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Endonuclease G, a Candidate Human Enzyme for the Initiation of Genomic Inversion in Herpes Simplex Type 1 Virus*

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The herpes simplex virus type 1 (HSV-1) *a* sequence is present as a direct repeat at the two termini of the 152-kilobase viral genome and as an inverted repeat at the junction of the two unique components L and S. During replication, the HSV-1 genome undergoes inversion of L and S, producing an equimolar mixture of the four possible isomers. Isomerization is believed to result from recombination triggered by breakage at the *a* sequence, a recombinational hot spot. We have identified an enzyme in HeLa cell extracts that preferentially cleaves the *a* sequence and have purified it to near homogeneity. Microsequencing showed it to be human endonuclease G, an enzyme with a strong preference for G+C-rich sequences. Endonuclease G appears to be the only cellular enzyme that can specifically cleave the *a* sequence. Endonuclease G also showed the predicted recombination properties in an *in vitro* recombination assay. Based on these findings, we propose that endonuclease G initiates the *a* sequence-mediated inversion of the L and S components during HSV-1 DNA replication.

The genome of herpes simplex virus type 1 (HSV-1)[1] consists of a linear 152-kb double stranded DNA molecule composed of two unique segments, U_L (unique long) and U_S (unique short), with each segment flanked by inverted repeated sequences. U_L is flanked by the *ab* and *b′a′* sequences, whereas U_S is flanked by *a′c′* and *ca* sequences, with the *a′* sequence shared by U_L and U_S (*a′*, *b′* and *c′* are inverted repeats of *a*, *b*, and *c*). The organization of the HSV-1 genome can therefore be delineated as *ab*-U_L-*b′a′c′*-U_S-*ca* (1). HSV-1 is known to freely invert the U_L and U_S segments relative to each other to generate four isomers (2–4), an event that is closely associated with viral DNA replication (5, 6). The four isomers exist in an equimolar ratio, indicative of 100% recombination frequency. Thus, underlying inversion is a very efficient recombination reaction. It has been proposed that the inversion event results from double strand break repair initiated by multiple double strand breaks in the inverted repeats of the HSV-1 genome (4). Although the manner in which double strand breaks are generated is unknown, the *a* sequence appears to be involved. The *a* sequence has been shown to be sufficient for the U_L-U_S inversion (7); however, it is not clear whether or not it is dispensable (8).

The *a* sequence is ~300 bp long, containing 83% G+C, and is itself composed of multiple repeated sequences (see Fig. 1) consisting of 20-bp direct repeats (DR1) at each end followed by two unique regions (U_b and U_c) separated by multiple copies of the 12-bp DR2 repeats (7, 9–11). In addition to its role in recombination, the *a* sequence also contains sites for cleavage and packaging of the concatameric product of rolling circle DNA replication (7, 12).

Recombination mediated by the *a* sequence could be reproduced in a plasmid-based recombination system, which demonstrated that the *a* sequence is a recombinational hot spot (13, 14). More importantly, studies with this system showed that HSV-1 infection resulted in high levels of *a* sequence-mediated recombination, analogous to HSV-1 genome inversion, that specifically required plasmid DNA replication initiated at an HSV-1 origin of replication (13, 14). However, repeated *a* sequences on the plasmid could also mediate recombination in the absence of HSV-1 infection. Although the levels of recombination were low, this finding revealed the intrinsic recombinogenic potential of the *a* sequence and the presence of a cellular recombinational mechanism that can drive *a* sequence-mediated recombination.

Enzymatic activities capable of cleaving the *a* sequence and, therefore, initiating *a* sequence-mediated recombination have been described in earlier studies. These include the HSV-1 alkaline nuclease (the product of the UL12 gene), the only known virally encoded enzyme capable of cleaving the *a* sequence. However, the alkaline nuclease was shown to be nonessential for HSV-1 genome inversion (15), possibly due to its late function during viral replication (16). Another enzymatic activity capable of specifically cleaving the *a* sequence was observed in mammalian cell extracts (17). Although this activity was not directly implicated in *a* sequence-mediated recombination (18), it was found to increase 35-fold after HSV-1 infection of susceptible cells. Finally, a partially purified cellular recombinase activity that mediated *a* sequence-dependent recombination *in vitro* was found to catalyze cleavage of the *a* sequence (19, 20).

In this report we describe the purification of a cellular enzyme, identified as endonuclease G, that preferentially cleaves the HSV-1 *a* sequence. Endonuclease G appears to be the only cellular enzyme capable of specifically cleaving the *a* sequence and is, therefore, responsible for the *a* sequence-cleavage activity observed with the less purified cellular fractions reported in the earlier studies. On the basis of these observations, we propose that endonuclease G serves to initiate the recombinational event that underlies HSV-1 genome inversion.

EXPERIMENTAL PROCEDURES

Construction of pKJH20—Plasmid pKJH20 was constructed in two steps. An approximately 340 bp *a* sequence-containing fragment was

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[1] The abbreviations used are: HSV, herpes simplex virus; DR, direct repeat; DTT, dithiothreitol; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside.

removed from pRD105 (13) by *Bam*HI digestion and inserted into the *Bam*HI site of pBluescript SK (+) (Stratagene). The 1.24-kb kanamycin cassette from pUC4K (Amersham Biosciences) was then inserted into the *Pst*I site of the resulting plasmid to generate pKJH20.

a Sequence-cleavage Assay—The *a* sequence-cleavage assay was modified from a method previously described (19). The reaction mixture (31 μl) contained 0.5 μl of pKJH20 linearized by *Eco*RI (400 ng/μl), 29.5 μl of ACE buffer, and the indicated amounts of enzyme diluted in the ACE buffer. ACE buffer was composed of 0.25 μl of bovine serum albumin (20 mg/ml, Roche Molecular Biochemicals), 0.25 μl of spermidine (300 mM) and 29 μl of buffer R (20 mM Hepes, pH 7.6, 45 mM NaCl, 0.2 mM EDTA, 1.2 mM $MgCl_2$, 5% glycerol, 1 mM dithiothreitol (DTT)). When examining enzymatic activities that function at low salt conditions, NaCl was removed from buffer R. After incubation for 2 h at 37 °C, stop buffer (5 μl) composed of 0.5% SDS and 20 mM EDTA was added. The reaction mixture was then extracted with a phenol-chloroform mixture (1:1) and analyzed by agarose gel electrophoresis (1.2–1.5% agarose). One unit of *a* sequence-cleavage activity is defined as 1 ng of the L-type digestion product (the 3–3.3-kb fragments) generated at 37 °C in 2 h. The amount of the L-type product was estimated by densitometry (Alpha Imager™2000, Alpha Innotech Corp.) after ethidium bromide staining.

Preparation of HeLa Cell Nuclear Extract—Nuclear extracts were prepared from frozen HeLa cell nuclei that had been extracted with 150 mM NaCl in 50 mM Hepes buffer, pH 7.5. The frozen nuclei were kindly provided by Dr. Paul Modrich (Duke University). One volume of frozen nuclei was thawed in 4 volumes of hypotonic buffer (20 mM Hepes, pH 7.6, 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, 10 mM sodium metabisulfite, pH 7.1, 0.5 μg/ml leupeptin, 0.5 μg/ml pepstatin A). After gently shaking at 4 °C for 2 h, the supernatant, which contained the *a* sequence-cleavage activity, was collected by centrifugation at 27,000 × *g* for 30 min. The *a* sequence-cleavage activity could be repeatedly extracted from the nuclei if the nuclei were frozen and thawed between each extraction. To remove nucleic acids and other negatively charged macromolecules from the supernatant, NaCl was added to 500 mM, and 200 μl of 50% Polymin P (for 47.5 mg of protein) was added gradually. After keeping the suspension on ice for 2 h, the clear supernatant was collected by centrifugation at 27,000 × *g* for 30 min.

