DNaseY: A Rat DNaseI-like Gene Coding for a Constitutively Expressed Chromatin-Bound Endonuclease‡

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ABSTRACT: A rat gene, designated DNaseY, encoding a 36 kDa endonuclease was identified and cloned. Sequence analysis of the cDNA showed it to be the rat homologue of human DNAS1L3. The DNaseY gene product had 42% identity to DNaseI, including conserved critical active site residues, the essential disulfide bridge, the calcium binding domain, and a signal peptide, as well as 2 of the 3 signature boxes. Significantly, DNaseY had 2 nuclear localization signals and was more basic (pI 9.5) than DNaseI (pI 4.8). The DNaseY gene contained a number of exons similar to that of DNaseI, separated by much larger introns, resulting in a gene of >17 kb compared to <4 kb gene of DNaseI. The 36 kDa DNaseY gene product was catalytically inactive but was converted to an active 33 kDa endonuclease following processing of the hydrophobic signal peptide. Antibody generated against peptides representing the predicted amino acid sequence of DNaseY cross-reacted with a 33 kDa nuclear protein which possessed endonucleolytic activity. The enzyme was active over a broad pH range (optimum pH 7−8), was Ca^{2+}/Mg^{2+}-dependent, was inhibited by Zn^{2+}, and was capable of both single- and double-stranded DNA cleavage, producing DNA fragments with 3′-OH ends. Furthermore, the DNaseY gene was expressed constitutively in all cells and tissues tested, but it was not transcriptionally up-regulated in apoptotic cells. All these features were consistent with a role in the early stages of apoptotic DNA fragmentation.

DNA fragmentation in apoptosis is primarily a two-stage process which probably involves more than one endonuclease (*1*−*8*). At the early stage of nuclear disassembly the chromatin is cleaved into large domains of 50−300 kilobase pairs (kb). This step is most likely catalyzed by an endonuclease that resides at the matrix attachment regions (MARs) where DNA binds to the nuclear scaffold (*9, 10*); hence, such an enzyme would be expected to be tightly associated with chromatin. The second stage involves more extensive DNA degradation and usually produces oligonucleosomal DNA fragments. As many as $(2-3) \times 10^6$ double-strand breaks are introduced into the DNA during the secondary stage of fragmentation, indicating a very substantial increase in the catalytic activity. This could be achieved either by activation of the same endonuclease with calcium ions or by the cooperation of additional calcium-requiring endonuclease activities. However, the second stage of DNA degradation is not required for cells to undergo apoptosis, and blocking of oligonucleosomal cleavage does not protect cells from death (*7, 11*−*13*). Therefore, only the high molecular weight stage of DNA fragmentation is considered to be essential for apoptosis.

In most cell types, DNA degradation in apoptosis occurs at or around neutral pH. Thus, endonuclease(s) involved in apoptosis would be expected to have a basal activity at neutral pH in the presence of magnesium ions and to be further activated by calcium ions. Zinc ions have been shown to be inhibitory, at least for the activities responsible for the secondary stages of DNA fragmentation. The fragment ends have, almost universally, been found to be 3′-OH and 5′-P. Many of these properties are found in DNaseI, and this enzyme has, indeed, been implicated in apoptosis (*1*). However, DNaseI is primarily a secretory protein, being released into the alimentary tract and bloodstream. In contrast, the enzyme responsible for the initial stages of DNA fragmentation would be expected to be localized in the nucleus and to be tightly associated with chromatin.

In this paper we describe the cloning and characterization of a DNaseI-related endonuclease, designated DNaseY. The DNaseY contained nuclear localization sequences directing it to the nucleus. The product of DNaseY, a 33 kDa active endonuclease, was isolated from nuclei of a variety of cells and tissues. The gene was constitutively expressed, and the protein was shown to be ubiquitously distributed. The enzyme had all of the properties required for the initial stages of DNA cleavage during apoptosis.

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EXPERIMENTAL PROCEDURES

Experimental Cell Systems. The following rat cell lines were used: hepatoma 5123tc (*14*); adrenal pheochromocytoma PC-12 (ATCC CRL 1722), liver epithelial T51B (*15*); and F111 and mT1 rat fibroblasts (*16, 17*). In addition, human teratocarcinoma NT-2 (Stratagene, San Diego, CA), prostatic carcinoma DU145 (ATCC HTB81), breast adenocarcinoma MCF7 (ATTC HTB 22), and acute T cell leukemia Jurkat cells (ATCC T1B152) were also used. The cells were propagated either according to the manufacturers' protocols or as previously described (*7, 18*).

Rat tissues were obtained from 5 week old, 150−200 g, male Sprague-Dawley rats bred in this Institute.

Apoptosis was induced either by addition of 10 μM VM26 (15 mM stock solution in ethanol) or by 800 rad of ionizing radiation (*19*).

Analyses of Genomic DNA. Bacteriophage clones harboring rat DNaseY genomic sequence were obtained by screening a rat λ FIX II genomic library (Stratagene, La Jolla, CA) using rat DNaseI cDNA as a probe. The screening procedure was as described previously (*20*). The DNA fragments containing portions of the rat DNaseY gene were sequenced in both directions from the double strand phage templates using a ABI 373A sequencer.

Northern Blotting. RNA was isolated according to Ausubel et al. (*21*) and poly A^+ RNA was purified using the PolyATrack mRNA system IV (Promega Corporation, Madison, WI). For Northern hybridization, 1 μg of poly A^+ RNA was separated on a 1.2% agarose/1.0 M formaldehyde gel and transferred onto a Nytran nylon membrane (Schleicher and Schuell Inc., Keene, NH). The membrane was UV cross-linked with a Stratalinker 2400 (Stratagene, La Jolla, CA), hybridized, and washed as previously described (*19, 22*).

RT-PCR and cDNA Sequence Analyses. Total RNA (5 μg) or poly A^+ RNA (1 μg) per 40 μL assay was reverse-transcribed by Superscript reverse transcriptase with 1 μg of oligo-dT primer (GIBCO-BRL, Bethesda, MD). The first-strand cDNA was purified by passing through a "Wizard PCR Preps" column (Promega Corporation, Madison, WI). After heat denaturation (5 min, 90 °C), 1 μL of the first-strand cDNA was used for PCR analysis. The primers used to detect DNaseY expression pattern were designed to avoid sequence similarities with DNaseI. They span 472 bp of the coding region of rat DNaseY cDNA and they were DNaseYF, 5′ AACTCACGAAGAAGCACGAC 3′, the sense primer; and DNaseYR, 5′ TGTCATAGGCACAGCTGGTG 3′, the antisense primer. The GAPDH control primers were 5′ GTGAAGGTCGGTGTCAACG 3′, the sense primer; and 5′ CATACTCAGCACCAGCATC 3′, the antisense primer. The PCR reaction was performed with one denaturation cycle at 94 °C for 1 min, followed by 35 cycles of 94 °C for 30 s, 60 °C for 1 min, and 72 °C for 50 s. In the last cycle the products were kept at 72 °C for an additional 5 min. One-fifth of each reaction was separated on a 2.5% Nuseive agarose gel, stained with ethidium bromide, and photographed. PCR products were purified from the agarose gel by Geneclean (Bio 101, Vista, CA), cloned into a TA cloning vector (Invitrogen, San Diego, CA), and sequenced in both directions using syntheticoligonucleotide primers and a ABI 373A sequencer. To obtain the missing 5′ end of the rat DNaseY transcript, we performed rapid amplifications of 5′ cDNA ends from a rat liver cDNA pool using a Marathon cDNA amplification kit (Clontech, Palo Alto, CA). The 3′ gene-specific antisense primer was DNaseYR3, 5′ CCACAGAGTTGACTATCTCTTG 3′, and the sense adaptor primer was provided by the manufacturer. The PCR product of expected size was purified as described above and cloned into a PUC18-TA vector which was prepared according to Ausubel et al. (*21*). The cDNA was sequenced as described above.

