We have previously implicated deoxyribonuclease II (DNase II) as an endonuclease responsible for DNA digestion during apoptosis. The full-length human cDNA has now been cloned. The cDNA contains an open reading frame of 1078 bases coding for a 40-kDa protein. This protein is 10 kDa larger than commercially supplied enzyme, which has been proteolytically cleaved at an internal aspartate residue. The gene is located at chromosome 19p13.2, and has no significant homology to other human proteins, but has >30% identity to three predicted genes in Caenorhabditis elegans. To determine whether overexpression of DNase II induces apoptosis in Chinese hamster ovary cells, the cDNA was co-transfected with a plasmid encoding green fluorescent protein. Within 24 h, a significant proportion of green fluorescent protein-positive cells contained condensed chromatin, whereas vector-only controls remained viable. Considering that DNase II is normally active only at low pH, it was surprising that transfection induced chromatin condensation. To confirm that transfection was not activating another endonuclease, cells were incubated with the caspase inhibitor benzoyloxycarbonyl-Val-Ala-Asp-(O-methyl)-fluoromethylketone; this failed to inhibit chromatin condensation induced by DNase II. These results demonstrate that DNase II acts downstream of caspase activation and that it may be activated by an as yet unknown mechanism to induce DNA digestion during apoptosis.

Apoptosis is a form of cell death utilized physiologically to maintain tissue homeostasis, as well as in response to various toxic stimuli, such as cancer chemotherapeutic agents. Apoptosis is characterized by cell shrinkage, membrane blebbing, condensation of the chromatin around the periphery of the nucleus, and DNA fragmentation (1). Many stimuli can induce apoptosis, such as CED-3 and CED-9 have been shown to have mammalian homologs that are implicated in apoptosis (2). For example, BCL-2 is the human homolog of CED-9, and is well known as a suppressor of apoptosis in both species (3). CED-3 is the prototype for a family of cysteine proteases now termed caspasases that cleave substrates at the carboxy side of an aspartate residue and function to transduce a signal that leads to the degradation of the chromatin (4).

The enzyme responsible for the DNA digestion observed in apoptosis has yet to be conclusively identified. It has frequently been suggested that this endonuclease is Ca2+- and Mg2+-dependent (5–9). This laboratory initially attempted to purify the Ca2+/Mg2+-dependent endonuclease from CHO cells, but no such endonuclease was detected. However, an acid-activated endonuclease was found in these cells. This enzyme was purified and identified as deoxyribonuclease II (DNase II) (10). DNase II is active only at low pH, and studies performed consistently showed that all cells undergoing apoptosis also undergo intracellular acidification (11–14). Endonuclease activity was also studied in many different leukemic cell lines, and internucleosomal DNA digestion was stimulated at an acidic pH (15). Additional studies show that cells can undergo apoptosis after the chelation of Ca2+ hence obviating an essential role for Ca2+ in DNA digestion (16, 17). These studies all suggest that DNase II may be involved in apoptotic DNA digestion.

To better study the role of DNase II in the apoptotic process we have cloned its cDNA. To accomplish this, we purified bovine DNase II and obtained the NH2-terminal amino acid sequence, facilitating cloning of the bovine cDNA. We have subsequently cloned the complete human cDNA. Full-length cDNA for the human enzyme was subcloned into an expression vector and found to induce apoptosis when transfected into cells.

**EXPERIMENTAL PROCEDURES**

Protein Purification and Sequencing—Bovine spleen DNase II was purified on a heparin-agarose column followed by a S-Sepharose column. Fractions were assayed for digestion of plasmid DNA at pH 5 (10). Following the second column, the active fractions were pooled, concentrated, electrophoresed on a 10% polyacrylamide gel, transfected to a Probloth membrane (Applied Biosystems), and stained with 0.1% Coomassie Blue. A single 31-kDa protein was observed, excised from the membrane, and sequenced on an Applied Biosystems 473A automated sequencer.

