

Herpes Simplex Virus Type 1 Recombination: the U_c-DR1 Region Is Required for High-Level *a*-Sequence-Mediated Recombination

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The *a* sequences of herpes simplex virus type 1 are believed to be the *cis* sites for inversion events that generate four isomeric forms of the viral genome. Using an assay that measures deletion of a β -galactosidase gene positioned between two directly repeated sequences in plasmids transiently maintained in Vero cells, we had found that the *a* sequence is more recombinogenic than another sequence of similar size. To investigate the basis for the enhanced recombination mediated by the *a* sequence, we examined plasmids containing direct repeats of approximately 350 bp from a variety of sources and with a wide range of G+C content. We observed that all of these plasmids show similar recombination frequencies (3 to 4%) in herpes simplex virus type 1-infected cells. However, recombination between directly repeated *a* sequences occurs at twice this frequency (6 to 10%). In addition, we find that insertion of a cleavage site for an *a*-sequence-specific endonuclease into the repeated sequences does not appreciably increase the frequency of recombination, indicating that the presence of endonuclease cleavage sites within the *a* sequence does not account for its recombinogenicity. Finally, by replacing segments of the *a* sequence with DNA fragments of similar length, we have determined that only the 95-bp U_c-DR1 segment is indispensable for high-level *a*-sequence-mediated recombination.

Herpes simplex virus type 1 (HSV-1) is an enveloped virus with a 152-kb linear duplex DNA genome. There are two unique regions in the HSV-1 genome, termed U_L (unique long) and U_S (unique short). These are flanked by repeated regions, such that the final structure can be represented as *a*_n*b*-U_L-*b*'*a*_m'*c*'-U_S-*ca* (41). Recombination occurs through the inverted repeats during HSV-1 infection, giving rise to four equimolar isomeric forms that differ in the orientation of their unique regions (18, 23, 45). There is considerable evidence that this inversion process occurs through the HSV-1 *a* sequences. Insertion of fragments containing an *a* sequence into the viral thymidine kinase gene produces new inversions in regions flanked by the inverted *a* repeats (32, 50) and deletions of regions with directly repeated *a* sequences at their ends (32, 50). In addition, the few noninverting mutants of HSV-1 that have been identified contain large deletions encompassing the *a* sequences at the U_L-U_S junction (25, 37).

The HSV-1 *a* sequence varies from 200 to 500 bp in length and is approximately 83% G+C. The *a* sequence is essential for cleavage and packaging of HSV-1 (35, 56) and also includes the promoter for the ICP34.5 gene (10). The *a* sequence contains 20-bp direct repeats (DR1) at each end and two unique segments (U_b and U_c) separated by arrays of direct repeats. The composition of the internal directly repeated elements of the *a* sequence varies from strain to strain (15, 31, 33, 55, 56), but all contain an array of 12-bp DR2 repeats. The DR2 repeats have several features that could potentially play a role in *a*-sequence-mediated recombination. They have been shown to adopt a novel DNA conformation under the influence of negative supercoiling (62, 63), and they are sites for cleavage by a HSV-1-induced endonuclease (61).

High-level recombination between either *a* sequences or other pairs of homologous sequences requires both HSV-1 infection and replication of the DNA containing the repeated

sequences. Recombination between inverted repeats of the *a* sequence that had been stably integrated into the cellular genome along with a flanking HSV-1 origin of replication was shown to occur only in conjunction with HSV-1-dependent amplification (34). Similarly, recombination between Tn5 repeats within plasmids was observed only in association with HSV-1 DNA replication (58). Our previous studies of recombination between *a* sequences in plasmids transiently introduced into Vero cells confirmed that high levels of recombination were seen only when the plasmids underwent replication in HSV-1-infected cells (20). Analysis of the time course of HSV-1 recombination and replication indicated that the two processes parallel each other, and direct quantitation of the products of recombination by Southern analysis demonstrated that recombination occurred during or subsequent to the last round of DNA replication (20).

The exact nature of the high-level *a*-sequence-mediated recombination remains unclear. HSV-1-infected cells show high levels of homologous recombination, and repeated regions such as the HSV-1 *Bam* L sequence (38), the HSV-1 *b* sequences (30), or the Tn5 repeats (58) can mediate inversion events. The *a* sequence does, however, appear to be more highly recombinogenic than other sequences: Weber and co-workers demonstrated that the 3.0-kb *b-a-c* junction was more recombinogenic than a 3.0-kb plasmid sequence (59), and our previous work showed that recombination between *a* sequences was twice as efficient as recombination between a different set of direct repeats of the same size (20). Recombination between *a* sequences could be enhanced either because it is a hot spot for homologous recombination or because it is acted on by a site-specific recombinase. The finding that the *a* sequences of HSV-1 and HSV-2, which have little homology, are incapable of recombining with each other argues against a site-specific mechanism (51). However, the simple conditions of an in vitro system for *a*-sequence recombination (7) are indicative of a site-specific mechanism.

In this report, we describe our continued studies of recom-

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bination with plasmids transiently introduced into Vero cells. We find that direct repeats from a variety of sources and with a wide range of G+C content give essentially identical recombination frequencies. However, directly repeated *a* sequences show recombination frequencies that are twice that seen with the other sequences. Introduction of a cleavage site for the *a*-sequence endonuclease identified by Wohlrab and coworkers (61) does not increase the frequency of recombination, indicating that the presence of cleavage sites for this enzyme cannot account for the recombinogenic nature of the *a* sequence. Finally, replacement of portions of the *a* sequence with DNA segments of similar length showed that the U_c -DR1 region contains the *cis* signals required for high-level *a*-sequence-mediated recombination.

