

# Action of Recombinant Human Apoptotic Endonuclease G on Naked DNA and Chromatin Substrates

COOPERATION WITH EXONUCLEASE AND DNase I\*

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**Endonuclease G (endoG) is released from mitochondria during apoptosis and is in part responsible for internucleosomal DNA cleavage. Here we report the action of the purified human recombinant form of this endonuclease on naked DNA and chromatin substrates. The addition of the protein to isolated nuclei from non-apoptotic cells first induces higher order chromatin cleavage into DNA fragments  $\geq 50$  kb in length, followed by inter- and intranucleosomal DNA cleavages with products possessing significant internal single-stranded nicks spaced at nucleosomal ( $\sim 190$  bases) and subnucleosomal ( $\sim 10$  bases) periodicities. We demonstrate that both exonucleases and DNase I stimulate the ability of endoG to generate double-stranded DNA cleavage products at physiological ionic strengths, suggesting that these activities work in concert with endoG in apoptotic cells to ensure efficient DNA breakdown.**

Apoptosis, or programmed cell death, plays an important role in both development and maintenance of tissue homeostasis (reviewed in Refs. 1 and 2). Two apoptotic pathways have been identified: the death-receptor pathway and the mitochondrial pathway (3). Mitochondria have been shown to harbor multiple apoptogenic factors including cytochrome *c*, procaspases, SMAC/DIABLO,<sup>1</sup> AIF, and endoG (4–10). Both cytochrome *c* and SMAC/DIABLO are involved in caspase activation, whereas AIF and endoG have been associated with one of the hallmarks of the terminal stages of apoptosis, DNA breakdown (11, 12).

Apoptotic cell genomic DNA cleavage occurs in at least two

stages: initial cleavage at intervals of  $\geq 50$  kb, consistent with the size of chromatin loop domains, followed by a second stage of internucleosomal DNA cleavage (also called DNA laddering) (13). AIF (8), topo II (14), and caspase-treated DFF/CAD-ICAD (15–17) have each been implicated in the higher order DNA cleavage reaction. Nucleosomal DNA laddering, on the other hand, has been associated with several endonucleases, including caspase-activated DFF/CAD-ICAD (18–25), endoG (9, 10), and DNase I (26).

Although some of the catalytic properties of endoG have been reported previously, nucleic acid, not chromatin substrates, had been employed. In addition, most of these studies used various partially purified forms of the protein from different tissue sources, and the possible contributions of impurities remain uncertain (27–31). Furthermore, this nuclease was originally thought to play a role in mitochondrial DNA replication (29), which seems unlikely because a yeast knockout exhibits no phenotype (32), and the enzyme co-localizes with cytochrome *c* in the intermembrane space as opposed to the matrix where DNA replication occurs (9). One newly recognized function for endoG is as a caspase-independent pathway for DNA breakdown during apoptosis (9, 10). Here we study the action of homogenous human recombinant endoG on DNA and chromatin substrates. We have found that the enzyme possesses novel properties including cooperation with exonuclease and DNase I for more efficient DNA breakdown under physiological ionic strengths.

## EXPERIMENTAL PROCEDURES

**Expression and Purification of Recombinant EndoG**—Full-length human endoG cDNA with an additional six histidine residues appended to its C terminus and cloned into pFastBac1 (Life Technologies, Inc.), was transformed into DH10Bac cells (Life Technologies, Inc.), and the recombinant viral DNA was purified according to the Bac-to-Bac baculovirus expression procedure. The purified bacmids were used to transfect Sf21 insect cells using CellFECTIN reagent (Life Technologies, Inc.). Transfected cells were grown in IPL41 medium with 10% fetal calf serum, 2.6 g/liter tryptose phosphate, 4 g/liter yeastolate, and 0.1% Pluronic F-68 plus penicillin (100 units/ml), streptomycin (100  $\mu$ g/ml), and Fungizone (0.25 g/ml). Forty milliliters of the amplified viral stock was used to infect 1 liter of cells at  $2 \times 10^6$  cells/ml. The infected cells were harvested 2 days later, and resuspended and homogenized in 5 volumes of buffer T (20 mM Tris-HCl (pH 8.0), 50 mM NaCl, 1 mM  $\beta$ -mercaptoethanol, and 0.1 mM phenylmethylsulfonyl fluoride) with 0.5% Nonidet P-40. These and all subsequent operations were conducted at 4 °C. The cell homogenate was centrifuged at  $10,000 \times g$  for 30 min, and the supernatant was loaded onto a 3-ml nickel affinity column. The column was washed with 30 ml of buffer T with 0.5% Nonidet P-40, then 30 ml of buffer T, and followed by 200 ml of buffer T plus 1 M NaCl. The column was washed once more with buffer T, and proteins were eluted with buffer T plus 250 mM imidazole. The eluted proteins were loaded onto a Superdex 200 column (Amersham Biosciences, Inc.) and eluted with buffer A (20 mM Hepes-KOH, pH 7.0, 10 mM KCl, 1.5 mM MgCl<sub>2</sub>, 1 mM NaEDTA, 1 mM NaEGTA, 1 mM dithio-

