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Molecular, Biochemical and Immunological Analyses of Canine Pancreatic DNase I

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The DNase I from canine pancreas was purified 260-fold to electrophoretic homogeneity with a 35% yield using three-step column chromatography. The activity of the purified enzyme was completely inhibited by 20 mM EDTA, an antibody specific to the purified enzyme and G-actin. A 1,373-bp cDNA encoding canine DNase I was constructed from the total canine pancreatic RNA using a rapid amplification of cDNA ends method, followed by sequencing. The mature canine DNase I protein was found to consist of 262 amino acids. A survey of DNase I in 13 different canine tissues revealed the highest levels of both DNase I enzyme activity and gene expression in the pancreas; therefore, the canine DNase I is of the pancreatic type. Phylogenetic and sequence identity analyses, studies of immunological properties and the tissue-distribution patterns of DNase I indicated that the canine enzyme is more closely related to the human DNase I than to other mammalian DNases I. Therefore, canine DNase I is found to be one of the best substitutes in studies of human DNase I.

Key words: DNase I, dog, molecular cloning, pancreas, purification.

Abbreviations: RACE, rapid amplification of cDNA ends; RT, reverse transcriptase; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SRED, single radial enzyme diffusion.

Deoxyribonuclease I (DNase I, EC 3.1.21.1) is an enzyme that preferentially attacks double-stranded DNA by Ca^{2+}- and Mg^{2+}-dependent endonucleolytic cleavage to produce oligonucleotides with 5′-phospho- and 3′-hydroxy-termini (*1*). It is generally considered to be a digestive enzyme, since, in mammals, it is secreted mainly from the pancreas, parotid glands or both (*2*). Recently, a report by Napirei *et al*. that a lack of, or decrease in, DNase I activity might be a critical factor in the initiation of human systemic lupus erythematosus has focused attention on the potential physiological role(s) of this enzyme (*3*). Yasutomo *et al*. described two patients with a heterozygous nonsense mutation in exon 2 of *DNASE1*, decreased DNase I activity and an extremely high titer of immunoglobulin G against nucleosomal antigens (*4*). We have previously demonstrated that human DNase I exhibits genetic polymorphism that is controlled by at least six alleles on chromosome 16p13.3 (*5–9*). DNase I polymorphism has been reported to be associated with diseases of the digestive system (*10*) and with gastric and colorectal carcinoma (*11*, *12*).

Dogs are commonly used as a nonrodent laboratory animal for medical and drug-safety experiments since they are similar to humans in many respects (*13*). In this context, information on canine DNase I may allow us to elucidate the potential physiological and etiological role(s) of the enzyme in humans. However, the biochemical, enzymatic and molecular-genetic properties of canine DNase I remain unknown. In this paper, we describe a novel purification procedure for canine DNase I and describe its biochemical and immunological similarities to human DNase I, as well as its cDNA structure and gene expression.

MATERIALS AND METHODS

Materials and Biological Samples—Phenyl Sepharose CL-4B and Superdex 75 were purchased from Amersham Biosciences (Tokyo). Concanavalin A (Con A)–agarose was obtained from Seikagaku Kogyo (Tokyo), and rabbit muscle G-actin from Sigma (St. Louis, MO, USA). Superscript II RNase H^- reverse transcriptase (RT) and 5′-and 3′-RACE kits were obtained from Life Technologies (Rockville, MD, USA). DNase I was purified from human (*14*) and mouse (*15*) urine samples, from porcine (*16*) and *Xenopus laevis* (*17*) pancreas, and from carp (*18*) hepatopancreas according to previously described methods. All other chemicals used were of reagent grade and were available commercially. Male purebred Beagle dogs (*Canis familiaris*, 12–15 months old, weight 10–12 kg) and rabbits (weight 5 kg) were obtained from SEASCO, Saitama. Dogs and rabbits were acquired, maintained and used in accordance with the Guidelines for the Care and Use of Laboratory Animals (NIH, USA; revised 1985).

Analytical Methods—DNase I activity was determined by either a test tube method (*14*) or the single radial enzyme diffusion (SRED) method (*19*, *20*), except that 0.1 M MES buffer, pH 6.75, containing 20 mM $MgCl_2$ and 2 mM $CaCl_2$ was substituted for the reaction buffer

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described in the previous reports. Briefly, in the former method, salmon testis DNA is digested with DNase I and soluble DNA fragments are then measured at 260 nm after precipitation with ethanol. The latter method is based on the radial diffusion of DNase I in a thin agarose gel plate containing salmon testis DNA; ethidium bromide forms a circular dark zone under UV light in proportion to the amount of DNase I added. Protein concentrations were measured using a protein assay kit (Bio-Rad, Hercules, CA, USA) with bovine serum albumin as the standard. The enzymatic and chemical properties of, inhibitory effects of G-actin on, and tissue distributions of the canine DNase I enzyme were examined as described previously (*21*). The purified enzyme was analyzed by 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (*22*). The electrophoresed proteins were stained with silver or anti-canine DNase I antibody, according to previously described methods (*14*). Activity staining for DNase I was performed using a DNA cast-PAGE method (*23*). DNase I, when electrophoresed in PAG containing salmon testis DNA and then incubated in an appropriate buffer containing ethidium bromide, forms a dark band under UV light. For localization analysis, samples of 13 different tissues were obtained from four dogs as soon as possible after slaughter under ether anesthesia.

Purification of DNase I from Canine Pancreas—A 1-g sample of canine pancreas was minced and homogenized in 10ml of 25 mM Tris-HCl buffer, pH 7.5, containing 1 mM phenylmethylsulfonyl fluoride. After centrifugation (15,000 ×*g*, 20 min), the supernatant (crude extract) was fractionated with ammonium sulfate. The precipitate formed at ammonium sulfate concentrations between 1.5 and 4.3 M was collected and dissolved in a small volume of 25 mM Tris-HCl buffer, pH 7.5, containing 1.5 M ammonium sulfate. This fraction was applied to a phenyl Sepharose CL-4B column (1.0 × 3 cm) pre-equilibrated with the same buffer, and the adsorbed materials were eluted with a linear reverse gradient of 1.5 to 0 M ammonium sulfate in the same buffer. The active fractions were collected and passed through a Con A-agarose column (1.6 × 7 cm) pre-equilibrated with 25 mM Tris-HCl buffer, pH 7.5, containing 10 mM $MgCl_2$, 1 mM $CaCl_2$ and 150 mM NaCl. The column was washed with the same buffer and the DNase I was eluted with 300 mM methyl-α-mannopyranoside in the same buffer. The active fractions were collected, concentrated and subjected to gel filtration through a Superdex 75 column (1.6 × 60 cm) pre-equilibrated with 25 mM Tris-HCl buffer, pH 7.5, containing 150 mM NaCl. The resulting active fractions were collected, pooled and used as the purified enzyme for the subsequent experiments.

