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Carp Hepatopancreatic DNase I: Biochemical, Molecular, and Immunological Properties

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A survey of DNase I in nine different carp tissues showed that the hepatopancreas has the highest levels of both DNase I enzyme activity and gene expression. Carp hepatopancreatic DNase I was purified 17,000-fold, with a yield of 29%, to electrophoretic homogeneity using three-step column chromatography. The purified enzyme activity was inhibited completely by 20 mM EDTA and a specific anti–carp DNase I antibody and slightly by G-actin. Histochemical analysis using this antibody revealed the strongest immunoreactivity in the cytoplasm of pancreatic tissue, but not in that of hepatic tissue in the carp hepatopancreas. A 995-bp cDNA encoding carp DNase I was constructed from total RNA from carp hepatopancreas. The mature carp DNase I protein comprises 260 amino acids, the same number as the human enzyme, however, the carp enzyme has an insertion of Ser59 and a deletion of Ala225 in comparison with the human enzyme. These alterations have no influence on the enzyme activity and stability. Three amino acid residues, Tyr65, Val67, and Ala114, of human DNase I are involved in actin binding, whereas those of carp DNase I are shifted to Tyr66, Val68, and Phe115, respectively, by the insertion of Ser59: the decrease in affinity to actin is due to one amino acid substitution, Ala114Phe. The results of our phylogenetic and immunological analyses indicate that carp DNase I is not closely related to the mammalian, avian or amphibian enzymes, and forms a relatively tight piscine cluster with the tilapia enzyme.

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

Abbreviations: RACE, rapid amplification of cDNA ends; R. catesbeiana, Rana catesbeiana; RT, reverse transcriptase.

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*). The 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 (*2*). 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 (*3*). Mammalian DNases I are classified into three types [pancreatic, parotid and parotid-pancreatic (mixed)], based on their main sources and acid-sensitivities (*4*). Because human and mouse DNases I are of the pancreatic and parotid types, respectively, it remains to be seen whether the findings of experiments using DNase I–deficient mice (*2*) can be extrapolated directly to explain the etiology of human systemic lupus erythematosus.

Although DNase I has been considered to be merely a digestive enzyme, the presence of its activity in mammalian tissues other than the digestive organs suggests that it might have another physiological function *in vivo* (*5*). Recently, we found that somatostatin induces, through effects on DNase I gene expression, a transient down-regulation of DNase I activity in the rat lower gut and serum, but not in the parotid gland, which exhibits the highest DNase I activity among rat tissues (*6*). The DNase I activities and amounts of gene transcript in the pituitary glands of humans aged from 1 month to 89 years are significantly age-dependent, with abrupt elevations after the neonatal and prepubertal periods regardless of gender, followed by a gradual age-dependent decline in males and a marked reduction in females in their post-reproductive period. These findings suggest that a tissue-specific up-regulation of DNase I gene expression in the pituitary gland may occur at the onset of puberty (*7*).

Human DNase I has been shown to exhibit genetic polymorphism controlled by at least six alleles on chromosome 16p13.3 (*8–12*). Of these, the *DNASE1*2* allele might be a risk factor for gastric carcinoma (*13*). In this context, it is important to carry out comprehensive studies of lower and higher animals to elucidate the intrinsic intracellular and extracellular functions of DNase I, as well as the phylogenetic origins of the DNase I family. Our comparative studies of the tissue distributions of

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DNase I in lower to higher vertebrates demonstrated that the enzyme also exists outside the exocrine glands of the alimentary tract, i.e., in the pancreas and/or parotid gland (*4*, *14–16*). The DNase I of a fish, tilapia, has been found to possess several structural differences from the DNases I of other vertebrates: deletion of two amino acid residues at positions 61 and 224 of the latter and no disulfide bond (Cys100–Cys104) which is conserved in all DNases I other than the tilapia DNase I (*17*). However, it remains unknown whether these differences are shared by other fishes. To address this question, a systematic survey of the piscine enzymes is required.

In this paper, we describe a novel purification procedure for carp DNase I and describe its biochemical and immunological properties, cDNA structure and gene expression.

MATERIALS AND METHODS

Materials and Biological Samples—Phenyl Sepharose CL-4B and Superdex 75 were purchased from Amersham Pharmacia Biotech (Tokyo). Concanavalin A (Con A)-agarose was obtained from Seikagaku Kogyo (Tokyo) and rabbit muscle G-actin was from Sigma (St. Louis, MO, USA). Superscript II RNase H^- reverse transcriptase (RT) and 5′- and 3′- rapid amplification of cDNA ends (RACE) kits were from Life Technologies (Rockville, MD, USA). All other chemicals used were of reagent grade and were available commercially. A carp (*Cyprinus carpio*) weighing 1.5 kg was obtained from Yamagishi Fish Farm (Maebashi). Rabbits were acquired, maintained and used in accordance with Guidelines for the Care and Use of Laboratory Animals (NIH, USA; revised 1985).

Analytical Methods—DNase I activity was assayed by the single radial enzyme diffusion (SRED) (*16*, *18*) or test tube (*19*) method, except that 50 mM Tris/HCl buffer, pH 8.0, containing 10 mM $MgCl_2$ and 1 mM $CaCl_2$ was substituted for the reaction buffer described in these reports. One unit (U) of activity was defined as an increase in absorbance of 1.0 at 260 nm. The enzymological and chemical properties of, inhibitory effects of G-actin on, and tissue distributions of the carp DNase I enzyme were examined as described previously (*20*). Protein levels were determined using a protein assay kit (Bio-Rad, Hercules, CA, USA) with bovine serum albumin as the standard. The N-terminal amino acid sequences of the purified enzymes were analyzed by Edman degradation (*21*), and each purified enzyme was analyzed by 12.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (*22*). The electrophoresed proteins were detected by staining with 0.2% (w/v) Coomassie Brilliant Blue R-250 (Bio-Rad) or an anti-carp DNase I antibody, as described previously (*4*). Activity staining for DNase I was performed using a DNA cast-PAGE method (*23*) and the β-galactosidase assay was performed by the conventional method (*24*). The presence of DNase I-specific mRNA was verified by reverse-transcriptase-mediated polymerase chain reaction (RT-PCR) amplification of the total RNA extracted from each carp tissue with sets of primers corresponding to the N- and C-terminal amino acid sequences of the enzyme (*25*). Various vertebrate DNases I were incubated in 50 mM Tris/HCl, pH 8.0, at 15–60°C in a water bath for 40 min to determine their heat-stabilities.