Recombinant DNaseY Protein. For in vitro transcription, translation, and the production of a GST fusion protein, the complete coding region of the DNaseY cDNA was obtained from rat liver mRNA by RT-PCR. The sense and antisense primers were adapted with a *Bam*HI and a *Eco*RI restriction site, respectively. The sense primer was DNaseYF1, 5′ TCTCTCGGATTCATGTCCCTGTACCCAGC 3′, and the antisense primer was DNaseYR1, 5′ TCTCTCGAATTCCTAGGAGCGACTGCCT 3′. The gel-purified PCR product was cloned downstream of the T7 promoter of the TA cloning vector for in vitro production of [^{35}S]-labeled protein. This cloned cDNA was transcribed and translated with the TNT T7 coupled reticulocyte lysate system (Promega Corporation, Madison, WI). Two and a half microliters of canine pancreatic microsomal membranes (Promega Corporation, Madison, WI) were added to the translation reaction when testing signal peptide function. For fusion protein production, the PCR product was digested with *Bam*HI and *Eco*RI and cloned into a pGEX-2T vector (Phamacia Biotech., Baie d′Urfe, PQ). The fusion protein was expressed in XL1-Blue cells and purified according to the manufacturer's protocol.

Extraction of a Chromatin-Bound Endonuclease. Cultured cells (10^8/preparation) were harvested and washed twice, and nuclei were isolated as previously described (*7*). The nuclei were washed four times with 10 mM Tris-HCl, pH 7.4, and 60 mM KCl, resuspended in a high salt extraction buffer (the same buffer containing 4.0 M NaCl), and sonicated for 20 s. Octyl-sepharose beads (2.0 mL, Pharmacia Biotech., Baie d′Urfe, PQ) were added to the nuclear extract, and the suspension was gently rotated at room temperature for 30 min. The beads were removed by centrifugation and discarded. The supernatant containing DNA and proteins that did not bind to the octyl-sepharose was dialyzed for 48 h at room temperature against 10 mM Tris-HCl buffer, pH 7.4, and supplemented with 5.0 mM $MgCl_2$, 1.0 mM $CaCl_2$, and 0.5 mM PMSF, followed by several hours against double-distilled water. The dialyzed fractions were freeze-dried and analyzed on a DNA-PAGE activity gel for the presence of endonuclease activity, as described below. For further purification of the p33 endonuclease protein, gel pieces corresponding to the active band were cut out, homogenized in 50 mM Tris-HCl, pH 7.4, 1.0% Triton X-100, and 0.5 mM PMSF, and incubated with rocking for 10 h at 37 °C. The extracted proteins were recovered and concentrated by acetone precipitation.

Nuclease Activity Gel Assay. Endonuclease activities present in various extracts were identified using a previously described DNA-PAGE activity assay (*7*). The nonradioactive gels were stained with ethidium bromide to visualize a band corresponding to active enzyme and were photographed on a UV transilluminator. The radioactive gels were dried

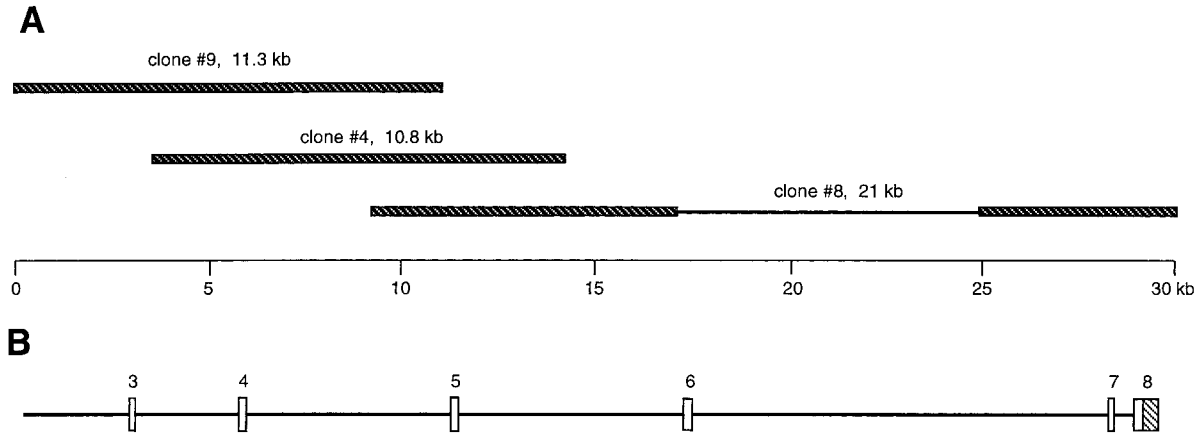

FIGURE 1: (A) The organization of a contig showing the three overlapping genomic clones. The hatched bars represent sequenced fragments. The solid line refers to an unsequenced region. The insert size of each clone is indicated above the fragment. (B) Physical map of the 17 kb genomic fragment showing the organization of the DNaseY exons. The coding exons are indicated by boxes. The hatched box represents the 3′ untranslated region of the last exon. The solid lines refer to introns.

and exposed to Kodak X-Omat film.

Two-Dimensional Gel Electrophoresis. Isoelectrofocusing (IEF) was performed using the Multiphor Electrophoresis unit (Pharmacia Biotech., Baie d'Urfe, PQ) with the experimental conditions suggested by the manufacturer. Briefly, samples of the gel-purified proteins were mixed with 240 μL of IEF loading buffer (8 M urea, 2%Triton X-100, 2% Pharmalytes pH range 3−10) and were absorbed onto Immobilin IEF strips pH 3−10 for 8 h. The proteins were subjected to overnight IEF at 15 °C for 20 000 Vh. After the IEF run was completed, the Immobilin strips were loaded onto 10% SDS−PAGE copolymerized with 30 μg/mL of sheared single-stranded salmon testis DNA and the proteins were separated in the second dimension. The endonucleolytic activity was reactivated and visualized as described above.

Plasmid DNA Digestion Assay. One microgram of plasmid DNA was added to the gel-purified p33 protein (approximately 0.5 μg/assay) and resuspended in 25 μL of 50 mM Tris-HCl buffer, pH 7.4, 5 mM $MgCl_2$, and 1 mM $CaCl_2$. The reaction mixture was incubated for 1 h at 37 °C, and the integrity of the plasmid DNA was analyzed by electrophoresis on a 0.8% agarose gel run in 40 mM Tris-acetate buffer, pH 8.5, and 2 mM EDTA at 20 V overnight. The gels were stained with ethidium bromide.