Purification of a Sequence-cleavage Enzyme—To purify the *a* sequence-cleavage enzyme, 3.6 g of nuclear extract (from nuclei of ~150 liters of HeLa cells) were processed in 7 batches. Purification of enzyme through the Mono S fraction from one batch (272 mg of nuclear extract) is summarized in Table I. Saturated ammonium sulfate was gradually added to 40% of saturation. The precipitate was collected by centrifugation at 27,000 × *g* for 30 min and dissolved in 10 ml of KP50K50 buffer (50 mM potassium phosphate, pH 7.0, 10% glycerol, 50 mM KCl, 2 mM DTT, 0.2 mM EDTA, 0.5 μg/ml leupeptin, 0.5 μg/ml pepstatin A, 10 mM sodium metabisulfite, pH 7.1, and 0.5 mM phenylmethylsulfonyl fluoride). Insoluble material was removed by centrifugation at 100,000 × *g* for 1 h. After filtering the supernatant through a 0.8-μm membrane filter (Corning Glass), the filtrate (12 ml) was applied to a 53-ml KP50K50 buffer-equilibrated desalting column (HiPrep 26/10, Amersham Biosciences) to remove residual ammonium sulfate. The de-salted solution was then applied to a 5-ml KP50K50 buffer-equilibrated SP-Sepharose column (HiTrap SP, Amersham Biosciences). The flow-through was collected and applied to a 5-ml KP50K50 buffer-equilibrated Heparin-Sepharose column (HiTrap Heraprin, Amersham Biosciences). A 60-ml linear gradient of TG50 to TG1000 buffer was then applied to the column. TG50 buffer contained 20 mM Tris-HCl, pH 7.5, at 25 °C, 10% glycerol, 50 mM NaCl, 2 mM DTT, 0.2 mM EDTA, 0.5 μg/ml leupeptin, 0.5 μg/ml pepstatin A, and 0.5 mM phenylmethylsulfonyl fluoride. TG1000 buffer was the same as TG50, except that the NaCl concentration was 1 M. Fractions eluting at 425–475 mM NaCl, which contained the majority of *a* sequence-cleavage activity, were pooled. The pooled fractions were applied to a 53-ml TG50 buffer-equilibrated desalting column (HiPrep 26/10) for buffer exchange, and the eluate was applied to a 1-ml Q-Sepharose column (HiTrap Q, Amersham Biosciences). The flow-through was collected and applied to a 53-ml desalting column (HiPrep 26/10, Amersham Biosciences) equilibrated in KP10 buffer for buffer exchange and then applied to a 1-ml KP10 buffer-equilibrated Mono S column (HR 5/5, Amersham Biosciences). KP10 buffer contained 10 mM potassium phosphate, pH 7.0, 10% glycerol, 2 mM DTT, 0.2 mM EDTA, 0.5 μg/ml leupeptin, 0.5 μg/ml pepstatin A, and 0.5 mM phenylmethylsulfonyl fluoride. A 20-ml linear gradient of KP10 to KP10K1000 buffer (KP10 buffer containing 1 M of KCl) was then applied to the column. The peak of *a* sequence-cleavage activity was present in fractions eluting at 97–134 mM KCl, with weaker activity trailing to the fraction corresponding to 313 mM KCl. The fractions with peak activity (Mono S fraction) were pooled and stored at −80 °C. When all seven batches of the 3.6 g of nuclear extract were processed through the Mono S fraction, they were pooled and applied to a 1-ml hydroxyapatite column (CHTII, Bio-Rad). Fifteen milliliters of a linear gradient, from KP10 buffer to KP300 buffer (KP10 containing 300 mM phosphate, pH 7.0) was applied to the column. The peak of *a* sequence-cleavage activity appeared in fractions eluting from 39 to 85 mM phosphate, with lower activity trailing to the fraction containing 174 mM phosphate.

Identification of Proteins with a Sequence-cleavage Activity—To analyze the protein profile of the hydroxyapatite fraction, 450 μl of hydroxyapatite fraction (~3 μg) were precipitated for 10 min at room temperature with 10% trichloroacetic acid plus 0.015% deoxycholate. After centrifugation, the pellets were neutralized in 1 M Tris-HCl, pH 8.0, buffer and subjected to SDS-polyacrylamide gel electrophoresis (4% stacking gel, 10% separating gel, Bio-Rad Mini-protein II system) followed by Coomassie Blue staining. To determine the identity of the protein components of the hydroxyapatite fraction, protein precipitation was performed as described above, and the precipitate was subjected to SDS-polyacrylamide gel electrophoresis at 200 V for 6 min. After the gel was stained with Coomassie Blue, the portion of the gel that contained all of the detectable protein was cut out and analyzed. Microsequencing of the proteins was performed by trypsin digestion followed by Mass spectrometry, performed by Harvard Microchemistry Facility, Cambridge, MA.

Preparation of Whole Cell Extract from HeLa Cells—To prepare the whole cell extract, 1 liter of frozen HeLa cells (about 2.5 ml) that had been washed twice with phosphate-buffered saline (20 mM sodium phosphate, pH 7.4, 150 mM NaCl) was thawed in 7.5 ml of hypotonic buffer. After 30 min at 0 °C, the cells were broken with a Dounce homogenizer (20 times with a tight pestle). Ten milliliters of extraction buffer (20 mM Hepes, pH 7.6, 2 M NaCl) were gradually added. After extraction for 20 min at 4 °C, the supernatant was collected by centrifugation at 27,000 × *g* for 30 min and then centrifuged again at 100,000 × *g* for 1 h. The clear supernatant was dialyzed against hypotonic buffer. After removing the precipitate, which formed during dialysis, by centrifugation at 27,000 × *g* for 30 min, the clear supernatant was used.

Preparation of Anti-endonuclease G—Rabbit anti-endonuclease G was produced by immunization with a 12-amino acid peptide (AELPPVPGGPRG) located at amino acid 49 to amino acid 60 of human endonuclease G (12-mer peptide). The choice of the peptide was based on the design described by Cote and Ruiz-Carrillo (21). Peptide synthesis and antibody preparation were performed by ResGen, Huntsville, AL. Anti-endonuclease G and preimmune serum were purified with a protein A-agarose affinity column following the method described by Sambrook *et al.* (22).

Immunoaffinity Purification of Endonuclease G—An anti-endonuclease G affinity column was prepared according to Harlow and Lane (23). Approximately 300 mg of filtered (0.45-μm filter, Corning) ammonium sulfate fraction of nuclear extract (see above) was applied to three tandemly connected columns in the following order:1 ml of protein A column (HiTrap™ protein A HP, Amersham Biosciences), 1 ml of pre-immune serum column (prepared by immobilizing preimmune serum in a 1-ml protein A column), and 0.2 ml of anti-endonuclease G affinity column. The anti-endonuclease G affinity column was then detached and washed with 6 ml of high salt buffer (20 mM Hepes, pH 7.6, 0.5 M NaCl) and 1 ml of buffer R. The bound proteins were eluted from the column with 1 ml of 12-mer peptide (3 mM in buffer R). The 12-mer peptide did not influence the *a* sequence-cleavage activity of endonuclease G. To remove the 12-mer peptide from the immunopurified endonuclease G, 200 ng of endonuclease G fraction was applied to a TG50 buffer-equilibrated heparin-Sepharose column (bed volume = 30 μl). After washing the column with 150 μl of TG100 (TG buffer containing 100 mM NaCl), TG600 (TG buffer containing 600 mM NaCl) was used to elute *a* sequence-cleavage activity. This method produced 12-mer free endonuclease G with a yield of ~3%.

