Herpes Simplex Virus Type 1 Recombination: Role of DNA Replication and Viral *a* Sequences

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During the course of infection, elements of the herpes simplex virus type 1 (HSV-1) genome undergo inversion, a process that is believed to occur through the viral *a* sequences. To investigate the mechanism of this recombinational event, we have developed an assay that detects the deletion of DNA segments flanked by directly repeated *a* sequences in plasmids transiently maintained in Vero cells. With this assay, we have observed a high frequency of recombination (approximately 8%) in plasmids that undergo replication in HSV-1-infected cells. We also found a low level of recombination between *a* sequences in plasmids introduced into uninfected cells and in unreplicated plasmids in HSV-1-infected cells. In replicating plasmids, recombination between *a* sequences occurs at twice the frequency seen with directly repeated copies of a different sequence of similar size. Recombination between *a* sequences appears to occur at approximately the same time as replication, suggesting that the processes of replication and recombination are closely linked.

Herpes simplex virus type 1 (HSV-1) is an enveloped DNA virus with a linear duplex genome of approximately 152 kbp. The genome is composed of two unique regions, termed U_L (unique long) and U_S (unique short), that are flanked by repeated regions, so that the final structure appears as $a_n b-U_L-b' a_m' c'-U_S-ca$ (27). In 1975, it was suggested that recombination could occur through the inverted repeats, giving rise to different isomeric forms of the virus (30). Subsequent work confirmed that plaque-purified HSV-1 exists as an equimolar mixture of the four isomers (6, 9), generated by inversion of one or both unique regions.

The HSV-1 *a* sequences appear to play an important role in inversion. Noninverting mutants of HSV-1 have been generated by deletion of a segment of DNA that included the *a* sequences (11, 23), and insertion of fragments containing an *a* sequence produced additional inversions in regions flanked by inverted *a* repeats (18) together with deletions of regions with directly repeated *a* sequences at their ends (32). Insertion of viral fragments other than the *a* sequence produced no such effects (18). On the other hand, the HSV-1 *Bam*HI *L* or *b* sequences can mediate a low level of recombination (14, 24), and transposon Tn5 inserted into the HSV-1 genome can undergo high-frequency homologous recombination (37). The exact relationship between these recombinational events and the high-frequency recombination associated with the *a* sequences is presently unknown.

The HSV-1 *a* sequence, 200 to 500 bp in length, is approximately 85% G+C. It contains 20-bp direct repeats on each end (DR1) and two unique segments (U_b and U_c) that are separated by internal directly repeated regions. The composition of these internal repeats varies among different HSV-1 strains, with some containing sequences not found in other strains (5, 19, 35). However, all strains contain a common set of 12-bp direct repeats (DR2), though the number of tandem copies differs. These DR2 sequences can adopt a novel DNA conformation under the influence of negative supercoiling (39, 40). In addition to its role in

inversion, the *a* sequence contains the signals for cleavage and packaging (21, 33, 35) and the promoter for the HSV-1 ICP34.5 gene (3).

trans-acting factors are required for inversion. Stably integrated inverted repeated *a* sequences flanked by an HSV-1 origin of replication undergo amplification and inversion only after HSV-1 infection (20). Moreover, Tn5 inversion within plasmids is only observed in association with HSV-1 DNA replication (37).

In this report, we describe our studies of recombination with plasmids transiently introduced into Vero cells. Recombination between two directly repeated copies of the *a* sequence results in the deletion of a DNA segment that carries *lacZ*, a screenable gene. This event can be determined quantitatively by subsequent transformation into *Escherichia coli*. We have used this system to examine the role of the viral *a* sequences and DNA replication in HSV-1-associated recombination. We have found that plasmid replication after HSV-1 infection leads to a high frequency of recombination between *a* sequences. However, recombination between *a* sequences can also occur in the absence of HSV-1 infection or replication of the plasmid. Recombination between *a* sequences is twice as efficient as recombination between a different set of direct repeats of equivalent size. Finally, in agreement with the report of Weber et al. (37), we have found recombination to be closely associated with HSV-1 DNA replication.

MATERIALS AND METHODS

Cells and viruses. All experiments were carried out with Vero cells (African green monkey kidney fibroblasts) obtained from the American Type Culture Collection. Cells were propagated in DMEM (Dulbecco's modified Eagle's minimal essential medium) supplemented with 10% fetal calf serum, 0.1 mM nonessential amino acids, and 2 mM glutamine. The HSV-1 strain Δ 305 (26) was used.