Labeling of Terminal Hydroxyl Ends of DNA. Digested plasmid DNA was radioactively labeled using either terminal deoxynucleotidyl transferase (TdT) or T4 polynucleotide kinase. The TdT labeling reaction was performed for 1 h at 37 °C in 50 μL of reaction buffer containing 25 mM Tris-HCl buffer, pH 6.6, 200 mM potassium cacodylate, 5 mM $CoCl_2$, 0.5 mM DTT, 0.25 mg/mL BSA, 5 μCi of α-[^{32}P] dATP (NEN Dupont, Oakville, ON), and 60 units of TdT (Gibco BRL, Burlington, ON). The T4 kinase labeling was performed at 37 °C for 1 h in 50 μL of reaction buffer containing 50 mM glycine−NaOH buffer, pH 9.2, 5 mM DTT, 10 mM $MgCl_2$, 10 mM Na_2HPO_4, pH 9.2, 5 μCi of γ-[^{32}P] ATP, and 50 units of T4 kinase (New England BioLabs, Mississauga, ON). The labeled DNA was purified using a G-50 spin column (Pharmacia Biotech, Baie d′Urfe, PQ) and analyzed on 0.8% agarose gels as described above. The gels were dried and exposed to Kodak X-Omat film.

Antibody Production and Immunoprecipitation. The predicted amino acid sequence of DNaseY was scanned for likely antigenic sites using the Jameson and Wolfe algorithm (*23*). Three peptide sequences corresponding to amino acids 32−40, 73−81, and 297−305 were identified and custom synthesized by Research Genetics Inc (Huntsville, AL). The same company produced rabbit polyclonal antibodies. The anti-DNaseY antibody was affinity-purified by binding to and stripping from nitrocellulose filters containing immobilized chromatin-bound p33 nuclease. Immunoprecipitation was carried out for 2 h at room temperature in 500 μL of 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, and 0.1% Triton X-100 containing approximately 10 μg of gel-purified p33 protein, the affinity-purified anti-DNase Y antibody (the amount equivalent to 1:500 dilution of the original serum), and 20 μL of Dynabeads coated with goat-anti-rabbit IgG (Dynal AS, Oslo, Norway). The beads were isolated and washed three times with the same buffer, and the bead-bound complexes were assayed by the DNA-PAGE.

Western Blotting. Proteins were separated by 10% SDS−PAGE (Minigel system, BioRad, Mississauga, ON) and were electrotransferred onto a Hybond-C nitrocellulose membrane (Amersham, Oakville, ON). The membranes were blocked with 5% nonfat milk powder in 20 mM Tris-HCl buffer, pH 7.4, and 200 mM NaCl (TBS), washed in 0.1% (v/v) Tween-20 in TBS, and incubated for 1 h either with anti-nuclease antibodies (dilution 1:1000) or with preimmune serum (1:1000) followed by incubation with appropriate secondary antibodies conjugated to alkaline phosphatase. The alkaline phosphatase-conjugated goat anti-rabbit IgG (Promega Corporation, Madison, WI) was used at a 1:5000 dilution. Blots were washed and developed by colorimetric reaction using BICP/NBT substrate (KPL, Gaithersburg, MA).

Immunofluorescence Staining. Cells grown on poly-L-lysine-coated glass coverslips were fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton X-100, and blocked with 2% donkey serum. Coverslips were then incubated either with anti-nuclease antibodies (1:300 dilution) or with preimmune serum at the same dilution, followed by incubation with FITC-conjugated anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA). Cells were counter-stained for 1.5 min with Hoechst 33258 dye (1 μg/mL in PBS) and photographed under an Olympus Bmax Microscope.

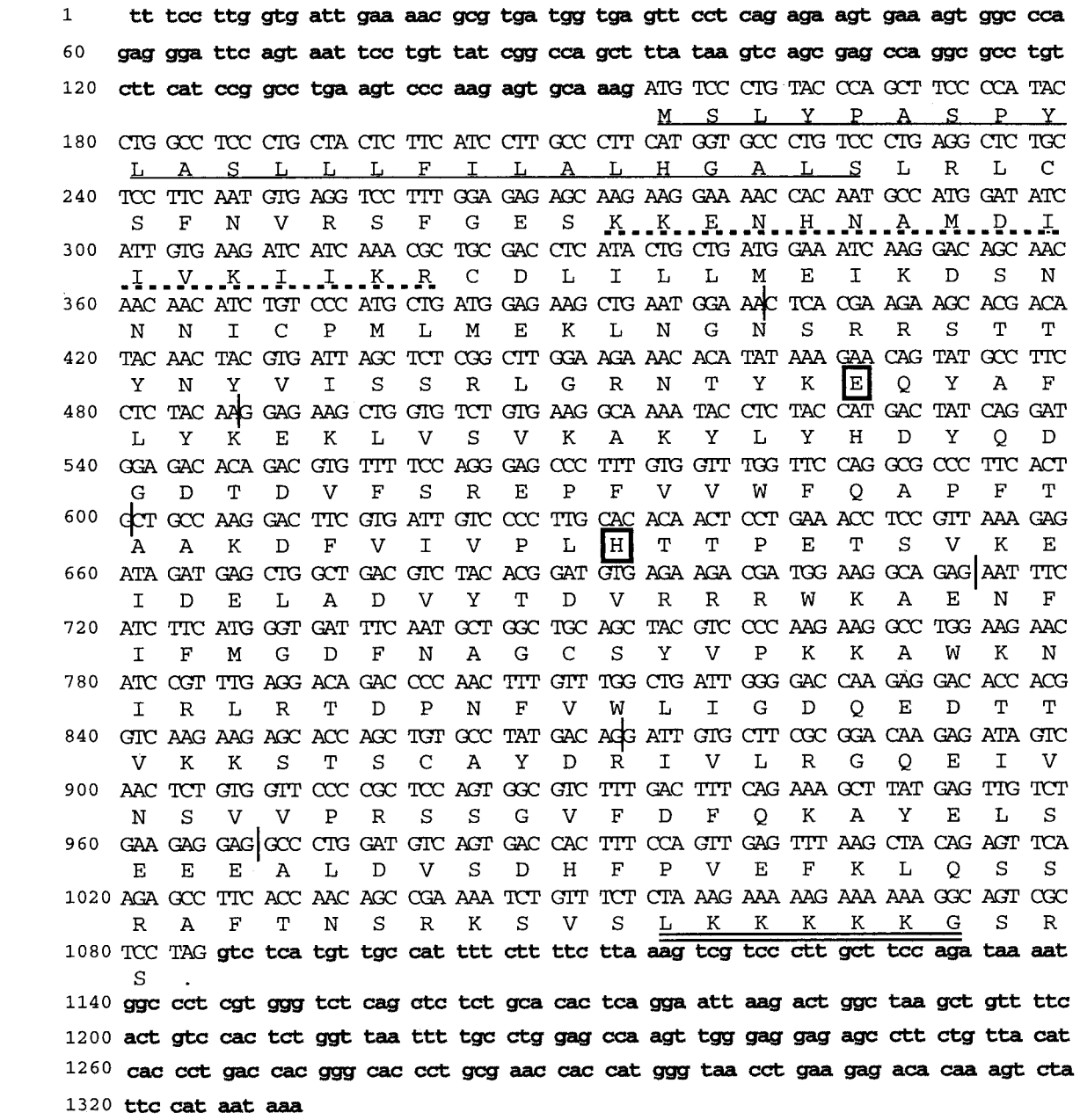

FIGURE 2: Nucleotide sequence of cDNA and deduced amino acid sequence of the encoded protein. Numbers in the left margin refer to nucleotide positions, and numbers in the right margin refer to amino acid residues. The signal peptide is underlined. The dotted underline indicates the bipartite nuclear targeting sequence. The active site amino acids are indicated by boxes. The SV40-type NLS is double underlined. The vertical bars represent exon boundaries.