Immuno-depletion of Endonuclease G from Whole Cell Extract—Whole-cell extract (27 μg) was mixed with 2.6 μg of purified anti-endonuclease G (or with 2.6 μg of purified anti-endonuclease G plus 11.7 μM 12-mer peptide or 2.6 μg of purified pre-immune serum) in a total volume of 49 μl, which also contained 0.5 μl of bovine serum albumin (20 mg/ml, Roche Molecular Biochemicals), 0.5 μl of spermidine (300 mM), and 42 μl of buffer R. After incubation on ice for 1 h, 10 μl of protein A-agarose (80% slurry stored in 5% nonfat dry milk) was added. To keep the protein A-agarose suspended, the final mixture was shaken vigorously for 5 h at 4 °C. Forty-two microliters of clear supernatant were collected after centrifugation (7200 × *g*) for 1 min (S

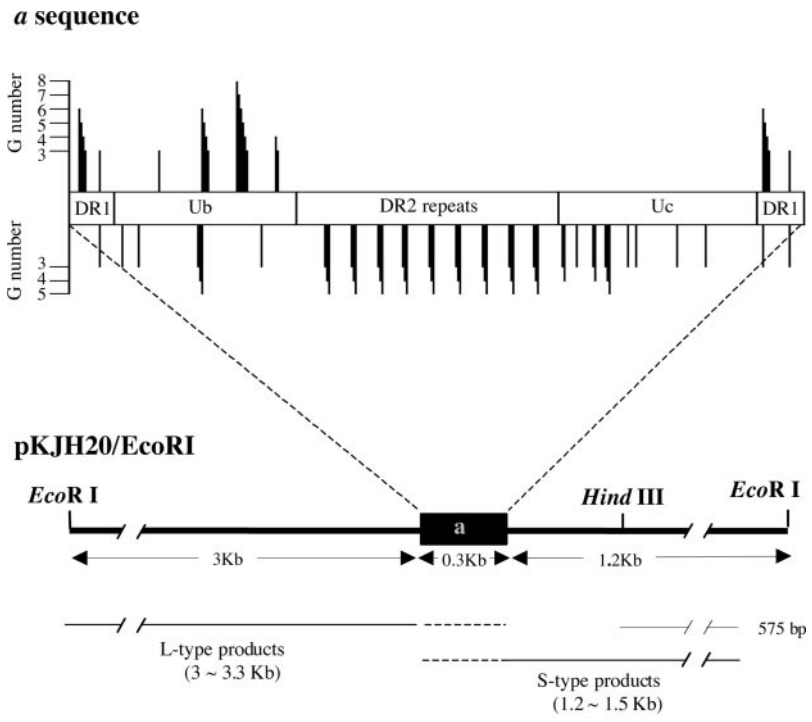

FIG. 1. **HSV-1 *a* sequence and *a* sequence-cleavage assay.** In the *upper panel*, the five regions of the HSV-1 *a* sequence, DR1, Ub, DR2 repeats, Uc, and DR1, are diagrammed on the *x* axis. Also depicted is the distribution of dG within these regions. The number of G residues (*G number*) that can form a continuous G-string at a particular site is shown on the *y* axis. Minor sites of endonuclease G cleavage are thought to be strings of G with 3–7 G residues. Sites cleaved with great efficiency are thought to have strings of 8 or more G residues. In the *bottom panel*, plasmid DNA pKJH20 was linearized with the *Eco*RI restriction enzyme to generate the substrate. Also shown is the *Hin*dIII site located 575 bp from the *Eco*RI site at the 3′ end of the substrate. Enzymes cleaving within the *a* sequence are expected to generate both the L type (3–3.3 kb) and the S type (1.2–1.5 kb) products.

fraction). After the remaining precipitate was washed 4 times with 60 μl of buffer R, the precipitate was resuspended in 60 μl of ACE buffer. While keeping the precipitate suspended in ACE buffer, 42 μl was collected (P fraction). In experiments testing the contribution of endonuclease R to *a* sequence-cleavage activity, immunoprecipitation experiments were performed as described above except that no NaCl was included in the ACE or and R buffers. To assay for *a* sequence-cleavage activity in the S and in P fractions, 20 μl of a substrate solution consisting of 1 μl of pKJH20/RI (400 ng/μl), 0.17 μl of bovine serum albumin (20 mg/ml), 0.17 μl of spermidine (300 mM in H_2O), 1 μl of the 12-mer peptide (700 μM in buffer R), and 18 μl of buffer R were added. The reaction proceeded at 37 °C for 570 min. The 12-mer peptide was included to avoid stimulation of *a* sequence-cleavage activity by residual anti-endonuclease G in the S fraction and to dissociate endonuclease G from the precipitate in the P fraction.

In Vitro Recombination Assay—The *in vitro* recombination assay was performed according to the procedure described by Bruckner *et al.* (20) with slight modification. The substrate used was plasmid pRD105 (see Fig. 9*A*), which has two direct repeats of the *a* sequence flanking a *lacZ* gene. Recombination between the *a* sequences is expected to result in deletion of *lacZ* and one *a* sequence. The resulting plasmid still contains the β-lactamase gene (for ampicillin resistance) and the plasmid replication origin. When transformed into a *lac*$^-$ *recA*$^-$ *Escherichia coli* strain (DH5α), this plasmid produces a white colony on a LB plate containing X-gal, isopropyl-β-D-thiogalactopyranoside, and ampicillin. Endonuclease G (0.3 ng) purified by anti-endonuclease G affinity chromatography was incubated with 2 μg of pRD105 in a volume of 60 μl containing 0.5 μl of bovine serum albumin (20 mg/ml), 0.5 μl of spermidine (300 mM), and 56 μl of buffer R. After incubation at 37 °C for 60 min, the reaction was terminated by extraction with 120 μl of a phenol/chloroform mixture (1:1). The DNA was precipitated and washed with 80% ethanol and finally dissolved in 50 μl of TE buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, pH 8.0). Two microliters of the dissolved DNA were incubated with 50 μl of competent *E. coli* cells (Library Efficiency DH5α, Invitrogen). The transformants (white colonies) were screened on LB plates containing carbenicillin (ampicillin analog), isopropyl-β-D-thiogalactopyranoside, and X-gal.

RESULTS

To detect cellular enzymes capable of cleaving the HSV-1 *a* sequence, we designed an assay in which a linearized plasmid DNA, pKJH20/*Eco*RI, containing a single *a* sequence serves as substrate (Fig. 1). The 3′ end of the *a* sequence was located ~1.2 kb from the *Eco*RI site of the linearized pKJH20. Thus, any enzyme that catalyzes cleavage of the DNA within the *a* sequence would be expected to generate both S-type (1.2–1.5 kb) and L-type (3–3.3 kb) products, as diagrammed in Fig. 1.

Detection of a Cellular Enzymatic Activity That Cleaves HSV-1 a Sequence—We examined the *a* sequence-cleavage activity in an extract prepared from frozen HeLa cell nuclei that had previously been extracted with a buffer containing 150 mM NaCl (see "Experimental Procedures"). As shown in Fig. 2, the nuclear extract generated a heterogeneous group of digestion products (*lane 4*). However, it also generated both S-type and L-type fragments, indicative of preferential cleavage of the HSV-1 *a* sequence. The more slowly migrating high molecular weight products resulted from DNA ligase activity present in the extract (data not shown). This cleavage pattern was confirmed by mapping the cleavage sites to the *a* sequence by digesting the products with the *Hin*dIII restriction enzyme (*lane 5*). Both L-type and S-type fragments were present in rather diffuse bands, indicating multiple, rather than single cleavage sites within the *a* sequence. These products closely resemble those observed with the mammalian nuclease activity previously reported by Wohlrab *et al.* (17).