MATERIALS AND METHODS

Cells and viruses. All tissue culture experiments were carried out with Vero cells (African green monkey kidney fibroblasts) obtained from the American Type Culture Collection. Cells were propagated in Dulbecco modified Eagle minimum essential medium supplemented with 10% fetal calf serum, 0.1 mM nonessential amino acids, and 2 mM glutamine. The HSV-1 strain F(Δ 305) (39) was used.

Plasmid construction. All restriction enzymes and linkers used in plasmid construction were obtained from New England Biolabs or the United States Biochemical Corp. Plasmids were prepared either by alkaline lysis followed by subsequent CsCl gradient purification (43) or by the alkaline lysis procedure followed by purification on Qiagen columns according to the manufacturer's instructions. The two methods gave essentially the same results in transfection experiments. All DNA samples for an individual transfection experiment were prepared by the same method.

Plasmid pRD107 (20) was used as the parent vector for all constructions. This plasmid contains the ampicillin resistance gene and the origin of replication of pUC19, permitting its propagation in *Escherichia coli*. It also contains the complete *lacZ* gene with ribosomal binding sites that allow transcripts made by runoff transcription to be translated. Upon transformation with pRD107, *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). In addition, pRD107 contains an HSV-1 origin of replication, *Orig*, which allows the plasmid to replicate in HSV-1-infected cells.

Plasmids containing direct repeats of homologous sequences were prepared by inserting a copy of the desired insert into both the *Xba*I and *Bam*HI sites of pRD107, so that the inserts were positioned in directly repeated orientation. In all cases, the inserts were prepared for insertion by preliminary restriction digestion, addition of either *Xba*I or *Bam*HI linkers, digestion with *Xba*I or *Bam*HI, purification on agarose-TAE (40 mM Tris-acetate, 1 mM EDTA) gels, and elution from the agarose gel with an Elutrap electrophoresis chamber (Schleicher & Schuell). For pRDMS2, the insert was a 374-bp *Pst*I-to-*Sac*II fragment from the mouse Na⁺/K⁺ ATPase gene, kindly provided by S. Ying Tam, Harvard Medical School. For pRDBR2, the insert was a 378-bp *Sph*I-to-*Eag*I fragment from pBR322 (nucleotides 562 to 939). This fragment contained no part of the parent vector, pRD107. pRD ϕ X2 contained 400-bp inserts generated by *Ssp*I-*Dra*I digestion of ϕ X174 (nucleotides 1007 to 1406; New England Biolabs). The 390-bp fragment in pRDU32 was from the yeast *URA3* gene, generated by a *Stu*I-*Nsi*I digest of yRP17 (nucleotides 3556 to 3945). pRDSVS2 contained a 370-bp insert from a *Hinc*II digest of simian virus 40 (nucleotides 2297 to 2666). For pRDUbx2, a 380-bp fragment from

an *Apa*I-*Pst*I digest of p(UT)₂(Hind)_D-33/t3CAT (27) was used. This insert contains multiple copies of the Ubx DNA binding site from *Drosophila melanogaster*. Finally, pRD110, as described previously (20), contains an insert from the d-signaling gene (*dsg*) of *Myxococcus xanthus*, provided by Yvonne Cheng and Dale Kaiser, Stanford University. pRD105, also described previously (20), contains two copies of the *a* sequence from HSV-1 strain KOS, provided by James Smiley, McMaster University.

To construct plasmid substrates containing cleavage sites for the *a*-sequence-specific endonuclease reported by Wohlrab et al. (61), (dG)₂₅ and (dC)₂₅ oligonucleotides flanked by *Xho*I sites were obtained from the PAN facility, Beckman Center, Stanford University School of Medicine. The oligonucleotides were annealed by mixing equal volumes, heating to 85°C, and then cooling to room temperature. The annealed oligonucleotides were then digested overnight with *Xho*I and purified on a 4% NuSieve low-melting-point agarose gel (FMC BioProducts). The vectors pRDMS1 (pRD107 containing only one mouse Na⁺/K⁺ ATPase insert) and pRD111 (pRD107 with one *dsg* insert) were prepared for insertion by cleavage with *Xho*I, which cuts once in the mouse and *dsg* inserts and for which there are no sites in the parent vector pRD107. Insertion of the (dG)₂₅·(dC)₂₅ oligonucleotide into the prepared vectors resulted in plasmids msendo1 and dsendo1. A second copy of the mouse and *dsg* inserts now containing the endonuclease recognition site was then added to the *Bam*HI site of the vectors such that the repeats were completely homologous and in the directly repeated orientation, creating plasmids msendo2 and dsendo2. Finally, plasmid dsmsendo was constructed by inserting a copy of the mouse Na⁺/K⁺ ATPase with inserted (dG)₂₅·(dC)₂₅ into the *Bam*HI site of dsendo1. Thus, this plasmid has two nonhomologous inserts, both containing the endonuclease site.

Plasmids for analysis of the recombinogenic properties of various regions of the *a* sequence were constructed by removing portions of the *a* sequence and replacing them with segments of the *dsg* sequence. The wild-type *a* sequence from HSV-1 strain KOS is 317 bp in length and contains both direct-repeat and unique regions such that its final structure is DR1-U_b-(DR2)₁₀-U_c-DR1 (56).