Plasmid construction. The plasmid pSE367 (Fig. 1A) was provided by Boyana Konforti (Stanford University) and used as the parent vector for all constructions. This plasmid

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- A. pSE367 - 6.4Kb. No Inserts.
 B. pRD100 - 6.7 Kb. Inserts: Site 3 - *a* seq.
 C. pRD102 - 7.1Kb. Inserts: Sites 2, 3 - *a* seq. (direct repeat).
 D. pRD107 - 6.6 Kb. Inserts: Site 1 - *ori*.
 E. pRD108 - 6.9 Kb. Inserts: Site 1 - *ori*, Site 3 - *a* seq.
 F. pRD105 - 7.3 Kb. Inserts: Site 1 - *ori*, Sites 2, 3 - *a* seq (direct repeat).
 G. pRD110 - 7.4 Kb. Inserts: Site 1 - *ori*, Sites 2,3 - *dsg* seq. (direct repeat).

FIG. 1. Plasmids used in this study. The parent plasmid is diagrammed, along with the three sites used for insertion. The three inserted sequences were the *a* sequence from HSV-1 strain KOS (320 bp); an origin of replication, *ori*_S, from HSV-1 (200 bp); and the *dsg* sequence from *M. xanthus* (370 bp).

is composed of the following segments (in order): *Pvu*II (nucleotide [nt] 628) to *Fsp*I (nt 1919) from pUC18, *Fsp*I (nt 3588) to *Bam*HI (nt 375) from pBR322, bp 18561 to 19400 from λ gt11, the complete *lacZ* gene, and 55 bp containing ribosome-binding sites and part of the polylinker from pUC19. It therefore contains the ampicillin resistance gene as well as the origin of replication from pUC19 that permits its propagation in *E. coli*. It also contains the complete *lacZ* gene with ribosome-binding sites that allow transcripts made by run-off transcription to be translated. Upon transformation with pSE367, *E. coli* DH5 α (*recA lacZ*) gives deep blue colonies when plated in the presence of ampicillin and 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal).

To construct the other plasmids, three additional components were introduced. First, the *a* sequence from HSV-1 strain KOS with *Bam*HI linkers on each end, obtained from the plasmids pUC18-19 and pUC18-23 (kindly supplied by James Smiley of McMaster University) was inserted once to form pRD100 (Fig. 1B) and twice in direct repeat orientation to create pRD102 (Fig. 1C). This *a* sequence is 317 bp in length, with the composition DR1-U_b-(DR2)₁₀-U_c-DR1 (35). Second, the *Sma*I fragment of the HSV-1 *ori*_S was added to pSE367, pRD100, and pRD102 to form the plasmids pRD107 (Fig. 1D), pRD108 (Fig. 1E), and pRD105 (Fig. 1F), respectively. The presence of *ori*_S allows each of these plasmids to replicate in HSV-1-infected cells. Third, two copies of the *dsg* (d-signaling) sequence from *Myxococcus xanthus* were added in direct repeat orientation, along with the HSV-1 *ori*_S, to obtain pRD110 (Fig. 1G). The *dsg* insert, which is 370 bp in length, was kindly provided by Yvonne Cheng and Dale Kaiser (Stanford University).

Transfection, infection, and DNA isolation. Actively growing Vero cells at a concentration of 5×10^6 cells per ml were electroporated (4) at 220 V with 20 μ g of the indicated plasmid. Cells were allowed to recover for 24 to 48 h. The transfected cells were then infected with 10 PFU of HSV-1 Δ 305 per cell in 3 ml of medium. Virus was removed after 1 h, the cells were washed twice with phosphate-buffered saline (GIBCO), and the medium was replaced.

Three different DNA extraction procedures were employed. For the rapid alkaline lysis procedure, the cells were scraped off the flask, centrifuged, and washed once with GET (50 mM glucose, 10 mM EDTA, 25 mM Tris [pH 8.0]). The cells were suspended in 0.35 ml of GET, 0.8 ml of 0.2 N NaOH-1% sodium dodecyl sulfate was added, and the suspension was left on ice for 5 min. Then, 0.6 ml of 3 M potassium acetate, pH 4.8, was added, the cells were incubated a further 5 min on ice, and the samples were centrifuged for 5 min in an Eppendorf centrifuge. The supernatant was then treated with an equal volume of isopropanol, and the precipitate was centrifuged. The pellet was washed with 70% ethanol and resuspended in TE (10 mM Tris-HCl [pH 8.0], 1 mM EDTA). The extracted DNA was incubated for 1 h with 100 μ g of RNase A per ml at 37°C and then with 1 mg of proteinase K per ml at 65°C for 2 h. The DNA was extracted first with phenol and then with chloroform and precipitated with ethanol. Cytoplasmic DNA extraction was performed as described before (18). Whole-cell extracts were prepared by the method of Maniatis et al. (15).

Transformation, miniprep isolation of DNA, and restriction analysis. Competent frozen *E. coli* DH5 α cells (0.2 ml) were prepared and transformed with 20 to 100 ng of DNA by the method of Hanahan (8) except that the growth medium contained 5 g of Bacto yeast extract, 20 g of Bactotryptone, and 5 g of MgSO₄ per liter. The cells were plated with carbenicillin (100 μ g/ml) and X-Gal (50 μ g/ml). Miniprep isolation of DNA from white colonies was performed by either the alkaline lysis (15) or modified boiling procedure (29), with 50 mM Tris-Cl (pH 8.0)-62.5 mM EDTA-0.4% Triton X-100-2.5 M LiCl used as the resuspension buffer. The DNA was digested with *Dra*I for 1 h and electrophoresed through 1% agarose gels in TAE (40 mM Tris-acetate, 1 mM EDTA).