Purification of a Sequence-cleavage Enzyme from HeLa Cell Nuclei—Purification of the *a* sequence-cleavage enzyme from a HeLa cell nuclear extract is summarized in Table I. The purification procedure included six steps and resulted in a 60,000-fold purification (see "Experimental Procedures"). As shown in Fig. 3*A*, the hydroxyapatite fractions generated both L-type and S-type DNA fragments, with fraction 13 containing the peak of activity. The purified enzyme in this fraction also generated a sharp, rapidly migrating product that presumably resulted from cleavage at a site that mapped to a high G+C-containing region in the backbone plasmid. Several heterogeneous digestion products were also generated, giving the gel a slightly smeared appearance. Thus, the purified *a* sequence-cleavage enzyme does not appear to be absolutely specific for the *a* sequence.

Identification of a Sequence-cleavage Enzyme as Endonuclease G—When the hydroxyapatite fraction of the *a* sequence-cleavage enzyme was examined by SDS-polyacrylamide gel electrophoresis followed by Coomassie Blue staining, three major bands appeared (Fig. 3*B*) with molecular masses of ~60, 35, and 31 kDa. Comparison of the profile of *a* sequence-cleavage

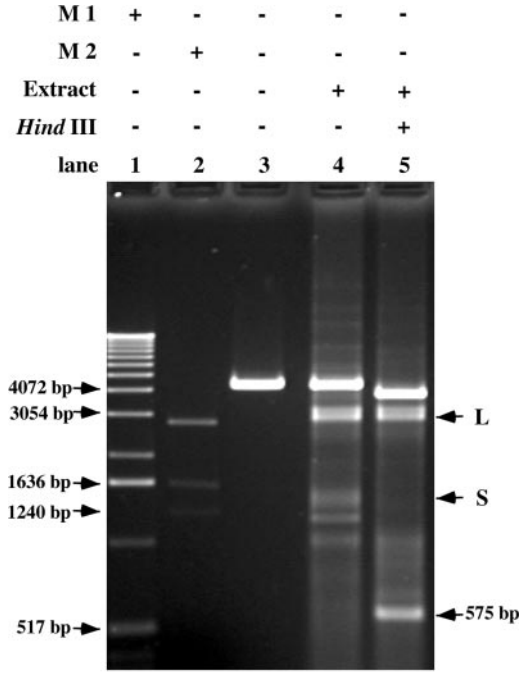

FIG. 2. ***a* sequence cleavage activity is present in HeLa cell nuclear extracts.** Twelve micrograms of HeLa cell nuclear extract were incubated for 1 h with 200 μg of substrate (pKJH20/*Eco*RI) in the *a* sequence-cleavage assay described under "Experimental Procedures." After extraction with phenol/chloroform, the reaction products were analyzed by agarose gel (1.5%) electrophoresis and stained by ethidium bromide. The L- and S-type products are indicated by *arrows*. Digestion of the purified reaction products by *Hin*dIII resulted in the generation of a 575-bp DNA fragment and the disappearance of the S type product (*lane 5*). Also shown in *lane 1* and *lane 2* are molecular weight markers M1 and M2.

TABLE I
Purification of a sequence-cleavage enzyme

Step	Total protein	Total activity	Yield	Enrichment
	mg	*units*	*%*	
Hypotonic extract	272	257,000	100	1
Ammonium sulfate	63	124,000	48	2.1
SP-Sepharose	39	128,000	50	3.5
Heparin-Sepharose	3.2	43,400	17	14
Q-Sepharose	0.43	40,750	16	101
Mono S	0.032	19,000	7	631
Hydroxyapatite				6,300[a]

[a] Estimate 10× increase from previous step.

activity across the hydroxyapatite peak (Fig. 3*A*) with the profile of protein bands on the SDS-polyacrylamide gel revealed that only the 31- and the 60-kDa proteins coincided with *a* sequence-cleavage activity. To identify these proteins, the hydroxyapatite fraction was microsequenced by trypsin digestion followed by mass spectrometry. Among the peptides identified, seven matched various portions of human endonuclease G (Fig. 4). The precursor form of human endonuclease G has a calculated molecular mass of 32.5 kDa. The predicted mature form of endonuclease G has a calculated molecular mass of 27.6 kDa. However, it would be expected to co-migrate with a 31-kDa protein in our electrophoresis system, which was influenced by the presence of 1 M Tris buffer in the sample preparation (see "Experimental Procedures"). Endonuclease G homologues of higher eukaryotes are known to generate single strand breaks in dG·dC homopolymer pairs (24). Comparison of the substrates of bovine endonuclease G with the HSV-1 *a* sequence suggested that bovine endonuclease G can cleave at many sites within the *a* sequence, leading to the generation of double strand breaks (Fig. 1). One might therefore expect that the human homologue of bovine endonuclease G (89.3% identity) possesses a similar activity and is responsible for cleavage of the *a* sequence.

We therefore asked whether the *a* sequence-cleavage activity of the hydroxyapatite fraction can be modulated by an antibody directed against human endonuclease G. The antibody (anti-endonuclease G) was raised against a peptide (12-mer peptide) consisting of amino acids 49 to 60 of human endonuclease G (Fig. 4). As shown in Fig. 5, *lane 3*, in the presence of anti-endonuclease G, the *a* sequence-cleavage activity of the hydroxyapatite fraction was significantly increased (~5-fold). Pre-immune serum had no effect (*lane 4*) nor did the unrelated antibody, anti-hemagglutinin (data not shown). The specific increase of *a* sequence-cleavage activity observed with the purified enzyme could also be observed with the relatively crude ammonium sulfate fraction (*lane 7*). Because the *a* sequence-cleavage activity can be precipitated by anti-endonuclease G (see Fig. 8), the increase of *a* sequence-cleavage activity must be due to stimulation rather than removal of an inhibitor. Anti-endonuclease G itself did not cleave DNA (*lane 5*), and endonuclease G is the only identifiable protein in the hydroxyapatite fraction that contains the 12-mer peptide, the antigen interacting with anti-endonuclease G. Thus, the stimulation of *a* sequence-cleavage activity must be the result of interaction between the endonuclease G present in the hydroxyapatite fraction and anti-endonuclease G. Endonuclease G must therefore be an essential component of the *a* sequence-cleavage activity.

Endonuclease G Is Both Necessary and Sufficient for a Sequence Cleavage—To examine further the association of *a* sequence-cleavage activity with endonuclease G, we purified endonuclease G from a HeLa cell nuclear extract to homogeneity to determine whether it is sufficient to cleave the HSV-1 *a* sequence. To purify endonuclease G, we used anti-endonuclease G to construct an antibody affinity column. A relatively crude fraction of *a* sequence-cleavage activity (ammonium sulfate fraction) was applied to the column, and the bound protein was eluted with the 12-mer peptide. As shown in Fig. 6*A*, the eluate contained only 1 major protein, the 27.5-kDa endonuclease G, as judged by SDS-polyacrylamide electrophoresis followed by silver staining. A trace amount of another protein with a mass of ~75 kDa was detectable. However, this protein is unrelated to the *a* sequence-cleavage activity because it was not present in the hydroxyapatite fraction described above.