Purification of DNase I from Carp Hepatopancreas—All procedures were performed at 0–4°C. Carp hepatopancreas (20 g) was minced and homogenized in 150 ml 10 mM Tris/HCl buffer, pH 7.5, containing 1 mM phenylmethylsulfonyl fluoride, centrifuged (15,000 ×*g*, 10 min), and the supernatant (crude extract) was fractionated with ammonium sulfate. The precipitate formed at ammonium sulfate concentrations between 2.3 and 6.1 M was collected and dissolved in a small volume of 10 mM Tris/HCl buffer, pH 8.0, containing 2.0 M ammonium sulfate. This fraction was applied to a phenyl Sepharose CL-4B column (1.6 × 15 cm) pre-equilibrated with the same buffer, and the adsorbed materials were eluted with a linear reverse gradient of 2.0 to 0 M ammonium sulfate in the same buffer. The active fractions were collected and passed through a Con A-agarose column (1.6 × 5 cm) pre-equilibrated with 10 mM Tris/HCl buffer, pH 8.0, containing 10 mM $CaCl_2$ and 150 mM NaCl. The column was washed with 300 mM methyl-α-D-mannopyranoside in the same buffer and the active fraction was collected, concentrated and subjected to gel filtration through a Superdex 75 column (1.6 × 60 cm) pre-equilibrated with 50 mM Tris/HCl buffer, pH 8.0, 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 carp 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 three occasions at 2 week intervals. The immunoglobulin fraction was purified from the antiserum using DEAE Affigel Blue (Bio-Rad) and used as the relevant antibody. The other antisera used were also produced in rabbits, as described previously (*4*, *14*).

Construction of Carp Full-Length DNase I cDNA and Analysis of Its Nucleotide Sequence—Total RNA was isolated from a piece of carp hepatopancreas by the acid guanidium thiocyanate-phenol-chloroform method (*26*). First, the DNA fragment corresponding to the 3′-end region of the carp DNase I cDNA was obtained by the 3′-RACE method using a 3′-RACE kit. 3′-RACE was performed using two degenerate primers based on the N-terminal amino acid sequence of carp DNase I (primer 1, 5′-AT(A/C/T)GGIGCITT(C/T)AA(C/T)AT(A/C/T)AA(A/G)-3′; primer 2, 5′-TT(C/T)GGIGA(C/T)GA(C/T)AA(A/G)(A/T)(C/G)(C/T)GA-3′), as described previously (*15*). Next, the 5′-end region of the cDNA was amplified by the 5′-RACE method using a 5′-RACE kit under the conditions described previously (*15*). The 3′- and 5′-RACE products obtained were subcloned separately into the TA cloning vector pCR II (Invitrogen, San Diego, CA, USA), and their 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) and all DNA sequences were confirmed by reading both DNA strands.

Table 1. **Summary of the purification of DNase I from carp hepatopancreas.**[a]

Step	Protein (mg)	Total activity ($\times 10^{-4}$ U)	Specific activity ($\times 10^{-4}$ U/mg)	Purification (-fold)	Yield (%)
Crude	4,100	6,800	1.7	1	100
Ammonium sulfate (2.3–6.1 M)	1,400	6,900	4.9	3	101
Phenyl Sepharose CL-4B	540	3,500	6.5	4	51
ConA-Agarose	5.0	2,500	500	300	37
1st Superdex75	0.13	2,400	18,000	11,000	35
2nd Superdex75	0.070	2,000	29,000	17,000	29

[a]Enzyme activity was measured in 50 mM Tris/HCl, pH 8.0, containing 10 mM $MgCl_2$ and 1 mM $CaCl_2$ as described in the text.

Expression of Carp DNase I cDNA in COS-7 Cells—A DNA fragment containing the entire coding sequence of carp DNase I cDNA was prepared from total RNA derived from the hepatopancreas by RT-PCR amplification using an Expanded High Fidelity PCR system (Roche Diagnostics, Tokyo) with a set of two primers (primer 3, 5′-GGATCCACAGCAGAATGAAGATTATT-3′; primer 4, 5′-CTCGAGTTTTTCACAGGAGCTGGAC-3′). The fragment was ligated into a pcDNA 3.1(+) vector (Invitrogen) to construct the wild-type expression vector DN1-p1. For site-directed mutagenesis of the Val68Ile and Phe115Ala substitutions, Ser59 deletion and A225insertion in the carp enzyme, mutant cDNA constructs were produced by splicing by overlap extension (*25*, *27*) using DN1-p1 as a template and named carp-sub (Val68Ile), carp-sub (Phe115Ala), carp-del (Ser59), carp-del (Ser59)/ins (Ala225), and carp-ins (Ala225), respectively. The other expression vectors with cDNA inserts encoding human, porcine and frog DNases I were prepared in the same manner (*14*). The nucleotide sequences of all constructs were confirmed by sequencing and then purified for transfection using a CONCERT High Purity Plasmid Midiprep System (Life Technologies). COS-7 cells were maintained under the conditions described previously (*14*) and transiently transfected with the required cDNA construct by the lipofection method using Lipofectamin-Plus reagent (Life Technologies) (*6*). In order to estimate the transfection efficiency, a mixture containing 2 μg DNase I cDNA construct and 600 ng pSV-β-galactosidase vector (Promega, Tokyo) was transfected into the cells and, two days later, the medium was recovered for subsequent analysis and the cells were harvested for assay of β-galactosidase activity. All transfections were performed in triplicate with at least two different plasmid preparations.