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<sup>1</sup> The abbreviations used are: SMAC/DIABLO, second mitochondria-derived activator of caspase/direct IAP binding protein with low pI; AIF, apoptosis-inducing factor; CAD, caspase-activated deoxyribonuclease (also termed DFF40); DFF, DNA fragmentation factor; ExoIII, exonuclease III; MNase, micrococcal nuclease; NP-40, Nonidet P-40; topo II, topoisomerase II; LTR, long terminal repeat; HIV-1, human immunodeficiency virus 1; EndoG, Endonuclease G; ICAD, inhibitor of CAD.

threitol, and 0.1 mM phenylmethylsulfonyl fluoride). The peak fractions were loaded onto a Mono S column (Amersham Biosciences, Inc.) and eluted with a 20-ml linear gradient from 0 to 300 mM NaCl in buffer A. The peak of endoG nuclease activity, eluting at ~80 mM NaCl, was stored at -20 °C in 50% glycerol. Protein purity was assessed by SDS, 15% polyacrylamide gel electrophoresis.

**Nuclease Substrates**—Plasmid pWLTR11 DNA (33),  $\phi$ X174 virion DNA (New England BioLabs), or high molecular weight RNA from wheat germ (Calbiochem) were employed as non-chromatin substrates. Nuclei were purified from HeLa S3 cells. Cells were lysed in a buffer consisting of 10 mM KCl, 0.25 M sucrose, 4 mM MgCl<sub>2</sub>, 1 mM dithiothreitol, 20 mM Hepes, pH 7.5, 0.5% Nonidet P-40, and Complete™ (Roche Molecular Biochemicals) protease inhibitors set and then washed two times in the same buffer without Nonidet P-40.

**Nuclease Cleavage**—One microgram of naked DNA was incubated for 30 min at 37 °C with endoG (final concentration: 0.5  $\alpha$  unit/ml) in buffer consisting of 10 mM KCl, 3 mM MgCl<sub>2</sub>, 0.5 mM dithiothreitol, 20 mM Hepes, pH 7.5 (final volume: 15  $\mu$ l), if not stated otherwise. Two micrograms of DNA (as nuclei) were incubated for varying times (5–45 min) at 37 °C with either recombinant purified endoG (final concentration 10 units/ml), recombinant purified activated DFF (50 units/ml, Ref. 24), MNase (Worthington, 40 units/ml), or DNase I (Worthington, 10 units/ml) in a buffer consisting of 100 mM KCl, 3 mM MgCl<sub>2</sub>, 1 mM CaCl<sub>2</sub>, 0.5 mM dithiothreitol, 20 mM Hepes, pH 7.5 (final volume 20  $\mu$ l). Nuclease reactions were terminated by mixing with one-half volume of stop solution (0.6% SDS, 50 mM EDTA, and 6-mg/ml proteinase K). To non-chromatin substrate reactions gel loading dye buffer was added, and samples were then run on 1.5% SeaKem-agarose gels, using 1 $\times$  (Tris, acetate-EDTA) as the running buffer and stained with ethidium bromide. Chromatin substrate reactions were incubated for 1 h at 42 °C with proteinase K, and then DNA was purified by phenol/chloroform extraction and ethanol precipitation. DNA was dissolved in Tris-EDTA buffer and incubated with a mixture of RNaseA and RNase T1. DNA was then separated on standard 1.5% agarose gels, two-dimensional (neutral/alkaline) agarose gels (23), 5% native polyacrylamide gels, or 25-cm long, 3-mm thick, 7.5% polyacrylamide, 7 M urea sequencing gels (10:1 proportion of acrylamide and bis-acrylamide), and stained with ethidium bromide.

