JOURNAL OF VIROLOGY, Oct. 1990, p. 5036–5050
0022-538X/90/105036-15$02.00/0

Vol. 64, No. 10

Sequence Requirements for DNA Rearrangements Induced by the Terminal Repeat of Herpes Simplex Virus Type 1 KOS DNA

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Received 9 May 1990/Accepted 10 July 1990

We investigated the sequence requirements for the site-specific DNA cleavages and recombinational genome isomerization events driven by the terminal repeat or *a* sequence of herpes simplex virus type 1 KOS DNA by inserting a series of mutated *a* sequences into the thymidine kinase locus in the intact viral genome. Our results indicate that sequences located at both extremities of the *a* sequence contribute to these events. Deletions entering from the Ub side of the *a* sequence progressively reduced the frequency of DNA rearrangements, and further deletion of the internal DR2 repeat array had an additional inhibitory effect. This deletion series allowed us to map the *pac1* site-specific DNA cleavage signal specifying the S-terminal cleavage to a sequence that is conserved among herpesvirus genomes. Constructs lacking this signal were unable to directly specify the S-terminal cleavage event but retained a reduced ability to give rise to S termini following recombination with intact *a* sequences. Deletions entering from the Uc side demonstrated that the copy of direct repeat 1 located adjacent to the Uc region plays an important role in the DNA rearrangements induced by the *a* sequence: mutants lacking this sequence displayed a reduced frequency of novel terminal and recombinational inversion fragments, and further deletions of the Uc region had a relatively minor additional effect. By using a construct in which site-specific cleavage was directed to heterologous DNA sequences, we found that the recombination events leading to genome segment inversion did not occur at the sites of DNA cleavage used by the cleavage-packaging machinery. This observation, coupled with the finding that completely nonoverlapping portions of the *a* sequence retained detectable recombinational activity, suggests that inter-*a* recombination does not occur by cleavage-ligation at a single specific site in herpes simplex virus type 1 strain KOS. The mutational sensitivity of the extremities of the *a* sequence leads us to hypothesize that the site-specific DNA breaks induced by the cleavage-packaging system stimulate the initiation of recombination.

The 152-kilobase (kb) genome of herpes simplex virus (HSV) consists of a long (L) and a short (S) segment flanked by large inverted repeats termed *b* and *c*, respectively. The smaller *a* sequence is directly repeated at both ends of the genome and is present in an inverted orientation at the junction between the L and S segments (reviewed in reference 28; Fig. 1). The L and S segments invert relative to each other, generating four isomeric forms of viral DNA (11, 30, 31, 46). As described below, the *a* sequence plays important roles in the HSV replicative cycle.

The herpes simplex virus type 1 (HSV-1) *a* sequence (Fig. 2) is flanked by direct repeats of a 20-nucleotide (nt) sequence termed direct repeat 1 (DR1) and is composed of two unique sequence arms, Ub and Uc, that are separated by internal repeated arrays that vary in copy number and sequence between HSV-1 isolates (6, 22, 41). The *a* sequences of HSV-1 strains USA8, KOS, 17, and F contain 8, 9 to 10, 18 to 21, and 18 to 21 tandem copies of the 12-nt DR2 repeat, respectively. In strain F, the DR2 repeats are followed by three tandem copies of a 37-nt sequence termed DR4; only one partial copy of the DR4 sequence is present in the Uc regions of KOS and strain 17. Strain USA8 contains a different sequence at this position, which is reiterated six times. The functional significance of these variations in repeat structure remains uncertain.

The most clearly defined function of the *a* sequence is that it contains the cleavage-packaging signal for encapsidation of progeny viral genomes (34, 37, 38). HSV DNA circularizes upon infection and then generates concatemeric forms, most likely by rolling-circle replication (12, 13, 15, 25, 42). The *a* sequence serves as the recognition sequence for the machinery that processes these concatemers to unit length during the encapsidation process (34, 37, 38, 41). The cleavage-packaging reaction involves two site-specific breaks that are made on either side of the *a* sequence, at defined distances from signals located in the Ub and Uc regions (41). Normally these two cleavages are made at the same position within each copy of the flanking DR1 sequences (6, 23); however, when the DR1 sequences are deleted, cleavage occurs at analogous positions within the flanking heterologous DNA sequences (41). The cleavage reaction appears to rely on localized DNA sequence amplification (7, 8, 41). Thus, substrates bearing a single *a* sequence are processed to yield two termini, each bearing a copy of the *a* sequence (8, 41; diagramed in Fig. 1), and *a* sequences become tandemly reiterated during propagation of viral stocks (43). It is not clear whether these sequence duplications precede the formation of termini; an alternative possibility is that the cleavage-packaging process itself duplicates the *a* sequence. Consistent with this latter hypothesis, heterologous sequences placed between the two cleavage sites are included in the reiterated unit and the endpoints of the resulting duplication correspond to the positions of site-specific cleavage (41). These data demonstrate that sequence reiteration is driven by the same site-specific DNA cleavages that give rise to the genomic termini.

Comparative analysis of the *a* sequences of diverse herpesvirus genomes has revealed two highly conserved sequence elements (termed *pac1* and *pac2*) that are likely

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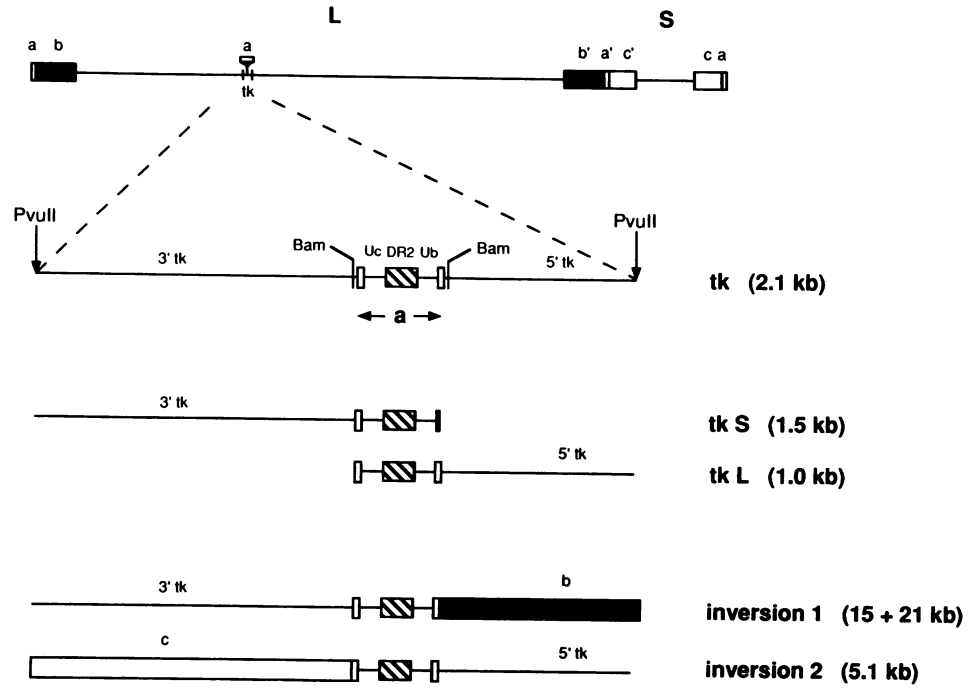

FIG. 1. Experimental design. *a* sequences were inserted into a *Bam*HI site that spans a previously described deletion in the *tk* gene of the intact HSV genome (41; diagramed in the upper portion of the figure). The lower portion of the figure shows the *tk* region at an expanded scale and diagrams the structures of the novel terminal and inversion fragments provoked by insertion of the *a* sequence. Cleavage of viral concatemers at the inserted *a* sequence gives rise to two novel termini, labeled *tk*-S and *tk*-L; these were detected by hybridization with plasmid clones bearing *Pvu*II-*Bam*HI fragments representing the 3′- and 5′-flanking *tk* sequences, respectively. Recombination between the inserted *a* sequence and those located at the L-S junction gives rise to novel inversion fragments that fuse *tk* sequences to the *b* and *c* repeats. In accord with convention, the viral genome is displayed in the prototype arrangement in this figure. In all other figures, the viral *tk* gene and associated *a* inserts are displayed in the opposite orientation (i.e., with the *tk* gene oriented 5′ to 3′).

candidates for the signals that direct site-specific cleavage (1, 2, 5, 10, 17, 18, 20, 39; Fig. 2). In the HSV-1 *a* sequence, *pac1* and *pac2* are located in the Ub and Uc regions, respectively. Thus, *pac1* is predicted to specify the location of the S-terminal cleavage event and *pac2* likely directs the L-terminal cleavage. Recent studies provide some evidence that these conserved sequences play functionally distinct roles in the cleavage-packaging process (7). Presumably, these elements are recognized by proteins that form part of the cleavage-packaging machinery. Consistent with this hypothesis, two HSV-1 polypeptides form a complex that binds to the *pac2*/DR1 region of the *a* sequence (4); the analogous region of the cytomegalovirus *a* sequence binds a cellular protein (14).

The inverted arrangement of the *a* sequences at the L-S junction relative to those at the genomic termini suggests an explanation for some, but not all, of the genome isomerization events; as detailed previously (41), circularization and concatemerization of viral DNA generates a structure with two potential cleavage phases defined by directly repeated *a* sequences separated by 152 kb. If cleavage can occur in either phase, then two isomeric forms of viral DNA will be produced from a concatemer derived from a single isomer. This hypothetical mechanism can account for half of the observed genome isomerization events. Recombinational mechanisms are required to account for the remainder.

It has been suggested that the *a* sequence serves as the target of a site-specific recombination system that drives these recombinational inversion events (3, 21–24). The initial evidence that led to this conclusion was that insertion of an additional copy of a fragment bearing the *a* sequence into the viral genome provoked rearrangements that could be accounted for only by recombination, while constructed inverted duplications of several other fragments of viral DNA did not induce analogous rearrangements (21). However, the conclusion that the recombination events provoked by the *a* sequence are site specific was weakened by later findings indicating that a variety of fragments lacking an *a* sequence are also able to recombine when they are duplicated in the viral genome (16, 26, 27, 41, 44). Perhaps the strongest evidence against site specificity was provided by the demonstration by Weber et al. (44) that the HSV DNA replication system is inherently recombinogenic. Those researchers found that constructed repeats of procaryotic DNA sequences are able to efficiently recombine by generalized recombination in HSV-based replicons in a process that depends only on the seven HSV genes that specify the viral DNA replication machinery.