Phylogenetic Analysis—The DNase I amino acid sequences of humans, mice, rats, rabbits, pigs, sheep (*28*), tilapia (*17*), cattle (*29*), hens, three Anura (*Xenopus laevis*, *Bufo vulgaris japonicus* and *Rana catesbeiana*), a newt (*Cynopus pyrrhogaster*), and a snake (*Elaphe quadrivirgata*) were obtained from the databases with the accession numbers described in the legend to Fig. 5. A CLUSTAL W program was used to construct a phylogenetic tree with the sequences aligned according to the neighbor-joining algorithm (*30*, *31*).

Histological Analysis—Fresh carp hepatopancreatic tissue was cut into small pieces, fixed with 4% (v/v) formaldehyde overnight, then embedded in paraffin wax, 4-μm-thick sections were cut and spread on poly-L-lysine-coated glass slides. Histochemical analysis was performed using the anti-carp DNase I antibody produced in this study, as described previously (*32*).

RESULTS AND DISCUSSION

Purification and Characterization of Carp Hepatopancreatic DNase I—A typical carp hepatopancreatic DNase I purification procedure is summarized in Table 1 and resulted in 17,000-fold purification with a 29% yield. The purified carp enzyme had a molecular mass of approximately 32 kDa, determined by both SDS–PAGE (Fig. 1) and gel filtration, which is similar to the molecular masses of mammalian DNases I, approximately 32–34 kDa (*1*, *33*). The N-terminal amino acid sequence up to the 29th residue of purified carp DNase I was: L^{1}LIGAFNIKS^{10}FG-DSKAXXAT^{20}LLDIITKVV^{29}, in which unidentified residues are indicated by X.

The catalytic properties of the purified enzyme closely resemble those of other vertebrate DNases I (*1*, *33*): 20 mM EDTA, 5 mM EGTA and the anti-carp DNase I antibody abolish the enzyme activity, and a divalent cation (Mn^{2+}, Mg^{2+}, or Ca^{2+}) is required for its activity. These biochemical characteristics are very similar to those of the mammalian enzymes from cattle (*1*), sheep (*28*), rats (*5*, *34*), mice (*20*), rabbits (*15*), pigs (*35*), and humans (*19*). However, it is worth noting that the pH-activity profile of

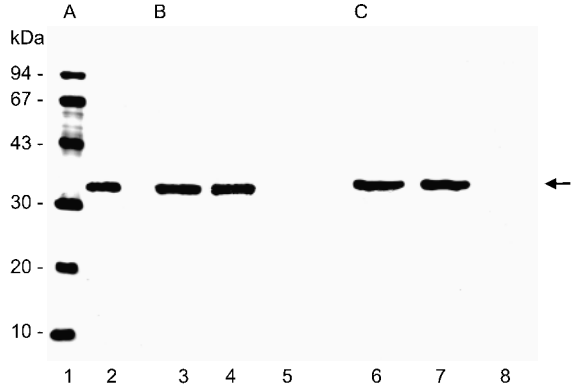

Fig. 1. **SDS–PAGE (A and B) and DNA-cast PAGE (C) patterns of the purified carp DNase I and recombinant proteins expressed in COS-7 cells.** Carp DNase I was detected by protein staining (A), immunostaining with an anti–carp DNase I antibody (B) and activity staining (C). The arrow indicates the band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

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

	DNase I activity (U)[a]		Gene transcript
	per g wet weight ($\times 10^{-2}$)	per mg protein ($\times 10^{-3}$)	
Brain	UD[b]	UD	–[b]
Heart	29 ± 41	13 ± 1.0	+[b]
Hepatopancreas	15,000 ± 8,600	1,500 ± 1,300	+
Kidney	160 ± 70	12 ± 4.0	+
Spleen	1,400 ± 960	320 ± 60	+
Small intestine	2,100 ± 480	690 ± 190	+
Gill filament	100 ± 64	10 ± 3.0	NT[b]
Gill raker	12 ± 4.0	14 ± 3.0	NT
Gas bladder	430 ± 490	33 ± 36	NT

[a]Values are the means ± SEM of duplicate tests on each extract from three carps. 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 carp DNase I. [b]UD, under the limit of detection; +, amplification of a 860 bp fragment specific to carp DNase I by RT-PCR using primers 3 and 4.; –, not amplified; NT, not tested.

the carp enzyme is bell-shaped with an optimal pH of 8.0, and in this respect resembles amphibian enzymes (*14*).

Heat denaturation profiles of three wild-type DNases I derived from the carp, human and frog (*R. catesbeiana*) expressed in COS-7 cells were determined (Fig. 2). The heat denaturation temperature ($T_{1/2}$) is defined as the temperature at which the DNase I activity is halved. The $T_{1/2}$ values were as follows: frog, approximately 37°C; carp, approximately 42°C; human, approximately 50°C. The low heat–resistance of amphibian enzymes in comparison with mammalian enzymes has been reported to be due to the insertion of a Ser residue into the Ca^{2+} site (*14*). Therefore, the low heat-resistance of the carp enzyme relative to mammalian enzymes might be caused by a minor conformational change following the substitution of an amino acid (*1*, *33*). Analysis of the causes of the heat-resistant and/or heat-sensitive properties of vertebrate DNases I is now in progress in our laboratory.

