the entire IS*50*$_R$ element through which recombination must take place. The *a* sequence derivatives illustrated in Fig. 1 and 2 were inserted in inverted orientation into the IS*50* elements of both pLR and pLΔR as shown, to generate a panel of pLR-*a* and pLΔR-*a* plasmids which were analyzed in Tn*5* inversion assays.

the DR2 sequences of these 440 derivatives could be similarly cleaved by nucleases present in HSV-1-infected cells and may therefore represent sites at which recombination events are initiated.

In addition, the ability of *a* sequence subfragments containing mutations in the pac1 and pac2 motifs to mediate cleavage/packaging events was examined by using an amplicon propagation assay. Defective HSV-1 plasmid vectors or amplicons minimally contain both a viral DNA replication origin and an *a* sequence and are replicated as large concatamers, cleaved, and packaged into virions in the presence of a helper virus (29). A number of 193 and 440 derivatives (Fig. 1 and 2) were therefore inserted into an HSV-1 origin-containing plasmid and tested for their abilities to be propagated as amplicons by cotransfection into Vero cells with infectious HSV-1 helper virus DNA. DNase-resistant virion DNA was prepared from the resulting viral stocks after multiple low-dilution serial passages and examined for the presence of plasmid amplicons by Southern blot analysis. Only those *a* sequence subfragments which contained both the pac1 and pac2 signals, 440 and 193, were observed to promote replication and packaging into virions with high efficiency and without rearrangement events (25). In contrast, mutant derivatives of 440 and 193 from which one or both pac elements had been deleted either failed to be propagated as amplicons or were propagated only after illegitimate recombination events had restored a complete set of pac elements to the defective *a* sequence. Thus, the double-strand break generated within the wild-type 440 and 193 subfragments during cleavage and packaging enabled their amplicon vectors to be passaged through multiple rounds of infection; these findings are in complete agreement with previous studies of *a* sequence mutants in amplicon propagation assays (6, 37). The possibility that this double-strand break may increase the frequency of recombination events at the *a* sequence by generating free DNA ends was explored in the transfection-superinfection assay described below.

Utilization of a quantitative Tn*5* inversion assay to analyze the recombinogenic properties of *a* sequence mutants. The relative contributions of DNA breaks in *a* sequence subfragments resulting either from anisomorphic structural conformations or from cleavage and packaging events to HSV-1 recombination were ascertained by using a Tn*5* shuttle plasmid system. This system relies on the ability of the Tn*5* transposon linked in *cis* to an HSV-1 origin of replication to undergo sequence inversion in a transient transfection-superinfection assay (39). Such inversion events are mediated by generalized recombination between the inverted repeat IS*50* elements of Tn*5*. To accurately quantitate increases in recombination frequency which would result from the insertion of various *a* sequence subfragments into the IS*50* elements of the transposon, a cotransfected standard plasmid (pGAL4-STD) possessing a constant recombination frequency was used as an internal reference. This plasmid contained two tandemly arranged copies of the yeast gene *GAL4* and an HSV-1 origin of replication (Fig. 4). In the absence of HSV-1 infection, pGAL4-STD yielded a linearized fragment that was 3.9 kb in size. However, in the presence of HSV-1 infection, one of the copies of *GAL4* was deleted by replication-mediated recombination. This resulted in the appearance of a linearized recombination product which was 3.4 kb in size (Fig. 4 and 5). The internal standard was tested in cotransfection experiments with the wild-type Tn*5*-containing plasmid pLR (Fig. 4), which yields two native fragments after *Nru*I-*Sst*I digestion as well as two novel fragments resulting from replication-mediated sequence inversion. Bands corresponding to the native fragments and recombination products of both pLR and pGAL4-STD could be readily detected in transfected-cell DNA (Fig. 5); *Dpn*I digestion was utilized to verify that only replicated species were analyzed.

The differences in the levels of inversion and deletion bands versus native bands between pLR and pGAL4-STD, respectively, remained relatively constant over repeated transfections. Inversion products in pLR were detected at a frequency of approximately 25% in repeated transfections. This indicated that the assay was only halfway to reaching recombinational equilibrium, such that system saturation would be marked by

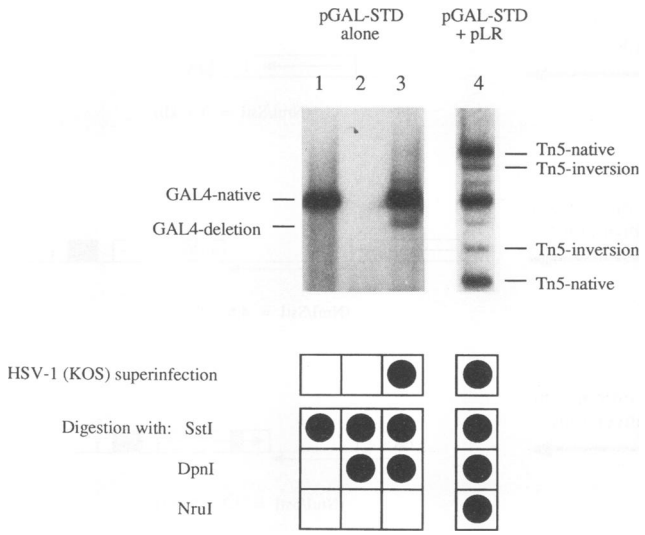

FIG. 5. Demonstration of the quantitative Tn*5* inversion assay. DNA was isolated from Vero cells which had been first transfected with pGAL4-STD alone or with both pGAL4-STD and pLR and then mock or HSV-1 infected. This DNA was digested with the indicated enzymes and then analyzed by Southern blot, using radiolabeled pUC19::Tn*5* DNA as a probe to detect both the native and recombination-derived bands of both plasmids.