These studies clearly demonstrate that genome isomerization can occur by generalized recombination between DNA fragments that lack an *a* sequence. However, several observations suggest that the *a* sequence may be considerably more recombinogenic than other DNA segments of equivalent length and provide some support for the hypothesis that these high-frequency recombination events result from specific mechanisms acting on the *a* sequence. First, Weber et al. (44) reported that the minimum length of homology required for detectable intramolecular recombination between DNA segments lacking the *a* sequence is approximately 600 base pairs in HSV replicons, while Chou and Roizman (3) found that smaller derivatives of the *a* sequence drove high levels of inversion. The latter researchers re-

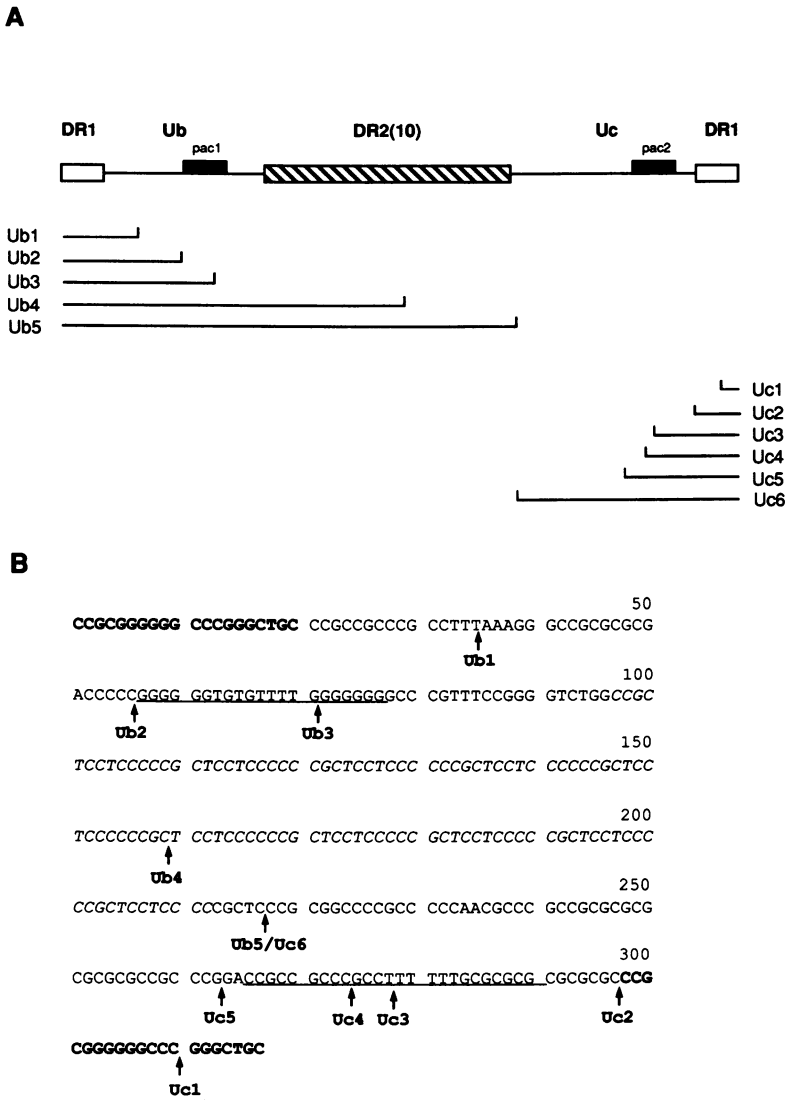

FIG. 2. Deletions of the *a* sequence. (A) The structure of an intact *a* sequence is diagramed, with the DR1, *pac1*, DR2, and *pac2* elements indicated. Bars represent the portions of the *a* sequence deleted from the various mutant constructs. (B) The sequence of the KOS *a* sequence is displayed, along with the deletion endpoints of the various constructs. The DR1 element is in boldface, the DR2 repeat array is italicized, and the *pac1* and *pac2* homologies are underlined.

ported that the recombinational activity of the *a* sequence is heavily dependent on the 37-nt DR4 sequence that is tandemly repeated three times in HSV-1 strain F. Second, Weber et al. (45) recently reported that a fragment spanning the L-S junction of HSV-1 DNA is considerably more recombinogenic than a procaryotic DNA segment of equivalent length when placed in a simian virus 40-based replicon. Third, Deiss, Chou, and Frenkel (8) found that a small deletion removing the *pac2* DNA cleavage signal from a plasmid-borne *a* sequence eliminated its ability to recombine with *a* sequences located in the viral genome. Those researchers interpreted their data to indicate that the site-specific cleavage induced by *pac2* is coupled to a specialized recombination system that is involved in the generation of termini. This study did not address the question of whether *pac2*-stimulated recombination contributes to HSV genome isomerization.

We investigated the sequence requirements for the DNA rearrangements driven by the *a* sequence of HSV-1 strain KOS. Our results demonstrate that relatively small deletions at either end of the *a* sequence inhibited DNA rearrangement, although completely nonoverlapping subfragments retained detectable activity. Our data suggest that inter-*a* recombination does not occur by breakage and ligation at a single specific site in HSV-1 KOS. The mutational sensitivity of the extremities of the *a* sequence leads us to hypothesize that the free DNA ends generated by the cleavage-packaging system stimulate the initiation of recombination.

MATERIALS AND METHODS

Virus and cells. HSV-1 strain KOS PAA^{r}5 (9) and Vero cells were used throughout this study. Some experiments used the previously described F10 recombinant (41), which bears the *Sma*I F subfragment of the *a* sequence inserted into the viral thymidine kinase (*tk*) locus.

Oligonucleotides. The following oligonucleotides were purchased from the Central Facility of the Institute of Molecular

Biology and Biotechnology, McMaster University: 3′ *tk*, 5′ CGCTGGCGTTTGGCCAGGCGGTCGA 3′; 5′ *tk*, 5′ TT CACGCCACCAAGATCTGCGGCAC 3′; probe 1, 5′ GGG CGCTTGTCATTACCACCGCCGC 3′; probe 2, 5′ CCGCC GCCCGCCTTTAAAGG 3′.

Construction of deleted *a* sequences. The mutated *a* sequences described in this study were derived from plasmids bearing an intact *a* sequence or the previously described *Sma*I F subfragment (41) inserted between the *Bam*HI sites of pUC7.

An intact *a* sequence lacking other flanking HSV-1 DNA sequences was constructed as follows. First, a synthetic copy of DR1 was cloned between the *Hin*cII sites of pUC7, as previously described (41). An *Xma*I partial digestion product corresponding to the remainder of the *a* sequence was then inserted into the *Xma*I site within DR1. The structure of the resulting complete *a* sequence was confirmed by Maxam-Gilbert sequence analysis (19). The *a* fragment was excised from pUC7 with *Bam*HI and recloned at the *Bam*HI site of pUC18.

Mutant Ub1 was constructed by ligating *Bgl*II linkers to the *Dra*I end of the larger *Dra*I-*Bam*HI subfragment of the *a* sequence and recloning the resulting *Bam*HI-*Bgl*II fragment. Ub2 was derived by ligating a larger *Bam*HI-*Ava*II subfragment of the *Sma*I F fragment to an *Ava*II-*Bam*HI fragment bearing the remainder of the *a* sequence. Ub3 and Ub4 were derived by BAL 31 digestion of a linearized plasmid bearing *Sma*I F, followed by repair with T4 DNA polymerase and ligation in the presence of *Bgl*II linkers. After nucleotide sequence analysis, these constructs were ligated to the appropriate *Bam*HI-*Ava*II fragment of the intact *a* sequence to generate variants deleted from the Ub side only. Ub5 was constructed by cloning the smallest *Sst*II subfragment of the *a* sequence into the *Sst*II site within DR1.

Uc1 was isolated by recombining the smaller *Bam*HI-*Ava*II fragment of *Sma*I F with the larger *Bam*HI-*Ava*II fragment of the intact *a* sequence. Uc2 was made by ligating the larger *Bam*HI-*Ava*II fragment of the intact *a* sequence to a synthetic oligonucleotide comprising the remainder of the Uc region linked to a terminal *Bam*HI site. Mutants Uc3, Uc4, and Uc6 were produced by exonuclease III/SI treatment of a pUC18 derivative bearing the *a* sequence at the *Bam*HI site. Following cleavage with *Pst*I and *Xba*I, the DNA was treated with *Exo*III/SI, repaired with T4 DNA polymerase, and religated in the presence of *Bgl*II linkers. Uc5 was derived by ligating *Bgl*II linkers to the *Ava*II end of the larger *Bam*HI-*Ava*II subfragment of the *a* sequence (after repair with T4 DNA polymerase) and recloning the resulting *Bam*HI-*Bgl*II fragment.

In each instance, the structure of the resulting construct was verified by Maxam-Gilbert sequence analysis (19). The mutated *a* sequences were then cloned into the middle of the HSV *tk* structural gene through a *Bam*HI site spanning a previously described 200-nt *tk* gene deletion (41). The *a* derivatives were all oriented with respect to the flanking *tk* sequences such that the Ub side of the insert was adjacent to the 5′ *tk* sequences.

Construction of recombinant viral strains. Viral recombinants bearing inserts of mutated *a* sequences at the *tk* locus were produced by in vivo recombination following cotransfection of Vero cells with infectious PAA^{r}5 DNA and plasmids carrying the appropriate *a* sequence insertion, as previously described (32, 41). *tk*-deficient viral mutants were isolated by plaque purification in the presence of 100 μg of 5-bromodeoxycytidine per ml, and those bearing the desired insertion were identified by Southern blot hybridization (33). Following plaque purification, isolates were amplified to give stocks derived from 10 150-cm^2 flasks of Vero cells. These were then used to prepare nucleocapsid DNA for analysis.

Cloning and sequencing of fragments from viral DNA. Recombinational inversion fragments from F10 viral DNA were cloned by ligating *Sst*I-cleaved nucleocapsid DNA into the *Sst*I site of pUC18. Clones bearing *tk*-related fragments were identified by colony hybridization by using a mixture of two synthetic 25-mers. One of these probes (3′ *tk*) annealed to the 3′-flanking *tk* sequences, and the other (5′ *tk*) was complementary to 5′ *tk* DNA. Positive colonies were purified, and the subset bearing inversion fragments was identified by failure to hybridize to one of the two *tk* probes. Following confirmation by restriction analysis, relevant regions of the clones were sequenced by chemical degradation (19), chain termination (29), or both methods.

A *tk*-S-terminal fragment from mutant Ub3 was recovered as follows. Viral DNA was treated with T4 DNA polymerase and then cleaved with *Eco*RI. Gel-purified *tk*-S-terminal fragments were cloned between the *Hin*cII and *Eco*RI sites on pUC18, and the desired recombinants were identified by colony hybridization with a 25-mer specific for 3′ *tk* sequences. The structure of the *tk*-S terminus was then determined by Maxam-Gilbert sequence analysis.

The integrity of construct Ub5 present in the corresponding viral strain was confirmed by recloning the Ub5 viral *Eco*RI *tk* fragment into the *Eco*RI site of pUC18. Following identification of an appropriate plasmid by colony hybridization with a 25-mer specific for 3′ *tk* sequences, the structure of the Ub5 *a* sequence insert was verified by Maxam-Gilbert sequence analysis.

Southern blots and hybridization. Viral DNA was isolated from cytoplasmic nucleocapsids as previously described (32, 43). Two-microgram samples were cleaved with the indicated restriction enzymes, and the fragments were separated by electrophoresis through agarose gels. Following transfer to nitrocellulose, blots were hybridized to denatured nick-translated plasmid DNA or 5′-end-labeled oligonucleotides in 4× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate–10% dextran sulfate–2.5× Denhardt solution containing 125 μg of denatured salmon sperm DNA per ml. For plasmid probes, hybridization was at 60°C, and the blots were washed three times with 2× SSC–0.1% sodium dodecyl sulfate at 68°C, followed by one wash with 0.1× SSC–0.1% sodium dodecyl sulfate at 54°C. Oligonucleotides were hybridized at 40°C, and the blots were washed four times with 2× SSC–0.1% sodium dodecyl sulfate at 50°C.