**Analysis of Cleavage Sites**—Detailed analysis of sequences at cleavage sites was performed as described (24). Briefly, a 177-base pair fragment of HIV-1 5'-LTR was excised from plasmid pWLTR11, 5'-end-labeled with T4 polynucleotide kinase, purified, and then incubated with either purified recombinant activated DFF or endoG. To analyze cleavage sites on both strands, DNA was digested with either *Sca*I or *Bsa*I enzymes. Digestion products were resolved on 6% polyacrylamide sequencing gels with the appropriate Maxam-Gilbert sequencing reactions.

**Pulsed-field Gel Electrophoresis**—Nuclei were incubated with purified recombinant endoG or activated DFF for 5, 15, and 45 min at 37 °C, and the nuclease reactions were terminated by adding aurintricarboxylic acid (final concentration: 0.1 mM). Alternatively, nuclei were incubated for 30 min in the presence of 0.1 mM topo II inhibitor VM-26. Reaction mixtures were embedded in low temperature melting-agarose plugs, and plugs were then incubated for 3 h at 37 °C in lysing solution (0.5% SDS, 20 mM EDTA, and 0.2 mg/ml proteinase K). After washing plugs with Tris-EDTA buffer, the DNA was separated in a CHEF Mapper pulsed-field gel electrophoresis system (Bio-Rad) and then stained with ethidium bromide.

**Mononucleosomal DNA Analysis**—Nuclei were incubated with either purified recombinant endoG, purified recombinant activated DFF, or MNase and then DNA was purified. Mononucleosomal DNA was isolated after electrophoresis on a low temperature melting agarose gel. DNA was <sup>32</sup>P 3'-end-labeled with terminal deoxynucleotidyl transferase (Sigma) and resolved on a 5% native polyacrylamide gel. Alternatively, DNA was 5' end-dephosphorylated with shrimp alkaline phosphatase (Roche Molecular Biochemicals), <sup>32</sup>P 5'-end-labeled with T4 polynucleotide kinase (Roche Molecular Biochemicals), and resolved on an 8% polyacrylamide, 7 M urea sequencing gel.

## RESULTS

**Expression and Purification of Recombinant Human EndoG**—We utilized a baculovirus expression system to produce a His<sub>6</sub>-tagged, full-length endoG protein, which was purified to homogeneity and free from insect cell endoG, by stepwise chromatography on nickel affinity columns, Superdex 200, and Mono S-columns, as demonstrated by SDS gel electrophoresis with Coomassie Blue staining (Fig. 1). The apparent molecular

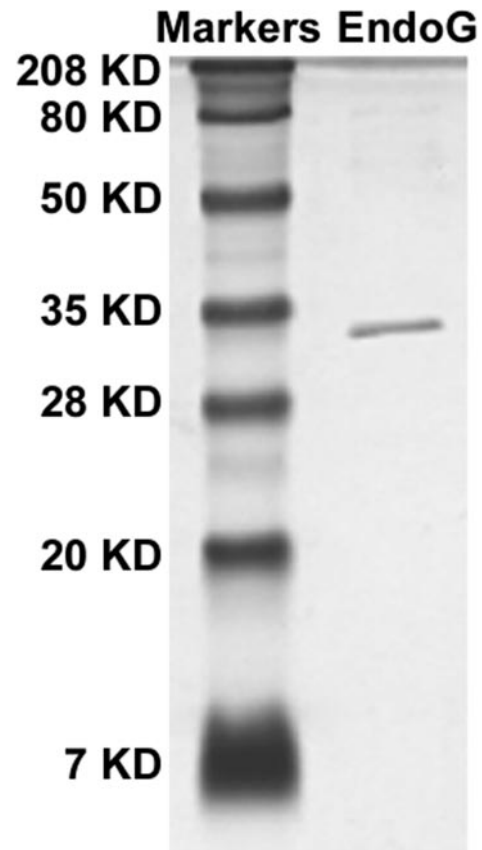


FIG. 1. Homogeneity of human purified recombinant endoG as revealed by SDS gel electrophoresis.

mass of the protein was 33.5 kDa, whereas the predicted size for the recombinant protein after targeting to the mitochondria and removal of the N-terminal leader sequence (29) is 28.6 K (including the His<sub>6</sub>-tag). However, we found that the leader sequence had been removed from the purified recombinant protein (data not shown), indicating that the mobility in SDS gels is aberrant.