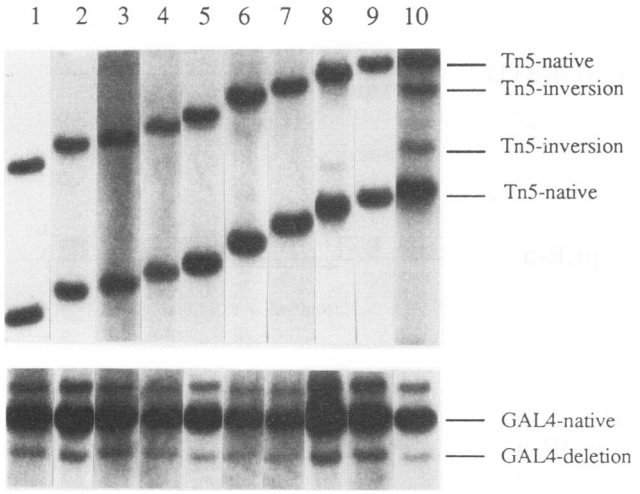

FIG. 6. Analysis of the recombinogenic properties of 440 and its derivatives in the pLΔR plasmid. Each pLΔR-440 derivative was cotransfected into Vero cells with pGAL4-STD, superinfected with HSV-1, reisolated, digested with *Nru*I-*Sst*I-*Dpn*I, electrophoresed on a 0.8% agarose gel, and blotted onto a GeneScreen Plus nylon membrane. Hybridizations were carried out with two different probes to circumvent the problem of quantitating overlapping pLΔR- and pGAL4-STD-derived bands. The blot was first probed with the radiolabeled 3.4-kb *Hin*dIII fragment of Tn*5* to visualize the pLΔR-specific bands (upper panel). This probe was then stripped, and the blot was reprobed with the radiolabeled 0.5-kb *GAL4* gene-containing *Hin*dIII-*Eco*RI fragment of pSG424 to visualize the pGAL4-STD-specific bands (lower panel). The native bands and the recombination products for both cotransfected plasmids are indicated on the right of the autoradiogram. Lanes: 1, pLΔR; 2, pLΔR-440Δ10; 3, pLΔR-440Δ14; 4, pLΔR-440Δ8; 5, pLΔR-440Δ13; 6, pLΔR-440Δ2; 7, pLΔR-440Δ12; 8, pLΔR-440Δ1; 9, pLΔR-440Δ11; 10, pLΔR-440.

the appearance of inversion products at a frequency of 50%. This inversion assay therefore represents a convenient model system for the quantitative analysis of HSV-1 replication-dependent recombination events and would be sensitive enough to detect any increase in Tn*5* inversion upon the insertion of a recombinational hot spot into the IS*50* elements of pLR. In other words, if any of the *a* sequence subfragments described above (Fig. 1 and 2) can function as hot spots for recombination, they should be able to promote a discernible increase in Tn*5* inversion frequency in pLR relative to the *GAL4* deletion frequency in pGAL-STD. Thus, by quantitating differences between the levels of pLR inversion products and pGAL-STD deletion products within a given experiment, an accurate assessment of the recombinogenic potential of any inserted *a* sequence subfragment can be made. Since the addition of identical *a* sequence derivatives into both IS*50* elements resulted in a small increase in the sequence homology between inverted repeats which could theoretically increase the frequency of generalized recombination events occurring in Tn*5*, the pLR derivative pLR-474, which contained a 474-bp insert of non-HSV-1 DNA (i.e., the yeast *GAL4* gene), was used as a control.

The recombinogenic properties of the *a* sequence mutants by themselves (i.e., in the absence of flanking IS*50* homology) were also examined by using the plasmid pLΔR, in which almost all of the IS*50*$_R$ element has been removed (Fig. 4). This plasmid was designed to directly identify isolated hot spots of recombination, since it lacks the IS*50* homology through which normal sequence inversion events are mediated, and a previous analysis demonstrated that Tn*5* inversion does not occur at detectable levels in constructs which contain regions of inverted repeat homology that are as small (i.e., 600 bp or less) as the inserted 440 or 193 subfragments (39). Thus, the extent to which an inserted *a* sequence mutant increases Tn*5* inversion above its normal level in pLR or allows the detection of Tn*5* inversion in pLΔR will be a direct indication of the relative ability of its anisomorphic DNA conformation or cleavage/packaging signals to enhance the frequency of existing homologous recombination events in HSV-1.

Analysis of the recombinogenic properties of the *a* sequence mutants in pLΔR. Duplicate copies of the 440 and 193 *a* sequence subfragments as well as their mutant derivatives (Fig. 1 and 2) were inserted in inverted orientation into pLΔR to assay their recombinogenicity in the transfection-superinfection system. Representative Southern blots of transfected pLΔR-440 or pLΔR-193 derivatives digested with *Nru*I-*Sst*I-*Dpn*I and hybridized with Tn*5*- and *GAL4*-specific probes are shown in Fig. 6 and 7, respectively. The intensities of bands in the autoradiograms were quantitated by densitometry to normalize the Tn*5* inversion levels to the levels of deletion in the internal standard pGAL4-STD; these values are expressed as a recombination index in Fig. 1 and 2.

The wild-type 440 insert was found to possess significant recombinogenic potential in that it conferred a 50-fold increase in inversion frequency over a pLΔR plasmid which lacked any inserts (Fig. 1). However, all of the deletion derivatives of 440 were defective for this enhancement in recombination activity in pLΔR, although a very low level of inversion could be detected in pLΔR-440Δ1, pLΔR-440Δ2, pLΔR-440Δ11, and pLRΔFsp, which represents a pLΔR plasmid that contains a 600-bp insert of non-HSV-1 DNA (Fig. 1). Similarly, pLΔR-193 also showed a 50-fold increase in inversion frequency over pLΔR which lacked an insert, but deletion of either or both pac sequences from the 193 insert resulted in the loss of most if not all of this activity (Fig. 2 and 7). These results demonstrated that both the wild-type 440 and 193