Microdensitometry. Southern blots were exposed to Kodak XAR5 film for various periods without an intensifier screen, and then the resulting autoradiograms were scanned with an LKB laser microdensitometer.

RESULTS

Experimental design. We wished to more precisely delineate the sequence requirements for the cleavage-packaging and genome isomerization events that are driven by the *a* sequence of HSV-1 strain KOS. In order to eliminate the possible contribution of sequences that flank the *a* sequence at normal L-S junctions, we first constructed an intact 317-nt *a* sequence flanked by *Bam*HI cleavage sites (Materials and Methods). We then derived two sets of mutated *a* sequences bearing deletions entering from the Ub and Uc sides, respectively (Fig. 2). Each of these mutant derivatives was transferred into the middle of the *tk* gene of the intact viral

genome by DNA-mediated marker rescue. The inserted *a* sequences were all oriented with respect to the flanking *tk* sequences such that the Ub portion of the insert was closest to 5′ end of the *tk* gene (Fig. 1).

Recombination between the test *a* sequences inserted at the *tk* locus and the additional *a* sequences present at their natural genomic sites is predicted to give rise to novel recombinant fragments in which *tk* sequences are fused to the *b* or *c* repeats (21, 32; diagramed in Fig. 1). The abundance of these fragments provides an estimate of the recombinational proficiency of the mutated *a* sequence. In addition, active *a* sequences provoke the formation of novel genomic termini. We have previously demonstrated that in some cases these termini arise directly through cleavage of viral concatemers at the *tk* locus rather than at normal L-S junctions (41; as diagramed in Fig. 1). However, as hypothesized by Diess, Chou, and Frenkel (7) and directly documented below, novel termini also arise following recombination between the test *a* sequence and the intact *a* sequences present at normal L-S junctions. Because these recombination events are able to restore sequences that were initially lacking from the test *a* sequence, the abundance of novel termini produced by mutant constructs is a function of both the ability of the altered *a* sequence to be acted upon directly by the cleavage machinery and its recombinational activity.

In order to detect the novel termini and junctions induced by the mutated *a* sequences, packaged viral DNA was cleaved with *Pvu*II and the resulting fragments were separated by electrophoresis through 1.4% agarose gels. The gels were blotted to duplicate nitrocellulose filters and then probed with cloned fragments representing the 5′- and 3′-flanking *tk* sequences. Various exposures of the resulting autoradiographs were analyzed by microdensitometry to provide a quantitative estimate of the relative abundance of the various *tk*-related fragments in each mutant isolate.

Activity of the intact *a* sequence. The intact *a* sequence present in strain A generated the predicted pattern of fragments (Fig. 3; see Fig. 8). Thus the 3′ *tk* probe detected one set of recombinant fragments representing 3′ *tk* sequences fused to the *b* repeats (ca. 15 and 21 kb; not resolved from each other in this gel system; additional data not shown), the unrearranged *tk* fragment (2.1 kb) and the *tk*-S terminus (1.5 kb). Similarly, the 5′ *tk* probe detected the other set of inversion fragments (5′ *tk* sequences fused to the *c* repeats; 5.1 kb), the *tk*-L terminus (1.0 kb), and the unrearranged *tk* fragment (2.1 kb). Bona fide L-S junctions and L termini bear varied numbers of tandemly reiterated *a* sequences (43) and hence migrate as a series of bands differing in size by ca. 300 nt. As expected on the basis of previous work (41), the *tk* and *tk*-L fragments present in strain A also displayed this characteristic pattern; the resulting series of related fragments are designated *tk*, *tk**, etc., in Fig. 3. By contrast, the *tk*-S-terminal fragment migrated as a single band, paralleling the behavior of bona fide S termini. The nucleotide sequence analysis of inversion fragments described below, in combination with our previously described sequence analysis of novel termini (41), confirmed our identification of each of the novel fragments displayed in Fig. 3 (see also Fig. 8).

The activity of the intact *a* sequence present in strain A served as a reference point for the evaluation of mutant phenotypes. The results obtained with the 5′ and 3′ *tk* probes were in good quantitative agreement (Table 1) and demonstrated that approximately two-thirds of the packaged strain A DNA molecules bore an intact *tk* gene (5′ probe: 67.1 ± 2.0% [standard deviation]; 3′ probe: 64.1 ± 2.3%, $n = 6$).

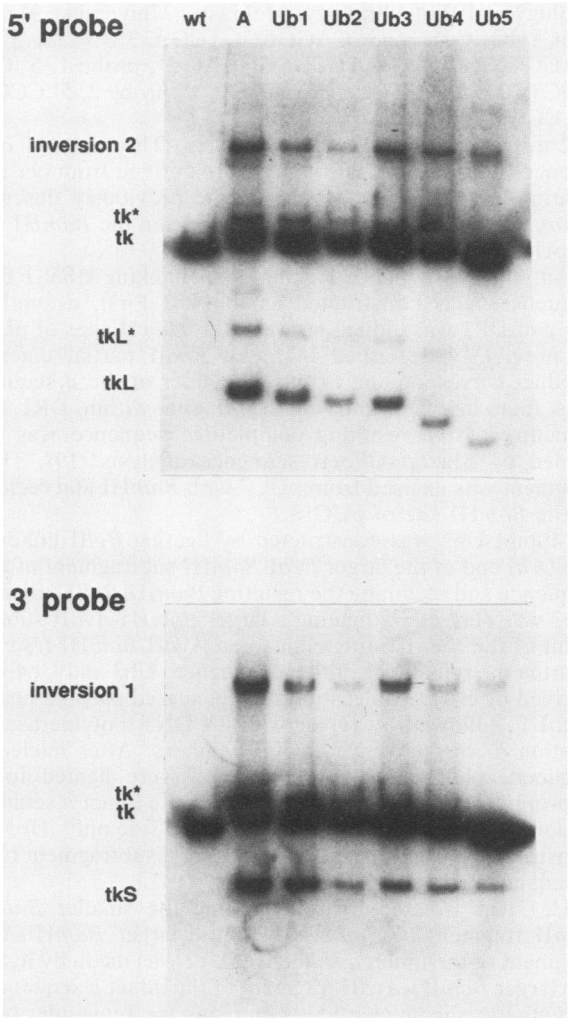

FIG. 3. Southern blot analysis of Ub deletion mutants. Packaged viral DNA prepared from the indicated viral strains was cleaved with *Pvu*II, and the fragments were separated by electrophoresis through a 1.4% agarose gel. Following transfer to duplicate nitrocellulose filters, the blots were hybridized to cloned *Pvu*II-*Bam*HI fragments representing the 5′- and 3′-flanking *tk* sequences. The various *tk*-related fragments are labeled (see Fig. 1 for more details concerning the structures of these fragments). wt, Wild-type KOS PAA^{r}5 DNA; A, DNA from a viral strain bearing an insert of the intact *a* sequence.

The remaining one-third of the molecules bore *tk* termini or novel inversion fragments or both. Although the two classes of novel termini and junctions were detected with nonoverlapping probes, the presence of a common hybridizing fragment (the intact *tk* gene) that comprised an equivalent proportion of the total signal obtained with each probe allowed a direct comparison of the data. The *tk*-L and *tk*-S termini were detected at approximately equal molar yields (*tk-L* + *tk-L**: 20.1 ± 2.3% of the 5′ *tk* signal; *tk*-S: 23.2 ± 3.8% of the 3′ *tk* signal, $n = 6$), as were the two classes of inversion fragments (inversion 1: 12.7 ± 2.1%; inversion 2: 12.9 ± 2.0%, $n = 6$).

Two features of these data deserve comment. First, the proportion of molecules bearing *tk* termini (ca. 20%) was somewhat lower than predicted on the assumption that introduction of an *a* sequence at the *tk* locus defines a third

TABLE 1. Frequency of rearrangements induced by *a* sequence mutants[a]

Probe	Mutant	Inversion	*tk*	Terminus
3′ probe	A	12.4/12.3	66.8/63.3	20.8/24.4
	Ub1	3.5/9.0	82.6/78.3	13.9/12.7
	Ub2	1.8/2.1	92.0/94.9	6.2/3.0
	Ub3	5.1/4.0	88.9/91.0	6.0/5.0
	Ub4	1.8/4.5	91.9/86.9	6.3/8.6
	Ub5	1.3/0.8	96.7/97.9	2.0/1.3
5′ probe	A	13.5/11.9	67.8/67.3	18.7/20.8
	Ub1	4.5/4.2	82.5/81.0	13.0/14.8
	Ub2	2.5/4.0	90.0/81.5	7.5/14.5
	Ub3	5.7/4.7	86.4/87.4	7.9/7.9
	Ub4	3.9/7.9	90.0/83.1	6.1/9.0
	Ub5	4.9/2.6	93.0/93.8	2.1/3.5
3′ probe	A	14.1/8.8/14.1/14.2	61.7/61.4/66.1/65.5	24.2/29.8/19.8/20.3
	Uc1	6.9/ 3.8/7.0/13.0	73.4/71.7/75.1/72.0	19.7/24.6/17.9/18.4
	Uc2	ND/ND/2.1/1.5	ND/ND/91.3/90.2	ND/ND/6.6/7.9
	Uc3	1.3/2.3/0.8/0.8	91.5/88.8/93.3/92.2	7.2/8.9/5.9/7.0
	Uc4	1.4/1.4	91.5/92.1	7.1/6.5
	Uc5	2.0/2.6	92.4/91.4	5.6/6.0
	Uc6	1.6/0.7	94.4/94.0	4.0/5.3
5′ probe	A	13.9/12.3/9.8/15.8	63.3/66.7/69.7/67.8	23.1/21.0/20.5/16.4
	Uc1	7.2/11.1/10.3/13.0	74.2/73.4/67.4/40.3	18.6/15.5/22.3/46.7
	Uc2	ND/ND/5.9/6.5	ND/ND/91.2/82.3	ND/ND/2.9/11.2
	Uc3	3.8/3.5/3.0/3.9	93.6/93.3/82.6/93	2.6/3.3/1.3/3.1
	Uc4	5.3/1.6	91.3/95.5	3.4/2.9
	Uc5	3.8/2.5	92.6/92.3	3.6/5.2
	Uc6	4.9/2.4	92.2/94.0	2.9/3.6

[a] Viral DNAs were cleaved with *Pvu*II, blotted to nitrocellulose, then probed with 5′- and 3′-flanking *tk* sequences as in Fig. 3 and 8. For each experiment, the proportion of the hybridization signal originating from the inversion, *tk*, and terminal bands is displayed. Observations separated by a shill are from independent experiments. ND, Not determined.

potential cleavage phase on viral concatemers; if this new phase was chosen with the same frequency as the two normal phases, then the simplest prediction is that one-third of viral DNA molecules would bear *tk* termini. One interpretation of this finding is that sequences flanking the *a* sequence at normal L-S junctions facilitate its recognition by the cleavage-packaging machinery. Second, the frequency of novel junction fragments was low (ca. 12%), suggesting that the system was far from recombinational equilibrium. (Insertion of the *a* sequence is predicted to generate 12 isomeric forms of intact viral DNA [21, 32], as well as novel deleted derivatives arising through recombination between directly repeated *a* sequences [32]. If the intact molecules were evenly partitioned between the 12 predicted isomers, then novel junctions would contribute one-third of the total signal obtained with each of the 5′ and 3′ *tk* probes [see reference 32 for a more detailed discussion]. The presence of deleted genomes would increase this fraction further.) The relatively low abundance of novel junctions observed in strain A DNA appears to contrast with the results obtained by Chou and Roizman (3) in similar experiments with the larger *a* sequence of HSV-1 strain F; although quantitative data were not reported, inspection of their published results suggests that novel junction fragments were at least as abundant as unrearranged *tk* genes in DNA prepared from their construct A. Thus, the *a* sequence of HSV-1 KOS may be less recombinogenic than the *a* sequence of HSV-1 F. As described above, the KOS *a* sequence lacks the DR4 repeats found in strain F; therefore, our data are consistent with the hypothesis of Chou and Roizman that the DR4 repeats enhance the recombinational activity of the HSV-1 F *a* sequence.