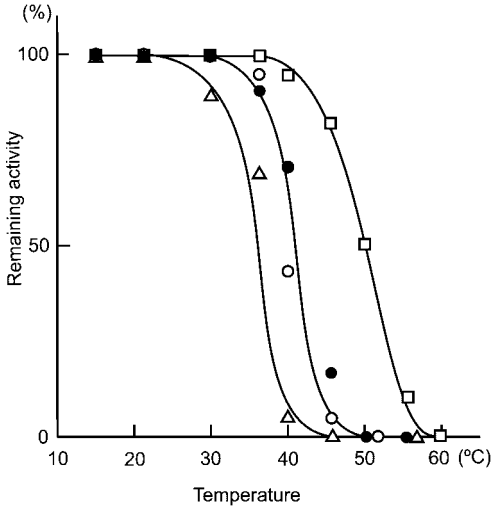

Fig. 2. **Heat denaturation profiles of wild-type carp, human and frog (*R. catesbeiana*) DNases I and several mutant DNases I expressed in COS-7 cells.** Purified enzymes (0.4 U) were incubated at each temperature indicated for 40 min in a water bath and the DNase I activities remaining were determined by the SRED method. Wild-type enzymes: carp (open circle); human (open square) and frog (open triangle) and mutant enzymes: carp-del (Ser59), carp-ins (Ala225) and carp-del (Ser59)/ins (Ala225) (solid cirlce) were tested (each of these mutants had a very similar profile).

Distributions of DNase I Activity and the DNase I Gene Transcript in Carp Tissues—Among the carp tissues analyzed, the highest DNase I activity was observed in the hepatopancreas and low activity was detected in kidney, small intestine, heart, spleen, gas bladder, gill raker and gill filament (Table 2). Under our assay conditions, the brain exhibited no DNase I activity. The enzyme activity was inhibited by 20 mM EDTA, 5 mM EGTA and the anti–carp DNase I antibody and, therefore, was confirmed to be DNase I. The presence of a DNase I–specific mRNA was verified by RT-PCR analysis of total RNA extracted from carp tissues. Using the specific primers 3 and 4 for the carp DNase I cDNA, an 860-bp fragment containing a region encoding the enzyme was amplified from the total RNA of carp heart, hepatopancreas, kidney, small intestine and spleen (Table 2). DNase I–specific mRNA expression in these tissues correlated well with the DNase I activity levels of the corresponding tissue extracts. As no DNase I activity has been detected in other vertebrate spleens (*4*, *14*, *33*, *35*), it appears to be a characteristic of the carp to exhibit a relatively high level of this enzyme activity in its spleen.

Immunohistochemical Analysis of DNase I in the Carp Hepatopancreas—Carp pancreatic tissues are widely scattered, like islands, among the hepatic tissues of the carp hepatopancreas (Fig. 3A). Immunohistochemistry using the anti–carp DNase I antibody revealed strong immunoreactivity in the cytoplasm of many pancreatic cells, but not in the hepatic cellular cytoplasm (Fig. 3B). In a number of different sections examined, no other organelles or components exhibited immunoreactivity with this antibody. Therefore, it is plausible that most of the DNase I enzyme activity, and the fragment specific to the carp DNase I gene that was detected in the hepatopancreas by RT-PCR amplification (Table 2), are derived from the pancreatic, not the hepatic, tissues of the carp hepatopancreas.

Molecular Cloning of a cDNA Encoding Carp DNase I and Expression in COS-7 Cells—Total RNA extracted from carp hepatopancreas was amplified by 3′- and 5′-RACE methods to construct a cDNA encoding carp DNase I. Using a set of specific primers designed from

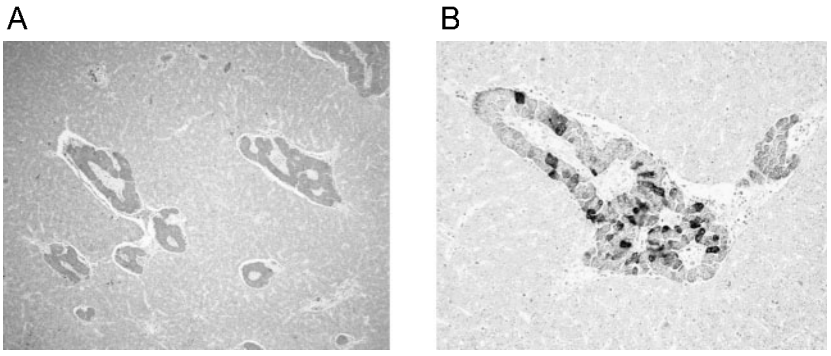

Fig. 3. **Expression of DNase I proteins detected in carp hepatopancreas.** Carp DNase I was detected with a rabbit anti-carp DNase I antibody in the cytoplasm of pancreatic, but not hepatic, tissue of the carp hepatopancreas (A: ×100, B: ×200).

the N-terminal amino acid sequence of the enzyme and nucleotide sequence determined in this study, we succeeded in amplifying two overlapping RACE products. Sequence analysis of these products revealed that the full-length 995-bp cDNA contained a 47-bp 5′-untranslated region, an 840-bp coding region and a 108-bp 3′-untranslated region (Fig. 4). The open reading frame started at position 48 with an ATG start codon and ended with a TGA stop codon at position 887. The putative upstream signal peptide was 19 amino acids long, the 3′-untranslated region was followed by a short poly(A) tail and a putative polyadenylation signal (AATAAA) was located 13 bp upstream from the poly(A) tail. The entire fragment carrying the coding sequence of carp DNase I was cloned into an expression vector, transiently expressed in COS-7 cells, and DNase I enzyme activity was detected in the conditioned medium of these cells. The mean (±SEM., $n = 6$) activity of the enzyme secreted into the medium was 1.3 ± 0.07 U/ml. In contrast, the human and frog (*R. catesbeiana*) DNase I vectors yielded activities of 2.2 ± 0.2 and 0.35 ± 0.10 U/ml respectively (*14*). Thus, the level of carp enzyme expressed in COS-7 cells is fairly similar to that of the human enzyme. These carp enzyme activities were abolished by the anti-carp

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-ttccctgtt ctcacagcat aagcgaaagc agagtcaaca acagcagaAT GAAGATTATT  59
                                                    Me tLysIleIle -16
ACTGCTATAG GTCTACTCCT GGTATCTGTC CACCTTGGAC ACTCCTTATT GATTGGAGCC 119
ThrAlaIleG lyLeuLeuLe uValSerVal HisLeuGlyH isSerLeuLe uIleGlyAla   5