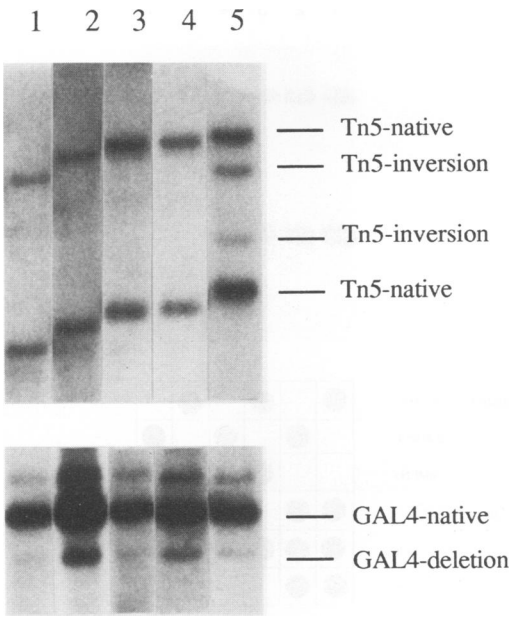

FIG. 7. Analysis of the recombinogenic properties of 193 and its derivatives in the pLΔR plasmid. Each pLΔR-193 derivative was cotransfected into Vero cells with pGAL4-STD, superinfected with HSV-1, reisolated, digested with *Nru*I-*Sst*I-*Dpn*I, electrophoresed on a 0.8% agarose gel, and blotted onto a GeneScreen Plus nylon membrane. Hybridizations were carried out with two different probes to circumvent the problem of quantitating overlapping pLΔR- and pGAL4-STD-derived bands. The blot was first probed with the radiolabeled 3.4-kb *Hin*dIII fragment of Tn5 to visualize the pLΔR-specific bands (upper panel). This probe was then stripped, and the blot was reprobed with the radiolabeled 0.5-kb *GAL4* gene-containing *Hin*dIII-*Eco*RI fragment of pSG424 to visualize the pGAL4-STD-specific bands (lower panel). The native bands and the recombination products for both cotransfected plasmids are indicated on the right of the autoradiograms. Lanes: 1, pLΔR-193Δ4; 2, pLΔR-193Δ1; 3, pLΔR-193Δ3; 4, pLΔR-193Δ2; 5, pLΔR-193.

inserts could indeed function as hot spots of recombination. Moreover, the mutational analyses of the 440 and 193 inserts indicated that the presence of two functional pac sequences was critical for enhancing *a* sequence-driven recombination; however, the DR2 array, with its associated anisomorphic DNA conformation, was dispensable for this activity.

Role of double-strand breaks generated during the cleavage/packaging process in promoting recombination at the *a* sequence. The finding that two functional pac sequences were both necessary and sufficient for mediating enhanced recombination at the *a* sequence suggested that the double-strand break generated at this site by the viral cleavage/packaging machinery was involved in this process. To explore this possibility further, recombination between *a* sequences was examined by using the quantitative Tn5 inversion assay utilized previously, except that the transfected cells were superinfected with an HSV-1 cleavage/packaging mutant, gCB, rather than the wild-type KOS strain. gCB lacks almost the entire UL28 open reading frame, which renders the virus completely defective for the cleavage and encapsidation of replicated viral DNA (32). Since the UL28 gene product is essential for virus propagation, stocks of the gCB mutant had to be grown in a UL28-expressing cell line prior to their use in experiments with noncomplementing Vero cells. Three constructs which had been employed in the experiments described above, pLR, pLΔR, and pLΔR-193 (Fig. 8A), were transfected into Vero cells, superinfected with gCB, and digested with *Nru*I-*Sst*I-*Dpn*I prior to Southern blot analysis; a representative blot of the transfected-cell DNA is shown in Fig. 8B.

a sequence-independent recombination was unaffected by the conditions employed in this experiment, since pLR exhibited the same high levels of inversion after gCB superinfection (Fig. 8B) that were observed after wild-type virus superinfection (Fig. 5). The finding that replication-dependent recombination was uninhibited for pLR in gCB-infected cells was not unexpected, since the major replication defect in this superinfecting virus is in the process of cleavage and packaging and not in DNA synthesis. However, no inversion events could be detected in pLΔR-193 superinfected with gCB (Fig. 8B); this

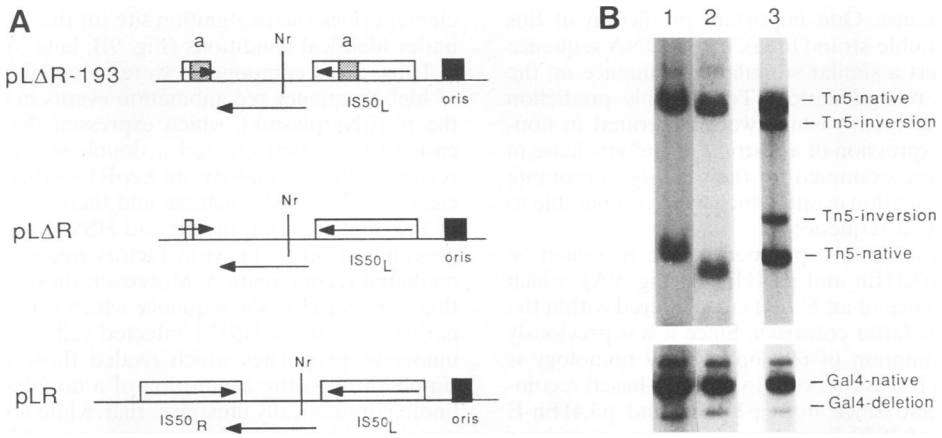

FIG. 8. Analysis of recombination in cells infected with an HSV-1 mutant defective for the cleavage/packaging process. (A) Structures of the transfected plasmids pLΔR-193, pLΔR, and pLR. Nr, *Nru*I. (B) The plasmids in panel A were individually cotransfected with pGAL4-STD into Vero cells, superinfected with the HSV-1 mutant gCB, reisolated, digested with *Nru*I-*Sst*I-*Dpn*I, electrophoresed on a 0.8% agarose gel, blotted onto GeneScreen Plus membrane, and hybridized with the radiolabeled 3.4-kb *Hin*dIII fragment of Tn5 (upper panel). This probe was then stripped, and the blot was reprobed with the radiolabeled 0.5-kb *GAL4* gene-containing fragment of pSG424 (lower panel). The native bands and the recombination products for both cotransfected plasmids are indicated on the right of the autoradiograms. Lanes: 1, pLΔR-193; 2, pLΔR; 3, pLR.