When the eluate was tested for *a* sequence-cleavage activity, the substrate DNA (pKJH20/*Eco*RI) was readily cleaved to generate both the S-type and the L-type fragments, indicative of specific *a* sequence cleavage (Fig. 6*B*). Thus, endonuclease G is sufficient for the specific cleavage of the HSV-1 *a* sequence. The eluate also generated a heterogeneous mixture of products when high levels of enzyme (66 pg) were used. As shown in Fig. 6*C*, the *a* sequence-cleavage activity was stimulated ~8-fold by antibody generated against human endonuclease G. These properties of the purified endonuclease G are the same as those observed with the hydroxyapatite fraction described earlier and confirm our conclusion that endonuclease G alone is sufficient for *a* sequence cleavage.

Endonuclease G Generates Multiple Single Strand Breaks within the HSV-1 a Sequence to Generate a Double Strand Break—As indicated above, human endonuclease G is likely to produce double strand breaks within the *a* sequence by introducing multiple single strand cleavages, a reaction analogous to that observed for the bovine and chicken endonuclease G with their preferred substrate, the dG·dC homopolymer pair (24, 25). We therefore measured endonuclease G cleavage of the *a* sequence in a supercoiled plasmid containing the *a* sequence,

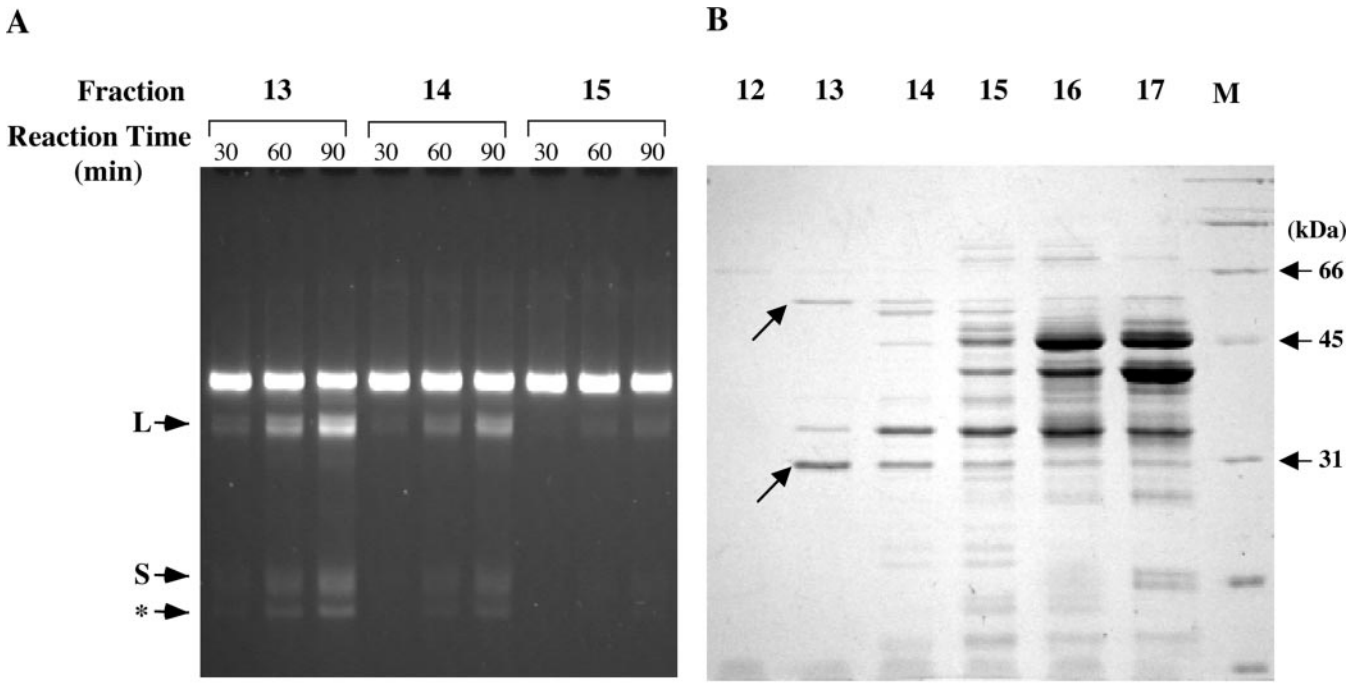

FIG. 3. ***a* sequence-cleavage activity and protein profiles of the hydroxyapatite fraction of *a* sequence-cleavage enzyme.** *A*, fractions were analyzed for *a* sequence-cleavage activity. Fraction 13 contains most of the activity, whereas fraction 12 shows no activity even after prolonged (19 h) incubation (data not shown). The L and S products are indicated by the *arrows*. The DNA band corresponding to cleavage at a site mapped to the plasmid backbone is indicated by an *asterisk*. *B*, proteins from the same fractions as in *panel A* were precipitated by trichloroacetic acid, dissolved in 1 M Tris buffer, pH 8.0, and analyzed by SDS-polyacrylamide gel electrophoresis followed by Coomassie Blue staining. *Arrows* point to the polypeptides that co-elute with *a* sequence-cleavage activity. Due to the presence of 1 M Tris buffer in the sample, which retarded protein migration in the gel, the actual molecular weight of each band is smaller than what it appears to be on the gel.

MRALRAGLTLALGAGLGAVVEGWRRRREDARAAPGLLGRLP

VLPVAAAAELPPVPGGPRGPGELA**KYGLPGLAQLK**SRESYV

LCYDPRTRGALWVVEQLRPERLRGDGDRRECDFREDDSVHA

YHRATNADYRGSGFDRGHXAAAANHRWSQ**KAMDDTFYLSNV**

APQVPHLNQNAWNNLEKYSRSLTRSYQNVYVCTGPLFLPRT

EADGKSYV**KYQVIGKNHVAVPTHFFKVLILEAAGGQIELRT**

YVMPNAPVDEAIPLERFLVPIESIE**RASGLLFVPNILAR**AG

SLKAITAGSK

FIG. 4. **Amino acid sequence of tryptic peptides from hydroxyapatite fraction.** The peptide fragments identified in microsequencing are in *bold*, and the 12-residue peptide used to raise anti-endonuclease G is *underlined*.

pKJH20. As shown in Fig. 7*A*, the relaxed form of the plasmid, resulting from a single strand break, was generated at low levels of endonuclease G (*lanes 1–5*). When higher levels of endonuclease G were used, the linear form of the plasmid appeared (*lanes 6–10*). To determine whether the double strand breaks were located within the *a* sequence, the reaction products (*lane 10*) were purified and digested with the *Eco*RI restriction enzyme. As shown in *lane 13*, DNA bands corresponding to L-type products and S-type products as well as the products resulting from cleavage within the plasmid backbone appeared, confirming that cleavage had occurred at the *a* sequence.

Quantitation of the bands corresponding to the relaxed and the linear form of the plasmids demonstrated that linear DNA was undetectable until the relaxed form of the plasmid reached 94% of maximum (Fig. 7*B*). At higher levels of endonuclease G, the amount of relaxed plasmid decreased with a corresponding increase in the linear form. Thus, human endonuclease G cleaves the HSV-1 *a* sequence by first introducing single strand breaks in the *a* sequence, leading to a double strand break when the requisite number of single strand breaks in sufficiently close proximity to each other had been introduced.