Preparation of Antisera—A specific antiserum against canine DNase I was prepared by emulsifying the purified enzyme with an equal volume of Freund's complete adjuvant (Difco, Detroit, MI, USA) and injecting the emulsion, containing about 50 μg purified enzyme, subcutaneously into a rabbit on five occasions at 2-week intervals. The immunoglobulin fraction was purified from the antiserum using DEAE Affigel Blue (Bio-Rad) and used as the canine DNase I-specific antibody. Antisera against DNases I from the other species investigated were also produced in rabbits as described previously (*14*, *16–18*).

Construction of a Canine Full-Length DNase I cDNA and Analysis of the Nucleotide Sequence—Total RNA was isolated from a piece of canine pancreas using ISOGEN (Nippon Gene, Tokyo). A full-length cDNA for the canine DNase I was constructed by the 3′- and 5′-RACE methods using 3′- and 5′-RACE kits, respectively, under the same conditions as those used previously (*24*). The 3′- and 5′-RACE products were separately subcloned into the TA cloning vector pCR II (Invitrogen, San Diego, CA, USA) and sequenced. The nucleotide sequences were determined by the dideoxy chain termination method using a sequencing kit (Dye Terminator Cycle, Applied Biosystems, Foster City, CA, USA). The sequencing run was performed on a Genetic Analyzer (model 310, Applied Biosystems). All DNA sequences were confirmed by reading both DNA strands.

Expression of Canine DNase I cDNA in COS-7 Cells—A DNA fragment containing the entire coding sequence of the canine DNase I cDNA was prepared from total RNA derived from the pancreas by reverse transcriptase-polymerase chain reaction (RT-PCR) amplification using an Expanded High Fidelity PCR system (Roche Diagnostics, Tokyo) with a set of two primers (primer 1, 5′-GGATCCACCTTCAGGATGAGGGGCGCC-3′; primer 2, 5′-CTCGAGAATTCCCCTCAGGCCCTCTT-3′). The fragment was ligated into the pcDNA 3.1(+) vector (Invitrogen) to construct an expression vector. The construct was confirmed by sequencing, and then purified for transfection using the CONCERT High Purity Plasmid Midiprep System (Life Technologies). COS-7 cells were maintained under the conditions described previously (*17*) and transiently transfected by the lipofection method using LipofectaminPlus reagent (Life Technologies) (*25*). A mixture containing 2 μg of the DNase I cDNA construct and 0.6 μg of pSV-β-galactosidase vector (Promega, Tokyo; for estimation of transfection efficiency) was transfected into the cells. Two days after transfection, the medium was recovered for analysis and the cells were harvested for assay of their β-galactosidase activity. Transfection was performed in triplicate, with at least two different plasmid preparations. The presence of DNase I-specific mRNA was verified by RT-PCR of total RNA extracted from seven different canine tissues using a set of two primers, 1 and 2 (*24*, *26*).

Phylogenetic Analysis—The amino acid sequences of the DNases I from humans, mice, rats (*27*), sheep (*28*), rabbits, pigs, cattle (*29*), fish (carp), hens, frogs (*Xenopus laevis*), newts (*Cynopus pyrrhogaster*) (*17*), and snakes (*Elaphe quadrivirgata*) (*30*) were obtained from the following databases with the following accession numbers: EMBL M55983, EMBL U00478, EMBL X56060, Swiss-Prot drn 1 sheep, EMBL D83038, EMBL AB048832, EMBL AB075779, EMBL AJ001538, EMBL AB013751, EMBL AB030958, EMBL AB041732, and EMBL AB046545. A phylogenetic tree was constructed, with the sequences aligned by the neighbor-joining algorithm of the CLUSTAL W program (*31*, *32*).

RESULTS AND DISCUSSION

Purification and Characterization of Canine Pancreatic DNase I—In this paper, we describe a three-step chromatographic procedure for the purification of canine

Table 1. **Summary of the purification of DNase I from canine pancreas.**[a]

Step	Protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (-fold)	Yield (%)
Crude extract	290	60	0.21	1	100
Ammonium sulfate (1.5–4.3 M)	170	58	0.34	2	97
Phenyl Sepharose CL-4B	74	47	0.64	3	78
Con A–Agarose	6.5	32	4.9	23	53
Superdex 75	0.39	21	54	260	35

[a]Enzyme activity was measured in 0.1 M MES, pH 6.75, containing 20 mM $MgCl_2$ and 2 mM $CaCl_2$ as described in the text.

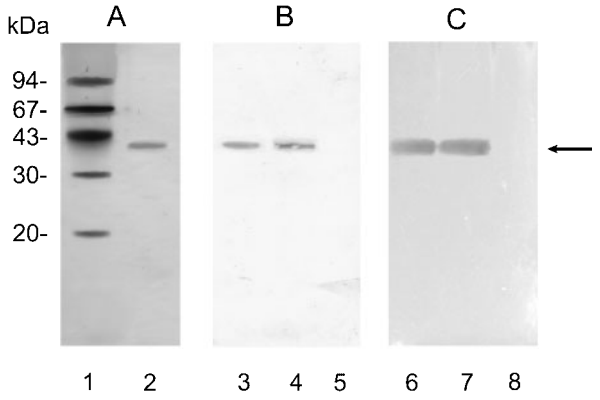

Fig. 1. **SDS-PAGE (A and B) and DNA-cast PAGE (C) patterns of the purified canine DNase I and recombinant proteins expressed in COS-7 cells.** Canine DNase I was detected by protein staining (A), immunostaining with an anti-canine DNase I antibody (B) and activity staining (C). The arrow indicates the band derived from canine DNase I. Lanes: 1, molecular mass markers; 2, 3 and 6, purified canine enzyme; 4 and 7, recombinant protein expressed in COS-7 cells; 5 and 8, medium from mock-transfected cells.