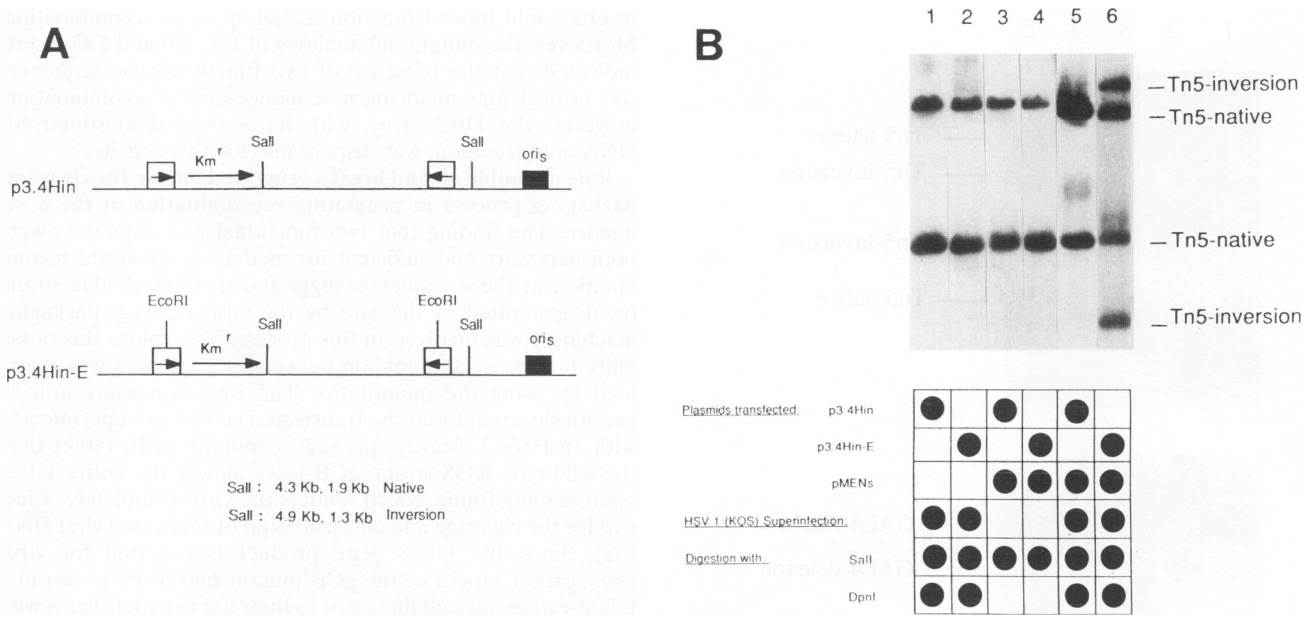

FIG. 9. Ability of a double-strand break generated by an in vivo-expressed restriction endonuclease to promote recombination in a Tn*5* inversion assay. (A) Structures and predicted restriction fragments of the transfected plasmids p3.4Hin and p3.4Hin-E. (B) The plasmids in panel A were individually transfected with or without the *Eco*RI-expressing plasmid pMENs into Vero cells, mock or HSV-1 infected, reisolated, digested with the enzymes indicated in the grid, electrophoresed on a 0.8% agarose gel, blotted onto a GeneScreen Plus membrane, and hybridized with the radiolabeled 1.3-kb *Hpa*I-*Bgl*II fragment of pUC19::Tn*5* DNA. This probe does not hybridize with the pMENs plasmid, thereby enabling visualization of only the test plasmids. The native bands and the recombination products are indicated on the right of the autoradiograms.

was in sharp contrast to the high levels of inversion observed for this same plasmid in the presence of superinfecting wild-type virus (Fig. 2 and 7).

Thus, enhanced recombinogenicity in an isolated *a* sequence requires not only the presence of both the pac1 and pac2 elements (Fig. 1 and 2) but also the complete complement of viral proteins required for cleavage and packaging (Fig. 8). These results strongly suggest that it is the double-strand break generated in the *a* sequence during the cleavage and packaging process, and not the sequences of the pac elements themselves, which is required for promoting high-frequency recombination events at the *a* sequence. One important prediction of this hypothesis is that a double-strand break in any DNA sequence should be able to exert a similar stimulatory influence on the initiation of HSV-1 recombination. To test this prediction directly, double-strand breaks which were generated in non-HSV-1 DNA by the expression of a restriction endonuclease in virus-infected cells were examined for their ability to promote frequencies of recombination events which were comparable to those observed for the *a* sequence.

The plasmids used in these experiments were two deletion derivatives of pLR, p3.4Hin and p3.4Hin-E (Fig. 9A), which differ only in the presence of an *Eco*RI site centered within the inverted repeats of the latter construct. Since it was previously established that a minimum of 600 bp of IS*50* homology is essential to detect an inversion event in the Tn*5*-based recombination assay (39) and since both p3.4Hin and p3.4Hin-E contain only 340 bp of IS*50* homology, it was not surprising that no sequence inversion events were detected in these plasmids by Southern blot analysis (Fig. 9B, lanes 1 and 2). However, pLΔR-193 contains approximately the same amount of sequence homology as the p3.4Hin plasmids but undergoes significant levels of sequence inversion by virtue of its recombinogenic double-strand break created during the cleavage/packaging process (Fig. 2 and 7). To generate a comparable double-strand break in p3.4Hin-E as a means of potentially stimulating recombination, use was made of pMENs, a plasmid which can transiently express the *Eco*RI restriction endonuclease gene in mammalian cells (18, 25). Cotransfection of pMENs with p3.4Hin-E was found to have no effect on recombination frequency in mock-infected cells (Fig. 9B, lane 4). However, the presence of both cotransfected pMENs DNA and HSV-1 superinfection resulted in a dramatic increase in the p3.4Hin-E inversion frequency (Fig. 9B, lane 6). This stimulatory effect was not observed with p3.4Hin, whose IS*50* elements lack the recognition site for the *Eco*RI endonuclease, under identical conditions (Fig. 9B, lane 5).