Effects of deletions of the Ub region. Deletions entering from the Ub side of the *a* sequence had two readily apparent effects. (i) Deletions removing increasing portions of the *a* sequence reduced the abundance of novel termini and junctions (Table 1). The data are summarized in graphic form in Fig. 4B; in order to simplify comparisons, we plotted the proportion of molecules bearing either type of sequence rearrangement against the position of the deletion endpoint. This presentation seemed appropriate because we were unable to discern any convincing differential effects of these mutations on formation of novel termini compared with novel junctions (Table 1). Rearranged fragments were present at approximately 1/2 the normal frequency in DNA samples prepared from mutant Ub1 and at roughly 1/5 to 1/10 the wild-type levels in isolate Ub5 (which lacks the entire Ub region and the DR2 repeat array). These results indicate that the integrity of the DR1/Ub region is required for normal levels of genome isomerization and cleavage and suggest that the DR2 array may also play a role in these processes. However, it is also clear from these data that neither the Ub region nor the DR2 array is absolutely required for detectable activity. Three points seem particularly noteworthy. First, the frequency of novel junctions and termini was significantly reduced by mutations that leave the *pac1* homology intact (Ub1 and Ub2). Second, deletion of *pac1* did not have a dramatic additional effect on rearrangement frequencies (Ub2 versus Ub3). Third, mutant Ub5 displayed detectable activity. We speculate on the significance of these observations in the Discussion.

The activity of Ub5 contrasts with the previous report of Chou and Roizman (3) that the analogous fragment of the HSV-1 strain F *a* sequence (their construct G) was incapable

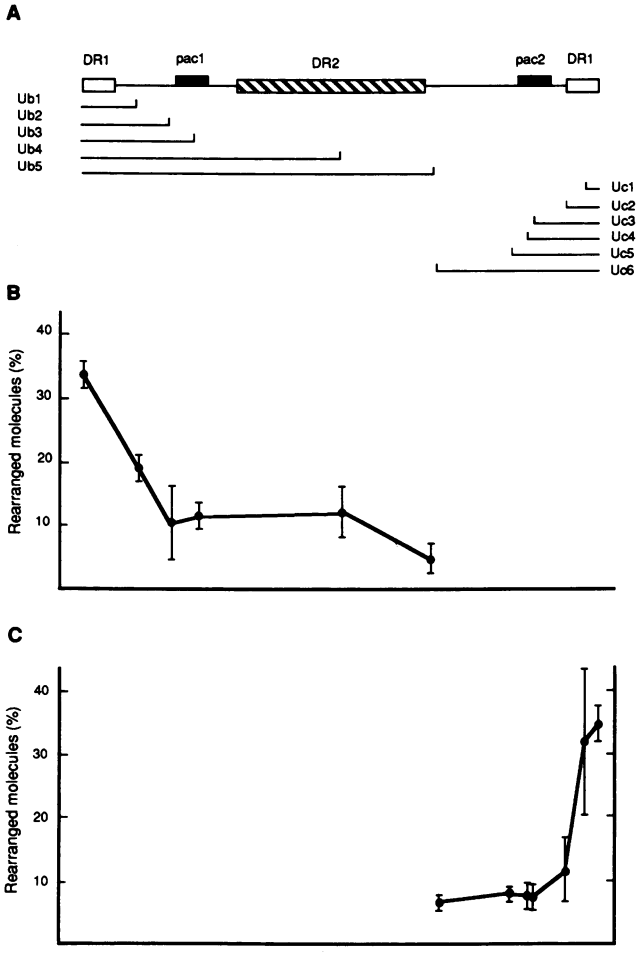

FIG. 4. Effects of deletions on the proportion of rearranged DNA molecules. (A) Bars represent the portion of the *a* sequence that is deleted from each construct. (B and C) The data displayed in Table 1 were used to compute the proportion of DNA molecules that either were cleaved at the *tk* locus or bore novel recombinant junctions. Error bars display the standard deviation of the data. (B) Ub deletions. (C) Uc deletions.

of generating novel termini. To test the possibility that sequence changes occurring after marker transfer might account for our discrepant results, we cloned and sequenced the mutant *a* sequence from the Ub5 recombinant viral strain and found that its structure corresponded to that initially constructed. We also generated an additional viral recombinant in which the Ub5 fragment was inserted in the other orientation relative to the *tk* gene and found that it displayed activity similar to that shown in Fig. 3 (data not shown).

(ii) The Ub deletions progressively reduced the length of the *tk*-L-terminal fragments, while the length of the *tk*-S termini was not affected (Fig. 3). The decreasing length of the *tk*-L termini suggested that these arose by cleavage at the correct position relative to the *pac2* signal (i.e., within the DR1 at the right end of the *a* sequence, as diagramed in Fig. 5); data that directly support this conclusion are presented below. As shown in Fig. 5, this process would produce *tk*-L termini that bear the initially constructed deletion of Ub sequences. The failure of the Ub deletions to affect the size of the *tk*-S termini was more difficult to explain. We have previously shown that a Ub-deleted construct that retains the *pac1* homology produces *tk*-S termini by cleavage within the flanking 5′ *tk* sequences at a fixed distance from *pac1* (41). This mechanism can account for the constant length of the *tk*-S terminal fragments in mutants retaining *pac1* (i.e., Ub1 and Ub2), but it does not explain the formation of *tk*-S ends in the mutants that lack this conserved sequence (Ub3, Ub4, and Ub5).

A recombinational pathway for formation of *tk*-S termini. We considered the possibility that *tk*-S termini might arise following recombination events between the mutated *a* sequence and one of the intact *a* sequences located elsewhere in the viral genome. Were this to occur, the resulting *tk*-S termini would be tipped with sequences donated by the intact *a* sequence (Fig. 5). In contrast, if the *tk*-S termini arose through the previously described mechanism involving cleavage at a fixed distance from *pac1*, then they would end in 5′-flanking *tk* sequences (Fig. 5; see reference 41 for further details). As one approach to distinguishing between these pathways, we generated two synthetic oligonucleotide probes that allowed us to examine the structure of the extreme terminal regions of the *tk*-S ends. One oligonucleotide (probe 1) consisted of 25 residues corresponding to the *tk* sequences immediately 5′ to the inserted *a* sequences (Fig. 5). We have previously shown that this sequence is included in at least some of the *tk*-S termini produced by the construct F10 (41), which bears the same Ub deletion endpoint as construct Ub2. Ub mutants that retain the *pac1* sequence are therefore predicted to produce *tk*-S termini that react with this probe. The other oligonucleotide (probe 2) was complementary to the first 20 residues of Ub, a region that is deleted from constructs Ub2 through Ub5. Putative *tk*-S termini arising following recombination with an intact *a* sequence are predicted to bear this sequence and would therefore hybridize to the probe (Fig. 5).

Viral DNA from selected Ub deletion mutants was cleaved with *Eco*RI, subjected to electrophoresis through a 1.4% agarose gel, transferred to nitrocellulose, and probed with oligonucleotide 1 (Fig. 6A). As a control, we included DNA from the previously described mutant F10, which produces at least some of its *tk*-S termini by direct cleavage within the 5′-flanking *tk* sequences (41). Strain F10 DNA displayed the predicted hybridization pattern: the probe hybridized to the *tk*-L terminus, the *tk*-S terminus, the unrearranged *tk* fragment, and a recombinational inversion fragment in which 5′-flanking *tk* sequences are fused to the *c* repeats. In addition, the probe also hybridized to a larger fragment of unknown origin. This latter band was also observed with wild-type DNA (not shown) and therefore most likely represents an unrelated viral DNA fragment that cross-hybridizes with the probe. The presence of hybridizing *tk*-S termini in F10 DNA confirmed our previous finding that strain F10 produces at least some *tk*-S termini by cleavage in the 5′-flanking *tk* sequences (41). Of the Ub mutants tested, only Ub2 (retaining the *pac1* homology) displayed detectable *tk*-S termini bearing 5′ *tk*-flanking sequences (Fig. 6A). This result, obtained in three independent experiments, indicated that mutants Ub3 and Ub4 (lacking *pac1*) were unable to produce *tk*-S termini through cleavage in the 5′-flanking *tk* sequences.

In order to test the hypothesis that *tk*-S termini can arise following recombination with intact *a* sequences, viral DNA was cleaved with *Pvu*II and probed with oligonucleotide 2 (Fig. 6B). The probe detected the intact *a* sequences located at the bona fide L-S junctions and termini. In addition, the probe also detected the *tk*, *tk*-L, and *tk*-S fragments in strain A DNA (the identification of these fragments was confirmed

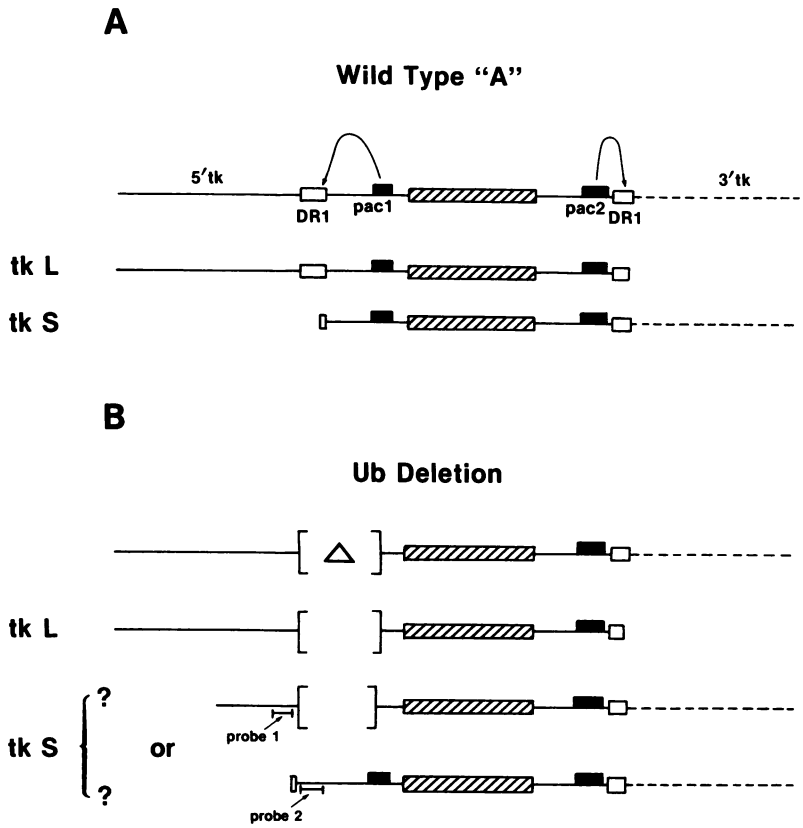

FIG. 5. Structures of novel termini produced by Ub deletion mutants. (A) The termini produced by a wild-type *a* sequence arise by two measured cleavages at fixed distances from the *pac1* and *pac2* signals; the position of the L-terminal cleavage is specified by *pac2*, and the position of the S-terminal cleavage is specified by *pac1*. The figure indicates that two termini, each bearing an *a* sequence, are generated from a substrate bearing a single *a* sequence, as previously documented (41). (B) Effects of Ub deletions. Deletions in the Ub region are predicted to reduce the size of *tk*-L termini, as diagramed. Those mutant constructs that retain *pac1* are predicted to produce *tk*-S termini by cleavage in the flanking *tk* sequences (41). These *tk*-S termini should therefore hybridize to probe 1 (representing 5'-flanking *tk* sequences). In contrast, constructs lacking *pac1* are postulated to generate *tk*-S termini only following recombination with intact *a* sequences. The resulting *tk*-S termini should therefore hybridize with probe 2 (representing a portion of the Ub region that is lacking from the mutant *a* sequence). Note that in both cases, the size of the *tk*-S-terminal fragment is not altered by the Ub deletion.