DNase I to apparent homogeneity. A typical canine pancreatic DNase I purification procedure is summarized in Table 1; this resulted in a 260-fold purification with a 35% yield. When the purified canine DNase I was subjected to SDS-PAGE followed by protein staining (Fig. 1A), immunostaining (Fig. 1B) and activity staining (Fig. 1C), only a single band was detected (Fig. 1, lanes 2, 3, and 6). The anti-canine DNase I antibody labeled a single, completely fused line of purified DNase I and canine pancreatic extract on a double immunodiffusion plate (data not shown). The purified DNase I had a molecular mass of approximately 41 kDa, as determined by both SDS-PAGE (Fig. 1A, lane 2) and gel filtration on a Superdex 75 column. The pH activity profile of the enzyme was bell-shaped and exhibited an optimal pH of 6.5 (data not shown). The addition of 20 mM EDTA or 5 mM EGTA to the reaction mixture completely abolished the activity of the enzyme. DNase I activity appeared in the presence of Mn^{2+}, Mg^{2+}, and Ca^{2+}, but was not detected in the absence of divalent cations when examined at pH 6.5 using 10 mM of each cation in the chloride form. Although G-actin potently inhibited the activities of the canine, rabbit (*24*), mouse (*21*) and human (*14*) DNases I, the activities of the porcine (*16*), *Xenopus laevis* (*17*), hen (*33*), and rat (*27*) enzymes were not inhibited by G-actin. These biochemical characteristics are very similar to those of mammalian DNases I, including those of humans (*14*), cattle (*29*), and mice (*21*).

Distribution of DNase I Activity and the DNase I Gene Transcript in Canine Tissues—The highest DNase I activity was observed in the pancreas. Low activity was also detected in kidney, liver, and small and large intestines (Table 2). Several tissues, including cerebrum, cerebellum, heart, lung, spleen and parotid gland exhibited no DNase I activity under our assay conditions. Mammalian DNases I are classified into three types (pancreatic, parotid and mixed types) based on differences in their tissue distribution patterns (*2*), therefore, these data show that, like the human and porcine DNases I, the canine DNase I is of the pancreatic type. The activity of the

Table 2. **Distribution of DNase I activities in canine tissues.**

Tissue	DNase I activity (U)[a]		Gene transcript
	per g wet weight ($\times 10^{-2}$)	per mg protein ($\times 10^{-5}$)	
Cerebrum	ND	ND	NT[b]
Cerebellum	ND	ND	NT
Heart	ND	ND	NT
Lung	ND	ND	NT
Stomach	5.1 ± 4.5	12 ± 9.9	NT
Liver	12 ± 9.3	10 ± 2.3	+[b]
Pancreas	49,000 ± 36,000	55,000 ± 9,400	+
Kidney	120 ± 100	110 ± 68	+
Spleen	ND	ND	–[b]
Small intestine	7.8 ± 2.0	26 ± 1.4	+
Large intestine	30 ± 18	74 ± 27	+
Parotid gland	ND	ND	–
Sublingual gland	5.8 ± 4.2	8.7 ± 3.4	NT

[a]Values are the means ± SD of duplicate tests on each extract from four dogs. DNase I activity in each tissue extract was measured by the SRED assay method and completely blocked by the addition of 20 mM EDTA and antibody specific to canine DNase I. ND, not detected under the assay conditions. [b]+, amplification of a 872 bp fragment specific to canine DNase I by RT-PCR using primers 1 and 2.; –, not amplified; NT, not tested.

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                CTGTGC TGGGACTCAG CTGCCGCTCT GCGTGGGTCT CTCACGCTCT   46
GCGGGTCTCT CACTACGTTA ACTCCCTTCC CTTTGTGGAT TATTTTTTCT CAAAGCAGCA  106
AAAGGCTAGT GTTGTTACTG ATCAGAGGAT AGCAGTTCCC GATTCTCATC CCAATCCCTG  166
TCCTCGGAGA AGCTGACCTT CAGGATGAGG GGCGCCAGGC TGATGGGGGC ACTGCTCGCC  226
                     MetArg GlyAlaArgL euMetGlyAl aLeuLeuAla   -11

CTGGCCGGCC TCCTGCAGGG GGCCCTGGCC CTGAGAATGG CGGCCTTCAA CATCCGGACC  286
LeuAlaGlyL euLeuGlnGl yAlaLeuAla LeuArgMetA laAlaPheAs nIleArgThr   10

TTTGGGGAGA CCAAGATGTC CAATGCCACT CTCTCCAAGT ACATCGTGCA GATCCTGAGT  346
PheGlyGluT hrLysMetSe rAsnAlaThr LeuSerLysT yrIleValGl nIleLeuSer   30

CGCTACGACG TTGCTGTAGT CCAGGAGGTC AGAGACAGCC ACCTGACAGC TGTAGGGAAG  406
ArgTyrAspV alAlaValVa lGlnGluVal ArgAspSerH isLeuThrAl aValGlyLys   50

CTGCTGGACA CACTCAATCA GGACGACCCC AATGCCTACC ATTATGTGGT CAGTGAGCCG  466
LeuLeuAspT hrLeuAsnGl nAspAspPro AsnAlaTyrH isTyrValVa lSerGluPro   70

CTGGGCCGCA GCAGCTACAA GGAACGCTAC CTCTTTCTGT TTAGACCTGA CCGCGTGTCC  526
LeuGlyArgS erSerTyrLy sGluArgTyr LeuPheLeuP heArgProAs pArgValSer   90

GTGCTGGACA GCTACCAGTA CGACGACGGC TGCGAGCCCT GCGGGAATGA CACCTTCAGC  586
ValLeuAspS erTyrGlnTy rAspAspGly CysGluProC ysGlyAsnAs pThrPheSer  110

CGCGAGCCTG CCATCGTCAG GTTCCACTCC CCGCTCACGG AGGTCAAGGA GTTCGCTGTC  646
ArgGluProA laIleValAr gPheHisSer ProLeuThrG luValLysGl uPheAlaVal  130