Southern blotting analysis. Southern blotting analysis was performed by the method of Maniatis et al. (15). Samples of whole-cell DNA (3 μ g) were digested with 10 U of the restriction enzyme *Ssp*I in the appropriate buffer for 5 h. Half of each sample was then phenol extracted, ethanol precipitated, resuspended in *Dpn*I buffer, and digested with 5 U of the *Dpn*I restriction enzyme overnight. The blot was probed with linear pRD102 labeled with ³²P by the random primer method (7).

DNA sequencing was performed by the method of Maxam and Gilbert (16).

RESULTS

Assay for recombination frequency. The assay used to measure recombination between *a* sequences is diagrammed in Fig. 2. The plasmid pDR105 (Fig. 1F) contains two *a* sequences in direct orientation, a plasmid origin, the ampicillin resistance and *lacZ* genes, and an HSV-1 origin of replication (*ori*_S). It produces dark blue colonies when transformed into *E. coli* DH5 α (*lacZ recA*) and plated in the presence of carbenicillin and X-Gal. Recombination between the two directly repeated *a* sequences results in a deletion that produces two smaller plasmids. One contains the ampicillin resistance gene and an origin permitting growth in *E. coli* but lacks the *lacZ* gene; it will therefore yield white colonies in the *lacZ* mutant strain. The second plasmid, which contains the *lacZ* gene, is unable to replicate in *E. coli* for lack of an origin and is consequently lost. The background recombination frequency in *E. coli* DH5 α for a plasmid containing two directly repeated *a* sequences (pRD102; Fig. 1C) was 3×10^{-5} (data not shown).

FIG. 2. Deletion assay for recombination. Construction of the test plasmid pRD105 is described in Materials and Methods. In all plasmids, ori refers to the plasmid origin of replication.

To assay recombination, plasmids were introduced into Vero cells by electroporation; the cells were infected with HSV-1 or mock infected, and the DNA was isolated. *E. coli* DH5 α was then transformed with the DNA. The numbers of blue and white colonies were scored to determine the frequency of *lacZ* deletion. Since events other than recombination between the *a* sequences can lead to loss of a functional *lacZ* gene, the DNA was isolated from some of the white colonies by the miniprep procedure and examined by restriction analysis.

Recombination in a replicating plasmid occurs at high frequency, is precise, and is dependent on the relative orientation of the *a* sequences. When pRD105 was allowed to replicate in HSV-1-infected cells and the products were transformed into *E. coli* DH5 α , 8.6% of the colonies formed were white (Table 1). DNA was isolated from a portion of the white colonies and examined by digestion with the restriction endonuclease *Dra*I. Digestion of pRD105 with *Dra*I produces one large and three small fragments (Fig. 3A and C, lane 14). The product containing the ampicillin resistance gene lacks the large fragment but will retain all three of the smaller fragments. Thirty-five of the 36 white colonies analyzed yielded a *Dra*I restriction digest pattern consistent with recombination through the *a* sequences (Fig. 3C, lanes 4 to 13). In subsequent studies, the correct deletion product was observed in approximately 80% of the white colonies screened.

A portion of the DNA obtained from white colonies which contained the correct deletion, as judged by *Dra*I analysis, were digested with *Bam*HI, which cleaves on either side of the *a* sequence (Fig. 3A) to check for small deletions or insertions (>10 bp) within the *a* sequence. Ninety-seven percent (68 of 70) had retained the full *a* sequence (Fig. 3D, lanes 9 to 18), as judged by comparison with the *a* sequences present in plasmid pRD105 (Fig. 3D, lane 19). Finally, five deletion products were purified on CsCl and subjected to *Bam*HI digestion (Fig. 3D, lanes 4 to 8), and their sequence

was determined by the Maxam-Gilbert procedure (16). Sequence analysis showed that no base changes had occurred in the *a* sequence of the deletion product (data not shown). Thus, the recombinational event is highly accurate.

Recombination through *a* sequences that are inverted relative to each other should lead to inversion of the DNA segment flanked by the *a* sequences. A plasmid identical to pRD105 except that the *a* sequences were inverted was therefore tested for its recombinational activity in HSV-1-infected cells. Very few white colonies were observed after transformation into *E. coli*, and none contained a plasmid whose restriction pattern was consistent with deletion through the *a* sequences. Since transformation of *E. coli lacZ* cannot differentiate between the parental construct and the inversion product, the DNA from approximately 150 blue colonies was examined directly by restriction analysis with the *Eco*RI restriction enzyme. The expected sizes of fragments obtained from the parent and recombinant are shown in Fig. 3B. Two colonies containing inversion products were identified (Fig. 3C, lane 2), confirming that the orientation of the *a* sequences determines the type of recombination observed. *Bam*HI digestion of the parental construct (Fig. 3D, lane 3) and one inversion product (Fig. 3D, lane 2) indicated that no significant change in the size of the *a* sequence had occurred during inversion. Sequence analysis of the inversion product confirmed that the *a* sequence had remained unchanged (data not shown). Because the number of colonies examined was small, further experiments will be needed to determine whether inversion truly occurs at a lower frequency than deletion in this system.