RESULTS

Isolation and Sequence Analysis of The DNaseY Gene. Since candidate apoptotic endonucleases share some characteristics of DNaseI, sequence homology with the DNaseI gene might be expected. Therefore, we screened a rat genomic library using the rat DNaseI cDNA as a probe under low stringency conditions. With this method we identified three clones giving weaker signals than the ones harboring bona fide DNaseI fragments (*20*). Restriction mapping showed that these three overlapping genomic fragments constituted an approximately 30 kb contig (Figure 1A). Sequence analysis and database searches of the sequenced contig revealed that it contained a gene encoding a protein with high sequence similarity to a human DNaseI homologue (DNAS1L3) cDNA (*5*). We designated this gene as DNaseY. Sequence comparison of our genomic contig with the DNAS1L3 cDNA and the rat DNaseI cDNA, as well as analysis of the intron/exon boundaries, enabled us to identify the last 6 coding exons in the gene spanning nearly 17 kb (Figure 1B). The 5′ coding region which likely contained the first two exons, previously identified in its human counterpart DNAS1L3 (*5*), was not found in the contig. DNaseY had a number of exons similar to that of DNaseI, but contained long introns which made the gene more than four times longer than the DNaseI gene (less than 4 kb, ref *20*).

On the basis of the sequence information from the contig, we designed an internal gene-specific primer to obtain the missing 5′ coding region. Ligation-mediated RT-PCR was performed with rat liver poly A^+ RNA by adding, prior to PCR, an adaptor to the 5′ end of the first cDNA strand (Marathon cDNA amplification). The sense primer was

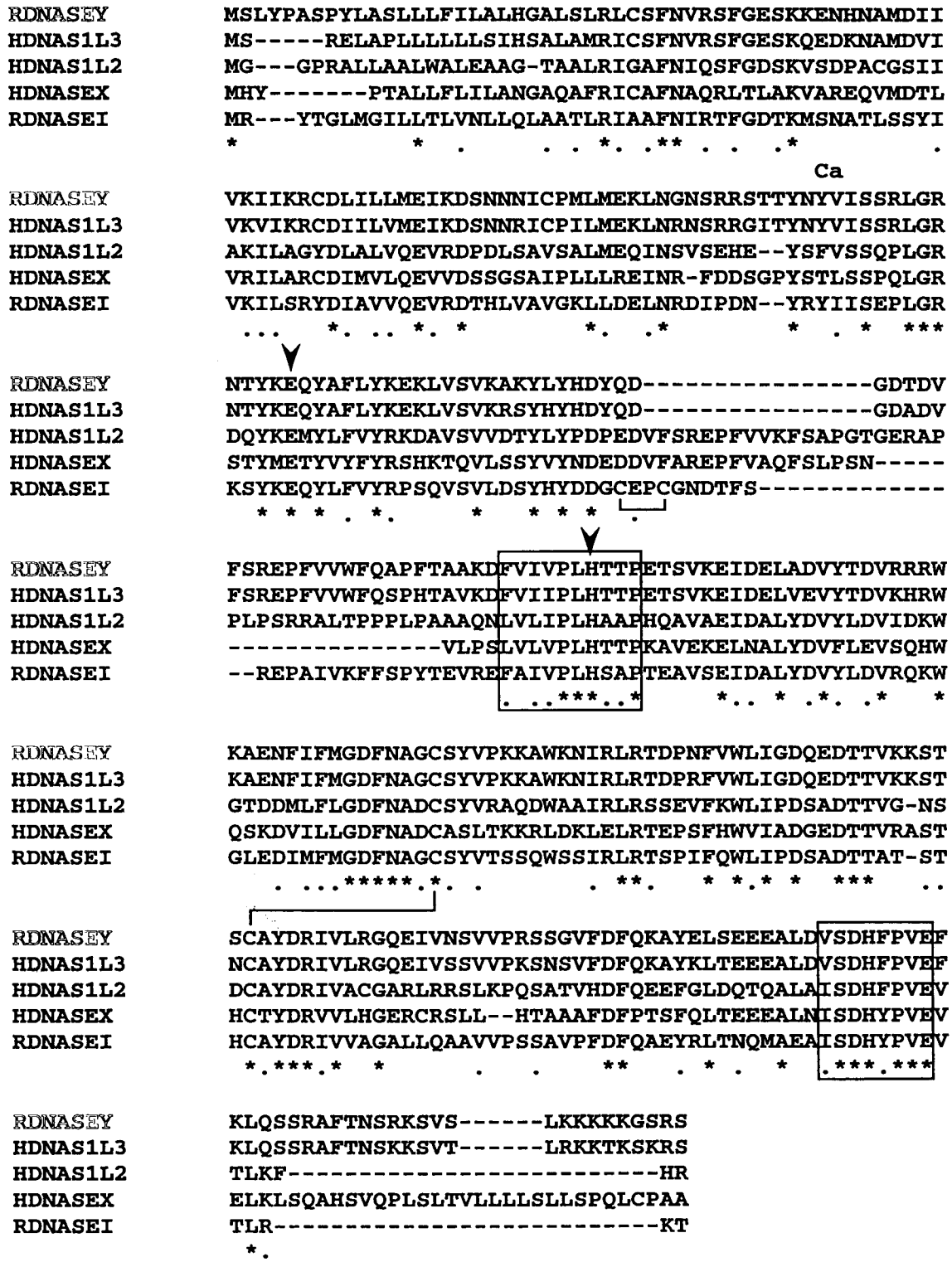

FIGURE 3: Comparison of rat DNaseY with known human and rat DNaseI-like proteins. The alignment was done using the ClustalV program: conserved residues (*), conserved substitutions (·), calcium binding site in DNaseI (Ca). Highly conserved regions characteristic of DNaseI are boxed. Arrowheads indicate active site residues. Cystine residues that are involved in disulfide bridge formation are also indicated: HDNAS1L3, human DNaseI-like gene 3; HDNAS1L2, human DNaseI-like gene 2; HDNASEX, human DNaseX.

complementary to the adaptor, and antisense gene-specific primer was as described in the Experimental Procedures. The PCR products which appeared as a short smear on an agarose gel were cloned and sequenced. Among the 4 clones obtained, the longest fragment gave a 909 bp cDNA sequence that extended 398 bp on the 5′ end of the first exon located at the beginning of the genomic contig (exon 3 shown in Figure 1B), and overlapped 511 bp with the existing cDNA sequence inferred from the genomic contig. From the beginning of this cDNA clone to the polyadenylation signal on the last exon of the contig, the combined cDNA sequence was 1331 bp in length. It contained a single open reading frame (ORF) encoding a protein of 310 amino acids with a calculated molecular mass of 35 685 Da (Figure 2). This ORF was preceded by 152 bp of 5′ leader sequence and was followed by 246 bp of 3′ untranslated region.