GTGCCCCTGC ATGCAGCCCC GCTGGACGCG GTGGCGGAGA TAGACGCGCT CTATGACGTC  706
ValProLeuH isAlaAlaPr oLeuAspAla ValAlaGluI leAspAlaLe uTyrAspVal  150

TACCTGGATG TCCAGCACAA GTGGGACCTG GAGGACATCG TGCTCATGGG CGACTTCAAC  766
TyrLeuAspV alGlnHisLy sTrpAspLeu GluAspIleV alLeuMetGl yAspPheAsn  170

GCCGGCTGCA GCTACGTGGC TGCCTCCCAG TGGTCCTCTA TCCGCCTGCG CACAAACCCG  826
AlaGlyCysS erTyrValAl aAlaSerGln TrpSerSerI leArgLeuAr gThrAsnPro  190

GCCTTCCAGT GGCTGATTCC CGACACCGCG GACACCACGT CCACATCCAC GCACTGTGCC  886
AlaPheGlnT rpLeuIlePr oAspThrAla AspThrThrS erThrSerTh rHisCysAla  210

TATGACAGGA TCGTGGTGGC AGGAAGTCAG CTGCAGCACG CCGTTGTGCC TGAATCAGCC  946
TyrAspArgI leValValAl aGlySerGln LeuGlnHisA laValValPr oGluSerAla  230

GCCCCCTTCA ACTTCCAGGT GGCCTACGGC CTCAGCAGCC AGCTGGCCCA GGCCATCAGT 1006
AlaProPheA snPheGlnVa lAlaTyrGly LeuSerSerG lnLeuAlaGl nAlaIleSer  250

GACCACTACC CCGTGGAGGT GACGCTCAAG AGGGCCTGAG GGGAATTGGG GCTGGCGCCC 1066
AspHisTyrP roValGluVa lThrLeuLys ArgAlaTerm                         262
CCCTAGAGCC TTTCCCTGTC TGCACAGTCA AGCATTGTGG ATGAGGTTTT CCTGAGCACC 1126
CTCTCCCCGT GGTCCCCTGA AATGTCACCA CAACTCAACT CAGCAACTCA ACTCAACTCG 1186
AGTTTGTGGA AGGAGGGATG GAGACTGGTC ACCTCCACTT CCAGTGGAAC ACGGGGTGGG 1246
GGTGTGGCAA GGGCACGCCC TCCGTACCTT CTCGGGCCAG ACAAAAGTTT AACATGGAGA 1306
TTGACAAATG CCTTTAATTT AAGTAAATAA AGCTCAAAGG GGTGAGTTGT CAAAAAAAAA 1366
AAAAAAA                                                          1373
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Fig. 2. **Nucleotide and deduced amino acid sequences of the cDNA encoding canine DNase I.** The nucleotides are numbered in the 5′- to 3′-direction and the deduced amino acid residues are shown under the corresponding nucleotide sequence. Amino acid position 1 was assigned after comparison with the chemically determined N-terminal amino acid sequence of the purified enzyme. The initiation and termination codons and the putative polyadenylation signal are single-underlined, the amino acid sequence of the putative signal peptide is underlined with a dotted line, and the *N*-glycosylation site is double-underlined. The nucleotide sequence of canine DNase I cDNA has been submitted to the DDBJ/EMBL/GeneBank nucleotide databases and is available at accession number AB113380.

canine enzyme detected in various tissues was inhibited by 20 mM EDTA, 5 mM EGTA and the anti-canine DNase I antibody. Therefore, the enzyme activity was confirmed to be that of DNase I. The presence of a DNase I-specific mRNA was verified by RT-PCR of total RNA extracted from canine tissues. Using primers 1 and 2, which are specific to the canine DNase I cDNA, an 872-bp fragment containing a region encoding the mature enzyme was amplified from the total RNA from canine pancreas, liver, kidney, small and large intestines; no amplified product was obtained from the total RNA from other tissues (Table 2). It is reasonable, therefore, to assume that the enzyme activity reflects DNase I gene expression in these tissues.

Molecular Cloning of a cDNA Encoding Canine DNase I and Expression in COS-7 Cells—Total RNA extracted from canine pancreas was separately amplified by the 3′- and 5′-RACE methods to construct a cDNA encoding canine DNase I. Using a set of specific primers based on the N-terminal amino acid sequence of the human enzyme, plus the nucleotide sequence obtained in our previous study (*6*), we succeeded in amplifying two overlapping RACE products. Sequencing analysis of these RACE products revealed that the full-length 1373-bp cDNA contains a 190-bp 5′-untranslated region, an 855-bp coding region and a 328-bp 3′-untranslated region (Fig. 2). The open reading frame starts at position 191 with an ATG start codon and ends with a TGA stop codon at position 1045. The putative upstream signal peptide is 22 amino acids long. The 3′-untranslated region is followed by a short poly(A) tail, and a putative polyadenylation signal (AATAAA) is located 21 bp upstream of the poly(A) tail. The 3′-untranslated region of the canine DNase I cDNA is considerably longer than those (about