**Catalytic Properties of Recombinant EndoG on Naked Nucleic Acids**—EndoG has biphasic pH optima for attacking double-stranded DNA at pH 9.0 and pH 7.0 (Fig. 2A). The higher pH optimum is probably accounted for by increased DNA breathing and the fact that endoG has much greater activity on single-stranded nucleic acid substrates (see below). In agreement with previous reports (27, 30), the enzyme requires either Mg<sup>2+</sup> or Mn<sup>2+</sup> and not Ca<sup>2+</sup> as its divalent cation and is inhibited about 15-fold at physiological ionic strengths (Fig. 2, B and C). The presence of Fe<sup>2+</sup> or Zn<sup>2+</sup> in combination with Mg<sup>2+</sup> also inhibits enzyme activity (Fig. 2B). In further agreement with previous publications (29–31), both single-stranded DNA and RNA are preferred substrates over double-stranded DNA (Fig. 2D). First-hit kinetics indicate that supercoiled plasmids are first relaxed by single-stranded nicking by endoG (not shown).

**Sequences at EndoG DNA Cleavage Sites**—We evaluated the endoG cleavage sequences for naked DNA cleavage in comparison with DFF as a control. The corresponding cleavage products of <sup>32</sup>P 5'-labeled HIV-1 5'-LTR DNA of the Watson and Crick strands were separated on sequencing gels. We analyzed 22 cleavage sites at the nucleotide level and found that unlike caspase-3-activated DFF, which generates primarily blunt-end DNA cleavages (Fig. 3A, gray arrowheads), endoG made numerous single-stranded nicks, primarily 5' of G residues (14/22) (Fig. 3A, black arrowheads), in agreement with a previous report (28). We also discovered cleavages 5' of C and A resi-