by rehybridizing the blot with a probe representing the 5'- and 3'-flanking *tk* sequences; data not shown). The pattern observed with mutants Ub2, -3, and -4 displayed three noteworthy features. First, the *tk*-L-terminal fragments produced by these mutants did not hybridize to the probe. This result confirmed that these termini retained the initially constructed deletions (which remove sequences homologous to the probe). Second, all of the constructs displayed hybridizing *tk*-S termini (Fig. 6B). As these *tk*-S termini bear sequences that are not present in the mutated test constructs, they must have arisen through recombination with intact *a* sequences. Third, faint hybridization was also observed to the *tk* and *tk** bands, suggesting that a subset of these fragments acquired an intact *a* sequence (discussed further below).

As an additional test of the hypothesis that *tk*-S termini can arise following recombination with an intact *a* sequence, we cloned and sequenced a *tk*-S-terminal fragment from mutant Ub3 (which lacks the *pac1* homology). The results demonstrated that this *tk*-S-terminal fragment ended in sequences derived from intact *a* sequences, exactly as predicted in Fig. 5B. Because the initially constructed mutation was absent from this terminal fragment, these data prove that *tk*-S termini can arise following recombination with intact *a* sequences.

We drew two conclusions from these data. First, Ub deletion mutants that retain *pac1* (for example, Ub2) can generate *tk*-S termini through two distinct mechanisms. One, previously described (41), involves direct cleavage at a fixed distance from *pac1*. The other involves recombination with intact *a* sequences. Second, within the limits of detection, mutants lacking *pac1* generated *tk*-S termini only through this latter recombinational pathway. This analysis provided direct evidence that the *pac1* signal overlaps the 15-nt interval between the Ub2 and Ub3 deletion endpoints. This interval includes a portion of the previously recognized sequence that is strongly conserved among herpesvirus genomes (GGGGGGTGTGTTTTGGGGGGGG).

Our data indicate that constructs lacking *pac1* produce *tk*-L termini that retain the initially constructed deletion and *tk*-S termini that carry an intact *a* sequence. One explanation of this finding is that these termini arise from *tk* fragments that bear a partial *a* sequence duplication, generated by inserting information derived from an intact *a* sequence at the site of cleavage directed by the *pac2* signal of the mutant construct (Fig. 7). We have previously described an analogous partial tandem *a* sequence in the F10 mutant (clone pRIaa3; described in Fig. 5 of reference 41), and Deiss, Chou, and Frenkel (7) found that similar events occur during the propagation of constructed defective viral genomes.

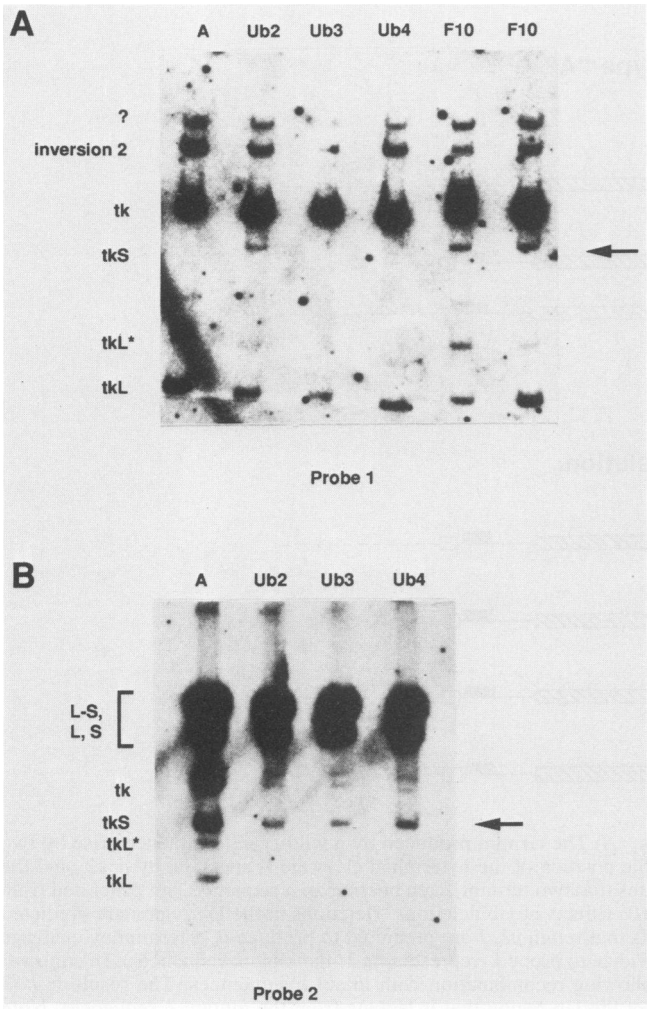

FIG. 6. Analysis of *tk-S* termini produced by Ub mutants. (A) Packaged viral DNA prepared from the indicated viral strains was cleaved with *Eco*RI, and the fragments were separated by electrophoresis on a 1.4% agarose gel. Following transfer to nitrocellulose, the blot was hybridized to a 5′-labeled synthetic 25-mer representing 5′ *tk*-flanking sequences (probe 1 in Fig. 5). The position of the *tk*-S-terminal fragment is highlighted by an arrow. ?, A cross-hybridizing fragment unrelated to the *a* sequence insertion. In the gel displayed in this figure, the *tk*-L-terminal fragments were run into a 5% polyacrylamide plug at the bottom of the gel; hence, these fragments are not resolved from one another. (B) DNA was cleaved with *Pvu*II, and the fragments were separated by electrophoresis through a 1.4% agarose gel. Following transfer to nitrocellulose, the blot was hybridized to a 5′-labeled synthetic 20-mer representing a portion of Ub that is deleted from constructs Ub2 to Ub5 (probe 2 in Fig. 5). The position of the *tk*-S-terminal fragment is highlighted by an arrow.

Cleavage between these tandem *a* sequence derivatives would directly generate the observed termini (Fig. 7). Alternatively, the *tk*-S termini might arise following cleavage of novel inversion fragments that arose by recombination with normal L-S junctions during genome isomerization events. The presently available data do not distinguish between these alternatives. Both of these mechanisms can explain the observed structures of the *tk*-L and *tk*-S termini, and the first directly predicts that the *tk** fragments will hybridize to probe 2. However, neither mechanism accounts for the hybridization of probe 2 to a subset of the *tk* fragments that bear a single *a* derivative. Further studies are required to clarify the nature of the events that give rise to this class of fragment.

Effects of Uc deletions. Deletions entering the *a* sequence from the Uc side reduced the frequency of terminal and inversion fragments (Fig. 8; quantitative data presented in Table 1 and summarized in Fig. 4). Thus, mutant Uc1 lacking 7 residues of DR1 retained close to full activity, while deletion of the remainder of DR1 in mutant Uc2 reduced the frequency of rearrangements approximately threefold. Further progressive deletion of the Uc region had a relatively minor additional effect. Thus, rearrangement frequencies were significantly reduced before the presumed *pac2* cleavage-packaging signal was removed. We were unable to discern any obvious differential effects of these mutations on inversions compared with formation of termini.

As described above, the Ub series of deletions progressively reduced the size of *tk*-L-terminal fragments; by analogy, we expected that the Uc deletions would reduce the size of the *tk*-S-terminal fragments. This reduction was not apparent in the *Pvu*II digests displayed in Fig. 8 because of the relatively small sizes of the Uc deletions compared with the length of the *Pvu*II *tk*-S termini. In order to test for the predicted size alterations, we cleaved viral DNA with *Pst*I (which produces considerably smaller *tk*-S-terminal fragments) and then probed with 3′-flanking *tk* sequences (Fig. 9). The probe hybridized with *tk*-related fragments of 2 kb and 840 nt in wild-type DNA; in addition, much weaker hybridization was observed to a ca. 1,800-nt fragment of unknown origin. The 840-nt *tk*-related fragment spans the site of *a* sequence insertion; hence, this fragment is replaced by larger fragments bearing the inserted *a* sequence in mutant DNAs. Mutant DNAs displayed the predicted recombinational inversion fragment (predicted size of 7.7 kb) and the *tk*-S-terminal fragment (500 nt for construct A). We found that the *tk*-S termini were progressively reduced in size by the Uc deletions. We interpreted this result to indicate that the *tk*-S termini were formed by cleavage at the predicted position dictated by the *pac1* signal and therefore retained the initially constructed deletions. Based on analogy with the Ub deletion mutants, we also postulate that *tk*-L termini arose at least in part through recombination with intact *a* sequences. However, we have yet to confirm these interpretations by direct analysis of the structures of the termini.