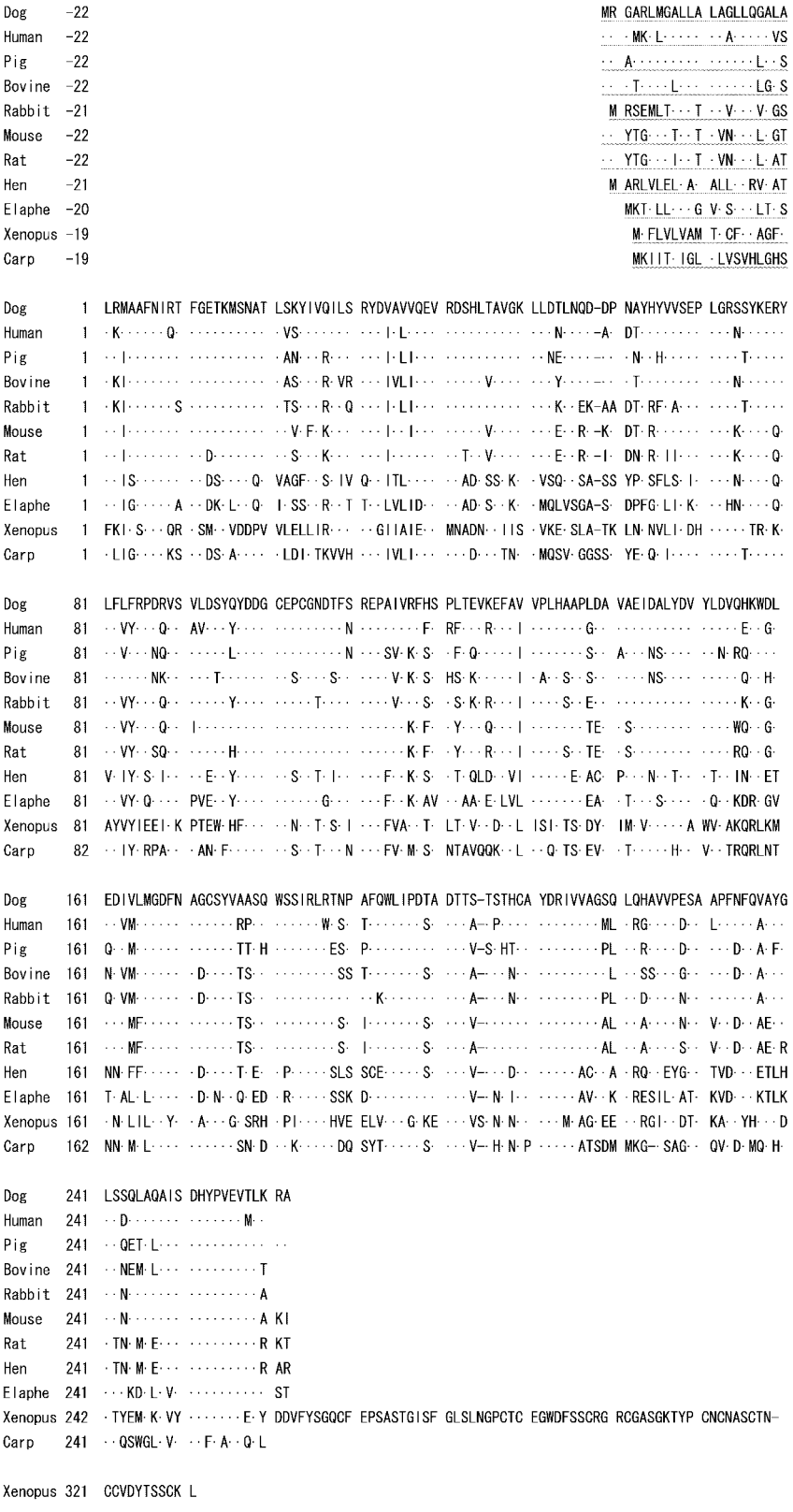

Fig. 3. **Comparison of the amino acid sequences of canine (this study) and other vertebrate DNase I enzymes.** The amino acid sequences of 11 vertebrate enzymes were obtained as described in the text. The amino acids are numbered starting at the N-terminus of each mature protein. The dots indicate that the residues are the same as those in canine DNase I, and the horizontal bars indicate deleted amino acid residues. The amino acid sequences of the putative signal peptides are underlined with wave lines.

150 bp) of the other cloned mammalian DNase I cDNAs. The fragment carrying the coding region of the canine DNase I cDNA was cloned into a mammalian expression vector and transiently expressed in COS-7 cells, and DNase I enzymatic activity was detected in the conditioned medium of these cells. The mean (±SEM., $n = 6$) activity of the enzyme secreted into the medium was 2.4 ± 0.45 U/ml. In contrast, the human, frog and carp DNase I vectors yield activities of 2.2 ± 0.24, 0.35 ± 0.10, and 1.3 ± 0.07 U/ml, respectively (*17*, *18*). Thus, the level of canine

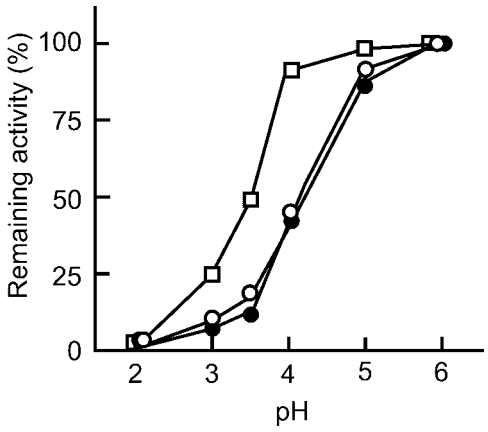

Fig. 4. **Comparison of the acid sensitivities of three mammalian DNases I.** Purified DNases I from the dog (open circles), human (solid circles) and rat (open squares) were incubated at 37°C for 10 min in 50 mM glycine-HCl at pH 2.0–3.0 or in 50 mM sodium acetate at pH 3.0–6.0, and the remaining DNase I activity was determined by the SRED method.

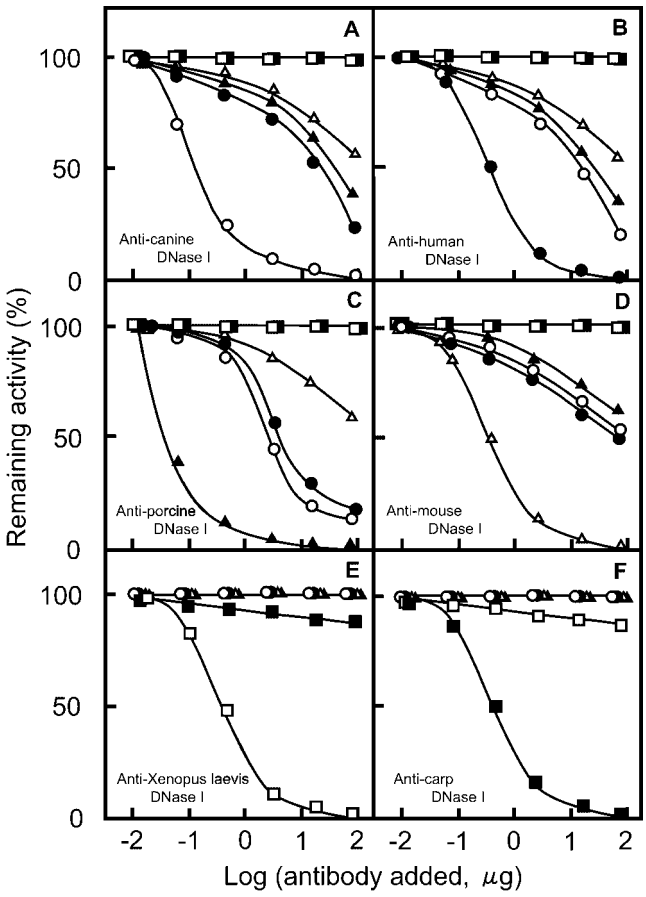

Fig. 5. **Mutual inhibitory effects of species-specific antibodies on the activities of vertebrate DNases I.** Each DNase I (0.4 unit) was incubated with an antibody specific to the canine (A), human (B), porcine (C), mouse (D), frog (*Xenopus laevis*) (E), or carp (F) DNases I, and the activities remaining were measured by the SRED method. The effects of each antibody on the activities of the purified canine (open circles), human (solid circles), porcine (solid triangles), mouse (open triangles), frog (open squares), and carp (solid squares) DNases I are shown.