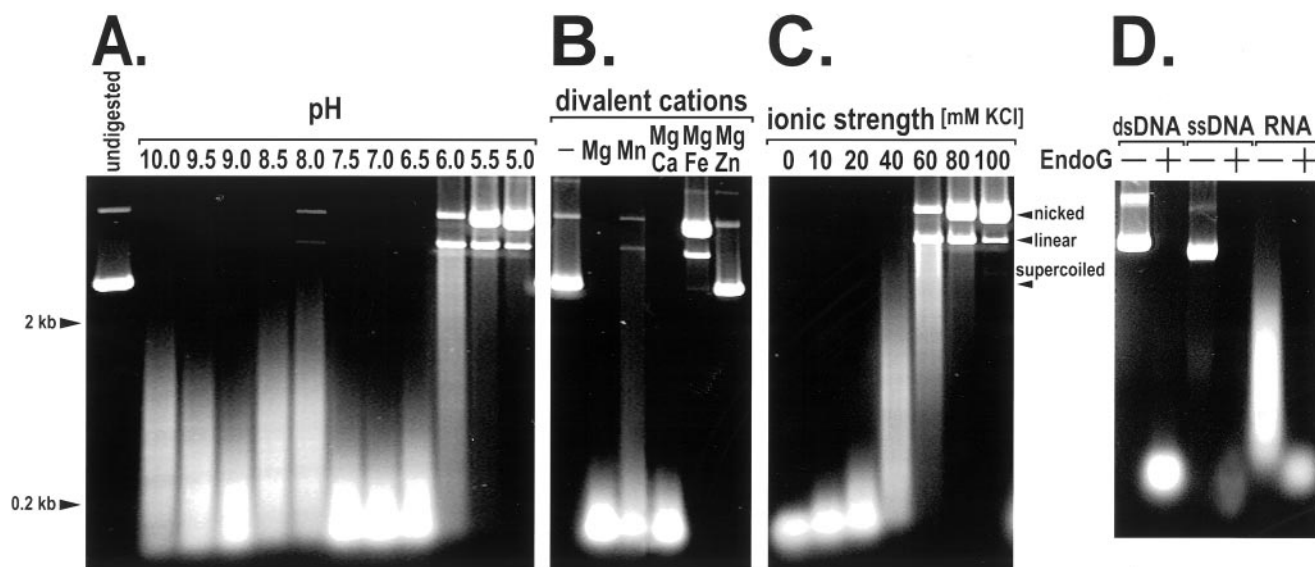


FIG. 2. The effects of reaction parameters on the activity of human purified recombinant endoG. *A*, pH values of reaction mixtures were adjusted using 40 mM Tris-HCl or sodium acetate. *B*, reaction mixtures contained 0.25 mM EDTA, 3 mM MgCl<sub>2</sub>, and 0.25 mM EGTA, 3 mM MnCl<sub>2</sub>, 3 mM MgCl<sub>2</sub>, 1 mM CaCl<sub>2</sub>, 3 mM MgCl<sub>2</sub>, and 1 mM FeCl<sub>2</sub>, or 3 mM MgCl<sub>2</sub>, and 1 mM ZnCl<sub>2</sub>. *C*, nuclease assay was performed at the indicated KCl concentrations. Arrowheads denote the positions of molecular size standards and different topological forms of the plasmid. *D*, endoG was incubated with the indicated substrates (double-stranded DNA, *ds*-DNA; single-stranded DNA, *ss*-DNA).

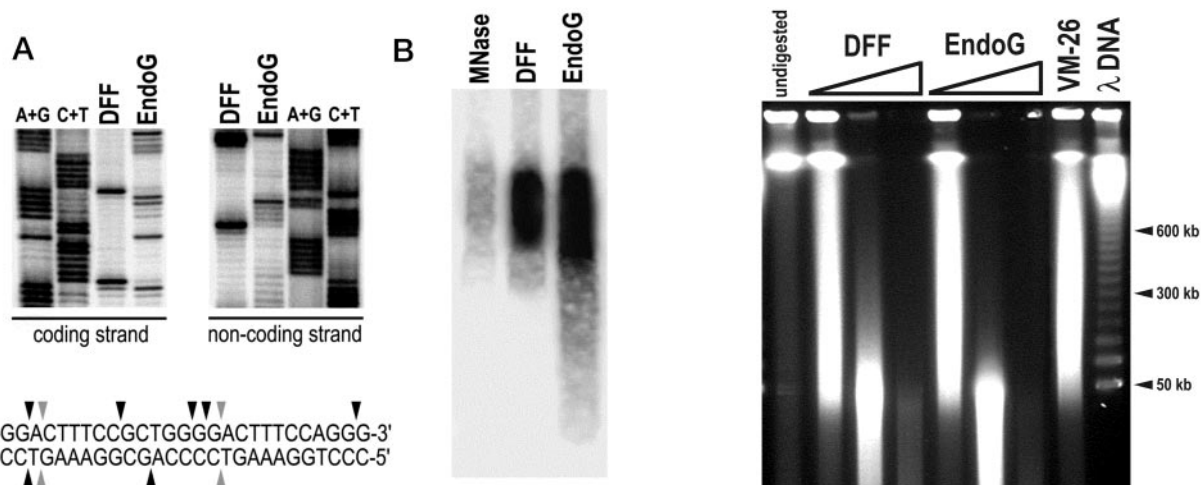


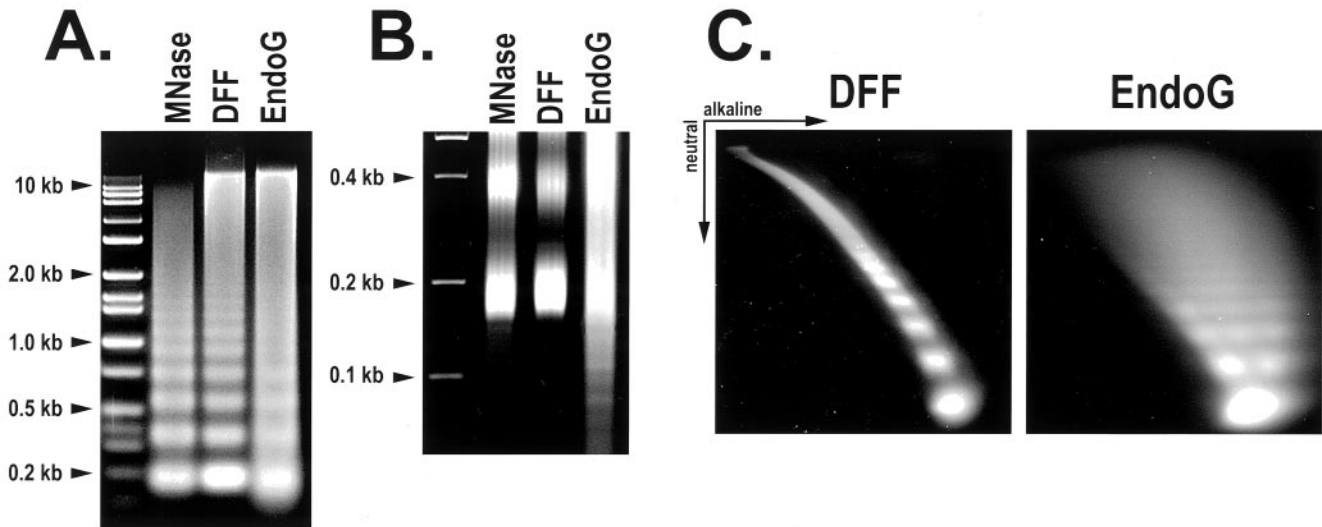
FIG. 3. Analysis of DNA sequences and ends at endoG cleavage sites. *A*, DNA fragments generated by either purified recombinant endoG or activated DFF were separated on a sequencing gel parallel to sequencing reaction products. Arrowheads (black for endoG, gray for DFF) depict the positions of DNA strand cleavage within the sequence. *B*, purified mononucleosomal DNA generated by either purified recombinant endoG, activated DFF, or MNase was 3' end-labeled with terminal deoxynucleotidyl transferase.