High frequency of deletion is independent of size and conformation of DNA recovered. To determine whether the high frequency of recombination between *a* sequences occurred in all or only some of the DNA molecules, three different procedures were used to extract the DNA from HSV-1-infected Vero cells following electroporation with pRD105. After extraction, the DNA was either transformed into *E. coli* directly or cut into unit-length molecules with the *Xmn*I restriction enzyme and then religated. This procedure was used because HSV-1 replicates primarily by a rolling-circle mechanism that produces concatameric DNA (1, 10). Digestion and ligation of long concatamers is therefore necessary to produce unit-length DNA molecules that can transform *E. coli* efficiently. Southern blot analysis of several samples confirmed that this procedure greatly increased the percentage of monomeric plasmids (data not shown).

The first method of extraction, rapid alkaline lysis, yielded DNA smaller than the HSV-1 genome. As shown in Table 2, the fraction of white colonies (9.5%) increased twofold upon restriction enzyme digestion and ligation. DNA obtained by the second extraction procedure, which yields cytoplasmic DNA, should consist of both input transfected DNA that had never penetrated the nucleus and replicated DNA that had been packaged into virions that accumulate in the cytoplasm. Most of the recombinants in this population were found to exist as concatamers that could transform *E. coli* only after digestion and ligation. This result is expected, since the plasmid contains both ori γ and the *a* sequence packaging signals, the two components required for propagation of the defective viral particles (33, 34, 36). The third method, the whole-cell DNA extraction procedure, yielded a significant percentage of recombinants in the initial samples and a fourfold increase in recombinants after digestion and ligation. The DNA extracted from the white colonies obtained from each of the three procedures was analyzed, and most (>75%) contained the correct recombination product.

TABLE 1. Recombination through directly repeated *a* sequences in pRD105 following HSV-1 infection^a

Total no. of colonies	No. of white colonies	% White colonies	SD
1,075	92	8.6	0.9

^a The assay for recombination was performed as described in the text. DNA was harvested 18 h after infection by the rapid alkaline lysis procedure. Standard deviation (SD) was calculated by the formula $SD = [(1 - \text{freq}) (\text{freq}) / (\text{number of colonies})]^{1/2}$.

FIG. 3. Restriction analysis of deletion and inversion products of recombination through *a* sequences. (A) *DraI* and *BamHI* digestion of pRD105 and the deletion product propagated in *E. coli*. *DraI* sites on each plasmid are shown, together with the predicted sizes of the restriction fragments. *BamHI* sites on each plasmid are also diagrammed. (B) *EcoRI* digestion of pRD104, in which the *a* sequences are in the inverted orientation, and the inversion product of recombination. *EcoRI* sites are indicated, and predicted fragment sizes are shown below. (C) 1% agarose gel showing restriction digest analysis. Lanes 1 and 15, 1-kb ladder; lane 2, *EcoRI* digest of inversion product (Inv. Prod.); lane 3, *EcoRI* digest of inversion substrate (Inv. Sub.); lanes 4 to 13, DNA from white colonies in the experiment shown in Table 1, digested with *DraI*; lane 14, *DraI* digest of pRD105, the deletion substrate (Del. Sub.). (D) 4% NuSieve agarose gel of *BamHI* digests. Lanes 1 and 20, 123-bp ladder; lane 2, CsCl-purified inversion product (Inv. Prod.); lane 3, pRD104, the inversion substrate (Inv. Sub.); lanes 4 to 8, CsCl-purified deletion products; lanes 9 to 18, DNA from white colonies obtained during experiments with pRD105; lane 19, pRD105, the deletion substrate (Del. Sub.).

Thus, the high frequency of recombinants is independent of the size and conformation of the DNA extracted from the infected cells. In all subsequent experiments, we have employed the rapid alkaline lysis procedure without digestion and ligation.

Recombination frequency is greatly reduced in plasmids lacking an HSV-1 origin. Plasmids that do not have an HSV-1 origin of replication and either lack (pSE367; Fig. 1A) or contain one *a* sequence (pRD100; Fig. 1B) yielded a low frequency of white colonies (Table 3), and the frequency appeared to be unchanged by HSV-1 infection. Restriction analysis of DNA isolated from these colonies demonstrated that a wide variety of deletion events had occurred. The frequency of random deletions observed is similar to that noted previously for plasmids transfected into mammalian cells (13, 17).