Plasmid pRDU_b⁻² was constructed in the following manner. The 317-bp *a* sequence was removed from pRD105 by *Bam*HI digestion, isolated on a 1% agarose-TAE gel, and purified by Elutrap. This fragment was then digested with the restriction enzyme *Eae*I, and the resulting 222-bp DNA fragment, with the sequence *Eae*I-(DR2-U_c-DR1)-*Bam*HI, was gel purified and isolated by Elutrap. The required *dsg* fragment was generated by digestion of pRD111 with *Csp*I, addition of *Eag*I linkers (*Eag*I and *Eae*I produce compatible cohesive ends), and digestion with *Bam*HI and *Eag*I. The resulting 120-bp fragment was purified by using a 1% agarose-TAE gel and Elutrap. This *Bam*HI-(*dsg*)-*Eag*I fragment was cloned into the Bluescript cloning vector (Stratagene) cut with *Bam*HI and *Eag*I to obtain sufficient quantities of DNA and again excised and purified prior to use. The *a* sequence and *dsg* fragments were joined by simultaneous insertion into the Bluescript vector previously digested with *Bam*HI, giving a 342-bp insert of structure *Bam*HI-(*dsg*-DR2-U_c-DR1)-*Bam*HI. This insert was placed into the *Xba*I site of pRD107 (*Xba*I linkers added prior to insertion) to give pRDU_b⁻¹ and then into the *Bam*HI site of pRDU_b⁻¹ to yield pRDU_b⁻².

To construct plasmid pRDR2⁻², two *a*-sequence fragments were needed. The first, containing the DR1-U_b portion, was generated by cleaving the purified 317-bp *a* sequence with *Eae*I and purifying the resulting 95-bp fragment through 1% aga-

rose-TAE and Elutrap, giving a fragment of structure *Bam*HI-(DR1-U_b)-*Eae*I. The DNA fragment containing the U_c-DR1 was generated by digestion of pRD105 with *Dra*I and *Sph*I, gel and Elutrap purification of the resulting 270-bp fragment, and digestion with *Mn*II for 6 h to remove the DR2 region. The resulting fragment was then filled in with the Klenow fragment of DNA polymerase I. *Xho*I linkers were added, and product was digested with *Bam*HI and *Xho*I. The final 100-bp fragment, with the structure *Xho*I-(U_c-DR1)-*Bam*HI, was gel and Elutrap purified. Finally, the desired dsG fragment was constructed by cleavage of pRD109 with *Bam*HI and *Stu*I, addition of *Eag*I linkers, digestion with *Xho*I and *Eag*I, and gel and Elutrap purification. This 126-bp fragment, *Xho*I-(dsG)-*Eag*I, was cloned into Bluescript previously digested with *Xho*I and *Eag*I in order to obtain sufficient quantities of the DNA. Once the three required fragments were obtained, the *Bam*HI-(DR1-U_b)-*Eae*I and *Eag*I-(dsG)-*Xho*I pieces were ligated by simultaneous insertion into Bluescript previously digested with *Bam*HI and *Xho*I. This plasmid was grown in large quantities, and the 221-bp insert was cut out with *Bam*HI and *Xho*I and purified. Next, this *Bam*HI-(DR1-U_b-dsG)-*Xho*I fragment and the *Xho*I-(U_c-DR1)-*Bam*HI DNA were joined by double insertion into Bluescript previously digested with *Bam*HI. This procedure generated the final 321-bp insert, *Bam*HI-(DR1-U_b-dsG-U_c-DR1)-*Bam*HI, which was subsequently cloned into the *Xba*I site of pRD107 (using *Xba*I linkers on the insert) to give pRDR2⁻¹ and then into the *Bam*HI site of pRDR2⁻¹ in direct orientation to give pRDR2⁻².

Three fragments were also required to generate pRDU_c⁻². The first, the 95-bp *Bam*HI-(DR1-U_b)-*Eae*I fragment, was the same as described for pRDR2⁻². The segment containing the DR2 region was isolated by cleavage of pRD105 with *Sst*II, purification of the 217-bp fragment generated, cleavage by *Dra*I and *Eae*I, and isolation of the resulting 122-bp fragment [*Eae*I-(DR2)-*Sst*II]. These two segments were ligated by simultaneous cloning into Bluescript previously digested with *Bam*HI and *Sst*II to give *Bam*HI-(DR1-U_b-DR2)-*Sst*II. Next, the dsG fragment described for pRDR2⁻² was inserted by blunt-end ligation into the *Sac*I site of the Bluescript vector containing the required portions of the *a* sequence. Cleavage of the resulting plasmid with *Bam*HI and *Rsa*I (which cleaves near the end of the inserted dsG sequence) yielded a 337-bp insert with structure *Bam*HI-(DR1-U_b-DR2-dsG)-*Rsa*I containing 12 bp of Bluescript vector between the DR2 and dsG sequences. This insert was cloned into the *Xba*I site of pRD107 (using *Xba*I linkers) to create pRDU_c⁻¹ and into the *Bam*HI site of pRDU_c⁻¹ in direct-repeat orientation to give pRDU_c⁻². The correct construction of the three replacement constructs was confirmed by DNA sequencing.

Transfection, infection, and DNA isolation. Actively growing Vero cells at a concentration of 5×10^6 to 7×10^6 cells per ml were electroporated (12) at 220 V with 20 μ g of the indicated plasmid. Cells were allowed to recover for approximately 24 h. The transfected cells were then infected with 10 PFU/cell HSV-1 F(Δ 305) in 3 ml of medium or mock infected with 3 ml of medium. Virus and medium were removed after 1 h, the cells were washed twice with phosphate-buffered saline (obtained from Gibco), and the medium was replaced.

DNA was extracted from the transfected cells using the RAPP procedure as described previously (20).

