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Genomic Organisation and Expression of Mouse Deoxyribonuclease I

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Deoxyribonuclease I (DNase I) has recently been implicated in cell death by apoptosis, a process which is frequently accompanied by chromatin DNA degradation. Despite extensive studies on DNase I, its genomic organisation remained unknown. Here we report for the first time on the intron-exon structure of the DNase I gene. The coding region of mouse DNase I is composed of eight introns and eight exons, spanning 2315 base pairs. The deduced protein sequence is 91.5% identical to its rat counterpart, but does not carry the two mutations (Glu13 to D and V67 to I) responsible for the decrease in actin-binding of rat DNase I. The enzymatic activity of mouse DNase I is found in striated muscle, kidney, intestine, liver, lymphnodes, but not in the heart, spleen or pancreas.

Deoxyribonuclease I (DNase I, E.C. 3.1.21.1.) first isolated and purified from bovine pancreas in crystalline form by Kunitz (1), is an endonuclease that hydrolyses double stranded DNA down to 5'-phospho- (tri -and/or tetra-) oligonucleotides. Its pH-optimum is around pH 7.5 and for its full enzymatic activity it requires divalent cations: Ca^{2+} and Mg^{2+} or Mn^{2+} in micromolar concentrations. Its endonucleolytic activity is inhibited by chelating agents, Zn^{2+} and actin (2). The mode of action and the specificity of the enzyme depend on the nature of the divalent cations present: in the presence of Ca^{2+} and Mg^{2+} it causes single-strand cuts of duplex DNA, while double-strand breaks in purified DNA are produced in the presence of Mn^{2+} (3,4). Although DNase I does not exhibit base- or sequence specificity, it preferentially cleaves at the 5'-end of pyrimidines and bonds with a high degree of local twist (5).

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The primary structure of bovine pancreatic DNase I was described by Liao *et al.* (6). Subsequent structural analysis to 2 Å resolution led to the correction of the sequence (4). DNase I is composed of a single polypeptide chain of 260 residues and a carbohydrate moiety of the high mannose type linked to Asn18. Isoforms of DNase I from the same species can differ in the length (normally 8 hexose units) and the composition of the attached glycan. Human urine, for instance, contains eight glycosylation isoforms of DNase I, as revealed by isoelectric focusing (7). DNase I contains two disulphide bridges between Cys98 to Cys101 and Cys170 to Cys206. Reduction of the latter (essential) disulphide bridge leads to the loss of enzymatic activity. Two tightly bound ($K_d = 10^{-5}$ M) structural Ca^{2+}-ions protect DNase I against proteolytic degradation at the C-terminal end and the essential disulphide bridge from reduction (8). More recently, the primary structures of pig (9), sheep (10), human (11) and rat (12) DNase I have been described, while cDNA sequences are only available for human (11) and rat DNase I (12).

Besides its role in the digestion of alimentary DNA, DNase I has recently gained much attention in connection with apoptosis. Cell death by apoptosis is involved in a large variety of developmental events and physiological processes requiring a reduction in cell count (13). Nuclear collapse, one of the first visible changes denoting irreversible commitment to cell death by apoptosis, is frequently accompanied by chromatin degradation into nucleosome-sized fragments and multiples thereof. Among other enzymes (14), DNase I has been proposed to be involved in this process (15,16). Here we report of the cDNA sequence, genomic organisation and the distribution of mouse DNase I enzyme activity.

MATERIAL AND METHODS

Polymerase chain reaction (PCR):

Oligonucleotides corresponding to the N- and C-terminus of rat DNase I including EcoR I sites were synthesised. The following primers were used : $^{5'}$-CGG AAT TCC GAC CAT GCT GAG AAT TGC AGC CTT CAA CA (N-terminus) and CGG AAT TCC GTC ATC TGA GTG TCA CCT CCA CTG GGT AAT G-$^{3'}$(C-terminus). First strand synthesis of mouse kidney RNA was done using poly-T primers using the cDNA cycle kit (Invitrogen). 1 µl of the resulting mixture was added to 50 µl Tris-HCl pH 8.3, containing 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTPs and 1µM of each oligonucleotide and was denatured for 5 min at 95°C. After addition of 2.5 units Taq polymerase (Amplitaq , Perkin Elmer-Cetus), and PCR was carried out for 30 cycles (95°C for 1 min; 50°C for 1 min; 72°C for 1 min) followed by a final elongation step of 7 min at 72°C. The PCR product was cut with EcoR I, purified by agarose gel electrophoresis (GeneCleanII, B101) and subcloned into the Bluescript SK- vector (Stratagene).