TTCAACATTA AATCCTTTG GGGATTCAAAA GCATCTAATG CCACCCTTCT TGACATCATT 179
PheAsnIleL ysSerPheG lyAspSerLys AlaSerAsnA laThrLeuLe uAspIleIle  25

ACCAAAGTAG TACACCGGTA TGACATTGTA CTCATCCAAG AAGTGAGAGA CAGTGATCTC 239
ThrLysValV alHisArgTy rAspIleVal LeuIleGlnG luValArgAs pSerAspLeu  45

ACTGCAACAA ATAAGCTCAT GCAAAGTGTG AATGGAGGTT CCTCTCCATA TGAATACCAG 299
ThrAlaThrA snLysLeuMe tGlnSerVal AsnGlyGlyS erSerProTy rGluTyrGln  65

TACATTGTAA GTGAGCCCTT GGGTAGGAGC ACTTATAAAG AGAGATATCT CTTTATTTAC 359
TyrIleValS erGluProLe uGlyArgSer ThrTyrArgG luArgTyrLe uPheIleTyr 85

AGACGTCAGG CAGTGTCCGT TGCAAACAGC TTCCAGTATG ATGATGGATG TGAATCCTGT 419
ArgArgProA laValSerVa lAlaAsnSer PheGlnTyrA spAspGlyCy sGluSerCys 105

GGTACTGATA CTTTCAATAG AGAACCCTTT GTTGTGATGT TCTCCTCTAA TACAGCTGTC 479
GlyThrAspT hrPheAsnAr gGluProPhe ValValMetP heSerSerAs nThrAlaVal 125

CAGCAGAAAT TTGCCCTTGT TCCTCAGCAT ACATCCCCAG AAGTCGCTGT GACGGAAATC 539
GlnGlnLysP heAlaLeuVa lProGlnHis ThrSerProG luValAlaVa lThrGluIle 145

GATGCCCTGC ATGATGTCGT CTTGGATACT AGACAACGTC TGAACACAAA CAACATCATG 599
AspAlaLeuH isAspValVa lLeuAspThr ArgGlnArgL euAsnThrAs nAsnIleMet 165

CTTTTGGGAG ACTTCAATGC TGGCTGTAGC TACGTTTCGA ATTCAGACTG GTCCAAAATA 659
LeuLeuGlyA spPheAsnAl aGlyCysSer TyrValSerA snSerAspTr pSerLysIle 185

CGCCTCCGCA CTGACCAAAG TTACACCTGG CTCATCCCTG ATAGTGCGGA CACAACTGTC 719
ArgLeuArgT hrAspGlnSe rTyrThrTrp LeuIleProA spSerAlaAs pThrThrVal 205

ACACACACCA ACTGCCCCTA CGACAGGATT GTGGCCACCT CTGACATGAT GAAAGGAGTG 779
ThrHisThrA snCysProTy rAspArgIle ValAlaThrS erAspMetMe tLysGlyVal 225

TCTGCTGGAT CAGCGCAGGT CTTTGACTTC ATGCAAGCCC ATGGATTGAG CCAGAGTTGG 839
SerAlaGlyS erAlaGlnVa lPheAspPhe MetGlnAlaH isGlyLeuSe rGlnSerTrp 245

GGCCTGGCAG TGAGCGATCA CTTTCCAGCG GAGGTCCAGC TCCTGTGAaa aacatcaggg 899
GlyLeuAlaV alSerAspHi sProProAla GluValGlnL euLeuTerm             260
ttgttccttc gcagcagata catcatgcaa tgctaatgct tgatgtgtag tgtcttagac 959
ttaataaaaa aaattacaac aaaaaaaaaa aaaaaa                           995
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Fig. 4. **Nucleotide and deduced amino acid sequences of the cDNA encoding carp DNase I.** Nucleotides are numbered in the 5′- to 3′-direction and the deduced amino acid residues are indicated under the corresponding nucleotide sequence. Amino acid position 1 was assigned after comparison with the N-terminal amino acid sequence, determined chemically, 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 carp DNase I cDNA has been submitted to the DDBJ/EMBL/GenBank nucleotide databases and is available at accession number AB075779.

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Carp    -19                                                                                    MKIITAIGL LLVSVHLGHS
Human   -22                                                                                 MR G··LLG·LLA ·AALLQGAV·
Bovine  -22                                                                                 MR GTRLMGLLLA ·AGLLQ··L·
Rabbit  -21                                                                                  M RSEML··LLT ·A·LLQVAG·
Hen     -21                                                                                  M ARLVLELLAA A·LLLRVAAT
Tilapia -26                                                                           MQTYRS R·HLVCSL·· F·TLL··SN·
Xenopus -19                                                                                    ·RFLVLVAM TACFLQA·FA

Carp    1   LLIGAFNIKS FGDSKASNAT LLDIITKVVH RYDIVLIQEV RDSDLTATNK LMQSVNGGSSP YEYQYIVSEP LGRSTYKERY
Human   1   ·K·A··QT·· ··E·K····· ·VSY·VQILS ····A·V··· ···H···VG· ·LDNL·QD-AP DT·H·V···· ···NS·····
Bovine  1   ·K·A····RT ··E··M···· ·ASY·VRI·R ·········· ···H·V·VG· ·LDYL·QD-D· NT·H·V···· ···NS·····
Rabbit  1   ·K·A····R· ··ET·M···· ·TSY·VRILQ ····A····· ···H···VG· ·LDKL·EK-AA DT·RFVA··· ··········
Hen     1   ·R·S····RT ·····M··Q· VAGF·VSIIV Q···T·V··· ··A··SSVK· ·VSQL·SA-·· ·P·SFLS·I· ···NS···Q·
Tilapia 1   ··L······· ···T······ ·MN····I·K ···VI····· ·····S··QT ··NY··KD-·P -Q·K······ ··A·······
Xenopus 1   FK·AS···QR ·SMT·VDDPV V·ELLIRILS ··G·IA·E·· MNA·N··IIS ·VKELSLA-TK LN·N·LI·DH ····STR·K·