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 (22) except that the growth medium consisted of 5 g of Bacto Yeast Extract, 20 g of Bacto Tryptone, and 5 g of MgSO₄ per liter. The cells were plated with 100 μ g

of carbenicillin and 50 μ g of X-Gal per ml. Miniprep isolation of DNA from white colonies was performed by the modified boiling procedure (43), with 50 mM Tris-Cl (pH 8.0)–6.5 mM EDTA–0.4% Triton X-100–2.5 M LiCl used as the resuspension buffer. The DNA was digested with *Dra*I for 2 h and electrophoresed through 1% agarose gels in TAE.

Cleavage of plasmid DNA in vitro. A 0.4 M NaCl extract was prepared from nuclei of HSV-1-infected Vero cells, centrifuged at 100,000 $\times g$, and precipitated with ammonium sulfate as previously described (7). The dissolved precipitate was then dialyzed and applied to a column of heparin-agarose and eluted with a 50 ml 0.1 to 1.0 M NaCl gradient. The 0.5 M NaCl heparin-agarose fraction contains the *a*-sequence-specific cleavage activity.

One microgram of plasmid DNA linearized with *Ssp*I was incubated with 500 ng of the heparin-agarose fraction for 10 min at 37°C in a buffer consisting of 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 7.6), 1 mM dithiothreitol, 1 mM spermidine, 2 mM MgCl₂, 1 mM EDTA, and 50 mM NaCl. EDTA (10 mM) was used to stop the reaction, after which the DNA was purified by using the Magic DNA Cleanup System (Promega, Inc.).

Two hundred nanograms of enzyme-treated DNA was digested with *Fsp*I and *Sca*I, precipitated, and resuspended in 80% formamide–1 \times Tris-borate-EDTA (TBE) loading buffer. The samples were boiled, chilled on ice, and loaded onto an 8 M urea–1.2 \times TBE 5% Long Ranger gel (J. T. Baker, Inc.). The gel was run with 0.6 \times TBE buffer at 27 W and 50 to 55°C. Following electrophoresis, the gel was equilibrated for 20 min in 1 \times NAQ buffer (80 mM Tris-HCl, 118 mM borate, 2.4 mM EDTA [pH 8.3]). The DNA was then transferred with 1 \times NAQ buffer onto a 0.22- μ m-pore-size Nytran membrane (Schleicher & Schuell) at 2.5 mA/cm² for 1 h, using a Graphite Electrobloater II (Millipore, Inc.). Following transfer, the membrane was rinsed with 5 \times SSC (1 \times SSC is 0.15 M NaCl plus 0.015 M sodium citrate), and the DNA was immobilized onto the damp membrane by cross-linking in a Stratalinker oven (Stratagene, Inc.) with a UV dose of 120 mJ/cm².

Bands were detected in the following manner. Oligonucleotides CGCGATCTGGCGACCACCCCGTCC and CCACAGGACGGGTGTGGTCGCCAGATCGCG were tailed at their 3' ends with digoxigenin-11-dUTP/dATP by using terminal transferase and used to probe the membrane according to instructions provided with Genius 6 kit (Boehringer Mannheim, Inc.). The membrane was then processed with anti-digoxigenin antibody coupled to alkaline phosphatase and Lumigen PPD chemiluminescent substrate, using the Genius 7 kit and instructions suggested by the manufacturer (Boehringer Mannheim). Following a 2-h incubation at room temperature in the dark, the membrane was exposed to Hyperfilm ECL X-ray film (Amersham, Inc.) for 30 min. The film was developed in an automated film developer (Eastman Kodak, Inc.).

Assay for recombination frequency. The assay used to measure recombination between sets of direct repeats has been previously described (20). Briefly, plasmids containing directly repeated sequences flanking a *lacZ* gene were electroporated into Vero cells, and the cells were infected with HSV-1 or mock infected. Eighteen hours after infection, the DNA was isolated by the RAPP procedure and then transformed into *E. coli* DH5 α . The number of blue and white colonies was scored to determine the frequency of *lacZ* deletion. Since events other than recombination between the direct repeats can lead to loss of a functional *lacZ* gene, the DNA was isolated from a fraction of the white colonies by the miniprep procedure and examined by restriction analysis.

TABLE 1. Comparison of recombination frequencies with plasmids containing direct repeats

Plasmid	Source of DNA insert	% G+C	HSV-1 infection ^a	% White colonies (SD ^b)			Total no. of colonies	Avg % white colonies
				Expt 1	Expt 2	Expt 3		
pRD105	<i>a</i> sequence	83	–	0.2 (0.1)	0.5 (0.2)	0.4 (0.2)	2,875	0.4
pRDMS2	Mouse Na ⁺ /K ⁺ ATPase	73	+	8.7 (0.8)	9.8 (1.9)	12.6 (1.0)	2,433	10.4
			–	0.2 (0.1)	0.3 (0.2)	0.2 (0.1)	3,181	0.2
pRD110	<i>M. xanthus</i> <i>dsg</i>	65	–	4.5 (0.4)	2.5 (0.6)	3.9 (0.5)	4,632	3.6
			+	0.4 (0.2)	0.2 (0.2)	0.6 (0.2)	2,759	0.4
pRDBR2	pBR322	60	–	3.4 (0.4)	5.7 (1.0)	3.3 (0.5)	3,696	4.0
			+	0.2 (0.1)	0.6 (0.3)	0.5 (0.2)	3,012	0.4
pRD ϕ X2	ϕ X174	45	–	3.7 (0.4)	3.1 (0.5)	5.9 (0.6)	4,926	4.2
			+	0.2 (0.1)	<0.4	0.4 (0.4)	1,996	0.3
pRDU32	Yeast <i>URA3</i> gene	42	–	3.5 (0.4)	3.5 (0.8)	6.7 (0.6)	4,602	4.6
			+	0.6 (0.3)	<0.4	0.6 (0.3)	2,062	0.5
pRDSVS2	Simian virus 40	40	–	4.7 (0.6)	2.2 (0.6)	3.0 (0.7)	2,261	3.3
			+	0.4 (0.2)	0.1 (0.1)	0.2 (0.1)	2,704	0.2
pRDUbx2	<i>D. melanogaster</i> Ubx binding sites	22	–	3.0 (0.5)	3.5 (0.5)	4.3 (1.0)	2,629	3.6
			+	<0.1	<0.1	0.4 (0.2)	3,030	0.2
				3.1 (0.6)	2.8 (0.5)	4.9 (0.6)	3,329	3.6

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

^b Calculated as [(1–frequency) (frequency)/number of colonies]^{1/2}.