Endonuclease G Appears to Be the Only a Sequence-cleavage Enzyme in HeLa Cells—Our finding that endonuclease G is capable of cleaving the *a* sequence raises the question of whether there are other enzymes with this activity in HeLa cells. The extract used was prepared from HeLa nuclei that had previously been extracted with 150 mM NaCl (see "Experimental Procedures"). Thus, it is possible that some other enzymes with *a* sequence-cleavage activity might have been extracted by the 150 mM NaCl. We therefore examined the *a* sequence-cleavage activity in a whole cell extract freshly prepared from

HeLa cells. Anti-endonuclease G was used to precipitate endonuclease G in the presence of protein A-agarose, and the resulting supernatant was assayed for *a* sequence-cleavage activity. As shown in Fig. 8, *lane 4*, the antibody precipitated all of the *a* sequence-cleavage activity in the whole cell extract; no detectable activity remained in the supernatant (*lane 3*). Furthermore, when the precipitate was treated with the 12-mer peptide, which specifically dissociates the endonuclease G-anti-endonuclease G complex, all of the activity was found in the supernatant (*lane 5*), with no activity in the precipitate (*lane 6*). This result strongly suggests that endonuclease G is the only cellular enzyme that is capable of specifically cleaving the HSV-1 *a* sequence.

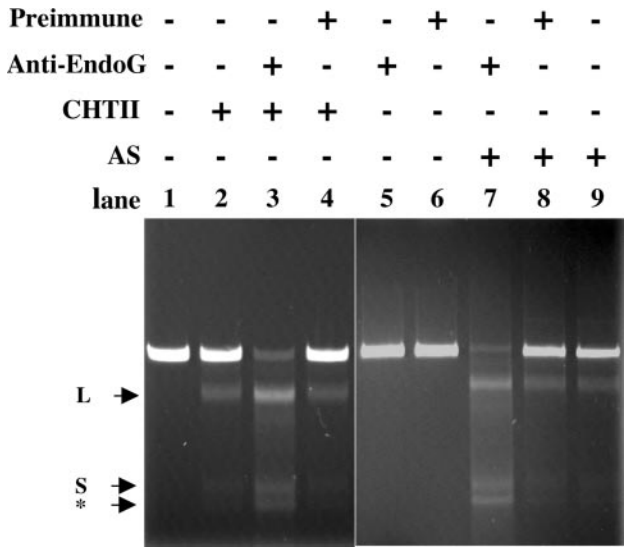

FIG. 5. **Antibody against endonuclease G (*Anti-EndoG*) interacts specifically with *a* sequence-cleavage enzyme.** Approximately 0.75 ng of hydroxyapatite (*CHTII*) fraction was used to measure *a* sequence cleavage activity in the absence (*lane 2*) or in the presence of 3.3 μg of anti-endonuclease G (*lane 3*) or 3.3 μg of preimmune serum (*lane 4*). Incubation was for 1 h, and *a* sequence-cleavage activity was measured as described under "Experimental Procedures." In *lanes 5–9*, the cleavage reactions were allowed to proceed for 11 h with 2.2 μg of ammonium sulfate fraction (*AS*).

Relationship of Endonuclease G to Recombinase Activity—Our earlier studies had identified a recombinase activity in extracts of HSV-1 infected and uninfected cells (20). This activity was shown to mediate recombination between repeated copies of the HSV-1 *a* sequence in the plasmid pRD105 (Fig. 9*A*) in an *in vitro* recombination assay. In this assay, the recombinase activity leads to deletion of a *lac*Z indicator gene situated between two directly repeated copies of the *a* sequence in pRD105. The recombinants were scored by the formation of white colonies when the recombination products were transformed into *lac*Z$^-$ *E. coli*. With this assay, limited purification of the recombinase activity was found to coincide with the purification of an *a* sequence-cleavage activity (19), suggesting that the two activities were related. We therefore wished to determine whether our near homogeneous *a* sequence-cleavage activity, now identified as endonuclease G, possessed an equivalent recombinase activity.

We found that endonuclease G treatment of pRD105 led to white colony formation in the *in vitro* recombination assay. The efficiency of white colony formation (4.8%) was more than 10 times higher than the background (0.35%). When the plasmid

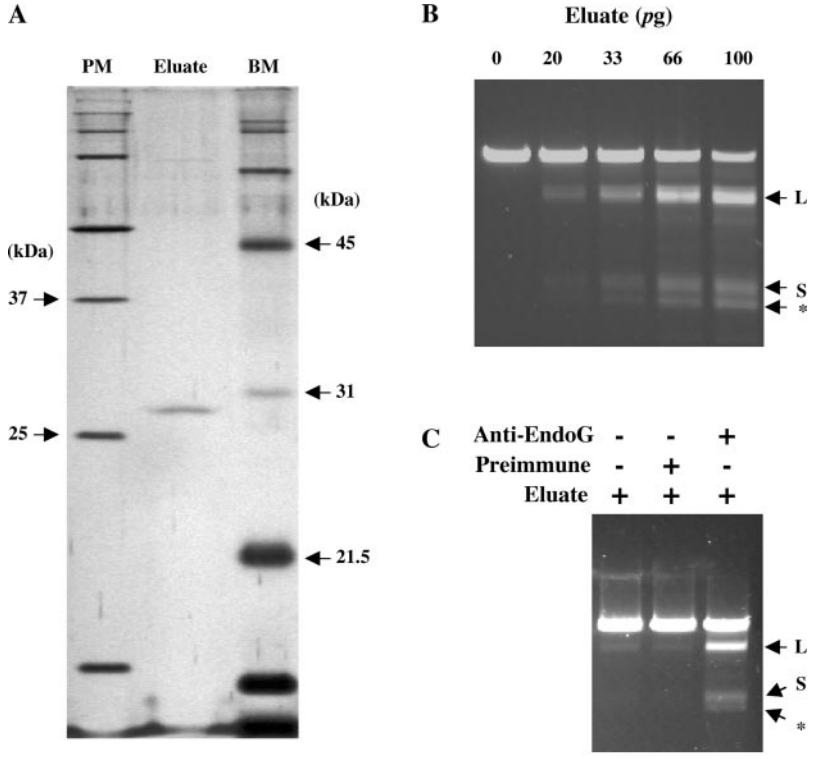

FIG. 6. **Purification of endonuclease G by anti-endonuclease G affinity chromatography.** *A*, 300 mg of ammonium sulfate fraction were loaded into an anti-endonuclease G affinity column prepared as described under "Experimental Procedures." Proteins eluted from the column by the 12-mer peptide (see "Experimental Procedures") were analyzed by SDS-polyacrylamide gel electrophoresis followed by silver staining. The eluate contains only one major protein, the predicted mature endonuclease G (27.6 kDa). *BM* and *PM* represent broad range and precision molecular weight markers (Bio-Rad). *B*, *a* sequence-cleavage activity of eluate from anti-endonuclease G affinity chromatogram. Assays were performed as described under "Experimental Procedures." The L and S products are indicated by the *arrows*. The DNA band corresponding to cleavage at a site mapped to the plasmid backbone is indicated by an *asterisk*. *C*, stimulation of the *a* sequence-cleavage activity of the eluate by anti-endonuclease G. Twenty picograms of 12-mer free eluate (see "Experimental Procedures") were tested for *a* sequence cleavage activity in the absence or presence of 2.6 μg of preimmune serum or 2.6 μg of anti-endonuclease G. The L and S products are indicated by the *arrows*.