Thus, three components were essential for the appearance of high-frequency recombination events in these experiments: the pMENs plasmid, which expressed the *Eco*RI restriction endonuclease that created a double-strand break within the region of IS*50* homology; an *Eco*RI restriction site, which was cleaved by the endonuclease and therefore represents the site of the double-strand break; and HSV-1 superinfection, which provides in *trans* the viral factors necessary for replication-mediated recombination. Moreover, these results demonstrate that a bacterial DNA sequence which was otherwise recombinationally inert in HSV-1-infected cells could acquire recombinogenic properties which rivaled those of the *a* sequence simply through the acquisition of a double-strand break. This finding dramatically illustrates that, while no specific sequences were required for high-frequency recombination to occur in HSV-1-infected cells, the creation of a double-strand break appeared to be critical. The implication of this result is that the *a* sequence is able to act as a hot spot for homologous recombination simply because it happens to coincide with the site of the double-strand break which is generated during the cleavage/packaging process, not because it contains discrete

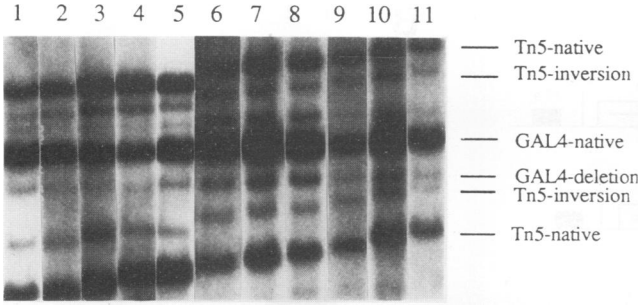

FIG. 10. Analysis of the recombinogenic properties of 440 and its derivatives in the pLR plasmid. Each pLR-440 derivative was cotransfected into Vero cells with pGAL4-STD, superinfected with HSV-1, reisolated, digested with *Nru*I-*Sst*I-*Dpn*I, electrophoresed on a 0.8% agarose gel, blotted onto a GeneScreen Plus nylon membrane, and hybridized with radiolabeled pUC19::Tn*5* DNA. The native bands and the recombination products for both cotransfected plasmids are indicated on the right of the autoradiogram. Lanes: 1, pLR; 2, pLR-440Δ10; 3, pLR-440Δ14; 4, pLR-440Δ8; 5, pLR-440Δ13; 6, pLR-440Δ2; 7, pLR-440Δ12; 8, pLR-440Δ1; 9, pLR-440Δ11; 10, pLR-440; 11, pLR-474.

sequences which are required for high-frequency inversion events.

Analysis of the recombinogenic properties of the *a* sequence mutants in pLR. Duplicate copies of the 440 and 193 *a* sequence subfragments as well as their mutant derivatives (Fig. 1 and 2) were inserted in inverted orientation into pLR to assay their recombinogenicities in the transfection-superinfection system. Representative Southern blots of transfected pLR-440 and pLR-193 derivatives digested with *Nru*I-*Sst*I-*Dpn*I and probed with pUC19::Tn*5* (39) sequences are shown in Fig. 10 and 11, respectively. The intensities of the bands in the autoradiograms were quantitated by densitometry to normalize the Tn*5* inversion levels to the levels of deletion in the internal standard pGAL4-STD; these values are expressed as a recombination index in Fig. 1 and 2.

Surprisingly, the introduction of the 440 and 193 inserts and their deletion derivatives into the intact IS*50* elements of pLR resulted in only a minimal increase (13% or less) in Tn*5* inversion frequency relative to the *GAL4* deletion frequency (Fig. 1 and 2). This slight stimulatory effect did not indicate the presence of a hot spot for recombination but rather appeared

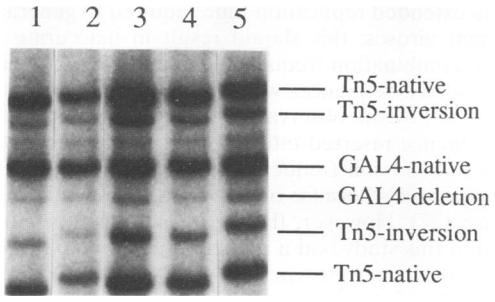

FIG. 11. Analysis of the recombinogenic properties of 193 and its derivatives in the pLR plasmid. Each pLR-193 derivative was cotransfected into Vero cells with pGAL4-STD, superinfected with HSV-1, reisolated, digested with *Nru*I-*Sst*I-*Dpn*I, electrophoresed on a 0.8% agarose gel, blotted onto a GeneScreen Plus nylon membrane, and hybridized with radiolabeled pUC19::Tn*5* DNA. The native bands and the recombination products for both cotransfected plasmids are indicated on the right of the autoradiogram. Lanes: 1, pLR-193Δ4; 2, pLR-193Δ1; 3, pLR-193Δ3; 4, pLR-193Δ2; 5, pLR-193.

to be the result of increasing the length of IS*50* homology through which recombination events could occur, since the plasmid containing the non-HSV-1 DNA insert (pLR-474) behaved similarly. Thus, the insertion of any *a* sequence derivative, even the wild-type 440 and 193 inserts, which were shown to be highly recombinogenic in pLΔR, had little stimulatory effect on the level of homologous recombination which was already occurring between the IS*50* elements of Tn*5* in pLR.

Analysis of the contribution of the *a* sequence to HSV-1 genome isomerization. The simplest interpretation of the results obtained with the pLR-*a* constructs is that inserts such as wild-type 440 and 193 could indeed function as hot spots of recombination when examined in an isolated state (i.e., in pLΔR) but that they lost this property when flanked by regions of extensive homology, such as the 1.3 kb of IS*50* sequences present in pLR. Since the *a* sequence is normally flanked by much larger (approximately 16-kb) tracts of *b* and *c* sequence homology at the L-S junction of the HSV-1 genome, this element may similarly fail to act as a recombinational hot spot in its natural context in the viral DNA. Unfortunately, it is not possible to test this hypothesis directly by generating a virus in which all of the *a* sequences have been deleted, since such a mutant genome would be incapable of normal propagation because of the loss of the pac signals. Moreover, any attempts to characterize a genome in which the *a* sequence has been removed from just the L-S junction would be hindered by the efficient repair of the deleted element by gene conversion-like processes (23, 36).