RESULTS

Recombination between *a* sequences occurs at twice the frequency of recombination between any other set of homologous sequences. We had previously shown that recombination between *a* sequences in replicating plasmids is twice as efficient as that seen between two directly repeated copies of *dsg*, a segment of DNA from *M. xanthus* of similar size but with no significant homology to the *a* sequence (20). To determine whether the high G+C content of the *a* sequence (83%) is responsible for the increased frequency of recombination, we constructed six plasmids with the repeats all 370 to 400 bp (compared with the 317-bp *a* sequence) but with G+C content varying from 22 to 73%.

In the absence of superinfection with HSV-1, all of the plasmids showed a low level of recombination (<1% white colonies), consistent with our previous finding (20), and there was no significant difference in the recombination frequencies of any of the plasmids (Table 1).

In the presence of HSV-1 infection, all plasmids showed a greatly increased frequency of recombination. As before, pRD105, containing the *a* sequences, gave an average of 10.4% white colonies, and pRD110, the plasmid with directly repeated *dsg* sequences, showed a recombination frequency less than half that of the *a*-sequence-containing plasmid (4%). All other repeated sequences recombined with a frequency similar to that of the *dsg* sequence, regardless of the source of the DNA or their G+C content. Restriction digest analysis of plasmid DNA from the white colonies of each sample showed that 60 to 80% contained a plasmid that was consistent with a deletion through the directly repeated sequences. Thus, we can conclude that the frequency of homologous recombination between these direct repeats in plasmids replicating in HSV-1-infected cells is 3 to 4% and is unaffected by G+C content ranging from 22 to 73%. Recombination between *a* sequences is at least twice as efficient, confirming the special recombinogenic properties of the *a* sequence.

Addition of sites for an *a*-sequence-specific endonuclease does not significantly increase recombination between direct repeats. An endonuclease that cleaves within the *a* sequence has been identified and partially purified by Wohlrab and

coworkers (61). It was suggested that this enzyme may play a role in *a*-sequence-mediated recombination by generating double-strand breaks that could be highly recombinogenic. To test this hypothesis, we constructed plasmids in which another substrate for the endonuclease, a duplex oligonucleotide, (dG)₂₅ · (dC)₂₅ (61), was inserted into both repeats of pRD110 and pRDMS2. To verify that these sequences were cleaved, a partially purified enzyme fraction from HSV-1-infected nuclei able to cleave the DR2 repeats within the *a* sequence (data not shown) and which is probably identical to the enzyme described by Wohlrab and coworkers (61) was incubated with the modified plasmid substrates. The DNA was then purified and analyzed by modified Southern analysis. In the absence of enzyme, little cleavage of the DNA occurred with any of the samples (Fig. 1, lanes 1, 3, 5, and 7). When enzyme was added, pRD110 and pRDMS2, which lack the endonuclease substrate insert, showed no significant cleavage (Fig. 1, lanes 2 and 6). However, the two plasmids with the (dG)₂₅ · (dC)₂₅ insert produced a 400-bp product for *dsgendo2* and a 250-bp product for *msendo2*, as predicted for cleavage at the inserted endonuclease site (Fig. 1, lanes 4 and 8). The amount of cleavage at the (dG)₂₅ · (dC)₂₅ insertion was similar to that for the *a* sequence (data not shown).

When recombination between direct repeats containing the (dG)₂₅ · (dC)₂₅ was examined in uninfected cells, a low frequency of white colonies was observed (Table 2). Four samples gave significantly higher values than normally seen. However, these higher values are randomly dispersed, and restriction analysis of the DNA isolated from the white colonies showed very few correct products.

After HSV-1 infection, there was a large increase in recombination frequency (Table 2). pRD105, the *a*-sequence-containing plasmid, showed the highest percentage of white colonies (5.8%). pRD110, with the *dsg* repeats, again gave white colonies at approximately half the frequency seen with pRD105. *dsgendo2*, formed by addition of the endonuclease substrate site to the *dsg* sequences, gave recombination frequencies that were very similar to those for pRD110. The data obtained with plasmids pRDMS2 and *msendo2* are somewhat more complex. pRDMS2 gave 3.0 to 4.4% white colonies in

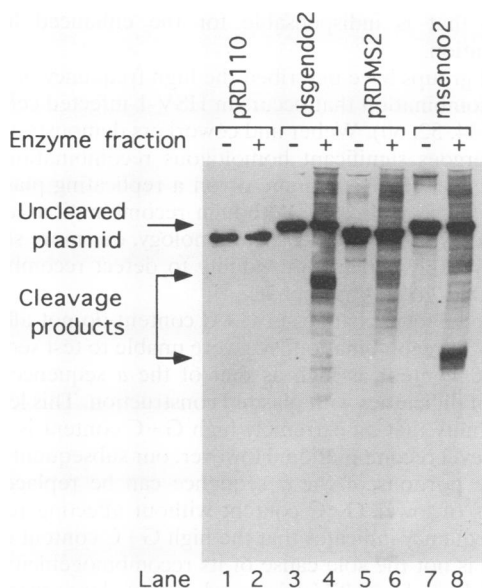


FIG. 1. Southern analysis of endonuclease cleavage of sites containing a (dG)₂₅ · (dC)₂₅ insert. The experiment was performed as described in Materials and Methods. Lanes 1, 3, 5, and 7, untreated DNA; lanes 2, 4, 6, and 8, DNA treated with enzyme fraction. Lanes 1 and 2, pRD110; lanes 3 and 4, dsgendo2; lanes 5 and 6, pRDMS2; lanes 7 and 8, msendo2.