Carp    82  LFIYRRPAVS VANSFQYDDG CESCGTDTFN REPFVVMFSS NTAVQQKFAL VPQHTSPEVA VTEIDALHDV VLDTRQRLNT
Human   81  ··V··PDQ·· AVD·YY···· ··P··N···· ···AI·RFF· RFTEVRE··I ··L·AA·GD· ·A····Y·· Y··VQEKWGL
Bovine  81  ··LF·PNK·· ·LDTY····· ·····N·S·S ···A··K··· HSTKVKE··I ·AL·SA·SD· ·A··NS·Y·· Y··VQ·KWHL
Rabbit  81  ··V··PDQ·· ·LD·YY···· ··P······S ···A··R··· PSTKVRE··I ··L·SA··D· ·A····Y·· Y··VQKKWGL
Hen     81  V····SDI·· ·LE·YY···· ·······I·S ····I·K··· P·TQLDEFVI ··L·AE·AC· PA··N··T·· YT·VINKWE·
Tilapia 80  ··L··EAL·· ·VK·YT···· P·ET·Q···S ·········· K·TAVKD·T· I·····DL· ·R·LN··Y·· ···V·A·W··
Xenopus 81  AYV·IEEI·K PTEWYHF··· ··N····S·I ·····AR·T· L·TV·KD··· ISI····DY· IM·V···Y·A WV·AK···KM

Carp    162 NNIMLLGDFN AGCSYVSNSD WSKIRLRTDQ SYTWLIPDSA DTTV-THTNCP YDRIVATSDM MKG-VSAGSA QVFDFMQAHG
Human   161 EDV··M···· ······RP·Q ··S···W·SP TFQ······· ···A-·P·H·A ·····VAGML LR·A·VPD·· LP·N·QA·Y·
Bovine  161 ·DV··M···· ·D····TS·Q ··S·····SS TFQ······· ···A-·S···A ·····VAGSL LQSS·VP··· AP···QA·Y·
Rabbit  161 QDV··M···· ·D····TS·Q ··S·····NP AFK·····T· ···A-·S···A ·····V·GPL LQDA·VPN·· AP·N·QA·Y·
Hen     161 ···FFM···· ·D····TAEQ ·PS····SLS ·CE······· ·····-S·D·A ······CGSA LRQA·EY··· T·DN·QETLH
Tilapia 160 ·D·V······ ·······G·A ·QQ··IF··K TFH···T·A· ····-SQ·V·· ·····V·T·· ·R·-VQN·· K·YNY·TDLN
Xenopus 161 E··LI··DY· ·A···GASRH ·PI····HVE ELV···G·KE ····STN···A ···M··GGEE LQRGIVPDT· KA·NYHVAYD

Carp    241 LSQSWGLAVS DHFPAEVQLL
Human   241 ··DQLAQ·I· ··Y·V··M·K
Bovine  241 ··NEMA··I· ··Y·V··T·T
Rabbit  241 ··NQLAQ·I· ··Y·V··T·A
Hen     241 ·TNQMAE·I· ··Y·V··T·R AR
Tilapia 240 ·K·DLA···· ····V··K·S
Xenopus 242 ·TYEMAK··Y ··Y·V··E·Y DDVFYSGQCF EPSASTGISF GLSLNGPCTC EGWDFSSCRG RCGASGKTYP CNCNASCTN-

Xenopus 321 CCVDYTSS CKL
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Fig. 5. **Comparison of the amino acid sequences of carp (this study) and other vertebrate DNases I.** The amino acid sequences of the DNases I of humans, mice, rats, rabbits, pigs, sheep (*27*), tilapia (*28*), cattle (*29*), hens, three Anura (*Xenopus laevis*, *Bufo vulgaris japonicus* and *Rana catesbeiana*), a newt (*Cynopus pyrrhogaster*), and a snake (*Elaphe quadrivirgata*) were obtained from the following databases with the following accession numbers: EMBL M55983, EMBL U00478, EMBL X56060, EMBL D82875, EMBL AB048832, Swiss-Prot drn 1-sheep, EMBL AJ001305, EMBL AJ 001538, EMBL AB013751, EMBL AB 030958, EMBL AB045037, EMBL AB038776, EMBL AB041732, and EMBL AB046545. The amino acid sequences of seven vertebrate enzymes were obtained from the data described above. The amino acid sequences of the putative signal peptides are underlined with waving lines. The amino acids are numbered starting at the N-terminus of each mature protein. The dots indicate residues that are the same as those in carp DNase I, and the horizontal bars indicate deleted amino acid residues.

DNase I antibody and 20 mM EDTA. The expressed carp DNase I had the same electrophoretic mobility as the carp hepatopancreatic enzyme (Fig. 1, lanes 3, 4, 6, and 7) and its N-terminal amino acid sequence up to the 29th residue, deduced from its cDNA data, exactly matched that determined chemically for the purified enzyme. These findings led us to conclude that the cDNA we isolated encodes the carp DNase I.

The coding region of the carp DNase I cDNA was translated into the corresponding amino acid sequence and compared with the other six available vertebrate DNase I sequences (*9*, *14*, *29*, *35–38*) (Fig. 5). The carp DNase I has a Ser59 insertion corresponding to the position between Asp58 and Ala59 in human DNase I and a deletion of one residue between Gly224 and Val225, corresponding to the position of Ala224 in human DNase I. The latter deletion is found in the tilapia enzyme (*28*) and thought to be common to piscine DNases I, although the Ser59 insertion was not found in the tilapia enzyme. In order to examine the biochemical significance of the insertion and deletion of one amino acid residue in the carp enzyme, we constructed a deletion mutant, carp-del (Ser59), an insertion mutant, carp-ins (Ala225), and a deletion/insertion double mutant, carp-del (Ser59)/ins

(Ala225), and compared their heat-stabilities (Fig. 2). As the heat denaturation profiles of these mutant enzymes and the wild-type carp enzyme were very similar, the deletion at position 59 and/or insertion at position 225 exerts no influence on the heat-stability of the carp enzyme. Furthermore, none of these changes resulted in any drastic alteration in the secondary structure predicted by the method of Ito et al. (*39*). In contrast, the insertion of Ser205 in the Ca^{2+}-binding region of the amphibian enzymes is essential for the generation of the active enzyme and heat-stability (*14*).