dues, and 3' of G residues (not shown). In addition, the DNA ends generated by endoG, like caspase-3 activated DFF but unlike those generated by MNase, possessed 3'-hydroxyl groups because they could be extended by terminal deoxynucleotidyl transferase (Fig. 3B), also in agreement with previous studies (28). In conclusion, our studies on naked nucleic acid substrates reveal that the catalytic properties of the recombinant protein are in general agreement with previous biochemical studies (27–31). We now focus on the previously uncharacterized action of the protein on chromatin substrates.

**EndoG Addition to Non-apoptotic Cell Nuclei Generates Higher Order Chromatin Cleavage**—During apoptosis, initial DNA cleavage occurs at intervals the size of chromatin loop domains,  $\geq 50$  kb (13). To determine whether endoG could catalyze such higher order cleavage events, we added the pro-

FIG. 4. Higher order DNA fragmentation induced by endoG addition to nuclei purified from non-apoptotic cells. High molecular weight DNA fragments generated by incubation of nuclei with either purified recombinant endoG, activated DFF (for 5, 15, and 45 min), or the topo II inhibitor VM-26, were analyzed by pulsed-field gel electrophoresis. Arrowheads depict the positions of molecular size standards.

tein to isolated HeLa cell nuclei and analyzed the cleavage products by pulsed-field gel electrophoresis. For positive controls, nuclei were also individually treated with caspase-3-activated DFF or with the topo II inhibitor VM-26, because each have been shown previously to generate higher order DNA cleavage (13, 17). Fig. 4 reveals that endoG also triggers higher order DNA cleavage in nuclei from non-apoptotic cells.

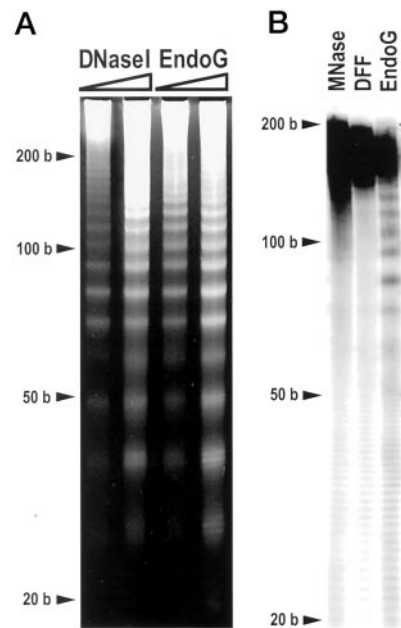


**FIG. 5. Oligonucleosomal DNA fragmentation and linker single-stranded DNA nicking induced by endoG addition to nuclei purified from non-apoptotic cells.** DNA was separated on either 1.5% agarose (A) or 5% polyacrylamide (B) gels. Arrowheads depict the positions of molecular size standards. C, DNA fragments generated by either purified recombinant endoG or activated DFF were separated on 1.5% agarose gels in 1× TAE buffer (neutral), and then gels were turned 90°, soaked in denaturing buffer (50 mM NaOH, 1 mM EDTA), and run in the same buffer for a second dimension of electrophoresis (alkaline).