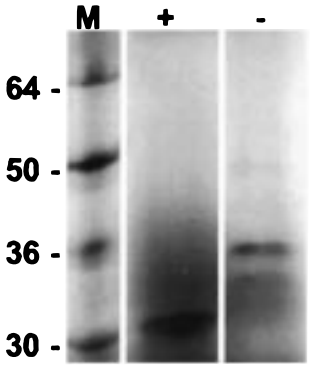

FIGURE 4: In vitro transcription and translation of recombinant DNaseY cDNA: lane M, standard prestained molecular size markers (Helixx Technologies Inc, Scarborough, ON); lane +, translation of DNaseY with microsomes; lane −, translation of DNaseY without microsomes.

Analysis of the amino acid sequence revealed that this protein had a typical eukaryotic signal peptide of 25 amino acids which includes a charged N terminus, a hydrophobic core, and a probable cleavage site "Ala-Leu-Ser" (*24*). Cleavage of this signal peptide would produce a 33 kDa mature protein. The sequence also contained two nuclear localization signals (NLS), one of which was a bipartite nuclear targeting sequence, starting 14 amino acids after the signal peptide cleavage site, and the other one was located at the C-terminal end of the protein, strongly resembling the SV40-type of NLS (*25*).

Alignment of the DNaseY sequence with all known rat and human DNaseI-like proteins is shown in Figure 3. It revealed that rat DNaseY is homologous to the human DNAS1L3 protein with 82.6% sequence identity and 10% conserved substitutions. In addition, both DNaseY and DNAS1L3 have exceptionally high isoelectric points (pI) with pH values of 9.46 and 9.78, respectively. The pI values of all other known DNaseI-like proteins are near or lower than 5. Comparison of DNaseY with rat DNaseI showed 42% sequence identity and 19% conserved substitutions. The active site residues (glutamic acid 105 and histidine 160) and the disulfide bridge essential for DNase activity are absolutely conserved in all five proteins. Two out of the three signature boxes of DNaseI and the signal peptide were also found in all sequences. However, the first disulfide bridge and the signature sequence surrounding it were no longer conserved in these proteins. Interestingly, the calcium-binding site is only conserved in DNaseI, human DNAS1L3, and the rat DNaseY.

In Vitro and In Vivo Characterization of DNaseY Protein. To establish whether the first 25 residues of the DNaseY gene product do function as a signal peptide, the full-length DNaseY cDNA was produced by RT-PCR and cloned into a TA vector containing a T7 promoter. The purified plasmids were transcribed and translated in a rabbit reticulocyte lysate system with or without microsomal membranes (Figure 4). Translation without microsomes gave a major protein band of 36 kDa, and inclusion of microsomes in the translation mixture yielded a protein of 33 kDa, indicating that the 25 amino acid sequence was cleaved during the translocation process. Since the full-length DNaseY gene product was catalytically inactive (data not shown), we tested whether processing would activate the enzyme. After digestion of microsomal membranes with proteinase K, we detected a strong nuclease activity inside the microsomes. However, we were unable to discriminate the activity corresponding to the DNaseY from contaminant endonucleolytic activities in the commercial preparations of microsomes (data not shown).

To further characterize the DNaseY protein, a rabbit polyclonal antibody was prepared. On a Western blot this antibody recognized the DNaseY-GST fusion product (Figure 5A, lanes 1 and 2) but did not cross-react with DNaseI protein (lane 3). In addition, the antibody recognized a protein band of molecular mass approximately 33 kDa that was isolated from the nuclei of rat 5123tc cells (lane 4). The same antibody cross-reacted with a 33 kDa protein band in all rodent and human cells tested, including 5123tc, Jurkat, and NT2 cells (Figure 5B, lanes 2−4, respectively). Endonuclease activity colocalized in the same region of the gel (Figure 5C, lanes 1−6). The activity band was detected both by autoradiography using [^{32}P]-labeled DNA as substrate (Figure 5C, lane 1) as well as by ethidium bromide staining of nonradioactive DNA (lanes 2−6). In separate experiments the anti-DNaseY antibody was able to immunoprecipitate the active p33 protein (Figure 5D).

The antibody was also used to establish the intracellular localization of the protein in 5123tc cells (Figure 6). Whereas the preimmune serum gave negligible background staining (Figure 6C), the anti-DNaseY antibody, under identical experimental conditions, reacted specifically with the nuclei of the cells (Figure 6F). A diffuse fluorescence staining pattern, indicative of immunoreactivity, was seen throughout the nucleoplasm, and no staining was observed in the nucleoli (Figure 6F).

The isoelectric point (pI) of the p33 endonuclease was also established using a combination of isoelectrofocusing in the first dimension and DNA-PAGE in the second dimension (Figure 7). Following renaturation and reactivation of the enzyme, a single activity spot corresponding to a 33 kDa protein was detected. It was calculated that pI of the enzyme was close to pH 9.5, identical to that calculated for the DNaseY gene product.

Biochemical Properties of DNaseY. The cation requirements for enzymatic activity of DNaseY are depicted in Figure 8A. The data showed that although the strongest in vitro activity was observed when both Mg^{2+} and Ca^{2+} ions were present in the assay, the DNA cleaving activity was also supported by either 1 mM $MnCl_2$ or $CoCl_2$ and was inhibited by 1 mM $ZnCl_2$ even in the presence of Ca^{2+} and Mg^{2+} (Figure 8A). It was active over a broad pH range, from 5.5 to 9.0, with the highest activity being observed in the neutral range, between pH 7 and 8 (Figure 8B). The enzyme was completely inactive in the presence of EDTA, indicating its absolute requirement for Mg^{2+} (Figure 8C). In the presence of EGTA and Mg^{2+} the enzyme could cleave both single-stranded (Figure 8A and B) as well as circular double-stranded DNA (Figure 8C). It should be noted that the additional band seen in panels A (Ca/Mg lane) and B (pH 7.4) does not represent enzymatic activity but a DNA-binding protein that quenched ethidium bromide, since it was not detected using radioactive substrate.

To determine the nature of cleaved DNA ends, digested plasmid DNA was end-labeled by either TdT or T4 kinase in the presence of radioactive nucleotides (Figure 8D). The