The four residues responsible for the catalytic activity of other vertebrate DNases I, Glu78, His134, Asp212, and His252, are conserved in the carp enzyme (*4*, *9*, *14*, *15*, *33–38*, *40–43*), although the first three are shifted to Glu79, His135, and Asp213 by the Ser59 insertion. The shift to a higher optimal pH might reflect conformational changes around the active site compared with the other mammalian enzymes, which have optimal pH values of 6.5–7.0 (*1*, *5*, *15*, *16*, *20*, *29*, *33*). Two residues, Cys173 and Cys209, which form the disulfide bond responsible for the stability of vertebrate enzymes (*40*), were also found in the carp enzyme, although shifted to Cys174 and Cys210, respectively, by the Ser59 insertion. The essential sites that mediate DNase I–DNA contact in other vertebrate DNases I include two well-conserved residues, Arg41 and Tyr76, that orientate the scissile phosphate relative to the enzyme (*41*). These two residues were found in the carp enzyme, although the latter was shifted to Tyr77. Mammalian DNases I possess two potential N-glycosylation sites, Asn18 and Asn106, that are well conserved and attached to carbohydrate chains (*1*, *33*). Although the former was found in the carp enzyme, the latter was shifted and substituted with Thr107. It is interesting that amphibian DNases I contain no carbohydrate (*14*). 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 (*33*). Both these sequences are well conserved in the carp enzyme, although their amino acid positions are shifted to 131–151 and 168–175, respectively.

Affinity of Wild-Type and Mutant DNases I for G-Actin—Carp wild-type DNase I activity was inhibited slightly by G-actin: the addition of 3 nM actin resulted in approximately 20% inhibition, whereas this concentration of actin resulted in 100% and 0% inhibition of the human and frog (*R. catesbeiana*) enzymes, respectively (Fig. 6). Although the DNase I activities of cow (*1*), rabbit (*15*), mouse (*20*), and human (*4*, *37*) are inhibited by G-actin, those of pig (*35*), frog (*14*), hen (*38*), and rat (*5*) are not. The lack of or reduction in the inhibition of DNase I by actin is due to amino acid substitution(s) at the actin-binding site, which, in human and bovine DNases I, comprises mainly three residues (Tyr65, Val67, and Ala114) (*4*, *28*, *37*, *44*, *45*). Rabbit DNase I, which retains two (Tyr65Phe and Val67Ala) of the three residues in the actin-binding site, is inhibited by actin to a lesser extent than human DNase I (*15*), whereas frog (*R. catesbeiana*) DNase I, in which all three residues (Tyr65Leu, Val67Ile, and Ala114Phe) are replaced, completely lacks affinity for actin (*14*). Actin-resistant human DNase I mutants, Tyr65Ala, Val67Arg, Ala114Glu, Ala114Met, Ala114Arg, and Ala114Tyr, bind significantly less well than the wild-type enzyme to actin (*43*). Tyr65 and Val67 in the human enzyme are conserved in the carp enzyme, although they are shifted to Tyr66 and Val68, respectively. However, in the carp enzyme, Ala114 is not only replaced by Phe but is also shifted to position 115 (Phe115) by the Ser59 insertion. In order to examine the biochemical significance of these amino acid replacements, we constructed two substitution mutants, carp-sub (Val68Ile), a frog-type change, and carp-sub (Phe115Ala), a human-type change, and compared their affinities for actin with those of wild-type carp, frog and human enzymes (Fig. 6). The affinity for actin of the carp-sub (Val68Ile) and carp-sub (Phe115Ala) mutant enzymes were reduced to approximately 10 and 0%, respectively, of the wild-type enzyme by the addition of 3 nM actin. According to the method of Ito *et al.* (*39*), the predicted secondary structure of each of these mutants is slightly altered in comparison with that of the wild-type carp enzyme, which may result in their reduced or absent affinity for actin.

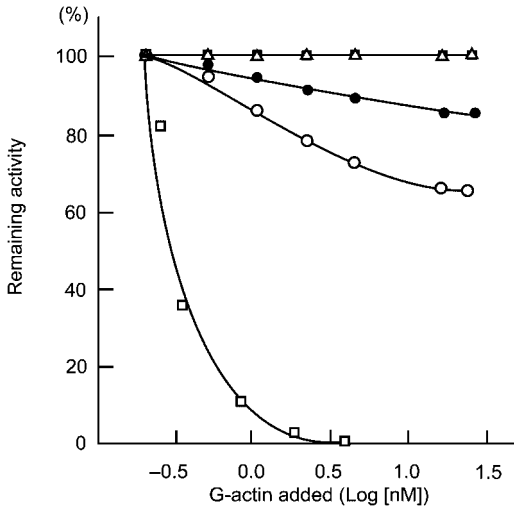

Fig. 6. **Actin-inhibition profiles of wild-type carp and human DNases I and several mutant DNases I expressed in COS-7 cells.** Wild-type enzymes: carp (open circle), human (open square) and frog (open triangle), and two substitution mutant enzymes: carp-sub (Val68Ile) (solid circle) and carp-sub (Phe115Ala) (solid square), were tested. Each purified enzyme (0.5 U) was incubated with G-actin and the activities remaining were determined by the test tube method.