five of the six samples. The reason for the high value in experiment 4 is unclear. The endonuclease insert in msendo appeared to give significant stimulation in one case (experiment 3) but otherwise gave values very similar to those for pRDMS2. Restriction digest analysis showed no significant difference in the percentage of correct deletion products obtained between plasmids with the endonuclease cleavage site and those lacking this site. Finally, plasmid dsgmsendo, which contains one dsg-endonuclease cleavage site insert and one mouse-endonuclease cleavage site insert, gave a very low frequency of white colonies and no correct products in restriction digest analysis, demonstrating that two homologous sequences are required even in the presence of the cleavage sites. We therefore conclude that the presence of a cleavage site for the *a*-sequence-specific endonuclease is not sufficient to ex-

plain the consistently high levels of recombination observed between repeated *a* sequences.

The U_c-DR1 region of the *a* sequence is responsible for the high level of recombination between *a* sequences. Our studies confirmed the highly recombinogenic nature of the *a* sequence but indicated that cleavage within the DR2 repeats by the *a*-sequence endonuclease was not sufficient. We therefore wished to determine which regions of the *a* sequence were necessary for the high recombinogenicity. Two groups had previously performed deletion analyses of the *a* sequence, with somewhat conflicting results (9, 49), possibly because of variations in the length of homologous sequences examined. There is significant evidence that in both prokaryotic and eukaryotic cells, homologous recombination is strongly dependent on the length of the homologous regions (3, 26, 29, 42, 46, 48, 53, 57, 64). Recombination between repeated sequences shorter than the minimal efficient processing segment (approximately 220 to 280 bp in mammalian cells) (29, 42) is very inefficient. Above this size, there is a linear dependence on length. Consequently, it is important that the constructs to be tested for their recombination frequency be of similar size. We therefore replaced portions of the *a* sequence with segments of the dsg sequence such that the length of homology was kept between 317 bp (the size of the wild-type *a* sequence) and 342 bp. The three constructs (Fig. 2) contain inserts which replace the DR1-U_b regions (pRDU_b⁻²), the DR2 repeats (pRDR2⁻²), or the U_c-DR1 regions (pRDU_c⁻²) of the *a* sequence.

In the presence of HSV-1 infection, the frequency of recombination with pRDU_b⁻², which lacks the DR1-U_b region, was similar to that seen with pRD105, which contains the wild-type *a* sequence (Table 3). pRDR2⁻², which lacks the DR2 repeats, gave an even higher level of recombination. Replacement of the U_c-DR1 region, however, reduced recombination to half of that obtained with the wild-type *a* sequence. The reduced level of white colony formation seen with pRDU_c⁻² was consistent through all of the experiments and was independent of the preparation of DNA (Table 3, experiment 5). Thus, when the total length of homology is kept constant, either the DR1-U_b region or the DR2 repeats can be replaced with no significant decrease in recombination frequency, indicating that they are not responsible for the increased frequency of recombination seen with the *a* sequence. Instead, the *cis* signal for high-level *a*-sequence recombination resides in the U_c-DR1 segment. Replacement of this segment reduces the recombination fre-

TABLE 2. Effects of insertion of *a*-sequence-specific endonuclease substrates on recombination

Plasmid	HSV-1 infection	% White colonies (SD)					Total no. of colonies	Avg % white colonies
		Expt 1	Expt 2	Expt 3	Expt 4	Expt 5 ^a		
pRD110	-	0.3 (0.2)	0.6 (0.2)	2.8 (1.0)	0.1 (0.1)	— ^b	4,338	0.9
	+	3.4 (0.6)	3.3 (0.4)	4.1 (1.2)	3.9 (0.4)	2.3 (0.4), 2.8 (0.4)	8,985	3.3
dsgendo2	-	3.3 (0.7)	0.5 (0.2)	—	0.3 (0.2)	—	2,667	1.4
	+	3.6 (0.5)	3.4 (0.6)	—	4.9 (0.4)	3.1 (0.4), 2.9 (0.4)	7,856	3.6
pRDMS2	-	1.8 (0.6)	0.2 (0.1)	0.5 (0.2)	0.3 (0.1)	—	4,308	0.7
	+	4.4 (0.7)	3.3 (0.4)	4.4 (0.6)	7.2 (0.8)	3.3 (0.4), 3.0 (0.4)	8,920	4.3
msendo2	-	1.9 (0.6)	0.2 (0.1)	<0.3	0.6 (0.2)	—	3,448	0.8
	+	3.5 (0.7)	4.7 (0.6)	6.4 (0.9)	6.8 (0.7)	3.3 (0.4)	5,635	4.9
dsgmsendo	-	0.4 (0.3)	0.3 (0.2)	0.1 (0.1)	—	—	2,494	0.3
	+	0.5 (0.2)	1.6 (0.3)	1.4 (0.4)	—	2.2 (0.3), 0.7 (0.2)	6,977	1.3
pRD105	-	0.3 (0.2)	0.2 (0.1)	0.7 (0.4)	<0.1	—	4,176	0.3
	+	5.8 (0.6)	4.0 (0.6)	6.8 (1.3)	8.0 (0.8)	5.0 (0.3), 5.1 (0.4)	8,356	5.8

^a HSV-1-infected samples were assayed in duplicate.
^b —, not done.