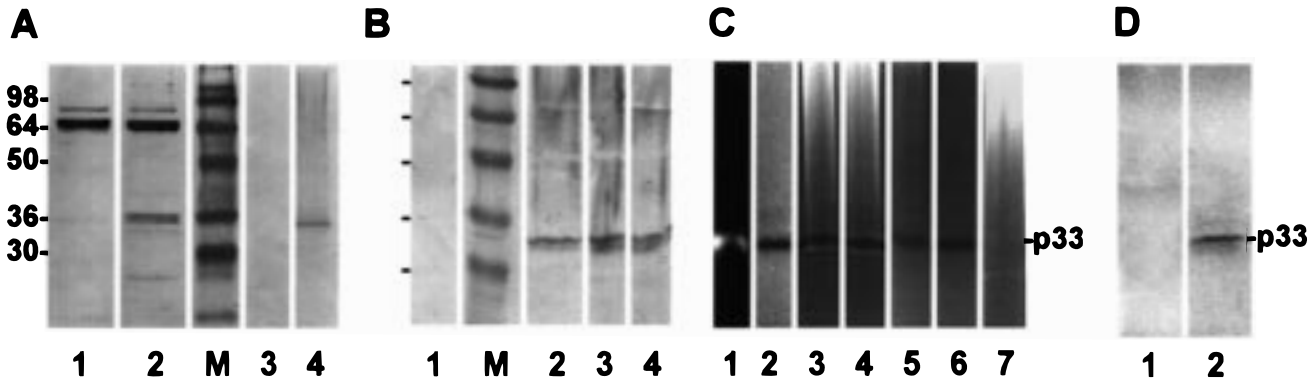

FIGURE 5: Identification of the DNaseY gene product. (A and B) Western blotting. Approximately 20 μg of proteins was separated on 10% SDS−PAGE, electrotransferred onto a nitrocellulose membrane, and immunoblotted with anti-DNaseY serum, followed by the detection with alkaline phosphatase-conjugated secondary antibody. (A) In lane 1, DNaseY-GST fusion protein; lane 2, DNaseY-GST fusion protein after digestion with thrombin; lane M, molecular size markers; lane 3, purified DNaseI protein; lane 4, purified p33 endonuclease from 5123tc cells. (B) Nuclear proteins isolated from lanes 1 and 2, 5123tc; lane 3, Jurkat cells; and lane 4, NT2 cells; were immunoblotted with either preimmune serum (lane 1) or with anti-DNaseY antibody (lanes 2−4). (C) Detection of endonuclease by the activity gel assay. Approximately 10 μg of nuclear protein/lane was prepared as described in the Experimental Procedures: in lane 1, Jurkat cells; lane 2, 5123tc; lane 3, T51B; lane 4, NT2 cells; lane 5, thymus; and lanes 6 and 7, liver tissue. A sample from liver was also prepared in the presence of 5 mM EDTA instead of Mg^{2+}/Ca^{2+} (lane 7). The radioactive gel (lane 1) was dried and autoradiographed on Kodak X-Omat film for 24 h at −70 °C. The nonradioactive gels (lanes 2−7) were stained with ethidium bromide and photographed on a UV transilluminator. (D)Immunoprecipitation of p33 endonuclease with anti-DNaseY antibody. Approximately 1 μg of gel purified p33 endonuclease was used for either a mock precipitation (lane 1) or immunoprecipitation with the affinity-purified anti-DNaseY antibody (lane 2). The immunoprecipitates were analyzed using a nonradioactive activity gel assay.

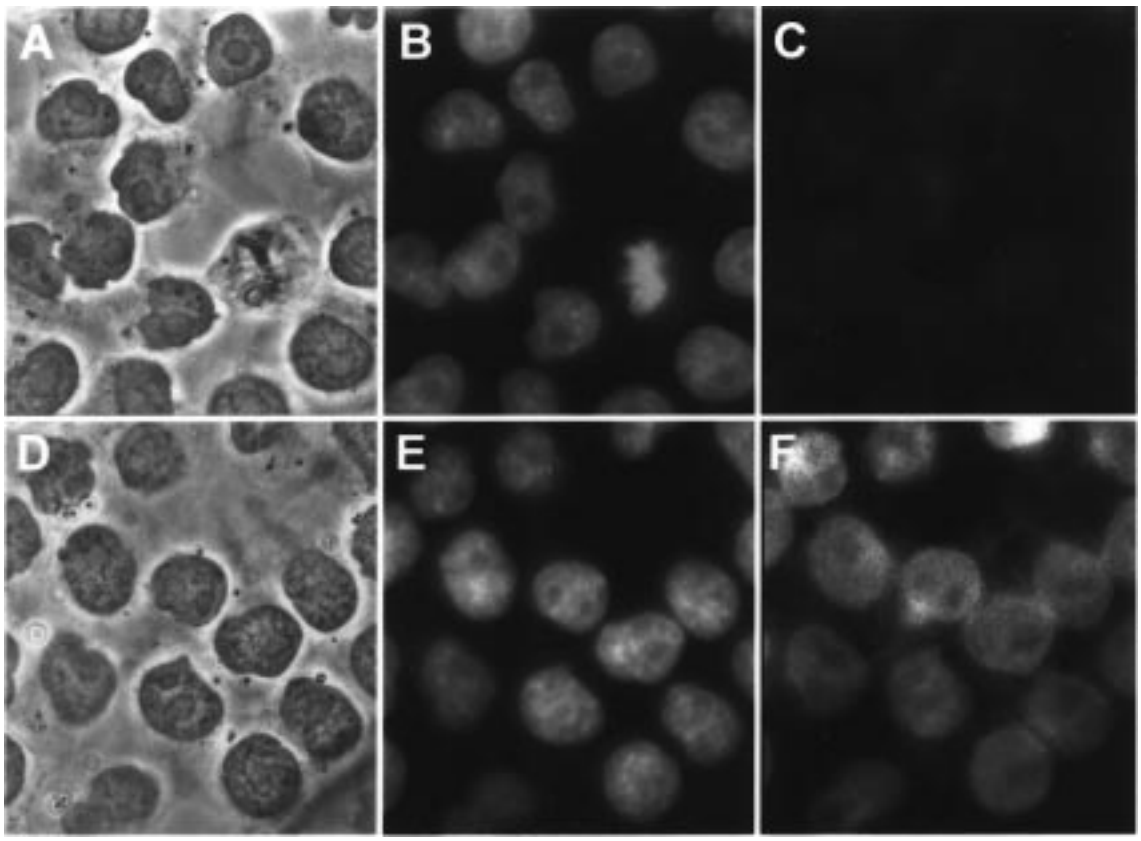

FIGURE 6: Immunofluorescence staining of 5123tc cells with anti-DNaseY antibody. Cells were grown on coverslips, fixed with paraformaldehyde, permeabilized, and stained with either preimmune serum (A, B, and C) or anti-DNaseY antibody (D, E, and F). FITC-conjugated anti-rabbit IgG was used to detect the specific immunostaining (C and F). Nuclei were counterstained with Hoechst 33258 dye (B and E). The slides were photographed with an Olympus Bmax fluorescence microscope using phase contrast (A and D) and epifluorescence (B,C, E, and F): magnification, 800X.

results indicated that at neutral pH and in the presence of both Ca^{2+} and Mg^{2+} the p33 endonuclease produced DNA fragments with only 3′-OH ends, as labeling was seen only in the TdT reaction and not in the T4 kinase reaction which would have labeled 5′-OH termini.

The Expression Pattern of the DNaseY Gene. The distribution of DNaseY mRNA was assessed by RT-PCR of poly A^+ RNA from various mammalian tissues and cell lines (Figure 9A). A single band of 472 bp product was detected in all the tissues and cell lines tested, indicating

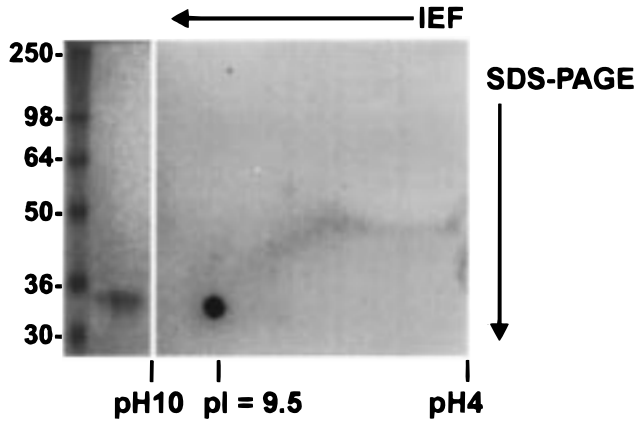

FIGURE 7: Isoelectrofocusing of p33 endonuclease. The endonucleolytic activity was reactivated and visualized as described in the Experimental Procedures. The isoelectric point of the endonuclease activity spot was calculated as suggested by the manufacturer. A single-dimensional DNA-substrate gel was run under identical conditions to indicate the position of the p33 endonuclease activity band (left-hand panel).

that this gene is ubiquitously expressed. We subsequently cloned and sequenced the single PCR band from rat liver to ensure that the product was indeed specific for the DNaseY transcript.