Sequence analysis of inversion products in mutant F10. The preceding mutational analysis suggested that sequences at both ends of the *a* sequence play important roles in generation of termini and genome isomerization. These results, in combination with our inability to mutationally separate generation of termini from genome isomerization, suggested that these processes might be closely linked. We therefore wished to determine whether the crossover events that give rise to inversions occur at the positions of the site-specific DNA cleavages that give rise to the genomic termini (note that all of the constructs tested retained at least one intact *pac* homology). In order to assess this possibility, we cloned and sequenced two inversion fragments from the mutant F10. The F10 *a* derivative bears deletions at both ends (41); the Ub endpoint is the same as in construct Ub2, and the Uc endpoint is the same as in construct Uc1. This particular strain was chosen because it retains functional *pac1* and *pac2* signals, and we have previously shown that both of the resulting site-specific cleavages occur in the flanking *tk* sequences (41). Thus, the cleavage sites are labeled with *tk*

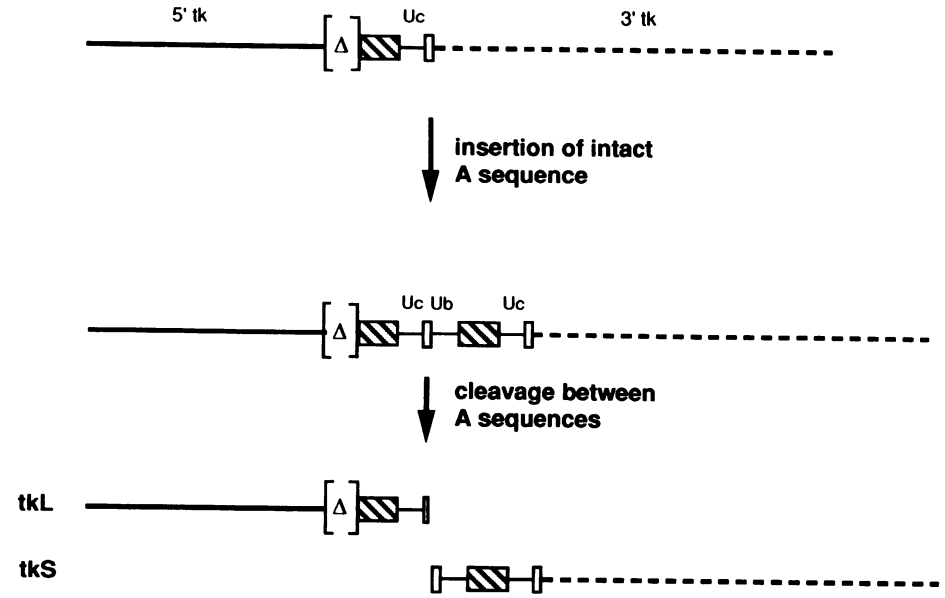

FIG. 7. One possible mechanism for generating the observed termini. In order to account for the production of *tk*-S termini bearing an intact *a* sequence and *tk*-L termini retaining the initially constructed deletion of Ub sequences, it is proposed that an intact *a* sequence is inserted into the DR1 of the mutated *a* sequence during the processing reaction. Cleavage within the DR1 element separating the tandem *a* sequences would then directly generate the observed termini.

DNA, allowing us to determine whether the crossover events that lead to recombination between this structure and intact *a* sequences take place at the positions of site-specific DNA cleavage (Fig. 10).

We first examined the structure of a cloned inversion fragment representing fusion of 5′ *tk* sequences to the *c* repeats of a normal L-S junction. Figure 10 illustrates the outcomes that are predicted if the recombination event that gave rise to this fragment occurred at either of the two sites of DNA cleavage. If the crossover took place at the cleavage site controlled by *pac1*, then 5′-flanking *tk* sequences distal to the cleavage site would be directly fused to a portion of the Ub-terminal DR1 contributed by the intact A sequence (case 1 in Fig. 10). Similarly, a crossover at the cleavage site controlled by *pac2* would join the 3′-flanking *tk* sequences proximal to the cleavage site to a partial copy of the Uc-terminal DR1 (case 2). Neither outcome was observed; the recombinant junction bore a single *a* derivative that began with the truncated *a* sequence derived from the F10 construct and ended in sequences derived from an intact *a* sequence (Fig. 10). Thus, this fragment arose through recombination at a site located somewhere within the region of homology between the F10 fragment and the intact *a* sequence rather than at either of the two positions of site-specific cleavage.

The structure of an inversion fragment fusing the *b* repeats to the 3′ *tk* sequences was more complex but led to the same conclusion (Fig. 11). This junction bore two tandem *a* derivatives. The *a* derivative closest to the *b* repeat had a recombinant structure analogous to that described above: it began in sequences derived from an intact *a* sequence and ended in sequences derived from the F10 construct. The other *a* derivative was identical to the F10 *a* construct. The junction between these two *a* derivatives had precisely the same structure as that previously observed for the most abundant class of *tk** fragments of F10 DNA (pRIaa6; described in Fig. 5 of reference 41): 3′- and 5′-flanking *tk* sequences were fused at the positions of the two site-specific cleavages. These data demonstrate that this recombinant junction was derived by recombination between a *tk* fragment bearing two tandem F10 *a* derivatives and an L-S junction bearing one or more *a* sequences. For simplicity, Fig. 11 assumes that the L-S junction involved bore two *a* sequences. The crossover that gave rise to the inversion fragment occurred between the 5′-most F10 *a* derivative and an intact *a* sequence, somewhere within the region of homology.

In summary, these two inversion fragments arose by recombination within the region of homology between the F10 *a* derivative and an intact *a* sequence. They did not arise by cleavage-ligation events at the positions of site-specific DNA breakage.

DISCUSSION

The results presented in this paper demonstrate that sequences at both ends of the *a* sequence play important roles in the generation of the termini of viral DNA and in the recombination events that give rise to genome isomerization. Our mutational analysis has so far failed to convincingly uncouple these two processes, suggesting that the required sequence elements overlap extensively. As discussed further below, this finding is consistent with the hypothesis that the recombinational activity of the *a* sequence of HSV-1 KOS depends on some of the same processes that give rise to the genomic termini.

We previously proposed that the site-specific DNA cleavage events that give rise to the S and L termini rely on measures taken from recognition signals located within the Ub and Uc regions, respectively (41). The present results support this hypothesis and map portions of the Ub signal to a 15-nt region that includes most of the *pac1* homology that is conserved between herpesvirus genomes. Thus, mutant Ub2 retains the *pac1* homology and produced some *tk*-S termini through the previously described "reach out and cut" mechanism. In contrast, Ub3, lacking *pac1*, failed to

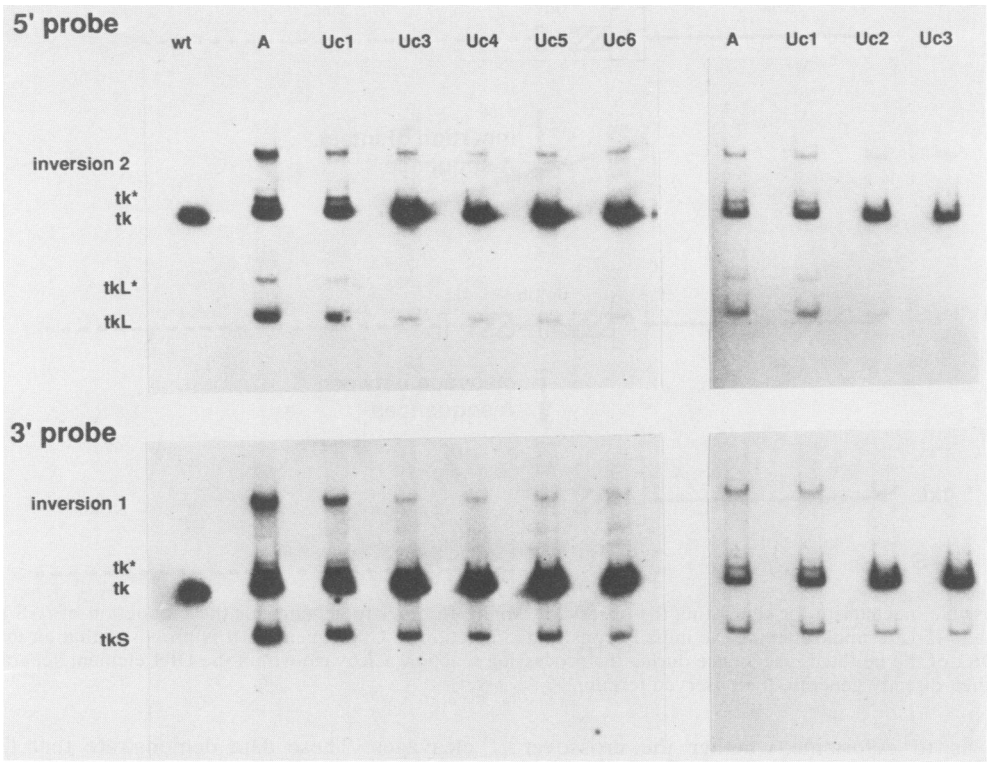

FIG. 8. Southern blot analysis of Uc deletions. Packaged viral DNA prepared from the indicated strains was cleaved with *Pvu*II, and the resulting fragments were separated by electrophoresis through a 1.4% agarose gel. Following transfer to nitrocellulose, the blots were probed with cloned fragments representing the 5′- and 3′-flanking *tk* sequences, as described in the legend of Fig. 3. The positions of the various *tk*-related fragments are indicated. The left and right panels present the results of two independent experiments.

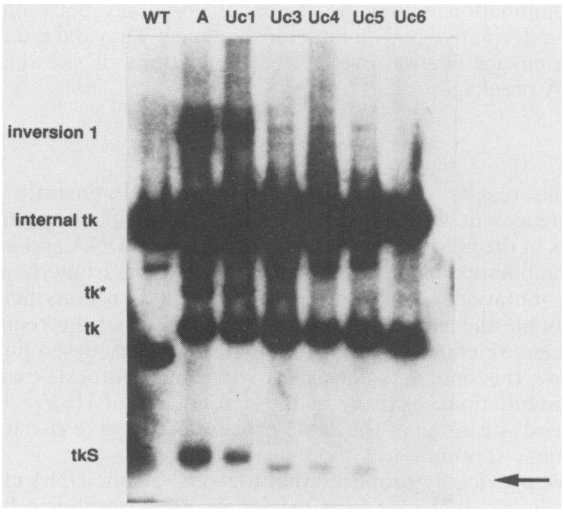

FIG. 9. Uc deletions reduce the sizes of *tk*-S termini. Packaged viral DNA prepared from the indicated viral strains was cleaved with *Pst*I, and the fragments were separated by electrophoresis through a 2.0% agarose gel. Following transfer to nitrocellulose, the blot was hybridized to a cloned *Pvu*II-*Bam*HI fragment representing 3′-flanking *tk* sequences, as described in the legend of Fig. 3. The positions of the various *tk*-related fragments affected by the *a* sequence insertion are indicated. Internal tk, a 2-kb *tk Pst*I fragment that is not affected by the *a* sequence insertion.

produce termini through direct distal cleavage. Rather, the *tk*-S termini present in this strain arose exclusively following recombination events with intact *a* sequences that restored the *pac1* sequence. These data directly demonstrate that the *pac1* homology is required for the S-terminal site-specific cleavage event. We presume that the *pac2* homology similarly directs the L-terminal cleavage, although we have yet to confirm this hypothesis through direct analysis of the termini produced by the Uc mutants that lack this conserved element.