Cloning and Characterizing DNase II cDNA—Polymerase chain reactions were performed with appropriate primers, and the products were cloned into the TA vector (Invitrogen). Plasmid DNA was sequenced with the PRISM Ready Reaction DyeDeoxy Terminator Cycle

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The abbreviations used are: CHO, Chinese hamster ovary; DNase, deoxyribonuclease; EST, expressed sequence tag; GFF, green fluorescent protein; xYAD-fmk, benzoyloxycarbonyl-Val-Ala-Asp-(O-methyl)-fluoromethylketone; bp, base pair(s); kb, kilobase; kbp, kilobase pair(s); CAD, caspase-activated DNase; ICAD, inhibitor of caspase-activated DNase.
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Sequencing kit followed by analysis on an Applied Biosystems 370 automated DNA sequencer. DNA probes were labeled with [32P]dCTP using the Random Primed DNA Labeling kit (Boehringer Mannheim) and used to screen cDNA libraries and mRNA. The Northern blots contained 20 μg each of total RNA from ML-1, HeLa, MCF7, MDA-MB-231, and T47D human cell lines.

Vector Construction and Transfection—The cloning strategy resulted in production of a Bluescript phagemid (Stratagene) that contained full-length DNase II. The insert was excised by digestion with Ncol and Xhol restriction endonucleases, electrophoresed on a 1% agarose gel, and purified using Geneclean II (Bio 101, Inc., Vista, CA). This fragment was ligated into the multiple cloning site of the pcDNA 3.0 vector (CLONTECH) that was first digested with the same enzymes and dephosphorylated with calf intestinal phosphatase (Boehringer Mannheim). The resulting plasmid was designated pDNase II.

The CHO cell line 5AHSmyc was maintained in a minimum essential medium with 5% fetal bovine serum plus antibiotics (18). CHO cells were cotransfected with 5 μg of either pcDNA 3.0 or pDNase II plasmids together with 1 μg of gS65T-C1 (CLONTECH) encoding the green fluorescent protein (GFP) using DOPSER Liposomal Transfection Reagent (Boehringer Mannheim). After 24–72 h, cells were incubated with 2 μg/ml Hoechst 33342 for 15 min, then scored for condensed chromatin and expression of GFP using fluorescent microscopy.

To confirm that overexpression of DNase II induces apoptosis downstream of caspases, the tripeptide inhibitor of caspases, zVAD-fmk (Enzyme Systems Products, Dublin, CA) was added at 10 μM to each sample, and the cells were then electrophoresed on a 12% polyacrylamide gel, transferred to Immobilon-P (Millipore), and probed with an antibody recognizing human D4-GDI.²

RESULTS

Cloning of Bovine DNase II cDNA—Bovine spleen DNase II was subjected to two column purification steps to yield an active protein that electrophoresed as a single band at 31 kDa. The NH₂-terminal amino acid sequence obtained from this protein was SSSRG HTKVQ LLLDQ EGGFW LIHSV PNFP. Degenerate oligonucleotide primers were designed to amino acids 4–10 and 15–21 and used to amplify a band of 53 bp to identical transfection experiments described above. As a marker of protein concentration, cells were also cotransfected with 1 μg of pHA-D4-GDI (also in pcDNA 3.0; gift from Dr. Dennis Danley, Groton, CT). After 48 h, cells were lysed in 2% SDS, 50 μM Tris, pH 6.8, 2 mM N-ethylmaleimide, 1 mM 1-(aminoethyl)benzenesulfonyl fluoride hydrochloride, 1 mM pepstatin A. Lysates were prepared on ice, sonicated to shear DNA, and frozen. An equal volume of loading buffer was added to each sample, and the cells were then electrophoresed on a 12% polyacrylamide gel, transferred to Immobilon-P (Millipore), and probed with an antibody recognizing human D4-GDI.²

Characterization of the Human DNase II Gene—The full-length cDNA was used as a probe for fluorescent in situ hybridization to human chromosome metaphase spreads. The probe was found to hybridize to chromosome 19p13.2 (data not shown) consistent with a previous report that DNase II activity was associated with chromosome 19 in somatic cell hybrid studies (22). These results were later confirmed by its presence in a 100 kbp contiguous sequence of human chromosome 19p13.2 deposited into the GenBank™ data base by Lawrence Livermore National Laboratories (Fig. 2). The sequence is identified as hypothetical human protein R31240_2. The cDNA is coded within a 7-kbp stretch of the genomic sequence containing 6 exons. The translated cDNA sequence predicted by Lawrence Livermore National Laboratories indicated a slightly different sequence than was observed in the cDNA clones obtained.