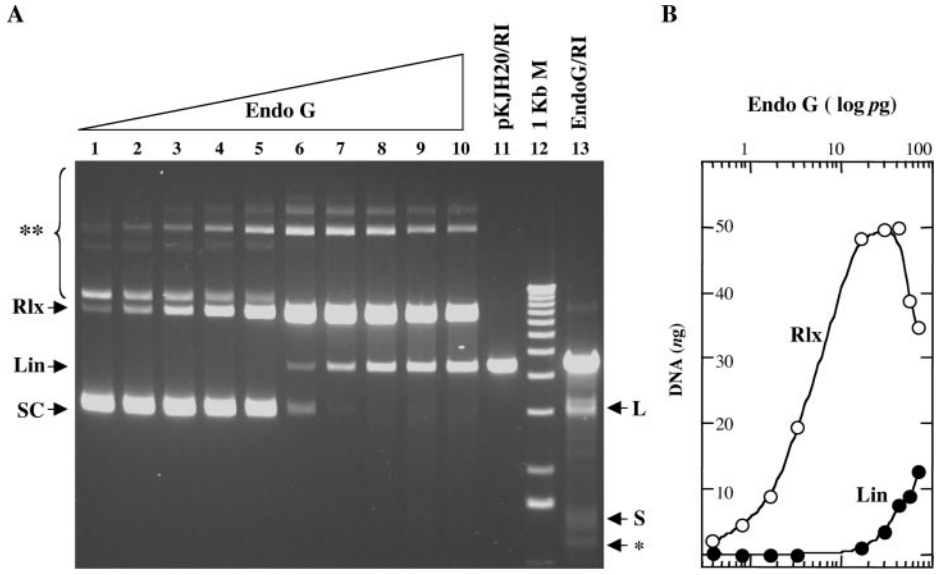

FIG. 7. **Human endonuclease G generates single strand, then double strand breaks in the *a* sequence.** *A*, 500 ng of pKJH20 were incubated with increasing amounts of affinity-purified endonuclease G (0, 0.4, 0.8, 1.7, 3.3, 6.7, 30, 43.3, 56.7, and 70 pg in *lanes 1–10*) in an *a* sequence-cleavage assay for 2 h. After extraction with phenol/chloroform, the reaction products were analyzed by agarose gel (1.2%) electrophoresis followed by staining with ethidium bromide. *Lane 11* is pKJH20-linearized by the *Eco*RI restriction enzyme. *Lane 12* is the 1-kb molecular weight (*M*) marker (Invitrogen). In *lane 13*, endonuclease G (*EndoG/RI*) reaction products from the same reaction as shown in *lane 10* were purified and digested with *Eco*RI. The migration positions of the relaxed form (*Rlx*), the linearized from (*Lin*), and the supercoiled form (*SC*) of pKJH20 monomer are indicated by the *arrows*. Also shown are the L- and S-type products (indicated by the *arrows*) and the DNA bands corresponding to cleavage at a site mapped to the plasmid backbone (indicated by an *asterisk*). Different conformations of multimers of pKJH20 were indicated by *double asterisks*. *B*, the amounts of the relaxed (*Rlx*) and the linearized forms (*Lin*) of pKJH20 from *lane 2* to *lane 10* were measured by densitometry (Alpha Imager™2000, Alpha Innotech Corp.) and plotted against the amounts of endonuclease G in each reaction.

DNA from the white colonies was examined by *Dra*I restriction enzyme digestion, which cleaves the *a* sequence (Fig. 9*A*), all of plasmids showed deletion of the *lac*Z gene (Fig. 9*B*, *lane 4–17*). Fifty-seven percent of the products (8 of 14, *lanes 4*, *5*, *11–15*, and *17*) showed a precise deletion of *lac*Z and contained one *a* sequence, a result expected from a perfect recombination. The remainder (43%, 6 of 14) showed additional deletions into the *a* sequence, as illustrated by the shortened 1.25- and 1.05-kb *Dra*I fragments. The only white colony obtained in the absence of endonuclease G treatment (Fig 9*B*, *lane 3*) showed the 4.3-kb *Dra*I fragment indicative of the presence of *lac Z*. Presumably this white colony is unrelated to the recombination reaction. Thus, endonuclease G is capable of initiating recombination between directly repeated *a* sequences.

DISCUSSION

The inversion of the L and S segments of the HSV-1 genome appears to result from a highly efficient recombinational event that is stimulated by the G+C-rich HSV-1 *a* sequence flanking the L and S segments. Because the initiation of recombination at the *a* sequence requires cleavage at this site, we sought and ultimately found a nuclease with a strong preference for the *a* sequence in HeLa cell nuclear extracts. Extensive purification of the enzyme showed it to be identical to the human homologue of endonuclease G, one of a group of widely distributed nucleases with a strong preference for the dG·dC homopolymer pair. Identification of the purified HeLa cell enzyme as endonuclease G was based on amino acid sequence analysis and the specific interaction of the HeLa cell enzyme with anti-endonuclease G. Surprisingly, interaction with the antibody resulted in stimulation rather than inhibition of enzyme activity. Possibly, binding of the endonuclease G to the two antigen binding sites of the IgG facilitated dimerization of endonuclease G, which has been shown to exist as a dimer in the bovine homologue (25). Alternatively, binding of the antibody might prevent formation of a less functional form of the enzyme, as has been described for the p53 tumor suppressor (26). We also noted that a protein with an apparent molecular mass of 60 kDa, tentatively identified as the human polypyrimidine track binding protein (PTB-2) (27), had a chromatographic profile on hydroxyapatite consistent with the *a* sequence-cleavage enzyme. However, this protein did not co-elute with the *a* sequence-cleavage activity of endonuclease G during antibody affinity chromatography and is, therefore, not an essential component of the *a* sequence-cleavage enzyme.

Endonuclease G appears to be the only enzyme in HeLa cells that is capable of cleaving the HSV-1 *a* sequence. Another G-specific mammalian endonuclease, endonuclease R, (28), can be excluded. Although endonuclease R would be expected to be inhibited at the ionic strength of our standard assay conditions (45 mM NaCl), the *a* sequence-cleavage activity in the whole cell extract at low monovalent ion concentrations (1.5 mM KCl) was completely depleted by anti-endonuclease G, which should not inhibit endonuclease R.[2] Based on this finding and the similarity of digestion products, the *a* sequence-cleavage activity reported by Wohlrab *et al.* (17) and by Zemelman (19) is probably endonuclease G.

Bovine endonuclease G and endonuclease G from chicken erythrocytes have been shown to generate single strand cleavages within the dG·dC homopolymer pair (21, 24, 25). Our study shows that human endonuclease G also generates multiple single strand cleavages in the *a* sequence before the formation of a double strand break. As judged by the heterogeneity of the products of endonuclease G cleavage, there are very likely multiple cleavage sites within the *a* sequence. The generation of single strand breaks is consistent with a similar finding with a recombinant human endonuclease G activity (29).

The *a* sequence does not appear to be a particularly good substrate for endonuclease G, which prefers a $G_n{\cdot}dC_n$ ho-

[2] K.-J. Huang, B. V. Zemelman, and I. R. Lehman, unpublished information.

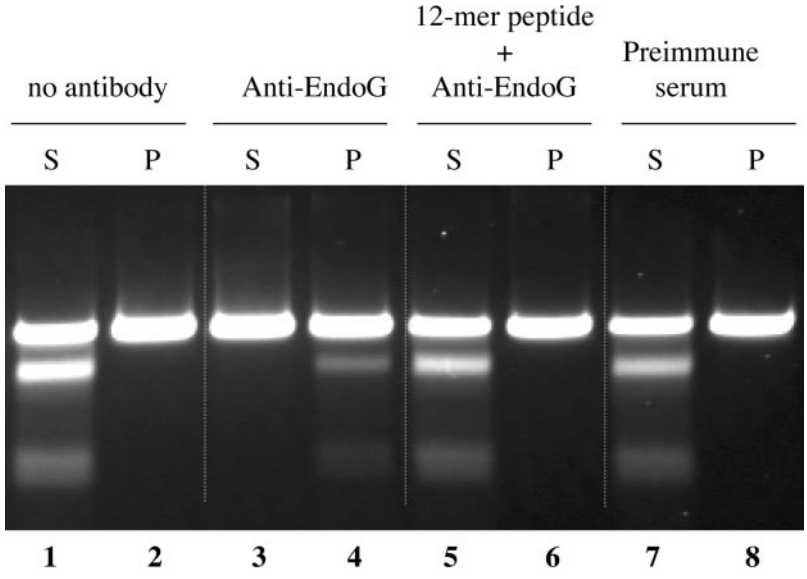

FIG. 8. **Immunodepletion of *a* sequence-cleavage activity from whole-cell extracts by anti-endonuclease G.** 27 μg of HeLa cell extract ("Experimental Procedures") were incubated with 2.6 μg of anti-endonuclease G or 2.6 μg of anti-endonuclease G containing 11.7 mM 12-mer peptide or 2.6 μg of preimmune serum. Protein A-agarose was added to remove the immuno complexes. The resulting precipitates (*P*) and supernatants (*S*) were examined for *a* sequence-cleavage activity as described under "Experimental Procedures." All assays except for the one shown in *lane 6* were performed in the presence of 11.7 mM 12-mer peptide. In *lane 6*, the assay was performed in the presence of 19.5 mM 12-mer peptide.