The highly diverged *a* sequences of various herpesvirus genomes contain *pac1* and *pac2* homologies located at analogous positions relative to the site-specific cleavages that give rise to the genomic termini (1, 2, 5, 10, 17, 18, 20, 39). This observation, coupled with the finding that various herpesvirus cleavage-packaging systems can cleave heterologous *a* sequences (35, 36), suggest that *pac1* and *pac2* serve as the primary recognition signals for the cleavage-packaging machinery. However, we found that several of our constructs that retain both *pac1* and *pac2* displayed a significantly reduced ability to produce novel termini (e.g., Ub1, Ub2, and Uc2). The mutationally sensitive intervals defined by these constructs (DR1 and a portion of Ub) are not strongly conserved among various herpesvirus genomes. One interpretation of this finding is that the activity of the HSV-1 cleavage-packaging system is boosted by specific recognition of one or more elements in addition to the *pac* homologies. Consistent with this hypothesis, Chou and Roizman (4) found that binding of a complex of viral proteins to the *pac2*/DR1 region of the HSV-1 *a* sequence required

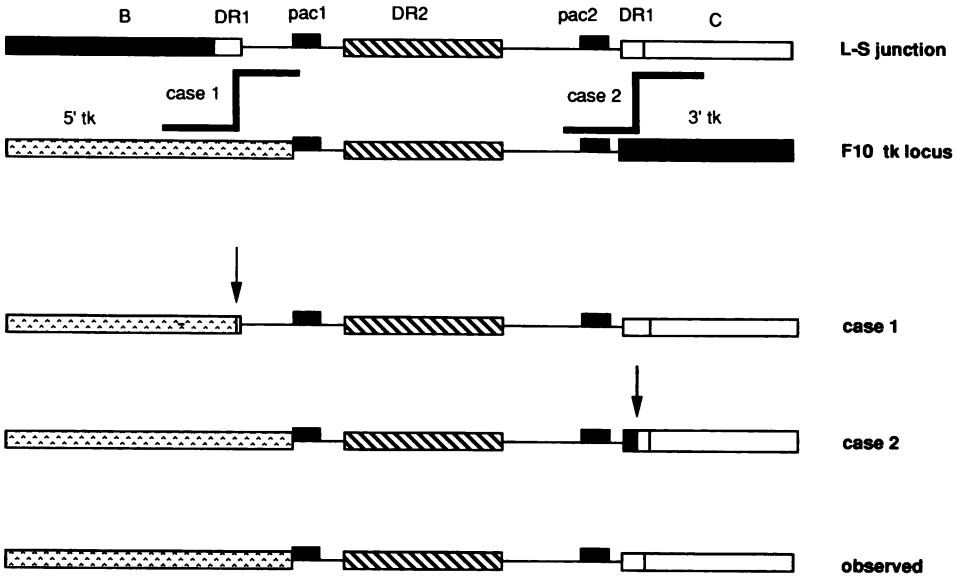

FIG. 10. Structure of an inversion 1 fragment isolated from strain F10. The upper portion of the figure compares the F10 insert in *tk* to an intact *a* sequence at a normal L-S junction. The *tk* termini produced by F10 arise by cleavage in the 5′- and 3′-flanking *tk* sequences, at the appropriate distances from the *pac* signals (41). If the recombination event that gave rise to the inversion fragment occurred by cleavage-ligation at the position of site-specific cleavage directed by *pac1* (case 1), then the 5′-flanking *tk* sequences lying between *pac1* and the cleavage site would be replaced by sequences derived from the intact *a* sequence in the resulting inversion fragment. Similarly, if the crossover occurred at the site of cleavage directed by *pac2* (case 2), 3′-flanking *tk* sequences would be fused to a partial copy of DR1 contributed by the intact *a* sequence. The lower portion of the figure compares the outcomes predicted for cases 1 and 2 with the structure of the cloned inversion fragment. The data demonstrate that it did not arise by recombination at either of the two positions of site-specific cleavage.

the integrity of both elements. This hypothesis predicts that heterologous *a* sequences are processed relatively inefficiently by the HSV-1 cleavage-packaging system. An alternative possibility is that the cleavage-packaging system is stimulated by homologous pairing of the DR1 elements located at either end of the *a* sequence. According to this view, deletions removing one copy of DR1 inhibit cleavage-packaging by preventing this interaction. This hypothesis implies that the DR1 element enhances the cleavage-packaging reaction by providing homologous sequences at the two cleavage sites, rather than by contributing a specific recognition signal. In this context, it is worth noting that although *a* sequences of diverse herpesviruses are often flanked by direct repeats, these are not obviously conserved in sequence.

In an earlier study we suggested that the Ub and Uc signals act in concert to generate termini (41). This hypothesis is supported by the present finding that deletions of either end of the *a* sequence reduced the frequency of both the *tk*-L and *tk*-S termini. As described above, the residual activity of constructs lacking *pac1* or *pac2* appears to rely on recombination events that restore the integrity of the *a* sequence. An interesting feature of this process is that it gives rise to one terminus bearing an intact *a* sequence and

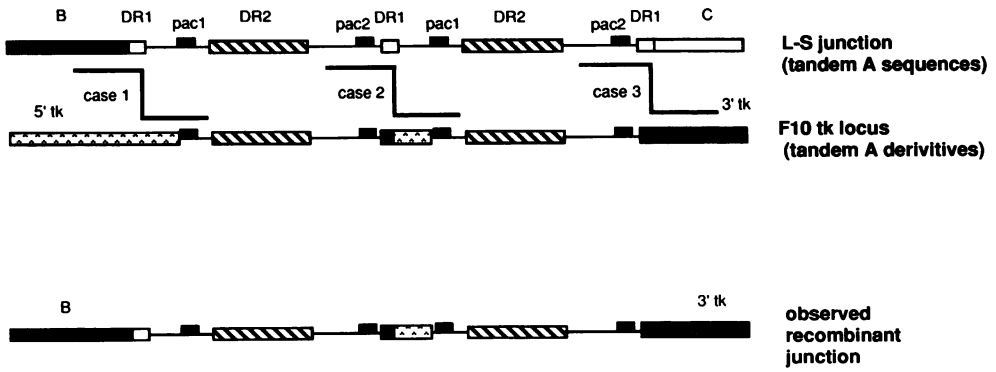

FIG. 11. Structure of an inversion 2 fragment isolated from strain F10. The figure compares the structure of an L-S junction bearing two tandem *a* sequences with a *tk** fragment of the F10 construct and indicates three crossovers that could be mediated by cleavage-ligation events at the positions of site-specific DNA cleavage (cases 1 to 3). As previously described (41), tandem *a* derivatives in the F10 construct are separated by 3′- and 5′-flanking *tk* sequences fused at the positions of site-specific DNA cleavage. The lower portion of the figure diagrams the structure of the cloned inversion fragment. The crossover event that gave rise to this fragment occurred within the region of homology between the left-most *a* derivatives, rather than at a position of site-specific DNA cleavage.

one bearing the initially constructed deletion. Although the precise nature of the recombination events underlying this process remains uncertain, the simplest interpretation of our data is that junctions bearing a mutated *a* sequence can give rise to a partial *a* sequence duplication by insertion of an intact *a* sequence into the remaining copy of DR1 (Fig. 7). We have previously described an analogous process in the F10 mutant (pRIaa3 in reference 41), and Deiss, Chou, and Frenkel (7) noted similar events during the propagation of constructed defective genomes. Those researchers ascribed these duplicative recombination events to a double-stranded gap repair mechanism that was suggested to be involved in the formation of the genomic termini. The data described in the present report are generally consistent with this hypothesis; however, we believe that other possibilities are not excluded by the available evidence.

Deiss, Chou, and Frenkel (7) reported findings that are similar to ours, with some differences in fine detail. They found that plasmids bearing *a* sequences lacking the *pac1* sequence were packaged into virions at a reduced frequency. In addition, those concatemers that were packaged contained a minority of *a* sequences derived from the helper virus, as well as more abundant input mutated *a* sequences. On the basis of this observation, they hypothesized that *a* sequences lacking *pac1* cannot be directly processed by the cleavage-packaging system but retain the ability to recombine with intact *a* sequences, which then provide the requisite packaging signal. Our data directly confirm this conjecture and further demonstrate that deletion of *pac1* eliminates the S-terminal cleavage event. Those researchers also reported that constructs lacking *pac2* could not be packaged into virions. On the basis of this observation, they concluded that *pac2* is required for the cleavage-packaging reaction and for recombination between *a* sequences. However, our data clearly demonstrate that constructs lacking *pac2* retain a reduced recombination activity and were able to form novel termini (presumably after recombination events had restored an intact *a* sequence). It is possible that these discrepancies are related to the greater sensitivity of assays conducted in the environment of the intact viral genome.

It has been proposed that the *a* sequence is acted upon by a site-specific recombination system that gives rise to genome isomerization (3, 21–24), and Chou and Roizman (3) suggested that the DR4 repeat arrays within the HSV-1 strain F *a* sequence serve as the target. As noted in the introduction, the KOS *a* sequence does not contain an intact DR4 element; instead, 27 nt of this sequence are present in one copy. A comparison of our results with those of Chou and Roizman (3) suggests that the KOS *a* sequence may be less recombinogenic than that of strain F. This possibility is consistent with the hypothesis that the DR4 element enhances the recombinational activity of the strain F *a* sequence (3). Our data argue against the hypothesis that the genome isomerization events driven by the KOS *a* sequence occur by cleavage-ligation reactions at a single specific site: we found that two completely nonoverlapping subfragments of the *a* sequence (Ub5 and Uc6) were able to give rise to detectable inversions.

Because our results appear to exclude a site-specific recombination system acting on a single site within the *a* sequence, it is worthwhile to consider alternative hypotheses. The simplest alternative is that recombination between KOS *a* sequences occurs by standard homology-dependent generalized recombination, without a requirement for particular DNA sequences. While this possibility has not been completely excluded, several observations argue against it. First, Weber et al. (44) reported that more than 600 nt of homology is required for detectable intramolecular generalized recombination in the HSV system. In contrast, we find that the 317-nt *a* sequence displayed easily detectable levels of recombination and the 100-nt Ub5 construct retained significant activity. We believe that the activity of short *a* derivatives in our assays is particularly noteworthy because these are separated from their closest potential recombination partners by over 40 kb of intervening HSV DNA. Second, the recombinational activity of *a* sequence derivatives was not directly related to their length: relatively short deletions at either extremity had a disproportionately large effect on recombination frequencies. This was most clearly demonstrated in the Uc series, where Uc1 retained close to full activity, and Uc2, lacking a further 13 nt, was significantly impaired. Further deletions in the Uc region had little additional effect. These data suggest that complete deletion of the Uc-terminal DR1 sequence perturbed a structural feature that is required for efficient inter-*a* recombination. It is important to stress that our data do not exclude a role for the internal repeat array in the recombination process. They do however demonstrate that in the case of the KOS *a* sequence, sequences at both extremities are needed for full activity.

In considering possible explanations for these data, we attached particular significance to our inability to mutationally uncouple the recombinational activity of the *a* sequence from its ability to contribute to the formation of genomic termini. In our view, the most straightforward explanation is that both types of events depend on the same machinery. We therefore hypothesize that the site-specific DNA cleavages directed by the *pac1* and *pac2* signals stimulate the recombination events that lead to genome inversions. Because we found that recombination does not invariably occur at the sites of DNA cleavage, we suggest that the free ends or nicks generated by cleavage stimulate the initiation of recombination (reviewed in reference 40) and that the actual crossovers can occur anywhere within the region of homology between the interacting DNAs. According to this model, the *a* sequence is a hot spot for the initiation of recombination, through specialized mechanisms. As noted above, a somewhat similar scheme was proposed by Deiss, Chou, and Frenkel (7) to account for *a* sequence duplication during the cleavage-packaging reaction. However, they did not consider the possibility that these cleavage-dependent recombination events result in genome isomerization.