A plasmid that contains two directly repeated *a* sequences but lacks *ori_S* (pRD102; Fig. 1C) also gave a low frequency of white colonies, and this percentage was unaffected by HSV-1 infection (Table 3). Restriction analysis showed that

approximately 30% of the white colonies resulting from the uninfected and 10% of those from the HSV-1-infected cell samples contained plasmids whose restriction pattern was consistent with deletion having occurred through the *a* sequences. As noted earlier, the background recombination frequency for this plasmid in *E. coli* DH5 α is only 3×10^{-5} , so that recombination in a plasmid containing two directly repeated *a* sequences but lacking *ori_S* is increased 15- to 50-fold by passage through Vero cells, and this frequency is unaffected by HSV-1 infection (Table 3). Finally, pRD105 with two *a* sequences showed a low frequency of recombination in the absence of HSV-1 infection. Thus, a low level of recombination between *a* sequences occurs in uninfected cells and in plasmids in infected cells that lack *ori_S* and are not undergoing replication. However, both an HSV-1 origin of replication and HSV-1 superinfection are required for high-frequency recombination between *a* sequences.

Insertion of a single *a* sequence into a replicating plasmid increases the frequency of random deletions. To verify that replication-enhanced recombination is dependent upon the

TABLE 2. Effect of size and conformation of DNA on recombination frequency

Extract	Digestion and ligation ^a	% White colonies (SD) ^b				Total no. of colonies ^c	Avg % white colonies	Increase after digestion and ligation (fold)
		Expt 1	Expt 2	Expt 3	Expt 4			
Rapid alkaline lysis	—	11.7 (0.8)	9.1 (0.8)	9.2 (0.6)	9.0 (0.7)	6,774	9.8	
	+	25.0 (0.9)	16.2 (0.8)	19.8 (1.3)	17.0 (0.8)	7,744	19.5	2.0
Cytoplasmic	—	2.3 (0.4)	1.0 (0.3)	3.6 (0.6)	2.6 (0.4)	5,013	2.4	
	+	18.9 (0.8)	17.8 (0.8)	19.8 (0.8)	22.0 (1.8)	5,762	19.5	8.1
Whole cell	—	4.3 (0.7)	4.1 (0.7)	5.0 (1.6)	2.4 (0.6)	1,835	4.0	
	+	10.3 (1.0)	13.4 (1.2)	24.1 (1.6)	16.9 (1.4)	3,024	16.2	4.0

^a DNA samples were transfected in the form present after extraction (—) or subjected to digestion with the restriction enzyme *Xmn*I and then religated (+).

^b Results from individual experiments together with the standard deviation for that experiment.

^c Total colonies counted in all experiments. Cells were infected with HSV-1 24 to 48 h after transfection, and DNA was harvested 18 h after infection.

presence of two copies of the *a* sequence, experiments were performed with plasmids that contain *ori*_S but either lack (pRD107; Fig. 1D) or contain one (pRD108; Fig. 1E) or two (pRD105; Fig. 1F) *a* sequences.

All three plasmids showed a low frequency of white colonies in the absence of HSV-1 superinfection (Table 4). As before, pRD105 produced a high frequency of white colonies after HSV-1 infection, and approximately 80% of the white colonies screened contained a plasmid whose restriction pattern was consistent with recombination having occurred between the two directly repeated *a* sequences. Both pRD107 and pRD108 also showed an increase in the frequency of white colonies after HSV-1 infection. pRD107, lacking an *a* sequence, gave 0.8% white colonies, and pRD108, with a single *a* sequence, produced 3.2% white colonies. Restriction analysis of the DNA from both pRD107 and pRD108 samples revealed a wide variety of deletions, with no one consistent product. It is therefore unlikely that the increase resulted from specific sites in the plasmids that recombined after replication. Instead, it appears that replication increases the frequency of the random deletions, and the presence of a single *a* sequence serves to make these events even more frequent. These deletions may arise in a manner similar to the random deletions noted in DNA transfected into mammalian cells (13, 17), a process that probably involves cellular nucleases and ligases. Alternatively, recombination may occur between homologous sequences on the long concatamers generated after rolling-circle replication. While correct resolution would simply reform the parent plasmid, aberrant resolution of the cross-over could lead to deletions. The increase in the frequency of these events in the plasmid containing one *a* sequence indicates that the *a* sequence is either a preferred target for

cellular nucleases or a preferred site for the initiation of recombination.

Recombination between *a* sequences occurs twice as frequently as between other homologous sequences in a replicating plasmid. To determine whether recombination is specific for *a* sequences, a plasmid (pRD110; Fig. 1G) was constructed in which the two directly repeated *a* sequences were replaced by two direct repeats of a sequence from the coding region of a *Mxyococcus xanthus* gene (*dsg*, a putative translation initiation factor). This sequence, which has no known role in recombination (1a), is 65% G+C (compared with 85% for the *a* sequence) and is 370 bp in length (compared with 317 bp for the *a* sequence). The two sequences are not highly homologous overall, and the largest stretch of homology between the *dsg* and *a* sequences is a 9-bp stretch, GCCCGGACC, with no homologous sequences flanking this region.