The 357-bp fragment was also used to probe total RNA from five human cell lines by Northern blotting. mRNA of approximately 2 kb was detected in the human myeloid cell line ML-1, and the human breast carcinoma lines MDA-MB-231, T47D, and MCF7 consistent with the size predicted by cloning the cDNA (Fig. 1). However, no signal was detected from the HeLa cervical carcinoma, a cell line that does not readily undergo DNA laddering during apoptosis (20, 21).

Dr. T. Y. Chang, Dartmouth). Two unique clones containing over 1.9 kb of sequence were found to contain identical open reading frames coding for 360 amino acids and a large 3’-untranslated region (GenBank™ accession number AF047016). There was high amino acid homology observed between bovine and human DNase II (72% identity, 82% similarity).

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The cDNA sequence obtained shows one base different from the genomic sequence; we observed a cytosine at position 507, whereas the genomic sequence showed a thymidine, although this does not change the amino acid at this site. A scan of three ESTs that span this region also showed a thymidine. However, a recent report of the DNase II cDNA also contained a cytosine (23), suggesting the possibility of a polymorphism at this site.

Homologs of DNase II were found in other species, including ESTs in mouse, Drosophila, and C. elegans. The only other proteins displaying significant homology to the human sequence were three genomic homologs identified in C. elegans, two located on chromosome III and the third on the X chromosome. The gene designated F09G8.2 appeared to have high homology to DNase II in additional sequences downstream of the termination site. This is probably due to a computer error in predicting the correct splice junctions; our suggested cDNA sequence utilizes two splice junctions that were identical to ones found in C07B5.5 (Fig. 3). DNase II shows 36% identity (53% similarity) with F09G8.2, 35% identity (56% similarity) with C07B5.5, and 30% identity (48% similarity) with K04H4.6. Some areas of extensive sequence identity are observed, including the putative active site of the enzyme. Another conserved area surrounds the aspartate residue that is known to be cleaved during apoptosis (24). CHO cells express no detectable D4-GDI, so we cotransfected these cells with pDNase II and a plasmid expressing human D4-GDI. A prominent 26-kDa band of full-length D4-GDI was detected (Fig. 5B). Upon transfection with pDNase II, a new 21-kDa cleaved band appeared, consistent with the activation of caspase 3. However, this band was also observed in cells transfected with a vector-only control showing that this cleavage is likely a result of toxicity of the transfection procedure rather than expression of DNase II. Addition of zVAD-fmk prevented this cleavage of D4-GDI, but did not prevent chromatin condensation. These results suggest that caspases can be activated as a consequence of the transfection procedure, but that other endonucleases that might be activated by caspases are not responsible for the chromatin condensation observed here.

DISCUSSION

Many different endonucleases have been proposed as candidates responsible for the internucleosomal cleavage of the genomic DNA observed during apoptosis. Originally, the apoptotic DNA degradation was attributed to a Ca$^{2+}$-activated endonuclease in thymocytes (5, 6, 25). Others have implicated various Ca$^{2+}$/Mg$^{2+}$-dependent endonucleases, including DNase I, Nuc18, and, more recently DNase γ, a DNase I homolog found in rat thymocyte nuclei (8, 9, 26–31). However, considerable evidence suggests that Ca$^{2+}$ is not required for DNA digestion (32). For example, depletion of Ca$^{2+}$ from cells is an effective means to induce internucleosomal DNA digestion (17).

This laboratory was the first to suggest that DNase II might be an alternate endonuclease involved in apoptosis (10). One criticism often made of the role of DNase II in apoptosis is the assumption that it is a lysosomal enzyme and not present in the nucleus, although we originally identified the acidic endonuclease activity in the nuclei of CHO cells. Other groups have also reported DNase II activity in the nucleus (33, 34), while others suggest that an acidic nuclease activity is translocated from the cytosolic compartment to the nucleus during apoptosis (15). Therefore DNase II appears to be present in the nucleus and more could be translocated from the cytosol during apoptosis.

Other reports have also implicated DNase II in the process of apoptosis. When lens fiber cells differentiate, they lose their
nuclei in a process that is very similar to apoptosis. The chromatin condenses and the cells degrade their genomic DNA. A nuclease present in fiber cells was found to be cation-independent, and the fragmented DNA had no 3'-hydroxy termini that would result from a DNase I-type nuclease (36). DNase II was found by immunocytochemistry to be localized in the cytoplasm but translocated to the nucleus of the fiber cell before degeneration (35). These findings suggest DNase II is the endonuclease responsible for the genomic degradation observed during lens nuclear degeneration.