This hypothesis can explain why certain deletions (such as Ub1, Ub2, and Uc2) inhibit recombination even though they do not directly affect the cleavage signals: Ub1 and Ub2 shift the position of the *tk*-S-terminal cleavage into 5′-flanking DNA sequences; if the initiation of recombination involves strand invasion, then these deletions could inhibit this process by placing heterologous sequences at the tip of the invading strand. Similarly, one predicts that progressively larger deletions entering from the Uc side would also reduce recombination before they impinge on the *pac2* sequence. While the Uc1 mutant (lacking 7 nt of DR1) displayed close to normal activity, construct Uc2 (lacking a further 13 nt) was significantly impaired. The model also predicts that internal deletions of the *a* sequence can interfere with recombination by reducing the extent of uninterrupted sequence homology between the potential recombination partners. However, it leaves open the possibility that certain internal sequences (for example, the DR2 [and, in strain F, DR4] repeat arrays) specifically enhance the recombinational activity of the *a* sequence.

Weber et al. (44) have shown in elegant studies that the seven HSV gene products that encode the HSV DNA replicative machinery specify the viral generalized recombination system. The hypothesis outlined above suggests that the higher rate of recombination between *a* sequences depends in part on additional viral polypeptides that are involved in the site-specific DNA cleavage reaction. We currently seek to test this model by assessing the effects of point mutations in the *pac1* and *pac2* signals on the inversion process. It will be interesting to learn whether other regions of the *a* sequence, such as DR1 and the internal repeat array, also play a direct sequence-specific role in stimulating recombination.

ACKNOWLEDGMENTS

We thank Suzanne Butler for constructing several of the plasmids used in this study.

This work was supported by the National Cancer Institute of Canada. J.R.S. is a Terry Fox Senior Scientist of the National Cancer Institute of Canada.

LITERATURE CITED

1. **Albrecht, M., G. Darai, and R. M. Flugel.** 1985. Analysis of the genomic termini of tupaia herpesvirus DNA by restriction mapping and nucleotide sequencing. J. Virol. **56**:466–474.
2. **Bankier, A. T., W. Dietrich, R. Baer, B. G. Barrell, F. Colbere-Garapin, B. Fleckenstein, and W. Bodemer.** 1985. Terminal repetitive sequences in herpesviruses saimiri virion DNA. J. Virol. **55**:133–139.
3. **Chou, J., and B. Roizman.** 1985. Isomerization of herpes simplex virus type 1 genome: identification of the cis-acting and recombination sites within the domain of the *a* sequence. Cell **41**:803–811.
4. **Chou, J., and B. Roizman.** 1989. Characterization of DNA sequence-common and sequence-specific proteins binding to *cis*-acting sites for cleavage of the terminal *a* sequence of the herpes simplex virus 1 genome. J. Virol. **63**:1059–1068.
5. **Davison, A. J.** 1984. Structure of the genome termini of varicella-zoster virus. J. Gen. Virol. **65**:1969–1977.
6. **Davison, A., and N. Wilkie.** 1981. Nucleotide sequence of the joint between the L and S segments of herpes simplex virus types 1 and 2. J. Gen. Virol. **55**:315–331.
7. **Deiss, L. P., J. Chou, and N. Frenkel.** 1986. Functional domains within the *a* sequence involved in the cleavage-packaging of herpes simplex virus DNA. J. Virol. **59**:605–618.
8. **Deiss, L. P., and N. Frenkel.** 1986. Herpes simplex virus amplicon: cleavage of concatemeric DNA is linked to packaging and involves amplification of the terminally reiterated *a* sequence. J. Virol. **57**:933–941.
9. **Hall, J. D., D. M. Coen, B. L. Fisher, M. Weisslitz, S. Randall, R. E. Almy, P. T. Gelep, and P. A. Shaffer.** 1984. Generation of genetic diversity in herpes simplex virus: an antimutator phenotype maps to the DNA polymerase locus. Virology **132**:26–37.
10. **Hammerschmidt, W., H. Ludwig, and H. J. Budk.** 1988. Specificity of cleavage in replicative-form DNA of bovine herpesvirus 1. J. Virol. **62**:1355–1363.
11. **Hayward, G. S., R. J. Jacob, S. C. Wadsworth, and B. Roizman.** 1975. Anatomy of herpes simplex virus DNA: evidence for four populations of molecules that differ in the relative orientations of their long and short segments. Proc. Natl. Acad. Sci. USA **72**:4243–4247.
12. **Jacob, R. J., L. S. Morse, and B. Roizman.** 1979. Anatomy of herpes simplex virus DNA. XII. Accumulation of head-to-tail concatemers in nuclei of infected cells and their role in the generation of the four isomeric arrangements of viral DNA. J. Virol. **29**:448–457.
13. **Kaerner, H., I. Maichle, A. Ott, and C. Schroeder.** 1979. Origin of two different classes of defective HSV-1 Angelotti DNA. Nucleic Acids Res. **6**:1467–1478.
14. **Kemble, G. W., and E. S. Mocarski.** 1989. A host cell protein binds to a highly conserved sequence element (pac-2) within the cytomegalovirus *a* sequence. J. Virol. **63**:4715–4728.
15. **Locker, H., and N. Frenkel.** 1979. Structure and origin of defective genomes contained in serially passaged herpes simplex virus type 1 (Justin). J. Virol. **29**:1065–1077.
16. **Longnecker, R., and B. Roizman.** 1986. Generation of an inverting herpes simplex virus 1 mutant lacking the L-S junction *a* sequences, an origin of DNA synthesis, and several genes including those specifying glycoprotein E and the α47 gene. J. Virol. **58**:583–591.
17. **Marks, J. R., and D. H. Spector.** 1988. Replication of the murine cytomegalovirus genome: structure and role of the termini in the generation and cleavage of concatenates. Virology **162**:98–107.
18. **Matsuo, T., M. Heller, L. Petti, E. O'Shiro, and E. Kieff.** 1984. Persistence of the entire Epstein-Barr virus genome integrated into human lymphocyte DNA. Science **226**:1322–1325.
19. **Maxam, A. M., and W. Gilbert.** 1980. Sequencing end-labelled DNA with base-specific chemical cleavages. Methods Enzymol. **65**:499–560.
20. **Mocarski, E. S., A. C. Liu, and R. R. Spaete.** 1987. Structure and variability of the *a* sequence in the genome of human cytomegalovirus (Towne strain). J. Gen. Virol. **68**:2223–2230.
21. **Mocarski, E. S., L. E. Post, and B. Roizman.** 1980. Molecular engineering of the herpes simplex virus genome: insertion of a second L-S junction into the genome causes additional genome inversions. Cell **22**:243–255.
22. **Mocarski, E. S., and B. Roizman.** 1981. Site-specific inversion sequence of the herpes simplex virus genome: domain and structural features. Proc. Natl. Acad. Sci. USA **78**:7047–7051.
23. **Mocarski, E. S., and B. Roizman.** 1982. Structure and role of the herpes simplex virus DNA termini in inversion, circularization and generation of virion DNA. Cell **31**:89–97.
24. **Mocarski, E. S., and B. Roizman.** 1982. Herpesvirus-dependent amplification and inversion of cell-associated viral thymidine kinase gene flanked by viral *a* sequences and linked to an origin of viral DNA replication. Proc. Natl. Acad. Sci. USA **79**:5626–5630.
25. **Poffenberger, K. L., and B. Roizman.** 1985. A noninverting genome of a viable herpes simplex virus: presence of head-to-tail linkages in packaged genomes and requirements for circularization after infection. J. Virol. **53**:587–595.
26. **Pogue-Geile, K. L., G. T. Y. Lee, and P. G. Spear.** 1985. Novel rearrangements of herpes simplex virus DNA sequences resulting from duplication of a sequence within the unique region of the L component. J. Virol. **53**:456–461.
27. **Pogue-Geile, K. L., and P. G. Spear.** 1986. Enhanced rate of conversion or recombination of markers within a region of unique sequence in the herpes simplex virus genome. J. Virol. **58**:704–708.
28. **Roizman, B.** 1979. The organization of the herpes simplex virus genome. Annu. Rev. Genet. **13**:25–57.
29. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
30. **Sheldrick, P., and N. Berthelot.** 1974. Inverted repetitions in the chromosome of herpes simplex virus. Cold Spring Harbor Symp. Quant. Biol. **39**:667–678.
31. **Skare, J., and W. C. Summers.** 1977. Structure and function of herpes virus genomes. II. EcoRI, XbaI, and HindIII endonuclease cleavage sites on herpes simplex virus type 1 DNA. Virology **76**:581–595.
32. **Smiley, J. R., B. Fong, and W.-C. Leung.** 1981. Construction of a double-jointed herpes simplex virus DNA molecule: inverted repeats are required for segment inversion and direct repeats promote deletions. Virology **113**:345–362.
33. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.
34. **Spaete, R. R., and N. Frenkel.** 1982. The herpes simplex virus amplicon: a new eucaryotic defective-virus cloning-amplifying vector. Cell **30**:295–304.
35. **Spaete, R. R., and N. Frenkel.** 1985. The herpes simplex virus amplicon: analysis of cis-acting replication functions. Proc. Natl. Acad. Sci. USA **82**:694–698.

36. **Spaete, R. R., and E. S. Mocarski.** 1985. The *a* sequence of the cytomegalovirus genome functions as a cleavage/packaging signal for herpes simplex virus defective genomes. J. Virol. **54:** 817–824.
37. **Stow, N., and E. McMonagle.** 1983. Characterization of the TRs/IRs origin of DNA replication of herpes simplex virus type 1. Virology **130:**427–438.
38. **Stow, N., E. McMonagle, and A. Davison.** 1983. Fragments from both termini of the herpes simplex virus type 1 genome contain signals required for the encapsidation of viral DNA. Nucleic Acids Res. **11:**8205–8220.
39. **Tamashiro, J. C., and D. H. Spector.** 1986. Terminal structure and heterogeneity in human cytomegalovirus strain AD169. J. Virol. **59:**591–604.
40. **Thaler, D. S., and F. W. Stahl.** 1988. DNA double-chain breaks in recombination of phage λ and of yeast. Annu. Rev. Genet. **22:**169–197.
41. **Varmuza, S. L., and J. R. Smiley.** 1985. Signals for site-specific cleavage of HSV DNA: maturation involves two separate cleavage events at sites distal to the recognition sequences. Cell **41:**793–802.
42. **Vlazny, D., and N. Frenkel.** 1981. Replication of herpes simplex virus DNA: localization of replication recognition signals within defective genomes. Proc. Natl. Acad. Sci. USA **78:**742–746.
43. **Wagner, M., and W. C. Summers.** 1978. Structure of the joint region and the termini of the DNA of herpes simplex virus type 1. J. Virol. **27:**374–387.
44. **Weber, P. C., M. D. Challberg, N. J. Nelson, M. Levine, and J. C. Glorioso.** 1988. Inversion events in the HSV-1 genome are directly mediated by the viral DNA replication machinery and lack sequence specificity. Cell **54:**369–381.
45. **Weber, P. C., M. Levine, and J. C. Glorioso.** 1990. Recombinogenic properties of herpes simplex virus type 1 DNA sequences resident in simian virus 40 minichromosomes. J. Virol. **64:**300–306.
46. **Wilkie, N. M.** 1976. Physical maps for herpes simplex virus type 1 DNA for restriction endonucleases *Hin*d III, *Hpa*-1, and *X. bad*. J. Virol. **20:**222–233.