mopolymer pair ($n > 8$). It contains only 1 string of 8 dGs in the Ub region and many short strings of dGs (dG_n, $n = 4–6$) located along the length of the *a* sequence in an asymmetric arrangement (Fig. 1), yet the *a* sequence is readily cleaved by endonuclease G. This efficiency of cleavage may be due to the nucleotide composition of the flanking sequences as described earlier (24). It is also possible that closely located minor cleavage sites force endonuclease G to function cooperatively, leading to higher *a* sequence-cleavage activity than expected from its short strings of G residues. Finally, the asymmetric distribution of G residues may make the *a* sequence a better endonuclease G substrate. The previously reported anisomorphic structure that the *a* sequence adopts under negative supercoiling (30) does not appear to play an obligatory role in the cleavage since the *a* sequence in our substrate is a linear duplex and is nevertheless readily cleaved by endonuclease G.

In addition to the *a* sequence, purified endonuclease G can also cleave a site within the pKJH20 backbone. This site contains a string of 12 G residues and could serve as a good endonuclease G substrate. We have also observed that high levels of purified endonuclease G can cleave the *a* sequence-containing substrate to form a collection of heterogeneous products (Fig. 6*B*). At very high levels of enzyme, the substrate can be completely degraded.[2] Thus, it appears that human endonuclease G can cleave sequences other than the *a* sequence but with lower efficiency. Alternatively, the enzyme may have an unrecognized intrinsic exonuclease activity.

In this study, endonuclease G was purified from HeLa cell nuclei. This cellular location is consistent with earlier studies (21, 24, 25) in which bovine and chicken endonuclease G were isolated from both nuclei and mitochondria. Although one study (31) suggested that the endonuclease G activity detected in the nucleus resulted from mitochondrial contamination, another study in which immunocytochemistry was coupled with confocal-microscopy indicated that some endonuclease G is normally localized in the nucleus (21). The existence of endonuclease G in the nucleus is consistent with the result of a more recent study in which cellular fractionation demonstrated the existence of rat endonuclease G but not another mitochondrial enzyme in both the nuclear and the mitochondrial fractions (32).

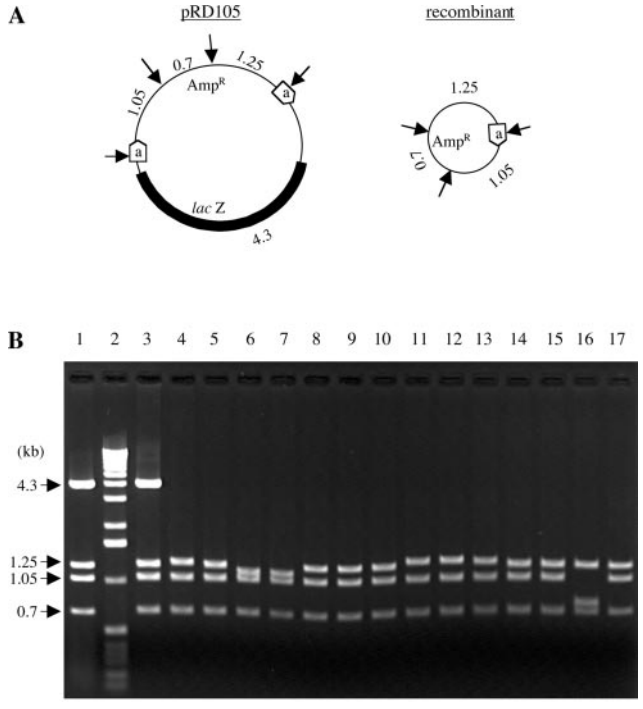

FIG. 9. **Recombinational activity of endonuclease G.** *A*, diagram showing the *Dra*I sites (indicated as *arrows*) in pRD105 and the predicted recombinant. The *Dra*I restriction fragments are shown in kb. *B*, restriction mapping of recombination products. Immuno-purified endonuclease G (0.3 *ng*) was used in the *in vitro* recombination assay (see "Experimental Procedures"). Plasmid DNA from 14 randomly picked white colonies from endonuclease G-treated reactions (*lanes 4–17*) and the only white colony from an untreated reaction (*lane 3*) were digested with the *Dra*I restriction enzyme and analyzed by 1.5% agarose gel electrophoresis and ethidium bromide staining. *Lane 1*, pRD105 digested with *Dra*I. The sizes of *Dra*I restriction fragments are shown in kb. *Lane 2*, the 1-kb molecular weight marker (Invitrogen). The loss of the 4.3-kb band, resulting from deletion of the *lac* Z gene, is indicated by the *arrow*.

It has been suggested that endonuclease G plays a role in mitochondrial DNA replication, DNA recombination, and genomic instability (21). Most recently, endonuclease G was

shown to participate in apoptosis (33, 34). In view of this multiplicity of functions, what is the significance of HSV-1 *a* sequence cleavage by human endonuclease G? The *a* sequence cleavage-activity reported by Wohlrab *et al.* (17) was found to be greatly stimulated by HSV-1 infection. Similarly *a* sequence-cleavage activity was found to coincide with a recombinase activity (19). Recombination is stimulated by double strand breaks (35), and endonuclease G appears to be the sole cellular enzyme capable of generating double strand breaks at the HSV-1 *a* sequence. Moreover, the only HSV-1-encoded endonuclease (the product of UL12) that can cleave the *a* sequence is clearly not required for genome inversion (15). It is therefore plausible that endonuclease G is the enzyme involved in the initiation of recombination at the HSV-1 *a* sequence. Indeed, the purified endonuclease G can generate the precise deletion products observed in our *in vitro* recombination system. Should endonuclease G prove to be responsible for the HSV-1-stimulated *a* sequence-cleavage activity reported by Wohlrab *et al.* (17), it is very likely responsible for the elevated *a* sequence-mediated recombination observed after HSV-1 infection. The ability of endonuclease G to cleave sequences other than the *a* sequence further suggests it may also be involved in the non-*a*-sequence mediated recombination reported by Dutch *et al.* (14, 18) and by Weber *et al.* (36).

In an earlier study, introduction of the $dG_{25}{\cdot}dC_{25}$ homopolymer pair into repeated non-*a* sequences within a plasmid, which would be expected to facilitate endonuclease G cleavage at these sites, did not result in a significant increase in recombination *in vivo* after HSV-1 infection (18). At present, our data cannot provide an explanation for this apparent discrepancy other than the possibility that the unique structure of the *a* sequence is involved. Future studies aiming at perturbing endonuclease G activity *in vivo* should define more precisely the role of endonuclease G in *a* sequence-mediated recombination and its relationship to HSV-1 genome inversion.

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