In the absence of HSV-1 superinfection, both the *a* sequence-containing (pRD105; Fig. 1F) and *dsg* sequence-containing (pRD110; Fig. 1G) plasmids showed low frequencies of white colony formation (Table 5). Products consistent with recombination between the direct repeats were seen with both plasmids. Thus, it appears that recombination in the absence of HSV-1 superinfection and subsequent replication is not specific to the *a* sequences but occurs with other homologous sequences.

When these plasmids were isolated from cells superinfected with HSV-1, the frequency of white colonies from the pRD110 samples increased to 4.3%, and approximately 80% contained a product whose restriction digest pattern was consistent with deletion through the repeats (Table 5). This frequency was approximately twofold lower than the 8.2% seen with pRD105.

TABLE 3. Recombination frequencies in plasmids lacking HSV-1 origin

Plasmid	No. of <i>a</i> sequences	HSV-1 origin	HSV-1 infection ^a	% White colonies (SD)			Total no. of colonies	Avg % white colonies	Correct deletion ^b
				Expt 1	Expt 2	Expt 3			
pSE367	0	—	—	2.8 (0.9)	0.2 (0.1)	<0.1	2,486	1.0	
			+	1.5 (0.8)	0.2 (0.1)	<0.5	1,153	0.7	
pRD100	1	—	—	0.9 (0.3)	0.2 (0.1)	<0.3	3,414	0.4	0/8
			+	0.8 (0.4)	0.5 (0.2)	0.1 (0.1)	2,176	0.5	0/16
pRD102	2	—	—	0.3 (0.2)	0.4 (0.2)	0.8 (0.3)	2,901	0.5	3/10
			+	0.6 (0.2)	0.4 (0.2)	0.4 (0.2)	4,222	0.5	2/21
pRD105	2	+	—	0.3 (0.2)	0.2 (0.2)	0.2 (0.1)	3,736	0.2	2/9
			+	10.5 (0.3)	8.4 (0.6)	8.5 (0.7)	9,073	9.1	45/58

^a Transfected cells were either infected with HSV-1 (+) or mock infected (—).

^b White colonies were screened by restriction digest analysis as described in the text. Those which yielded a pattern consistent with deletion through the *a* sequences are listed as a correct deletion.

TABLE 4. Effect of number of *a* sequences on recombination frequency in plasmids containing an HSV-1 origin of replication

Plasmid	No. of <i>a</i> sequences	HSV-1 infection	% White colonies (SD)				Total no. of colonies	Avg % white colonies
			Expt 1	Expt 2	Expt 3	Expt 4		
pRD107	0	—	0.3 (0.2)	0.1 (0.1)	<0.1	0.3 (0.1)	4,766	0.2
		+	0.9 (0.2)	1.0 (0.3)	0.7 (0.2)	0.5 (0.2)	6,975	0.8
pRD108	1	—	0.3 (0.2)	— ^a	0.2 (0.1)	0.4 (0.2)	3,621	0.3
		+	3.6 (0.5)	—	2.9 (0.3)	3.2 (0.4)	6,624	3.2
pRD105	2	—	0.1 (0.1)	0.1 (0.1)	0.1 (0.1)	0.1 (0.1)	4,555	0.1
		+	7.8 (0.7)	7.4 (0.8)	6.7 (1.0)	7.3 (0.8)	4,092	7.3

^a —, not done.

The time courses of replication and recombination are parallel. To investigate the relationship between replication and recombination in the HSV-1-infected cells, the two processes were analyzed in Vero cells transfected with pRD105 throughout 18 h of HSV-1 infection. DNA samples were extracted at 2-h intervals after infection, and portions of the DNA were digested with the restriction enzyme *Mbo*I or *Dpn*I. These enzymes recognize and cut at the same site but have different methylation requirements. *Dpn*I cleaves DNA that is fully methylated and will leave intact DNA that has undergone replication. Conversely, *Mbo*I only cleaves unmethylated DNA and will therefore leave unreplicated DNA intact.

As shown in Fig. 4A, the unreplicated (*Mbo*I-digested) pRD105 DNA gave no significant increase in recombinants during the 18-h period of infection. With both the undigested and *Dpn*I-digested samples, recombination began at approximately 8 h and peaked at 16 h postinfection. The samples treated with *Dpn*I showed a greater percentage of recombinants than the untreated samples, probably because digestion of the unreplicated DNA removes the background of plasmids that are not available for either replication or recombination.

To measure replication of the plasmid, equal amounts of each DNA sample were left uncut or digested with *Dpn*I. The DNAs were then transformed into *E. coli* DH5 α , and the number of colonies was determined. Plasmid transformation of *dam*⁺ strains of *E. coli*, such as DH5 α , is unaffected by the state of methylation of the plasmid (28). The percent replicated DNA can therefore be determined by dividing the number of colonies that appear after *Dpn*I treatment by the number appearing in the absence of *Dpn*I digestion. The resulting curve, shown in Fig. 4B, together with the curve for recombinants in the undigested samples of Fig. 4A make it clear that the processes of replication and recombination parallel each other closely.