*EndoG Addition to Non-apoptotic Cell Nuclei Generates Oligonucleosomal DNA Ladders with Single-stranded Nicks in the Linker Regions*—To determine the ability of endoG to generate oligonucleosomal DNA cleavage, we compared its action with that of MNase and caspase-3-activated DFF. As shown in Fig. 5A, digestion of isolated HeLa cell nuclei with MNase or DFF resulted in oligonucleosomal DNA ladders much sharper than those generated by endoG, as judged by oligonucleosomal multimer band sharpness and the interband background between successive oligonucleosomal multimers. Furthermore, cleavage within nucleosome core particles was detectable for endoG digestion products, which exhibited subnucleosomal DNA fragments (Fig. 5B). To investigate the degree and pattern of single-stranded nicking, we performed two-dimensional gel electrophoresis. After running the digestion products on a non-denaturing gel in the first dimension, a second dimension of electrophoresis was performed under denaturing conditions. As shown in Fig. 5C, this analysis revealed that in contrast to caspase-activated DFF, which generates predominantly double-stranded oligonucleosomal DNA fragments lacking internal single-strand nicks, endoG generates oligonucleosomal DNA fragments containing internal single-strand nicks spaced at oligonucleosomal intervals. For example, a significant fraction of fragments migrating as trinucleosomal in length in the first dimension possess nicks in their linker regions, thereby generating under denaturing conditions single-stranded mono- and dinucleosomal-length DNA fragments (Fig. 5C).

*EndoG Also Makes Single-stranded Nicks Within Nucleosomes at About a 10-base Periodicity*—To investigate further the action of endoG at the subnucleosomal level, we separated HeLa cell nuclei DNA digestion products on a high resolution sequencing gel. As shown in Fig. 6A, endoG cuts chromatin with the same periodicity as DNase I, namely at about 10.4 base multiples (34, 35). Proof that this cleavage occurs within the nucleosome core was obtained by first isolating mononucleosomal DNA fragments by non-denaturing electrophoresis and then end-labeling the material for visualization on a sequencing gel. As shown in Fig. 6B, material that was cut with endoG exhibits internal nicks spaced at about 10-base intervals, although such nicks are largely absent from MNase or DFF cut material.

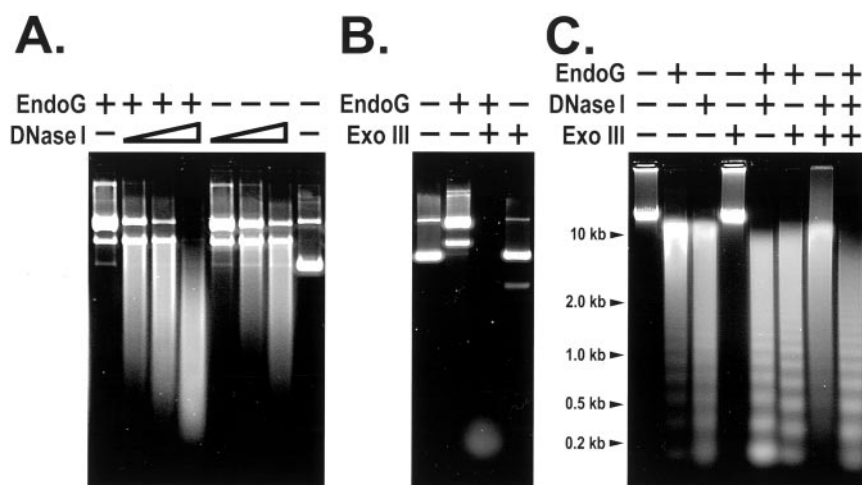
*EndoG Cooperates with Exonuclease and DNase I for DNA and Chromatin Cleavage*—A number of observations suggest



**FIG. 6. Intra-nucleosomal DNA fragmentation induced by endoG addition to nuclei purified from non-apoptotic cells.** A, 40  $\mu$ g of DNA purified from nuclei treated with either DNase I (15 and 30 min) or purified recombinant endoG (20 and 40 min) was separated on a sequencing gel. B, purified mononucleosomal DNA generated by purified recombinant endoG, activated DFF, or MNase was 5' end-labeled with polynucleotidyl kinase and separated on a sequencing gel. Arrowheads depict the positions of molecular size standards.

that other proteins may facilitate the ability of endoG to fragment DNA during apoptosis. First, the activity of the enzyme is quite low at physiological ionic strengths (Fig. 1C and Refs. 27 and 30). Second, the enzyme activity is elevated markedly on single-stranded nucleic acids (Fig. 1D and Refs. 30 and 31). Third, there is a high internucleosomal DNA background upon endoG digestion of isolated nuclei, not completely characteristic of the DNA laddering pattern seen during apoptosis in DFF knockout cells (9). Fourth, DNase I knockout cells fail to ladder chromatin under apoptotic conditions that block DFF activation (26). Fifth, the ladder generated by DNase I digestion of