enzyme expressed in COS-7 cells is very similar to that of the human enzyme. The canine enzymatic activity was completely abolished by the anti-canine DNase I antibody and 20 mM EDTA. The expressed canine DNase I showed the same electrophoretic mobility as the canine pancreatic enzyme (Fig. 1, lanes 3, 4, 6, and 7), and the N-terminal amino acid sequence up to the 10th residue (determined chemically) of the purified enzyme exactly matched that deduced from the cDNA data (data not shown). These findings led us to conclude that the cDNA we isolated encodes the canine DNase I.

The coding region of the canine DNase I cDNA was translated into the corresponding amino acid sequence, which was compared with the other 10 available vertebrate DNase I sequences (*6*, *16–18*, *24*, *27–30*, *33*) (Fig. 3). All four residues (Glu78, His134, Asp212, and His252) postulated to be involved in the active site (*34*, *35*), two residues (Cys173 and Cys209) that form the disulfide bond responsible for enzyme stability (*8*), and two other residues (Arg41 and Tyr76) that mediate DNase I–DNA contact (*8*) in other mammalian DNases I were also found in the canine enzyme. G-actin has been identified as a potent DNase I inhibitor and four amino acid residues (Glu13, Tyr65, Val67, and Ala114) are thought to be responsible for the binding of actin to human and bovine DNases I (*35–37*). All four of these residues are conserved in canine DNase I. Two potential N-linked glycosylation sites in the canine enzyme, Asn18 (Asn-Ala-Thr) and Asn106 (Asn-Asp-Thr), are both well conserved in other mammalian enzymes (*6*, *16*, *24*, *29*, *38*). However, the canine enzyme does not contain the Ser59 insertion found in the carp enzyme (*18*), or the additional cysteine-rich C-terminal region found in the amphibian enzymes (*17*). A sequence motif search on GenomeNet revealed that two motifs identified as "DNase I signature sequences," $(L/I/A/M)_2(A/P)LH(S/T/A)_2PX_5E(L/I/V/M)(D/M)XLX(D/E)V$ and GDFNAXC(S/A), in which arbitrary residues are indicated by X, have been identified at positions 130–150 and 167–174 in all vertebrate DNase I sequences determined so far (*8*). Both of these sequences are well conserved in the canine enzyme, although the Ile at position 130 in the other mammalian enzymes is substituted by Val in the canine enzyme. These findings indicate that, with respect to their structures, the canine enzyme is distantly related to the fish and amphibian enzymes, but closely related to other mammalian enzymes. Furthermore, the amino acid sequence of the canine DNase I exhibits the highest homology (82.7%) to that of the human enzyme among all mammalian enzymes determined so far (range 82.7–78.2%).

Acid Sensitivity of Canine DNase I—We examined acid sensitivity by incubating the purified canine, human and rat enzymes at 37°C for 10 min at various pH. As shown in Fig. 4, the activities of the human and canine enzymes (both pancreatic type enzymes) were reduced to about 35% of the initial level after incubation at pH 4, whereas the activity of the rat enzyme (parotid type) was reduced to about 85% under the same conditions. These results indicate that the human and canine enzymes are more sensitive to acidic conditions than the rat enzyme. This may reflect the fact that the canine and human DNases I are secreted into the intestine and work at neutral pH,

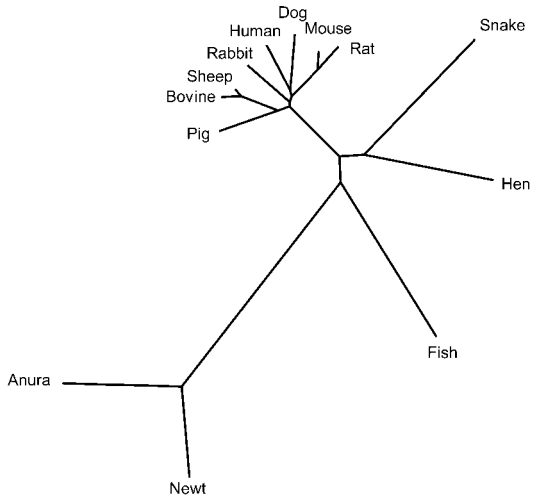

Fig. 6. **Phylogenetic analysis of the DNase I family.** The amino acid sequences of the mature DNase I proteins of various species were subjected to phylogenetic analysis. An unrooted phylogenetic tree was constructed using the neighbor-joining method. References and accession numbers for the DNase I sequences are described in the text.

whereas rat DNase I is secreted into the oral cavity and must pass through the acidic pH of the stomach intact. It is plausible that these different characteristics were acquired during the course of evolution. It has been shown that the acid-sensitive form of mammalian DNase I (pancreatic type: human and porcine) is transformed into the acid-resistant form (parotid type: rat or mixed type: bovine) by substitution of Ser for Asn at position 110, and *vice versa* (*2*, *16*). However, canine DNase I is acid-sensitive but has a Ser residue at position 110 (Fig. 3). These findings suggest the influence of another mechanism in the acquisition of acid sensitivity by mammalian DNases I. Research into a possible mechanism is now in progress in our laboratory.