Since the DNaseY protein had biochemical properties consistent with those of an apoptotic endonuclease, one might expect to detect its upregulation during apoptosis. We tested the expression of DNaseY in VM26-treated rat F111 and mT1 fibroblasts (Figure 9B). The cells were exposed to 10 μM teniposide for 48 h, and it was established, on the basis of trypan blue viability assay, that this treatment killed 15% of F111 and 40% of mT1 cells (data not shown). The expression of DNaseY in VM26-treated F111 cells was slightly higher (lane 2) than in control untreated cells (lane 1); however, the same treatment of mT1 cells did not alter DNaseY expression (Figure 9B, lanes 3 and 4), even though these cells were more sensitive to the VM26 treatment. Furthermore, the expression of DNaseY was also tested in rat thymocytes induced to undergo apoptosis by 800 rad of ionizing radiation (Figure 9C). Messenger RNA isolated at different time points after irradiation was subjected to Northern hybridization with the DNaseY and α-tubulin probes. As shown in Figure 9C the expression of both DNaseY and α-tubulin genes actually decreased within 4 h following irradiation. It was shown previously that this treatment kills 100% of thymocytes within 12 h (*19*).

DISCUSSION

We have cloned and sequenced a rat endonuclease gene, DNaseY, which was highly homologous to human DNAS1L3 (*5*). The amino acid sequence of DNaseY also showed 42% homology with the DNaseI protein, primarily in the amino acids coding for the catalytic and regulatory sites. The DNaseY gene contained a signal peptide, conserved active site residues, the important disulfide bridge, and the Ca^{2+}-binding domain, suggesting that DNaseY would have a cationic dependency and an overall mechanism of action similar to that of DNaseI (*1*). The nonconserved regions of DNaseY contained a high proportion of basic residues and dual NLSs capable of directing the protein into the nucleus and bringing about its association with chromatin. The C-terminal NLS was shown to be functional since it was capable of directing the nuclear uptake of albumin (data not shown). DNaseI, on the other hand, is a secretory protein and has not been shown to be present in the nucleus (*26*).

The antibody raised against antigenic peptides from the predicted amino acid sequence of DNaseY specifically cross-reacted with a 33 kDa nuclear protein that possessed an endonucleolytic activity. This enzyme was present in a variety of mammalian cells and rat tissues (Figure 5). The antibody, however, did not react with DNaseI protein. In addition, the isoelectric point of the DNaseY, calculated to be at pH 9.5, was very different from the acidic range (pH 3.9−4.3) of DNaseI (*27*). On the other hand, the molecular mass and the isoelectric point of the p33 nuclease (Figure 7) matched perfectly with that of the DNaseY gene product. Thus, the biochemically purified p33 endonuclease and the DNaseY gene product were identical and distinct from DNaseI.

Biochemical studies revealed that the p33 enzyme, which was tightly associated with chromatin, had biochemical properties similar to those of DNaseI. Thus, like DNaseI, the p33 enzyme was found to be capable of cleaving both single- and double-stranded DNA in the activity gel and plasmid digestion assays (Figure 8). It required both Ca^{2+} and Mg^{2+} for full activity, although it was active in the presence of Mg^{2+}, Co^{2+}, or Mn^{2+} alone, and like DNaseI, it was fully inhibited by Zn^{2+} (Figure 8). Furthermore, at neutral pH, it produced DNA fragments with 3′-OH ends which are typically observed in apoptotic cells and which form the basis of the widely used TUNEL assay (*28*−*30*).

The cDNA coding for DNaseY had 42% sequence identity with DNaseI and a similar cleavable N-terminal signal peptide. The DNaseY gene had the same number of exons, but the gene was much larger than DNaseI due to interruption by long introns. This suggested that DNaseY may have arisen from an early gene duplication of DNaseI or a DNaseI-like gene and later evolved and adopted distinct functions. The presence of both a signal peptide, which usually determines ER processing and export from the cell, and a nuclear localization signal in the same protein is unusual. The N-terminal hydrophobic signal peptide of DNaseY was shown to be cleaved in the microsomes, and the mature 33 kDa active DNaseY was smaller than the 36 kDa pre-protein (Figure 4). This suggested that the protein must pass through the ER membranes before being imported into the nucleus. It also implied that after the signal peptide was cleaved the mature protein must be released back to the cytoplasm and then transported to the nucleus through the nuclear pore. A precedent of a protein containing both a functional N-terminal hydrophobic signal peptide and a NLS is the Hepatitis B virus precore protein (*31*). In this case, after removal of the signal peptide in the ER, 70% of the mature protein is released back to the cytoplasm and is then translocated to the nucleus. This nuclear targeting is due to the presence of the NLS in the viral protein (*31*). The complicated translocation process of the Hepatitis B virus precore protein and DNaseY is likely to be less efficient, and the two NLSs of DNaseY may improve this efficiency. It has been reported that multiple NLSs, indeed, contribute to the efficient and quick transport of a protein into the nucleus (*32*).

The endonuclease activities responsible for DNA fragmentation during apoptosis are generally considered to reside