We have observed intracellular acidification in numerous models of apoptosis, which is consistent with the involvement of DNase II (11–14). However, we have shown recently that low intracellular pH is not required for DNA digestion during apoptosis.
optosis and furthermore that low pH can actually suppress apoptosis by preventing activation of caspases (12, 37). It was therefore surprising that transfection of pDNase II induced apoptosis in cells grown under normal culture conditions, suggesting that this protein has activity at neutral pH. All previous analysis of the catalytic activity of DNase II has been performed with a 31-kDa protein, which we now show is truncated from the 40-kDa full-length form. It is possible that this larger protein has different catalytic requirements, and in particular, it may be active at neutral pH. Unfortunately, attempts so far to produce recombinant forms of this full-length protein have been compromised by the lethality of the constructs.

An alternate explanation for the chromatin condensation appearing after transfection with pDNase II is that another endonuclease is activated. The proteolytic cascade of apoptosis mediated by caspases is normally required for DNA digestion; this can be suppressed by the caspase inhibitor zVAD-fmk. In these studies, we showed that zVAD-fmk did not prevent chromatin condensation following transfection with pDNase II, suggesting that other endonucleases were not responsible. Accordingly, it appears that the chromatin condensation is a direct consequence of the action of DNase II.

Several caspases have been implicated in apoptosis, and numerous substrates have been reported. The caspase family of proteases cleave their substrates on the carboxy side of an aspartate residue. The coding sequence of DNase II contains a potential caspase cleavage site immediately upstream of the terminal serine in the 31-kDa bovine protein that was originally purified and sequenced. This indicated that the protein was produced as a larger precursor and subsequently modified to the acid-active 31-kDa protein by proteolytic cleavage at an aspartate residue. The cleavage site is ESQD in bovine and KSQD in human. Both of these potential recognition sequences have charged residues in the P4 position. The amino acid Lys at position P4 is not a known caspase cleavage site, but Glu at this position is found in a number of substrates of caspase 3 (38).

Lysosomal proteins are commonly cleaved after translocation to the lysosomes. The amino terminus of DNase II contains a stretch of hydrophobic amino acids which, by comparison with other lysosomal proteins, is likely to be a signal sequence. Cleavage of this signal sequence is postulated to follow the translocation of the protein to the lysosome, and is required for the lysosomal activity of the enzyme (39–41). Apart from caspases, the only other protease known to cleave at aspartic acid is a lymphocyte granule protease called granzyme or fragmentin (42). It is interesting...
that an earlier report of DNase II suggested it was a dimer of 35 and 10 kDa, which could represent the two fragments of the protein predicted here (19). However, the same report suggested both fragments were required for activity. During our purification over the S-Sepharose column, we observed a 10-kDa fragment that separated from DNase II but did not result in loss of DNase II activity (data not shown).

The gene for DNase II was originally assigned to chromosome 19 using somatic cell hybrid studies in which mouse cells with a human chromosome 19 displayed higher acidic endonuclease activity (22). The exact localization of this gene has been found to be chromosome 19p13.2. The genomic sequence analysis predicted one splicing junction of DNA digestion as exemplified by the conservation of two splice junctions in the cloned cDNA. This computer prediction is unlikely to be correct, since the ESTs found thus far confirm the cloned cDNA sequence. The human genomic sequence was compared with the genomic sequences of the three C. elegans homologs. There was significant conservation observed between human and the three C. elegans proteins. The 30–36% identity between DNase II and the three C. elegans homologs is comparable with the 35% identity between caspase 3 and CED-3 (43) and considerably greater than the 23% identity between BCL-2 and CED-9 (44). It is also interesting that no significant homology is observed above that Ca\(^{2+}\) concentration, the genomic structure, and homology of other species. The cloning of the cDNA will facilitate studies of its activity as well as establishing its biological functions and localization in cells. The transfection experiments presented here clearly show the potential of this enzyme to induce the DNA degradation observed during apoptosis. Future studies will be aimed to definitively establish any role in this process.

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