To circumvent these difficulties in working with the viral genome, use was made of a modified Tn*5* inversion assay in which the two IS*50* elements of the transposon were replaced by either of two inverted segments of the L-S junction. One L-S junction fragment contained 698 bp of the *b* sequence, the entire 572-bp *a* sequence, and 1,437 bp of the *c* sequence; two copies of this segment were used to construct pBaC (Fig. 12A). The other L-S junction fragment was identical to that in pBaC except that the *a* sequence had been deleted from both *b-a-c* repeats; two copies of this segment were used to create pBΔaC (Fig. 12A). These two plasmids were cotransfected into Vero cells with the internal-standard plasmid pGAL4-STD, superinfected with HSV-1, reisolated, cleaved with *Nru*I-*Bam*HI-*Dpn*I, and analyzed by Southern blotting (Fig. 12B). Quantitation of Tn*5* inversion events after normalizing for pGAL4-STD deletion events revealed that the recombination indices for pBaC and pBΔaC were nearly identical (3.68 and 3.62, respectively). Thus, the loss of the *a* sequence from the L-S junction repeats in pBΔaC had no effect on the ability of this plasmid to undergo recombination, and conversely, as in the experiments utilizing pLR-*a*, the presence of the *a* sequence in pBaC failed to increase the frequency of existing sequence inversion events. The simplest interpretation of these results is that high-frequency recombination which arises during DNA replication through the *b* and *c* sequences of the L-S junction is fully capable of diluting out any recombinogenic contributions of the *a* sequence. Thus, the presence of this element most probably adds very little to the overall pool of recombination events which are responsible for HSV-1 genome isomerization.

DISCUSSION

The phenomenon of genome isomerization is one of the most unique and yet enigmatic aspects of the life cycle of HSV-1. The L and S components of the HSV-1 genome are able to invert relative to each other at a high frequency,

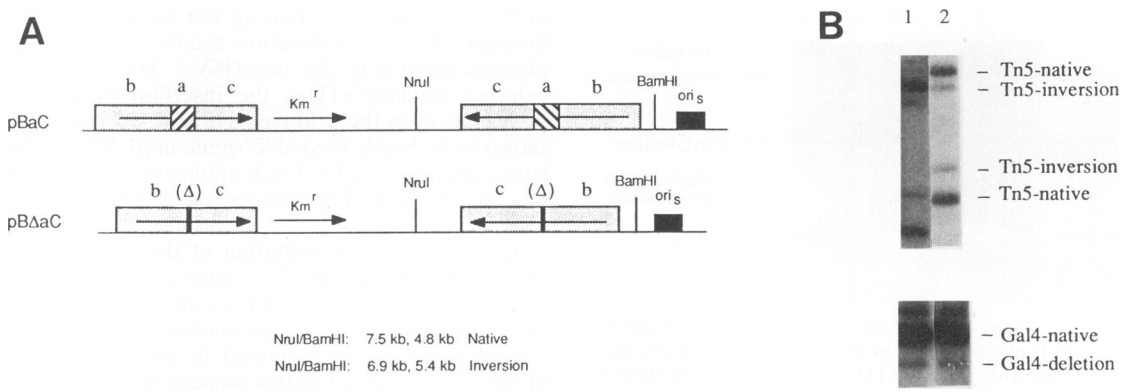

FIG. 12. Analysis of the contribution of the *a* sequence to recombination between duplicated L-S junctions of the viral genome. (A) Structures of the transfected plasmids pBaC and pBΔaC. The predicted restriction fragments for pBaC are listed; each of the corresponding fragments in pBΔaC will be 0.5 kb smaller because of the deletion of the *a* sequences from this plasmid. (B) The plasmids in panel A were individually cotransfected with pGAL4-STD into Vero cells, superinfected with HSV-1, reisolated, digested with *Nru*I-*Bam*HI-*Dpn*I, electrophoresed on a 0.5% agarose gel, blotted onto a GeneScreen Plus membrane, and hybridized with the radiolabeled 2.8-kb *Bgl*II fragment of Tn*5* (upper panel). A second aliquot of the same infected-cell DNA was digested with *Sst*I-*Dpn*I, electrophoresed on a 0.8% agarose gel, blotted onto a GeneScreen Plus membrane, and hybridized with the radiolabeled 0.5-kb *GAL4* gene-containing fragment of pSG424 to determine the deletion frequency of pGAL4-STD (lower panel). The recombination index values for the two pairs of plasmids were then determined as described in the legend to Fig. 1; these are listed in the text. The native bands and the recombination products for both cotransfected plasmids are indicated on the right of the autoradiogram. Lanes: 1, pBΔaC; 2, pBaC.

resulting in the appearance of four equimolar isomeric populations of DNA (7, 9). Although a great deal has been learned in recent years about HSV-1 genome propagation, the mechanism by which the process of isomerization occurs has been somewhat controversial. The earliest studies suggested that these inversions were due to homologous recombination between the entire set of inverted repeats flanking the two components (26), but later investigators implicated the *a* sequence as a *cis*- acting element through which site-specific recombination events occurred (4, 14, 15, 17). The most recent studies, however, have indicated that the *a* sequence may be sufficient but is not necessary for genome isomerization. The overwhelming evidence in support of this conclusion includes a description of *a* sequence-independent genome isomerization (12), the characterization of genome recombination events that are mediated by HSV-1 sequences which lack any homology to the *a* sequence (10, 11, 20–22, 37), the inability to map discrete elements within the *a* sequence which are absolutely required for the alleged site-specific recombination event (4, 27), the inability of an isolated *a* sequence to promote the same high frequency of inversion events as the intact L-S junction (4, 27), the lack of enhanced intermolecular recombination events between L-S junction sequences compared with that between the nonreiterated sequences of the viral DNA (35), and finally, the demonstration that inversion events in the HSV-1 genome can be directly mediated by the viral DNA replication machinery and not a site-specific recombinase and can occur in the complete absence of sequence specificity (39).