**FIG. 7. Stimulation of endoG DNA cleavage by DNase I and exonuclease III.** *A*, plasmid DNA was incubated with purified recombinant endoG (0.5 units/ml) and DNase I (0.005, 0.010 and 0.015 units/ml). *B*, plasmid DNA was incubated with endoG (0.5 units/ml) and exoIII (100 units/ml). *C*, purified nuclei were incubated with endoG (2.5 units/ml), DNase I (5 units/ml), and exonuclease III (100 units/ml). All reactions were for 30 min at 37 °C in the presence of 100 mM KCl.



chromatin also exhibits a high internucleosomal DNA background, which is not nearly as sharp as the reported DNase I-dependent apoptotic ladder (26). Taken together, these observations prompted us to test whether nicks generated by DNase I would be targets for endoG action because of their single-stranded character, and whether exonuclease gapping of nicks generated by endoG or DNase I would also stimulate DNA processing under physiological ionic strengths.

Fig. 7A shows that the DNA digestion products are processed more than additively upon co-digestion of naked DNA with DNase I and endoG. Furthermore, ExoIII stimulates endoG activity by orders of magnitude on a naked DNA substrate (Fig. 7B). Finally, on chromatin substrates co-digestion again leads to more than additive DNA processing. In particular, the ladder of nucleosomal fragments is much sharper when either ExoIII, DNase I, or both were combined with endoG (Fig. 7C). As expected, ExoIII did not stimulate DNase I digestion (Fig. 7C), because DNase I does not preferentially attack single-stranded DNA like endoG. In conclusion, these results may provide insight into new molecules that are predicted to participate in DNA processing during apoptosis.

#### DISCUSSION

EndoG is released from the intermembrane space of mitochondria during apoptosis in a caspase-independent fashion and represents a novel pathway for nuclear DNA breakdown (9, 10). It should be appreciated that the *in vitro* properties of endoG cleavage largely fit the phenotype of the DNA products generated by apoptosis *in vivo*. Specifically, first higher order DNA cleavage into fragments >50 kb followed by nucleosomal DNA laddering, with fragments bearing 3'-hydroxyl groups. These features have been routinely used in bioassays for cells undergoing apoptosis.

We have found that the action of human recombinant endoG on naked nucleic acid substrates is in close agreement with previous reports (27–31), but in contrast to what might be observed from previous publications (27, 29), we demonstrate that the sequence specificity of endoG cleavage is clearly broad enough to attack essentially any DNA sequence, a feature required for efficient genome breakdown in apoptotic cells. However, the action of endoG on chromatin substrates had not been previously studied and represents the major focus of our investigation. We demonstrate that endoG catalyzes higher order DNA cleavage when added to nuclei isolated from non-apoptotic cells, just like topo II (13, 14) or DFF (15–17). These higher order cleavage events may represent an attack of preformed hypersensitive sites demarcating the boundaries of chromatin domains, such as those associated with domain in-

ulators or locus control regions (36–39). Upon further digestion of chromatin, we found that endoG generates single-stranded nicks at nucleosomal (~190 bases) and subnucleosomal (~10 bases) intervals. These cleavage patterns are somewhat similar to those of DNase I digestion products (34, 35). However, unlike DNase I, endoG preferentially attacks single-stranded regions, allowing for targeting by single-stranded nicks for adjacent strand cleavage to generate double-stranded nucleosomal length fragments.

Two features of the catalytic properties of endoG were not optimized for nucleosomal DNA breakdown: the preferential attack of single-stranded nucleic acids and maximal activity at less than 10 mM monovalent cations. Because these properties are clearly not optimal for physiological double-stranded DNA processing, we reasoned that additional proteins may participate with endoG for facilitating efficient DNA breakdown. Indeed, we demonstrated that exonuclease and DNase I each can cooperate with endoG to facilitate DNA processing. It is significant that DNase I recently has been reported to be required for DNA laddering under certain apoptotic conditions that are DFF independent (26). Taken together with our results, we suggest that endoG may participate with DNase I (and possibly exonucleases) *in vivo* for apoptotic DNA processing. Perhaps another important *in vivo* function for endoG is RNA breakdown during apoptosis.

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