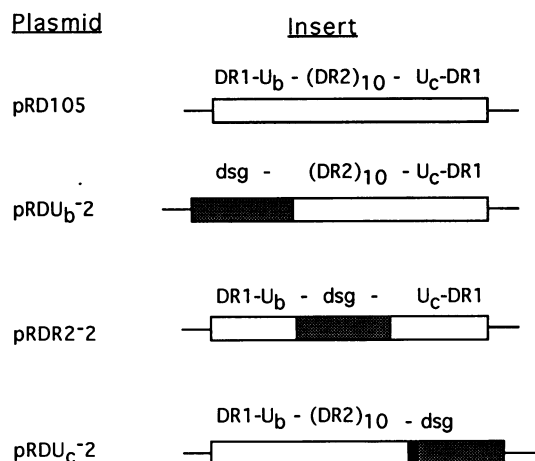


FIG. 2. Constructs with *a*-sequence replacements. Inserts are shown in correct size proportion, with total lengths being 317 bp for pRD105, 342 bp for pRDU_b-2, 321 bp for pRDR2-2, and 337 bp for pRDU_c-2. Constructions are described in Materials and Methods. □, segments from the HSV-1 strain KOS *a* sequence; ■, segments from the *M. xanthus* *dsg* gene; ▨, 12-bp fragment from the polylinker region of the Bluescript cloning vector.

quency to the level observed between any set of non-*a*-sequence direct repeats.

DISCUSSION

Our findings shed new light on several aspects of recombination in HSV-1-infected cells. First, the very similar recombination frequencies observed with direct repeats in which G+C content varied from 22 to 73% indicates that there is a significant level of homologous recombination in replicating plasmids that is independent of G+C content. Second, recombination between *a* sequences occurs at twice the level observed with these other sequences. Third, addition of a cleavage site for an *a*-sequence-specific endonuclease does not significantly increase the frequency of recombination, indicating that cleavage of the *a* sequence by this enzyme cannot account for the high recombinogenicity of the *a* sequence. Finally, the U_c-DR1 region is the only segment of the *a*

sequence that is indispensable for the enhanced level of recombination.

Several groups have described the high frequency of homologous recombination that occurs in HSV-1-infected cells (5, 6, 8, 14, 24, 44, 52, 60). Weber and coworkers demonstrated that Tn5 undergoes significant homologous recombination when present in the HSV-1 genome or on a replicating plasmid in HSV-1-infected cells (58). Although recombination could be detected only down to 600 bp of homology, our more sensitive assay very likely explains our ability to detect recombination between 350- to 400-bp repeats.

As noted above, changes in G+C content do not affect the frequency of recombination. We were unable to test sequences with G+C content as high as that of the *a* sequence (83%) because of difficulties with plasmid construction. This left open the possibility that an extremely high G+C content is needed for high-level recombination. However, our subsequent finding that large portions of the *a* sequence can be replaced with sequences of lower G+C content without affecting recombination frequency indicates that the high G+C content of the *a* sequence is not the sole cause of its recombinogenicity.

The finding of an HSV-1-induced endonuclease that specifically cleaves within the *a* sequence (61) led to speculation that cleavage by this enzyme was responsible for the high level of recombination. There is considerable evidence that double-strand breaks in DNA can stimulate recombination (for reviews, see references 21 and 54). However, it is clear that cleavage by the *a*-sequence-specific endonuclease is not responsible for the recombinogenicity of the *a* sequence. Possibly recombination occurs with such high frequency in HSV-1-infected cells that double-strand breaks do not significantly influence the total signal. It is also possible that cleavage by the *a*-sequence-specific endonuclease, while readily seen in vitro, did not occur within the infected Vero cells. However, our finding that the DR2 region of the *a* sequence is not required for high-level recombination reinforces our conclusion that cleavage within the DR2 repeats by the *a*-sequence endonuclease is not the cause of the high level of *a*-sequence-mediated recombination.

Replacement of portions of the *a* sequence was used to determine whether any regions within the *a* sequence were dispensable. Simple deletions decrease the amount of homology, and as stated earlier, there is considerable evidence that the frequency of homologous recombination is directly related

TABLE 3. Effects of replacement of regions of the *a* sequence on recombination

Plasmid	HSV-1 infection	% White colonies (SD)					Total no. of colonies	Avg % white colonies
		Expt 1	Expt 2	Expt 3	Expt 4 ^a	Expt 5 ^b		
pRDU _b -2	-	0.1 (0.1)	0.2 (0.1)	0.1 (0.1)	— ^c	—	3,830	0.1
	+	5.7 (0.5)	4.8 (0.5)	8.5 (0.7)	3.5 (0.4), 6.2 (0.8)	6.0 (0.5), 9.1 (0.8)	12,267	6.3
pRDR2-2	-	<0.1	0.2 (0.1)	0.8 (0.2)	—	—	4,362	0.4
	+	7.3 (0.7)	6.8 (0.6)	7.6 (0.7)	3.5 (0.3)	9.4 (0.7), 12.4 (0.7)	11,458	7.8
pRDU _c -2	-	0.6 (0.2)	0.4 (0.2)	0.2 (0.1)	—	—	3,950	0.4
	+	3.8 (0.4)	3.0 (0.4)	4.1 (0.5)	3.0 (0.5), 2.5 (0.3)	3.0 (0.3), 2.5 (0.4), 2.9 (0.4), 3.0 (0.5), 1.9 (0.2)	19,814	3.0
pRD105	-	0.2 (0.1)	<0.1	0.3 (0.1)	—	—	5,805	0.2
	+	5.8 (0.6)	5.7 (0.7)	5.5 (0.6)	7.9 (0.9), 5.6 (1.0)	7.2 (0.7), 9.7 (0.6)	9,206	6.8

^a HSV-1-infected samples were assayed in duplicate.