The two products of the deletion reaction are formed in equimolar amounts. To determine the relative timing of replication and recombination, whole-cell extracts from HSV-1-infected Vero cells that had been electroporated with pRD105 were examined directly by Southern blotting analysis. Since pRD105 contains *ori*_S, it will replicate during HSV-1 infection, as will the smaller deletion product, which also contains *ori*_S (Fig. 5A). The larger, *lacZ*-containing deletion product, however, lacks an HSV-1 origin and is therefore incapable of replication in Vero cells. Thus, if recombination occurred prior to replication, the smaller product would be replicated and amplified, while the larger product would not. On the other hand, if recombination occurred following replication, the two products should be present in equimolar amounts.

pRD105 is cleaved by the restriction enzyme *Ssp*I into two large fragments of 3,500 and 3,800 bp (Fig. 5A). The two recombination products are each cut once by *Ssp*I to yield 3,000-bp and 4,300-bp fragments. As shown in Fig. 5B, both fragments are clearly evident. Inspection of the autoradiograph indicated that the 3,000-bp and 4,300-bp products had similar intensities and hence were present in equimolar amounts. Some linear and higher concatameric products were also observed.

Each sample was subjected to digestion with the *Dpn*I restriction enzyme to verify that the bands represent DNA that had undergone replication. Control experiments indicated that the conditions used allowed complete digestion of the unreplicated DNA in the whole-cell extracts (data not shown). *Dpn*I treatment did not affect the amount of either the parent plasmid (pRD105) or the two products, demonstrating that these bands contain mainly replicated DNA. Thus, the parallel accumulation of the two products suggests that recombination occurs during or subsequent to the last round of DNA replication.

TABLE 5. Comparison of recombination frequencies in plasmids containing directly repeated *a* or *dsg*^a sequences

Plasmid	Direct repeats	HSV-1 infection	% White colonies (SD)						Total no. of colonies	Avg % white colonies	Deletion through repeats ^b
			Expt 1	Expt 2	Expt 3	Expt 4	Expt 5	Expt 6			
pRD110	dsg	—	0.9 (0.3)	0.2 (0.2)	1.0 (0.3)	<0.2	1.0 (0.4)	1.6 (0.6)	4,077	0.8	10/30
		+	4.7 (0.5)	3.6 (0.4)	3.8 (0.5)	3.4 (0.5)	4.4 (0.6)	5.7 (0.5)	9,854	4.3	47/60
pRD105	<i>a</i>	—	0.2 (0.2)	0.2 (0.2)	0.4 (0.2)	0.6 (0.6)	0.5 (0.5)	— ^c	2,559	0.4	1/8
		+	7.3 (0.6)	6.2 (0.6)	8.6 (0.7)	10.7 (0.8)	8.4 (0.8)	7.8 (0.9)	8,138	8.2	44/58

^a The *dsg* (d-signalling) sequence is 370 bp of DNA from the coding region of an *M. xanthus* gene. It is 65% G+C and contains no significant homology to the *a* sequence.

^b The DNA from a portion of the white colonies was subjected to restriction analysis. Those whose restriction pattern was consistent with deletion occurring through the repeated regions, leaving one complete repeat on the deletion product, were counted as deletions through the repeats.

^c —, not done.

FIG. 4. Time course of recombination and replication. (A) Time course of recombination. Samples were extracted at 2-h intervals after HSV-1 infection from cells transfected with pRD105. Digestion with the *DpnI* and *MboI* restriction enzymes was performed as described in Materials and Methods. (B) Time course of recombination and replication. Measurements of DNA replication were performed as described in the text. The curve of recombination frequency is for undigested DNA.

DISCUSSION

Deletion assay. The deletion assay that we have used to study recombination between *a* sequences has several important advantages. First, it scores both the substrate and products of recombination and hence permits quantitation of the frequency of recombination. Second, the assay is highly sensitive and can detect such low-frequency events as recombination in the absence of HSV-1 infection or replication. Third, the deletion assay permits examination of single recombinants after isolation and restriction analysis of the DNA from individual colonies.

It is clear that the recombination measured in the deletion assay occurs after introduction of the test plasmids into Vero cells and not after transfection of the products into *E. coli*. (i) The recombination frequencies measured are much higher than the background of 0.003% observed with the same plasmids in *E. coli* DH5 α . (ii) The recombinants occur in various forms of extracted DNA, including concatamers and virions. (iii) The recombination frequencies are greatly influenced by events within the Vero cells, in particular HSV-1 infection. (iv) The products of recombination can be observed directly by Southern blotting.

