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) 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 a 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 non-essential 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
removed from pRD105 (13) by BamHI digestion and inserted into the BamHI site of pBluescript SK (+) (Stratagene). The 1.24-kb kanamycin cassette from pUC4K (Amersham Biosciences) was then inserted into the PsiI site of the resulting plasmid to generate pKJH20.

a Sequence-cleavage Assay—The sequence-cleavage assay was modified from a linear gradient-depletion assay (19) previously described. A 10-μl reaction mixture (31 μl) contained 0.5 μl of pKJH20 linearized by EcoRI (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 MgCl2, 1.5% agarose). One unit of the L-type digestion product (the 3'-5' fragment) 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'-5'3-3.5-fragment) generated at 37°C in 2 h. The amount of the L-type product was estimated by densitometry (Alpha Imager™, 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 by 40% KCl, 0.5 mM phenylmethylsulfonyl fluoride, 10 mM sodium metabsulfite, pH 7.1, 0.5 μg/ml leupeptin (A), and other negatively charged macromolecules from the supernatant, which contained the majority of the sequence-cleavage enzyme, 3.6 g of nuclear extract (from nuclei of 5 liters of HeLa cells) were processed in 7 batches. Purification of enzyme activity was present in fractions eluting at 97–100°C, with weaker activity trailing to the fraction corresponding to 313 mM KCl. The fractions with peak activity (Mono S fraction) were collected 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). Fifty milliliters of buffer R (20 mM Hepes, pH 7.6, 2 mM MgCl2, 100 mM NaCl) 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 sequence-cleavage enzyme, 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 ml 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 mM NaCl) were gradually added. After extraction for 30 min at 4 °C, the supernatant was collected by centrifugation at 27,000 × g for 30 min.

Purification of a Sequence-cleavage Assay—To purify the 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 activity was present in fractions eluting at 97–100°C, with weaker activity trailing to the fraction corresponding to 313 mM KCl. The fractions with peak activity (Mono S fraction) were collected 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). Fifty milliliters of buffer R (20 mM Hepes, pH 7.6, 2 mM MgCl2, 100 mM NaCl) 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.

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Preparation of Anti-endonuclease G—Rabbit anti-endonuclease G was produced by immunization with a 12-amino acid peptide (AEPL-PVPGGPRG) 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, Alabama. Preimmune serum was collected, and preimmune serum and peptide were purified with a protein A-agarose affinity column following the method described by Sambrook et al. (22).

Immunopurification 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 tandem columns (Protein A—Protein G—Protein A) following the procedure described above. 1 ml of protein A column (HiTrap™ protein A HP, Amersham Biosciences), 1 ml of preimmune 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 mM NaCl) and 1 ml of buffer B. The bound proteins were eluted from the column with 1 ml of 12-mer peptide (3 mM in buffer B). 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%.

Immunodepletion 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 μl 12-mer peptide or 2.6 μg of purified pre-immune serum) in a 1-ml linear gradient-depletion assay (a linear gradient 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 (58
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 HindIII 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. 3A, 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. 3B) with molecular masses of ~60, 35, and 31 kDa. Comparison of the profile of a sequence-cleavage enzyme with those of DNA polymerase I, III, and IV showed that the purified enzyme closely resembled the tetrameric enzyme, suggesting that the enzyme is Endo G.
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activity across the hydroxyapatite peak (Fig. 3A) 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 (Fig. 4). The precursor form of human 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 1M 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 α sequence suggested that bovine endonuclease G can cleave at many sites within the α 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 α sequence.

We therefore asked whether the α 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 α 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 5). Because the α 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 α 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 α 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. 6A, 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 α 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/EcoR1) was readily cleaved to generate both the S-type and the L-type fragments, indicative of specific α sequence cleavage (Fig. 6B). Thus, endonuclease G is sufficient for the specific cleavage of the HSV-1 α sequence. The eluate also generated a heterogeneous mixture of products when high levels of enzyme (66 pg) were used. As shown in Fig. 6C, the α 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 α Sequence to Generate a Double Strand Break—As indicated above, human endonuclease G is likely to produce double strand breaks within the α 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 α sequence in a supercoiled plasmid containing the α sequence,

TABLE I

Purification of a sequence-cleavage enzyme

| Step                     | Total protein | Total activity | Yield | Enrichment |
|--------------------------|---------------|----------------|-------|------------|
| 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           |               |                |       | 6,300a     |

* Estimate 10× increase from previous step.
pKJH20. As shown in Fig. 7A, 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 \( \text{EcoRI} \) restriction enzyme. As shown in lane 13, DNA bands corresponding to \( \text{L-type} \) products and \( \text{S-type} \) 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. 7B). 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. The extract was 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.

**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**.

### 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.

### 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**.
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.

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) in an in vitro recombination assay. In this assay, the recombinase activity leads to deletion of a lacZ 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 lacZ+ 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...
DNA from the white colonies was examined by Dral restriction enzyme digestion, which cleaves the a sequence (Fig. 9A), all of plasmids showed deletion of the lacZ gene (Fig. 9B, lane 4–17). Fifty-seven percent of the products (8 of 14, lanes 4, 5, 11–15, and 17) showed a precise deletion of lacZ 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 Dral fragments. The only white colony obtained in the absence of endonuclease G treatment (Fig. 9B, lane 3) showed the 4.3-kb Dral 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 polyuridimidine 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. 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+dC homologous sequence-cleavage activity. The finding with a recombinant human endonuclease G activity. The finding with a recombinant human endonuclease G activity.
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. 6B). At very high levels of enzyme, the substrate can be completely degraded. 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. 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.

**Fig. 9.** Recombinational activity of endonuclease G. A, diagram showing the DraI sites (indicated as arrows) in pRD105 and the predicted recombinant. The DraI 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 DraI restriction enzyme and analyzed by 1.5% agarose gel electrophoresis and ethidium bromide staining. Lane 1, pRD105 digested with DraI. The sizes of DraI 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 lacZ 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 recombination 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 5'-sequence mediated recombination reported by Dutch et al. (14, 18) and by Weber et al. (36).

In an earlier study, introduction of the dG23dC25 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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