Immunological Comparison of Vertebrate DNases I—Six different antibodies specific to canine, human (*14*), porcine (*16*), mouse (*21*), frog (*Xenopus laevis*) (*17*) and carp (*18*) DNases I were tested for mutual cross-inhibition of purified canine, human, porcine, mouse, frog and carp DNases I. The anti-canine DNase I antibody completely blocked the activity of the canine enzyme, and weakly inhibited those of the human, porcine and mouse enzymes, but was totally ineffective against the frog and carp enzymes (Fig. 5A). The anti-human DNase I antibody completely blocked the activity of human DNase I and weakly blocked those of the canine, porcine and mouse enzymes (Fig. 5B), while the anti-porcine DNase I antibody completely blocked the activity of porcine DNase I and weakly blocked those of the canine, human and mouse enzymes (Fig. 5C). Both of these antibodies were ineffective against the frog and carp enzymes. Conversely, the anti-frog DNase I and anti-carp DNase I antibodies completely blocked the activities of the frog and carp enzymes, respectively, but were completely ineffective against the other five enzymes (Figs. 5, E and F). These findings show that, from an immunological standpoint, canine DNase I resembles other mammalian (human, porcine and mouse) enzymes, but bears little or no resemblance to the frog and carp enzymes.

Phylogenetic Analysis of Vertebrate DNase I—The amino acid sequences of 13 different mature DNase I enzymes were aligned in order to construct a phylogenetic tree for the DNase I family (Fig. 6). The mammalian enzymes from dogs, humans, pigs, rabbits, mice, rats, sheep and cattle formed a relatively tight cluster, whereas the hen, snake, fish and amphibian enzymes were situated independently, far from the mammalian enzyme cluster. Within the mammalian enzyme cluster, the canine and human enzymes formed a tight group. The degree of intra-class similarity for the mammalian DNase I group (average 80% in this study) was higher than those for the amphibian and picine DNase I groups (65% and 70%, respectively) (*17*, *18*), suggesting that the former group exhibits greater similarity than the latter two groups from the standpoint of molecular evolution. The results of our phylogenetic analysis of canine DNase I are consistent with those of our immunological analysis. This is a characteristic common to all DNases tested and described previously (*14*, *16–18*, *20*, *24*, *30*).

In conclusion, this study clarifies that, compared with the vertebrate DNases I tested and reported to date, canine DNase I exhibits the greatest similarity to the human enzyme with regard to organ distribution, protein structure, enzymatic and immunological properties, and phylogenetic situation. Based on the analytical data for canine and human DNases I, these findings suggest that dogs are the most suitable laboratory animals for use in the elucidation of the physiological and etiological role(s) of DNase I in humans.

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REFERENCES

1. Moore, S. (1981) Pancreatic DNase in *The Enzymes*, 3rd ed. (Boyer, P.D., ed.) Vol. **14**, pp. 281–296, Academic Press, New York
2. Takeshita, H., Mogi, K., Yasuda, T., Nakajima, T., Nakashima, Y., Mori, S., Hoshino, T., and Kishi, K. (2000) Mammalian deoxyribonucleases I are classified into three types: pancreas, parotid and pancreas-parotid (mixed), based on differences in their tissue concentrations. *Biochem. Biophys. Res. Commun.* **269**, 481–484
3. Napirei, M., Karsunky, H., Zevnik, B., Stephan, H., Mannherz, H.G., and Möröy, T. (2000) Features of systemic lupus erythematosus in Dnase1-deficient mice. *Nat. Genet.* **25**, 177–181
4. Yasutomo, K., Horiuchi, T., Kagami, S., Tsukamoto, H., Hashimura, C., Urushihara, M., and Kuroda, Y. (2001) Mutation of *DNASE1* in people with systemic lupus erythematosus. *Nat. Genet.* **28**, 313–314
5. Kishi, K., Yasuda, T., Ikehara, Y., Sawazaki, K., Sato, W., and Iida, R. (1990) Human serum deoxyribonuclease I (DNase I) polymorphism: pattern similarities among isozymes from serum, urine, kidney, liver and pancreas. *Amer. J. Hum. Genet.* **47**, 121–126
6. Yasuda, T., Kishi, K., Yanagawa, Y., and Yoshida, A. (1995) Structure of the human deoxyribonuclease I (DNase I) gene:

identification of the nucleotide substitution that generates its classical genetic polymorphism. *Ann. Hum. Genet.* **59**, 1–15
7. Yasuda, T., Nadano, D., Iida, R., Takeshita, H., Lane, S.A., Callen, D.F., and Kishi, K. (1995) Chromosomal assignment of the human deoxyribonuclease I gene, DNASE1 (DNL1), to band 16p13.3 using the polymerase chain reaction. *Cytogenet. Cell Genet.* **70**, 221–223
8. Kishi, K., Yasuda, T., and Takeshita, H. (2001) DNase I: structure, function, and use in medicine and forensic science. *Legal Med.* **3**, 69–83
9. Yasuda, T., Takeshita, H., Iida, R., Kogure, S., and Kishi, K. (1999) A new allele, *DNASE1*6*, of human deoxyribonuclease I polymorphism encodes an Arg to Cys substitution responsible for its instability. *Biochem. Biophys. Res. Commun.* **260**, 280–283
10. Yasuda, T., Nadano, D., Tanaka, Y., Tenjo, E., Takeshita, H., Nakanaga, M., and Kishi, K. (1993) Survey of the association of deoxyribonuclease I polymorphism with disease. *Hum. Hered.* **43**, 205–208
11. Tsutsumi, S., Asao, T., Nagamachi, Y., Nakajima, T., Yasuda, T., and Kishi, K. (1998) Phenotype 2 of deoxyribonuclease I may be used as a risk factor for gastric carcinoma. *Cancer* **82**, 1621–1625
12. Tsutsumi, S., Takeshita, H., Yasuda, T., Kuwano, H., and Kishi, K. (2000) Association of DNase I phenotype 2 with colorectal carcinoma risk in Japanese populations. *Cancer Lett.* **159**, 109–112
13. Hullinger, R.L. (1979) The heart and arteries in *Miller's Anatomy of the Dog.* 2nd edn. (Evans, H.E. and Christensen, G.C., eds.) pp. 632–756, W.B. Saunders, Philadelphia-London-Toronto
14. Yasuda, T., Awazu, S., Sato, W., Iida, R., Tanaka, Y., and Kishi, K. (1990) Human genetically polymorphic deoxyribonuclease: purification, characterization and multiplicity of urine deoxyribonuclease I. *J. Biochem.* **108**, 393–398
15. Yasuda, T., Takeshita, H., Nakajima, T., Hosomi, O., Nakashima, Y., and Kishi, K. (1997) Purification of deoxyribonuclease I using two-step chromatography. *Anal. Biochem.* **245**, 255–257
16. Mori, S., Yasuda, T., Takeshita, H., Nakajima, T., Nakazato, E., Mogi, K., Kaneko, Y., and Kishi, K. (2001) Molecular, biochemical and immunological analyses of porcine pancreatic DNase I. *Biochim. Biophys. Acta* **1547**, 275–287
17. Takeshita, H., Yasuda, T., Iida, R., Nakajima, T., Mori, S., Mogi, K., Kaneko, Y., and Kishi, K. (2001) Amphibian DNases I are characterized by a C-terminal end with a unique, cysteine-rich stretch and by the insertion of a serine residue into the Ca^{2+}-binding site. *Biochem. J.* **357**, 473–480
18. Mogi, K., Takeshita, H., Yasuda, T., Nakajima, T., Nakazato, E., Kaneko, Y., Itoi, M., and Kishi, K. (2003) Carp hepatopancreatic DNase I: biochemical, molecular, and immunological properties. *J. Biochem.* **133**, 377–386
19. Nadano, D., Yasuda, T., and Kishi, K. (1993) Measurement of deoxyribonuclease I activity in human tissues and body fluids by a single radial enzyme-diffusion method. *Clin. Chem.* **39**, 448–452
20. Yasuda, T., Takeshita, H., Nakazato, E., Nakajima, T., Hosomi, O., Nakashima, Y., and Kishi, K. (1998) Activity measurement for deoxyribonucleases I and II with picogram sensitivity based on DNA/SYBR Green I fluorescence. *Anal. Biochem.* **255**, 274–276
21. Takeshita, H., Yasuda, T., Nakajima, T., Hosomi, O., Nakashima, Y., and Kishi, K. (1997) Mouse deoxyribonuclease I (DNase I): biochemical and immunological characterization, cDNA structure and tissue distribution. *Biochem. Mol. Biol. Int.* **42**, 65–75
22. Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685
23. Nakajima, T., Yasuda, T., Nakashima, Y., Hosomi, O., Takeshita, H., and Kishi, K. (1998) Two novel screening methods for selecting monoclonal antibodies which specifically inhibit DNase I enzyme activity. *Immunol. Invest.* **27**, 145–152
24. Yasuda, T., Takeshita, H., Nakajima, T., Hosomi, O., Nakashima, Y., and Kishi, K. (1997) Rabbit DNase I: purification from urine, immunological and proteochemical characterization, nucleotide sequence, expression in tissues, relationships with other mammalian DNases I and phylogenetic analysis. *Biochem. J.* **325**, 465–473
25. Yasuda, T., Takeshita, H., Ueki, M., Iida, R., Nakajima, T., Mori, S., Mogi, K., Kaneko, Y., and Kishi, K. (2001) Tissue-specific *in vivo* inhibition of DNase I gene expression by somatostatin. *Biochem. Biophys. Res. Commun.* **283**, 287–291
26. Yasuda, T., Takeshita, H., Iida, R., Nakajima, T., Hosomi, O., Nakashima, Y., and Kishi, K. (1998) Molecular cloning of the cDNA encoding human deoxyribonuclease II. *J. Biol. Chem.* **273**, 2610–2616
27. Takeshita, H., Yasuda, T., Nadano, D., Iida, R., and Kishi, K. (1995) Deoxyribonuclease I from rat urine: affinity purification, characterization, and immunochemical studies. *J. Biochem.* **118**, 932–938
28. Paudel, H.K. and Liao, T.-H. (1986) Comparison of the three primary structures of deoxyribonuclease isolated from bovine, ovine and porcine pancreas: derivation of the amino acid sequence of ovine DNase and revision of the previously published amino acid sequence of bovine DNase. *J. Biol. Chem.* **261**, 16012–16017
29. Chen, C.-Y., Lu, S.-C., and Liao, T.-H. (1998) Cloning, sequencing and expression of a cDNA encoding bovine pancreatic deoxyribonuclease I in *Escherichia coli*: purification and characterization of the recombinant enzyme. *Gene* **206**, 181–184
30. Takeshita, H., Yasuda, T., Nakajima, T., Mogi, K., Kaneko, Y., Iida, R., and Kishi, K. (2003) A single amino acid substitution of Leu130Ile in snake DNase I contributes to the acquisition of thermal stability: a clue to the molecular evolutionary mechanism from cold-blooded to warm-blooded vertebrates. *Eur. J. Biochem.* **270**, 307–314
31. Saitou, N. and Nei, M. (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* **4**, 406–425
32. Thompson, J.D., Higgins, D.G., and Gibson, T.J. (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* **22**, 4673–4680
33. Nakashima, Y., Yasuda, T., Takeshita, H., Nakajima, T., Hosomi, O., Mori, S., and Kishi, K. (1999) Molecular, biochemical and immunological studies of hen pancreatic deoxyribonuclease I. *Int. J. Biochem. Cell Biol.* **31**, 1315–1326
34. Pan, C.Q., Ulmer, J.S., Herzka, A., and Lazarus, R.A. (1998) Mutational analysis of human DNase I at the DNA binding interface: implications for DNA recognition, catalysis and metal ion dependence. *Protein Sci.* **7**, 628–636
35. Ulmer, J.S., Herzka, A., Toy, K.J., Baker, D.L., Dodge, A.H., Sinicropi, D., Shak, S., and Lazarus, R.A. (1996) Engineering actin-resistant human DNase I for treatment of cystic fibrosis. *Proc. Natl Acad. Sci. USA* **93**, 8225–8229
36. Hitchcock, S.E. (1980) Actin-deoxyribonuclease I interaction: depolymerization and nucleotide exchange. *J. Biol. Chem.* **255**, 5668–5673
37. Mannherz, H.G. (1992) Crystallization of actin in complex with actin-binding proteins. *J. Biol. Chem.* **267**, 11661–11664
38. Peitsch, M.C., Irmler, M., French, L.E., and Tschopp, J. (1995) Genomic organization and expression of mouse deoxyribonuclease I. *Biochem. Biophys. Res. Commun.* **207**, 62–68