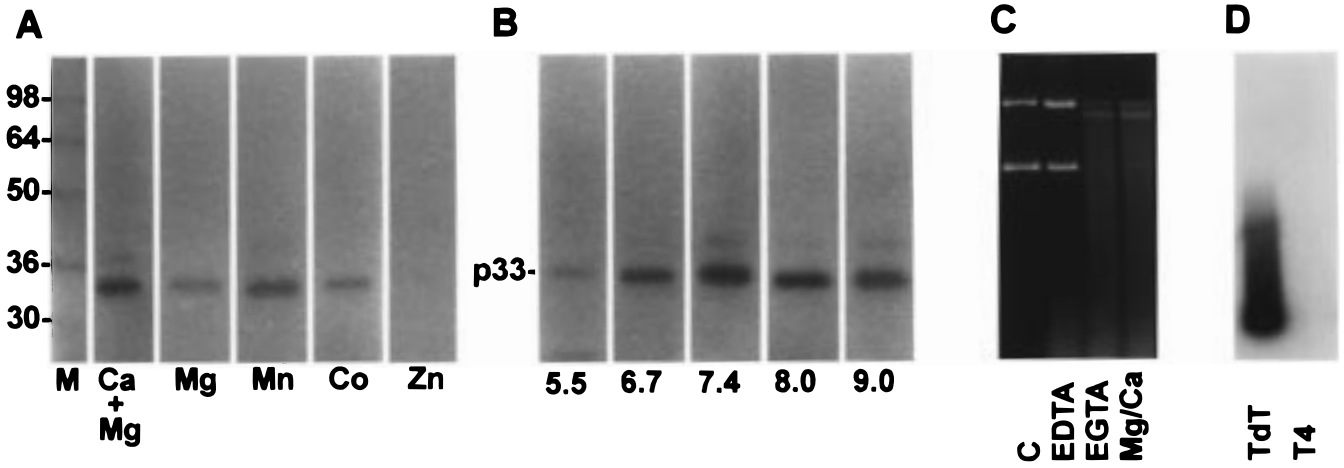

FIGURE 8: Biochemical properties of p33 endonuclease. (A) Cationic requirements. Approximately 0.5 μg/lane of gel-purified protein from 5123tc cells was analyzed by the activity gel assay in the presence of 5 mM $MgCl_2$ and 2 mM $CaCl_2$ (lane Ca/Mg), 5 mM $MgCl_2$ and 2 mM EGTA (lane Mg), 1 mM $MnCl_2$ (lane Mn), 1 mM $CoCl_2$ (lane Co), or 1 mM $ZnCl_2$ in the presence of 5 mM $MgCl_2$ and 2 mM $CaCl_2$ (lane Zn). (B) The pH profile. The enzymatic activity of p33 endonuclease (0.5 μg/lane gel-purified protein from 5123tc cells) was assessed by the activity gel assay following incubation with either 100 mM sodium acetate buffer, pH 5.5, or 50 mM Tris-HCl buffers of pH 6.7, 7.4, 8.0, and 9.0, as indicated under the lanes. All reaction buffers contained 5 mM $MgCl_2$ and 2 mM $CaCl_2$. (C) Plasmid DNA digestion by p33 endonuclease. One microgram of plasmid DNA and approximately 0.5 μg of gel-purified p33 endonuclease were incubated for 1 h in 25 μL of 10 mM Tris-HCl buffer, pH 7.4, containing 2 mM EDTA, but no divalent cations (lane EDTA), 5 mM $MgCl_2$ and 2 mM EGTA (lane EGTA), or 5 mM $MgCl_2$ and 1 mM $CaCl_2$ (lane Mg/Ca). Plasmid DNA incubated without the enzyme is shown in lane C. DNA was resolved on an agarose gel, stained with ethidium bromide. (D) Detection of free 3′-hydroxyl ends generated by p33 endonuclease. Plasmid DNA digestion was carried out in the presence of Mg^{2+} and Ca^{2+} as described above, and the digested DNA was end-labeled using radioactive nucleotides and either terminal deoxynucleotidyl transferase (lane TdT) or T4 polynucleotide kinase (lane T4). The samples were subjected to agarose gel electrophoresis; the gels were dried and autoradiographed as described in the Experimental Procedures.

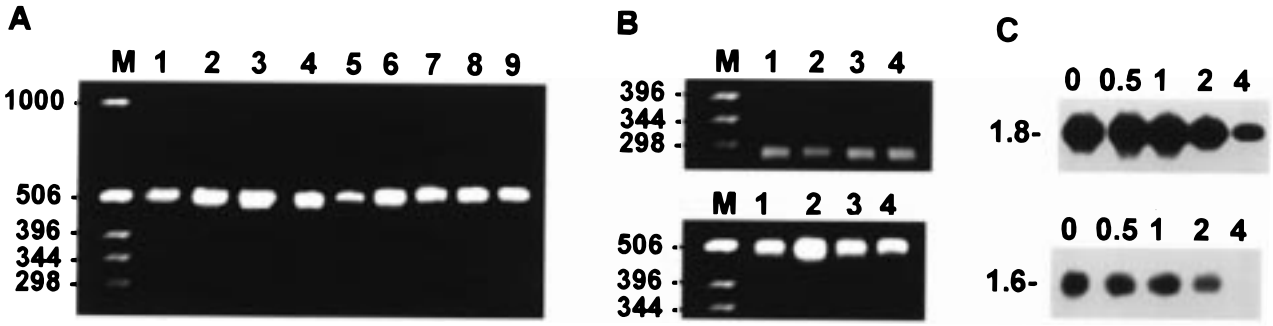

FIGURE 9: The expression pattern of the DNaseY gene. (A) Ethidium bromide stained agarose gel of RT-PCR products from various mammalian tissues and cell lines: lane M, molecular size markers; lane 1, rat brain; lane 2, rat liver; lane 3, rat thymus; lane 4, PC12; lane 5, 5123tc; lane 6, DU145; lane 7, Jurkat; lane 8, MCF7; and lane 9, NT2 cells. (B) RT-PCR products of GAPDH (top panel) and DNaseY (bottom panel) from control and VM26 treated rat fibroblasts: lane 1, F111 fibroblasts; lane 2, F111 cells 48 h after VM26 treatment; lane 3, mT1 cells; lane 4, mT1 cells 48 h after VM26 treatment. (C) Northern analysis of DNaseY expression in irradiated thymocytes. Poly A^+ RNA (1 μg/lane) was prepared from thymocytes at different times after irradiation. The numbers on top of each lane indicate the hours after treatment. Radiolabeled α-tubulin (top panel) and the DNaseY (bottom panel) cDNAs were used as probes. The α-tubulin blot was exposed for 15 h and the DNaseY 7 days.

constitutively in the nucleus, since nuclei isolated from normal tissues can reproduce all of the aspects of DNA fragmentation seen during apoptosis. The enzyme responsible for the initial high molecular weight fragmentation stage appears to be tightly bound to chromatin, whereas the activity responsible for internucleosomal DNA fragmentation is more loosely bound (*7*). The DNaseY gene was found to be expressed in all cells and tissues tested, and the endonuclease appears to be produced constitutively. Therefore, it is unlikely that during apoptosis this gene is regulated at the level of transcription. In fact, its mRNA was degraded in apoptotic thymocytes in parallel to other genes such as α-tubulin (Figure 9C). Furthermore, given that there were no cleavage sites characteristic of the Caspase family of proteins (*33*, *34*), the cleavage of the signal peptide was sufficient to generate an active endonuclease. No additional processing of the enzyme seems necessary as proposed for DNaseX (*4*) since smaller fragments were also not seen. It is likely, therefore, that if it plays a role in apoptosis its regulation is via post-translational modification of pre-existing protein.

In summary, DNaseY seemed to possess all the requirements of an apoptotic endonuclease. It was constitutively expressed in a variety of cultured cells (of rat and human origin) and in tissues. It was localized in the nucleus where it was strongly associated with chromatin, and to our knowledge, it is the first endonuclease shown to contain NLS sequences. This enzyme was active in the presence of Mg^{2+} alone and was capable of generating single-strand DNA breaks, which have been shown to occur at the onset of DNA fragmentation in apoptosis (*35*).

The activation mechanism of p33/DNaseY during apoptosis is currently being studied in our lab. However, it is clear that since the enzyme was present constitutively in

nuclei, it must be prevented from gaining access to DNA and therefore must undergo some modification(s) to modulate its endonuclease activity in normal cells. Alternatively, changes in the chromatin structure itself, due to proteolysis of chromatin-associated proteins, may render DNA accessible to the endonuclease at early stages of apoptosis. Degradation of nuclear matrix and DNA-associated proteins has been observed at early stages of apoptosis of thymocytes and Jurkat cells (ref 36, Sikorska et al., unpublished experiments), and this may support the latter hypothesis.

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