FIG. 5. Southern analysis of products of recombination. (A) Diagrammatic representation of *SspI* digests of parental plasmid and deletion products. Band sizes obtained after digestion are shown below. (B) Whole-cell extracts were subjected to Southern blotting with labeled pRD102 as the probe. The autoradiograph was exposed for 6 h. Digestion with the *SspI* restriction enzyme was performed for 5 h. Digestion with *DpnI* was performed overnight. Lanes 1, 3, and 5, *SspI* digestion. Lanes 2, 4, and 6, *SspI* and *DpnI* digestion. Markers shown are derived from a 1-kb ladder run on the same gel.

Relationship between replication and recombination. Recombination between two directly repeated *a* sequences can occur in the absence of HSV-1 infection or replication. The host cell machinery must therefore be capable of promoting the recombination. However, the frequency of recombination is greatly increased by replication. It is unlikely that replication selectively amplifies previously formed recombinants. Analysis of the time course of recombination revealed no pool of unreplicated recombinants at any point in the course of infection. Moreover, Southern blotting analysis showed that both recombination products could be recov-

ered in equivalent amounts. Recombination must therefore occur either during or subsequent to replication.

The increased frequency of recombination could result from a preference for the concatamers that are generated during DNA replication. However, the time course of recombinant formation showed that the processes of replication and recombination occurred in parallel. Thus, if a high affinity of the recombinase for the concatamers is responsible for the increase, recombination must somehow be shut off promptly upon completion of replication. It is possible that recombination is terminated by the packaging of a sequence-containing concatamers into virions. Time course experiments coupled with Southern analysis of recombination in plasmids with other homologous sequences should clarify this point, since such plasmids do not contain the requisite packaging signals and cleavage sites found in the *a* sequence and therefore would not be packaged.

The most likely explanation for the increase in recombination frequency during replication is that the two processes are tightly coupled. Thus, an HSV-1-encoded or host cell recombinase could be recruited to the replicating DNA, either by the complex of replication enzymes or by changes in the DNA. Replication introduces single-strand breaks, and such breaks are known to be recombinogenic (12, 17, 22). The breaks could be repaired slowly after replication, a process that could account for the narrow time frame during which recombination occurs (i.e., following replication but not too long after its completion). Alternatively, replication could induce transient alterations in DNA structure, making certain sites highly recombinogenic. The *a* sequence DR2 repeats adopt a novel DNA structure but only under the influence of negative supercoiling (39, 40).

Role of the *a* sequence. It has been suggested that the *a* sequences are the sites for a site-specific recombination event (18) that leads to the inversion of the *U_L* and *U_S* sequences. Thus far, deletion analyses have been unable to define clearly a recombination site within the *a* sequence. Chou and Roizman (2) concluded that the DR4 repeats are necessary for the inversion, while a more recent study (31) indicated that the ends of the *a* sequence are important. Both of these studies, however, were hampered by the complexities involved in using the whole virus genome for the inversion analysis. The simpler plasmid system used here may help to clarify this point.

There is some evidence consistent with the notion that *a* sequence inversion is mediated by general rather than site-specific recombination. Sequences other than the *a* sequence have been shown to be recombinogenic when placed within the HSV-1 genome, possibly because they function as sites for homologous recombination. Sections of both the *b* sequence (14) and *c* sequence (35) can lead to low-frequency inversions. Similarly, the *Bam*HI L fragment and the HSV-2 glycoprotein C gene lead to genomic rearrangements (24, 25). Finally, the transposable element Tn5 has been shown to undergo high-frequency inversion events when inserted into the HSV-1 genome, in a process that requires at least 600 bp of homology (37).

Our findings thus far suggest that homologous recombination is indeed increased in plasmids that are undergoing replication in HSV-1-infected cells. However, it is clear that the *a* sequence is of special significance. The presence of only a single *a* sequence in a replicating plasmid significantly increases the frequency of illegitimate recombination. This finding suggests that the *a* sequence is either a better site for nuclease action or a preferred site for initiation of recombination on the replication-generated concatamers, a process

that could lead to a *lacZ* deletion if the crossover is incorrectly resolved. Experiments by Weber and coworkers (38) have shown that the 3.0-kb *b-a-c* junction region of HSV-1 is more recombinogenic than a 3.0-kb plasmid sequence. In our work, recombination between the 320-bp *a* sequences in a replicating plasmid is twice as efficient as that seen between repeats of a different sequence of similar size. Comparison with other control sequences is presently being carried out to determine whether G+C content or repeat structures affect the level of recombination seen.

It is not possible at this point to decide whether the increase in recombination frequency due to the *a* sequence occurs because it serves as a recognition site for a site-specific recombinase (in addition to undergoing homologous recombination) or whether it is solely a hot spot for homologous recombination. This question warrants further study. Few site-specific recombinational events have been well characterized in eukaryotes, and homologous recombination in eukaryotes is poorly understood. A sequence that functions as a strong recombinational hot spot or a target for site-specific recombination could aid both in the discovery of the enzymatic components involved and in the identification of other preferred DNA targets.

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