If HSV-1 genome isomerization is indeed the result of generalized recombination between the inverted repeats which flank the L and S components, the idea that the *a* sequence acts as a site-specific recombination signal should be abandoned. However, if genome isomerization is mediated strictly by generalized recombination events which occur between extensive diploid sequences, it is unclear how an element as small as the *a* sequence can function as an apparent hot spot for recombination by directing homologous recombination events over such great distances in the viral genome (4, 27, 37). To resolve this apparent paradox, a novel quantitative recombination assay which utilizes the inversion of bacterial transposon sequences by viral DNA replication-mediated homologous recombination was employed to analyze the role of the *a* sequence in HSV-1 genome isomerization.

Since this approach utilized a shuttle plasmid and not actual viral DNA as a recombination template, care must be exercised in extrapolating the behavior of the *a* sequence in this situation to that in the viral genome. However, shuttle plasmids and viral DNA are indistinguishable in that they both contain viral origins of replication, both are replicated to form a linear concatameric state, and both are acted upon by the same set of proteins which mediate HSV-1 DNA replication and recombination. Moreover, the use of transient transfection assays employing shuttle plasmids minimizes the extent to which *a* sequence mutants might recombine with and be repaired by a wild-type *a* sequence present at the L-S junction, which has been frequently observed when they are incorporated into the viral genome (6, 27). Genomic *a* sequence derivatives which have undergone repair will become amplified in the DNA pool during the extended replication time required to generate such recombinant viruses; this should result in inaccurate assessments of recombination frequency, since the mutant character of these inserted sequences would only be temporary. This is best illustrated by the observation that pac element mutations in an *a* sequence inserted into the viral genome resulted in a reduction in inversion frequency which was only one-third of the wild-type levels because of recombination with a wild-type *a* sequence (27). However, the set of pac mutants which was employed in this study had a reduction in inversion frequency of up to 1/50 of the wild-type levels (Fig. 1 and 2), since they maintained the integrity of their deletions in a short-term assay.

The HSV-1 *a* sequence contains two features which were postulated to enhance the frequency of homologous recombination events in the viral genome: the anisomorphic conformations of the DR2 array and the pac1 and pac2 elements. An extensive array representing all possible combinations of anisomorphic, nonanisomorphic, cleavage/packaging-proficient, and cleavage/packaging-deficient derivatives of the *a* sequence

(Fig. 1 and 2) was characterized in this work. Analysis of these subfragments in the pLΔR construct, which lacked flanking IS*50* sequence homology, established that the enhanced recombinational activity of the *a* sequence was not dependent upon the length of the subfragment tested or its ability to form anisomorphic DNA but only on the presence of both cleavage and packaging signals. This conclusion is especially clear from the results obtained from the pLΔR-193 mutants, which demonstrate that the absolute minimal sequences which are capable of mediating a cleavage and packaging event (19) are indistinguishable from those required for detection of high-frequency recombination events (Fig. 2). These findings are supported by the earlier results of Smiley et al. (27), who showed that recombinant viral genomes containing insertions of mutant *a* sequence derivatives underwent a significant decrease in DNA rearrangements when one or the other pac element was deleted from the inserted *a* sequence. Thus, those mutations which reduced the ability of an *a* sequence to mediate cleavage and packaging events caused a concomitant decrease in its ability to promote recombination events (27).

Together, these results suggested that the double-strand break which is created within the pac2-DR1-pac1 arrangement during cleavage and packaging was critical for stimulating the initiation of recombination events. This interpretation was supported by the findings that the enhanced recombinogenic properties of the *a* sequence, but not normal replication-mediated recombination, disappeared in cells which were infected by an HSV-1 cleavage/packaging mutant (Fig. 8) and that a non-HSV-1 DNA sequence, whose only distinct feature was that it was cleaved in vivo by a restriction endonuclease, possessed the same high recombinogenic activity which was previously thought to be associated only with the *a* sequence (Fig. 9). The latter observation was especially remarkable in that it demonstrated that the viral *cis*- and *trans*-acting components which were 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. These results strongly suggest that a double-strand break, and not specific viral sequences (such as the pac elements of the *a* sequence), is all that is required for converting a segment of the HSV-1 genome into a hot spot for homologous recombination.

Thus, recombination between *a* sequences occurs via the DNA replication-mediated homology-dependent mechanism postulated previously (39) but can be stimulated by the free ends generated during the cleavage and packaging process. This conclusion is strengthened by the recent demonstrations that the actual site of crossover between two *a* sequences undergoing recombination maps in a region of homology which is distinct from the cleavage site (27) and that the ability of the cleavage and packaging process to enhance recombination is not manifest in the absence of homologous DNA sequences (28). It is therefore unnecessary to evoke additional models such as a site-specific recombination system to explain the unique recombinogenic properties of the *a* sequence. Moreover, these results raise the interesting possibility that HSV-1 genome isomerization may be yet another biological system which utilizes double-strand break repair (31) as a means of recombination; in this model, free ends generated by a double-strand break are able to recognize an intact homologous sequence and utilize it as a template for repair synthesis. Such a mechanism could also explain the high frequency of gene conversion-like repair which is commonly observed in HSV-1-infected cells (23, 36). It is interesting that under certain conditions, the cleavage and packaging site of the bacteriophage lambda genome can similarly behave as a hot spot for homologous recombination; this is also thought to involve a double-strand break repair-like mechanism (30, 33, 34).