Screening of a mouse heart cDNA library:

A commercially available mouse heart λ-ZAP-II cDNA library (Stratagene, Heidelberg) was screened in duplicate using the rat cDNA coding for DNase I (12) and the mouse kidney PCR amplification product. Recombinant plaques (3×10^5) with 10^4 plaques per 15 cm plate were analysed. Plaques were lifted onto nitrocellulose filters, denatured in 0.5 M NaOH/1.5 M NaCl, and neutralised in 0.5 M Tris-HCl pH 7.0, containing 1.5 M NaCl.

After drying, the filters were baked for 2 hrs at 80C in a vacuum oven. cDNA radiolabling was performed using the random priming cDNA labelling kit (Boehringer, Mannheim) in the presence of α-[^{32}P]CTP. Hybridisation of nitrocellulose filters was performed as described elsewhere (17). Double positive colonies were rescreened with the same probes until no negative plaque was detected.

DNA preparation, analysis of cloned DNA and sequencing:

Plasmid and bacteriophage λ DNA were purified by standard techniques (18). Restriction enzyme digestions were performed under conditions recommended by the manufacturer. Sequencing of both PCR products and cloned DNase I cDNA was performed according to the dideoxy chain-termination method (19) using the Sequenase Kit (US Biochemicals). The given sequences have been determined by sequencing both strands.

DNA zymography:

DNA zymography was carried out as previously described (20). Fresh mouse tissues were extracted by disrupting 1 gr tissue in 2 ml of 50 mM Tris-HCl , pH 7.5 containing 0.1 % Nonidet P-40 in a Dounce homogeniser at 4°C. 15 μg protein were then separated by SDS-PAGE in the presence of immobilised double-stranded DNA (10 μg/ml gel). The removal of SDS from the gel after electrophoresis allows the renaturation of DNase I and was performed in 25 mM Tris-HCl pH 7.5, containing 2 mM of both $CaCl_2$ and $MgCl_2$ and 5% non-fat powdered milk (5 changes within 5 hrs). Digestions were carried out over night at room temperature in the same buffer. The gels were then immersed for 10 min in water containing 10 μg/ml ethidium bromide and photographed on a UV transiluminator.

Sequence analysis:

Nucleic acids and protein sequence analysis was performed with the BLAST (21), FASTA (22) and the GCG package (23). The release 28 of the SwissProt database was used as a source for the other mammalian DNase I sequences (24).

RESULTS AND DISCUSSION

Mouse deoxyribonuclease I cDNA and genomic sequences:

The cDNA for mouse deoxyribonuclease I (DNase I) was amplified from mouse kidney cDNA using polymerase chain reaction (PCR) as described in Material and Methods. An amplification product of 799 bp was obtained and used for the screening of a commercial mouse heart cDNA library already available in the laboratory. Seven clones (later determined to be identical), reactive with both the rat DNase I cDNA and the mouse PCR amplification product were isolated and analysed by nucleotide sequencing. Surprisingly, the sequence of the isolated heart cDNA was 2667 bases long (more than twice the length of the rat cDNA for DNase I), and showed intermittent but highly significant (BLAST unlikelyhood probability of $3.1E^{-185}$, 21) similarity with both rat DNase I cDNA sequence and the mouse PCR amplification product. The alignment of these three sequences allowed the identification of eight introns, the first of which ends two bases before the coding region (Fig. 1). Although we screened a heart cDNA library, we may have cloned an unspliced nuclear RNA, or alternatively a fragment of chromatin DNA contaminating the library. In order to assess the presence of all introns interrupting the coding region in the cloned cDNA, we performed PCR amplifications of mouse genomic DNA and our cDNA clone (as described in

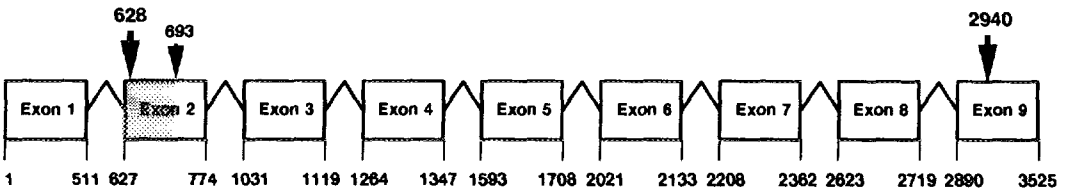

Figure 1. The intron-exon structure of mouse DNase I (Genbank entry U00478). Numbers below the boxes symbolising the nine exons of the mouse DNase I gene indicate the first and last base of the exon. The grey area of exon two represents the region coding for the signal peptide. The first and last bases of the coding region are shown by large arrows.