^b HSV-1-infected samples for pRDU_b-2, pRDR2-2 and pRD105 were assayed in duplicate; HIV-1-infected samples for pRDU_c-2 were assayed five times, using three different preparations of DNA.

^c —, not done.

to the length of the repeated regions (3, 26, 29, 42, 46, 48, 53, 57, 64). Moreover, the 317-bp length of the *a* sequence from strain KOS is quite close in size to the minimal efficient processing segment size of 250 to 280 bp seen in mammalian cells (29, 42). Since recombination frequencies between segments smaller than the minimal efficient processing segment show an exponential decrease, any significant shortening of the *a*-sequence repeats might be expected to greatly affect that portion of the signal generated by homologous recombination.

Our replacement analysis indicates that both the DR1- U_b and DR2 regions of the *a* sequence are dispensable for high-level recombination. In fact, pRDR2⁻², which lacks the array of DR2 repeats, gives the highest recombination frequency. It is possible that these repeats, which are known to adopt novel DNA structures (62, 63), actually repress *a*-sequence-mediated recombination. Further experiments will be needed to test this hypothesis.

Our most significant finding is that the U_c -DR1 region is the only segment of the *a* sequence that is indispensable for high-level recombination. Repeats lacking this region consistently recombine at half the frequency seen with the full *a* sequence. The fact that recombination is reduced to the level seen with any other set of homologous repeats indicates that removal of the U_c -DR1 region eliminates the special recombinogenicity associated with the *a* sequence. The construct pRDU_c⁻² does contain a short section of Bluescript cloning vector next to the *dsg* region, but the lack of sequence effects on the level of homologous recombination makes it unlikely that it is of any significance.

The finding that the U_c -DR1 region is indispensable is at variance with one deletion analysis. Chou and Roizman (9), studying the *a* sequence from HSV-1 strain F, reported that deletion of the U_b or U_c domain did not affect inversion, while DR4 repeats were important and DR2 repeats were essential for recombination through *a* sequences. Several factors complicated their study. First, use of the 153-kb HSV-1 genome makes quantitation very complex. More importantly, the seven constructs examined varied considerably in size, from less than 100 bp to well over 500 bp. Thus, it is difficult to differentiate between changes in background levels of homologous recombination and decreases due to removal of important portions of the *a* sequence. It is interesting that insertion of a fragment containing only the U_c -DR1 segment gave little inversion in the Chou and Roizman study. It is possible that the difference in our finding results from the use of *a* sequences from different strains of HSV-1. Specifically, the strain KOS *a* sequence used in our study does not contain the DR4 repeats, but a portion of one DR4 repeat is present in our U_c region. Further experiments will be needed to determine whether it is this segment of the U_c that contains the *cis* signals for high-level recombination.

Smiley and coworkers also performed a deletion analysis of the *a* sequence and concluded that the sequences at the ends were most important for inversion (49). Though again complicated by the use of the whole viral genome and decreasing size of homology, their results are consistent with our findings. Deletions from the U_c end of the *a* sequence decreased recombination much faster than deletions from the U_b terminus, and a fragment of less than 100 bp that contained the U_c -DR1 (their U_b5 construct) was capable of driving inversions. Both of these findings are consistent with our conclusion that the U_c -DR1 region contains the *cis* site for enhanced *a* sequence recombination.

The U_c region of the *a* sequence contains *pac2*, one of the two *cis* sequences essential for cleavage and packaging of HSV-1. However, it is unlikely that cleavage and packaging

play a role in recombination. Cleavage and packaging should also require the *pac1* sequence found in the U_b region (16, 56). Deiss and coworkers (16) found that plasmids with only U_c could be packaged, but examination of the final products revealed the presence of U_b regions that had been added by recombination with the helper virus. In our study, miniprep and restriction digest analysis of white colonies from pRDU_b⁻² indicate that no additions occurred, demonstrating that enhanced recombination can be seen in the absence of the *pac1* signal found in U_b . In addition, there is considerable evidence that cleavage and packaging are coupled processes. Disruption of packaging, caused by deletions in the packaging signals (17) or mutations in any of nine different HSV-1 genes (1, 2, 4, 19, 36, 40, 47), also prevents processing of high-molecular-weight viral DNA into unit-length monomers. Thus, it is likely that once the processing of DNA for packaging has begun, the DNA becomes unavailable for recombination. Finally, we have previously demonstrated that recombination parallels replication through the course of HSV-1 infection, with a significant signal seen by 6 h postinfection (20). Thus, both processes should be well under way before packaging has begun.

Several proteins that bind in the *a* sequence have been identified. Dalziel and Marsden (13) noted the binding of 21- and 22-kDa proteins to the *a* sequence. Kemble and Mocarski purified a host cell protein that binds to the highly conserved *pac-2* element in the cytomegalovirus *a* sequence (28). Finally, Chou and Roizman demonstrated sequence-specific binding to the U_c -DR1 region of the *a* sequence by two larger proteins, which they speculate to be ICP1 and ICP7 (11). The relationship between these proteins and *a*-sequence recombination remains to be determined.

The important question of whether the *a* sequence is a hot spot for homologous recombination or a *cis* signal for a site-specific enzyme remains unresolved. However, we have now narrowed the region of importance to the 95-bp U_c -DR1 region. It should now be straightforward to examine further the DNA requirements of the system.

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