The analysis of the 440 subfragment and its mutant derivatives in the pLΔR construct clearly indicated that the ability of the *a* sequence to enhance the frequency of recombination events in the HSV-1 genome is independent of its ability to form anisomorphic DNA. This is perhaps best illustrated in a comparison of the recombinogenic properties of pLΔR-440 and pLΔR-440Δ1. The latter plasmid exhibits only a residual level of inversion compared with the former, yet they both retain an identical DR2 array (Fig. 1) and therefore an identical propensity to form anisomorphic DNA in supercoiled plasmids (Fig. 3). Furthermore, the presence of anisomorphic DNA even failed to enhance the stimulatory effects of the pac elements on recombination, since the recombination indices for pLΔR-440, which contains the two pac elements and an anisomorphic DR2 array, and for pLΔR-193, which contains the same pac elements but lacks the DR2 repeats, were nearly identical (Fig. 1 and 2). Although there has been much speculation as to the role that this DNA conformation plays in recombination and although a host cell nuclease has been identified which specifically cleaves this DNA structure (41), the results in this work suggest that the anisomorphic DNA present in the *a* sequence, as well as any nucleases which recognize it, plays little if any role in HSV-1 genome isomerization. This is perhaps not surprising, since the *a* sequence is observed to form anisomorphic DNA only in a supercoiled circular DNA template, which represents a situation that is clearly distinct from the normal linear state of the HSV-1 genome. These results do not preclude the possibility that this unusual DNA conformation plays a role in recombination in other biological systems; for example, the DR2 repeat array appears to promote elevated levels of intramolecular recombination in *E. coli* plasmids (25) and may account for the elevated levels of intramolecular recombination observed in supercoiled circular simian virus 40 minichromosomes containing the L-S junction of HSV-1 (40).

It is important to note that the enhanced recombinogenicity which is associated with the *a* sequence is manifest only when it is examined in the absence of its normal extensive flanking sequence homology. In contrast to their behavior in pLΔR, the 440 and 193 inserts in pLR only nominally increased the frequency of Tn*5* inversion over that of the original pLR construct (Fig. 1 and 2). These results were unexpected, since a number of earlier studies (4, 27, 37) as well as the analysis of the 440 and 193 subfragments in pLΔR (Fig. 1 and 2) had suggested that the *a* sequence can indeed act as a recombinational hot spot. However, these investigations assayed isolated *a* sequences rather than those which are flanked by large regions of homology, such as the IS*50* elements of pLR or the large inverted repeats of the HSV-1 genome. Indeed, the presence of 1.3 kb of IS*50* homology in pLR (Fig. 1 and 2) and 2.2 kb of *b* and *c* sequence homology in pBaC (Fig. 9) was sufficient to abolish the enhanced recombinogenic properties of the *a* sequence. Since the *a* sequence is flanked by a much longer (approximately 16-kb) *b* and *c* sequence homology at its natural site in the HSV-1 genome, it is most probable that the high recombinogenic activity observed for isolated *a* sequences also fails to manifest itself in the viral genome. The experiments described in this work therefore effectively resolve the controversy over what role the *a* sequence plays in HSV-1 genome isomerization, because they directly demonstrate that the *a* sequence fails to increase the frequency of prevailing replication-dependent recombination events at the L-S junction of the viral genome. Thus, recombination arising from

DNA replication through the large *b* and *c* repeats probably accounts for most if not all of the genome isomerization observed for HSV-1, which eliminates the need to evoke models of site-specific recombination in which the *a* sequence acts as the primary recognition element. A previous demonstration of *a* sequence-independent genome isomerization (12) is consistent with this interpretation.

The exact mechanism by which the recombinogenic properties of the *a* sequence are abrogated in the presence of extensive flanking DNA sequence homology is unclear, but it probably involves the process of replication-mediated recombination itself. Studies on bacteriophage lambda and other systems have shown that a replicating rolling-circle template can generate numerous initiating sites (i.e., double-strand breaks) for the formation of recombination intermediates (30, 33, 34). These sites may be repaired without incident, or, when they map in regions of extensive homology, they may lead to recombination events prior to repair. The HSV-1 DNA replication machinery contains proteins which would be well suited for carrying out such processes, including a DNA polymerase for mediating repair synthesis and a single-stranded-DNA-binding protein which possesses a RecA-like ability to promote homologous pairing and strand transfer reactions (1). By virtue of its pac elements, an isolated intact *a* sequence was observed in this study to promote the formation of such initiating breaks at a much higher frequency than other DNA segments of equivalent size. However, when the same sequence was examined within the large inverted repeat segments of the L-S junction, the stimulatory effects of these breaks were effectively diluted out against the high background of initiating sites which resulted from DNA replication through the flanking *b* and *c* sequences. Thus, although the *a* sequence has long been regarded as a hot spot for recombination in HSV-1 and sometimes even as a recognition element for a virus-encoded site-specific recombination system, it is clear from examining this element in its natural context of flanking sequence homology that this reputation is unwarranted.

Finally, an in vitro recombinase assay which is purported to mediate site-specific recombination between duplicated *a* sequences was recently described (2). However, this system yielded several results which were inconsistent with what is known about HSV-1 recombination. First, the recombinase in question is present in mock-infected cells at high levels (i.e., only one-third less than in infected cells), yet numerous studies have shown that in vivo recombination is typically not observed in mock-infected cells (8, 14, 39, 40). Second, the alleged specificity of this recombinase for the *a* sequence in vitro (i.e., a difference of 20-fold in recombination frequency between plasmids with and without *a* sequences) is not reproducible in vivo (only a 2-fold difference between the same plasmid substrates in a transfection-superinfection assay) (8). Third, these experiments utilized a supercoiled circular template, which is topologically distinct from the linear state that the viral genome or a transfected shuttle plasmid assumes during infection. Fourth, only half of the recombination products generated in the assay underwent the desired recombination event. Finally, and perhaps most importantly, this in vitro recombination assay fails to recreate the strict requirements for viral gene products, and viral DNA replication in particular, that are necessary for HSV-1 genome inversion events (8, 14, 39). For these reasons, it is not clear what role, if any, the mammalian cell recombinase described by Bruckner et al. (2) plays in HSV-1 genome isomerization.

ACKNOWLEDGMENTS

We thank F. Homa for use of the HSV-1 mutant gCB prior to publication and for advice on purifying encapsidated viral DNA; N. Lill for DNA sequencing advice; W. Morgan, M. Ptashne, and B. Roizman for plasmids; and J. Smiley and F. Homa for helpful discussions.

This work was supported by Public Health Service grant AI-29961 from the National Institutes of Health (to P.C.W.) and by funds from The Pennsylvania State University, The Pennsylvania Research Corporation, and The Ciba-Geigy Corporation (to P.C.W.).

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