Material and Methods). This experiment yielded PCR products of approximately 2200 bp (as judged by agarose gel electrophoresis) from both DNA samples. We then compared the band patterns obtained by digesting both amplification products with a variety of restriction enzymes including Hinf I, Xba I, Sty I, Pvu II, Nco I, EcoR V and Pst I (Fig. 2 shows data for Hinf I and Pvu II). Since all restriction patterns were identical for both PCR products, we concluded that our cDNA clone contains all introns interrupting the coding region. The nucleotide sequence was thus submitted to Genbank by direct submission (Authorin, which can be obtained via anonymous ftp from ncbi.nlm.nih.gov) and received the accession code: U00478. The coding region of mouse DNase I was translated into the corresponding amino acid sequence and aligned with all other known mammalian DNase I protein sequences (Fig. 3). The pairwise levels of identities were calculated and are reported in Table I.

In 1974, Lazarides and Lindberg (2) identified actin as the naturally-occurring inhibitor of DNase I. DNase I is able to depolymerise F-actin (25,26) leading to the formation of stable 1:1 complexes of actin and DNase I. In this complex DNase I activity is inhibited (about 95 % inhibition at equimolar ratio), and the actin is unable to repolymerise even in the presence of high salt and other actin binding proteins known to induce actin polymerisation. The binding constant of skeletal muscle actin to bovine pancreatic DNase I

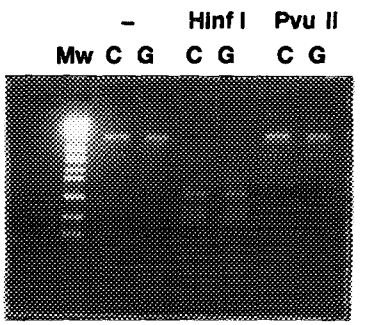

Figure 2. Polymerase chain reaction and restriction digest of mouse DNase I. The cloned cDNA for mouse DNase I (C) and genomic mouse DNA (G) were amplified by PCR as described in Material and Methods. Both amplification products were then digested with Hinf I and Pvu II. The resulting digestion products were separated by 1.8 % agarose gel electrophoresis. The molecular weight markers (Mw) indicate from bottom to top the relative migration of 0.37 to 8.1 kbp.

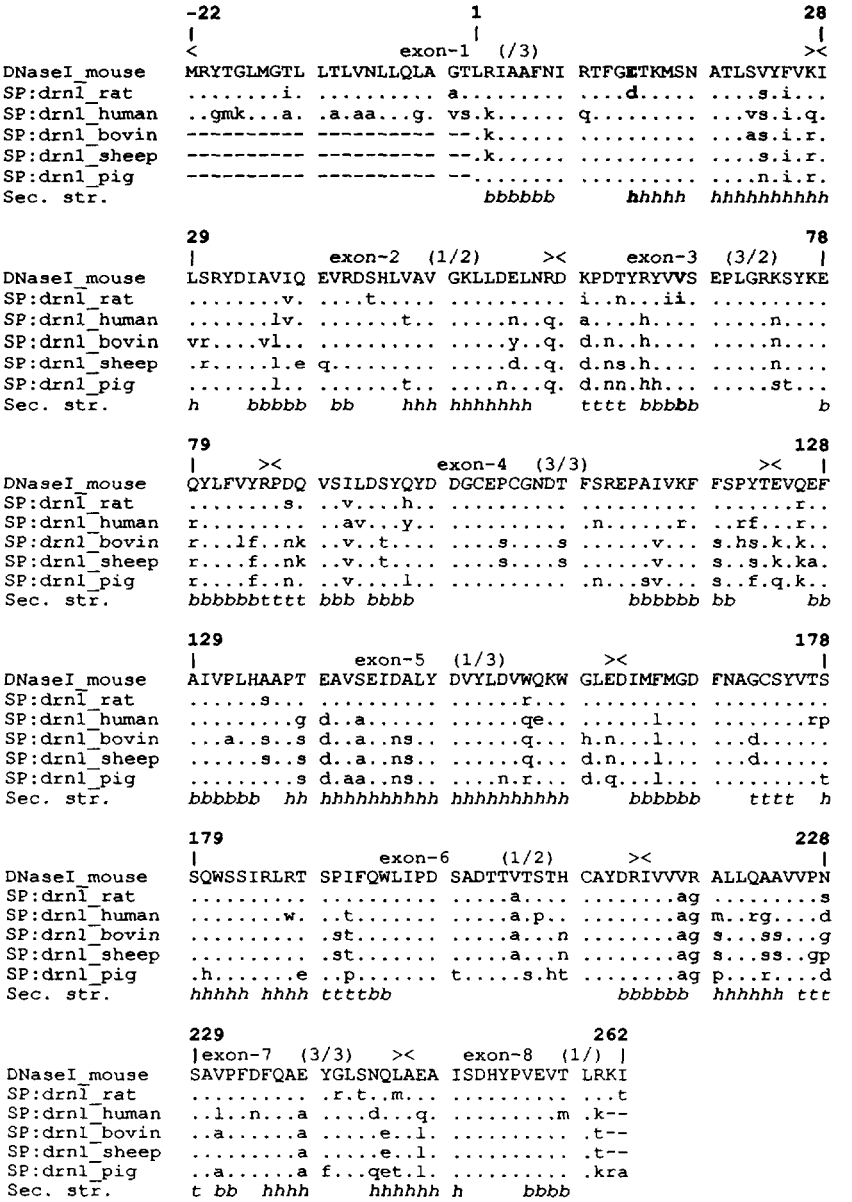

Figure 3. Alignment of the amino acid sequences of the known mammalian DNase I molecules. Residues differing from the mouse sequence are shown in lower case, while identities are displayed by dots. The secondary structure was deduced from the crystal structure of DNase I (Brookhaven database entry 1ATN) and is shown on the last line of the alignment (*h*: α-helix; *b*: β-strand; *t*: turn). The exon boundaries are shown above the sequence alignment and the position within the codon of the first and last base of the exon are indicated between brackets. Residues implicated in actin binding are shown in boldface (Glu-13 and Val-67). The SwissProt identification codes are shown for each DNase I in the database (release 28, March 1994). The amino acid sequence of mouse DNase I was deduced from the Genbank entry U00478.

was determined to be $K_a=10^9$ M^{-1} (27). However, the rat parotid DNase I binds to G-actin with an affinity lower by three orders of magnitude (28). and is therefore not able to depolymerise F-actin. The change in affinity was later attributed to the following amino acid

Table I

Relative degree of homology among all known DNase I sequences

Species	Mouse	Rat	Human	Bovine	Sheep	Pig
Mouse	100 (284)	91.5(283)	78.7(282)	77.6(259)	78.8(259)	77.4(261)
Rat		100(284)	77.7(282)	77.2(259)	78.8(259)	76.0(262)
Human			100(282)	78.4(259)	77.6(259)	77.3(260)
Bovine				100(260)	93.8(260)	80.7(259)
Sheep					100(260)	81.9(259)
Pig						100(262)

The six known DNase I protein sequences were aligned pairwise with LFASTA and their degrees of sequence identity determined (expressed in percent). The number of residues spanned by the identical region is given in parentheses; mouse, rat and human DNase I sequences included the signal peptide. Database accession codes: mouse, Genbank:U00478; rat, SWISS-PROT:drn1_rat; human, SWISS-PROT:drn1_human; bovine, SWISS-PROT:drn1_bovin; sheep, SWISS-PROT:drn1_sheep; pig, SWISS-PROT:drn1_pig.

changes: Glu-13 to Asp and Val-67 to Ile (12); these changes remain unique to rat DNase I, since mouse DNase I (its closest homologue) does not carry these mutations (Fig. 3).

Distribution of deoxyribonuclease I enzymatic activity in mouse tissues:

The distribution of mouse DNase I was analysed by DNA zymography and largely confirmed the distribution previously observed for the rat enzyme (29). Large amounts of DNase I are expressed in the kidney, the only organ positive in a Northern blot analysis involving total RNA (data not shown). Striated muscle, intestine and lymphnodes produce significant amounts of DNase I, while its activity is not detectable in heart, spleen and pancreas. The liver produces low amount of DNase I, which is just detectable under the experimental conditions used (Fig. 4). As positive controls, we used 300 picog of bovine pancreatic DNase I. Furthermore, we found human seminal plasma to contain large amounts of the endonucleolytic activity, which is most probably due to DNase I since it co-migrates with the bovine and mouse enzyme on SDS-PAGE (Fig. 4) and can be completely inhibited by rabbit muscle actin (data not shown). This finding is in contrast with the notion that seminal plasma contains a Ca^{2+}, Mg^{2+}-dependent endonuclease distinct from DNase I (30). Interestingly, mouse kidney, intestine and human seminal plasma contain at least two glycosylation isoforms distinguishable by their molecular weights, whereas lymph node and liver contain mainly the larger molecular weight species, and the striated muscle the lighter isoform (which corresponds to the bovine pancreatic molecular weight). The presence of DNase I mRNA has previously been reported for mouse lymphnodes and thymus (15) by PCR amplification.

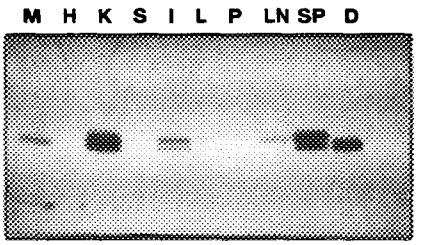

Figure 4. DNA zymography of mouse tissue extracts. 15 µg protein extract of striated muscle (M), heart (H), kidney (K), spleen (S), intestine (I), liver (L), pancreas (P), lymph nodes (LN) and human seminal plasma (SP) were analysed by DNA zymography. 300 picog bovine pancreas DNase I (D) was used as a control. The bands indicate the location of DNase I enzymatic activity.

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