Immunological Comparison of Vertebrate DNases I—Four different specific antibodies against carp, human, porcine and frog (*R. catesbeiana*) DNases I were tested for mutual cross-inhibition the COS-7 cell–expressed DNases I of carp, human, porcine and frog. The anti-carp DNase I antibody abolished the carp enzyme activity and weakly inhibited the frog enzyme activity, whereas it had no effect on the human and porcine enzymes (Fig. 7A). The anti-human DNase I and anti-porcine DNase I antibodies abolished human and porcine DNase I activities, respectively, and weakly inhibited the porcine and human enzymes, respectively, but were ineffective aga-

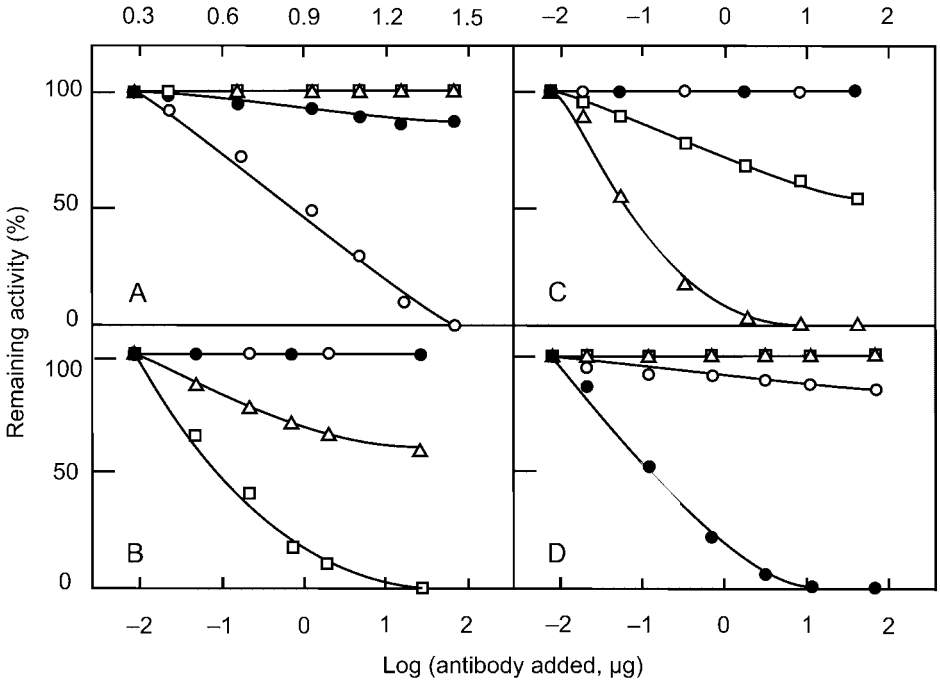

Fig. 7. **Mutual inhibitory effects of species-specific anti–DNase I antibodies on the activities of vertebrate purified DNases I.** Each DNase I (0.4 U) was incubated with the specific antibodies against the DNase I from carp hepatopancreas (A), human urine (B), porcine pancreas (C), or frog (*R. catesbeiana*) pancreas (D), and the activities remaining were measured by the test tube method. The effects of each antibody on the activities of purified carp (open cirlce), human (open square), porcine (open triangle), and frog (solic circle) DNases I expressed in COS-7 cells are shown.

inst the carp and frog enzymes (Fig. 7, B and C). The anti–frog DNase I antibody abolished the frog enzyme activity and weakly inhibited the carp enzyme, whereas it was ineffective against the human and porcine enzymes (Fig. 7D). These findings show that, from an immunological standpoint, carp DNase I resembles the frog enzyme slightly, but bears little or no resemblance to the human or porcine enzymes.

Phylogenetic Analysis of the Vertebrate DNase I Family—The amino acid sequences of 15 different mature DNase I enzymes were aligned in order to construct a phylogenetic tree for the DNase I family (Fig. 8). Carp DNase I is situated at an independent position far from the positions of mammalian, avian, reptilian and amphibian DNases I, whereas the carp and tilapia enzymes form a relatively tight cluster as a piscine group. The phylogenetic tree constructed for the DNase I family shows carp DNase I to be located far from the mammalian, avian, reptile and amphibian enzymes. When determined separately, the degree of intra-class similarity for the piscine DNase I group (65%) was lower than that for the mammalian DNase I group (79.8 ± 1.4%), suggesting that the former exhibits greater diversity than the latter from the standpoint of molecular evolution. More systematic surveys, not only of carp DNase I but also of other animal DNases I, might help to elucidate the inherent, but as yet unknown, physiological role(s) of members of the DNase I family. On the basis of the immunological properties of vertebrate DNases I, the carp enzyme slightly resembles the amphibian enzyme, but is far removed from the mammalian enzymes. The results of our phylogenetic analysis of carp DNase I are consistent with those of our immunological analyses and previous reports (*4*, *5*, *14*, *15*, *20*, *32*, *33*).

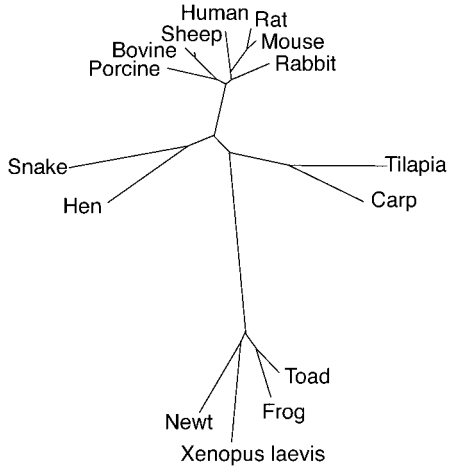

Fig. 8. **Phylogenetic analysis of the vertebrate DNase I family.** The amino acid sequences of the mature DNase I proteins of various species were subjected to phylogenetic analysis. The neighbor-joining algorithm of the CLUSTAL W program was used to construct a phylogenetic tree by aligning the sequences. The accession number of the amino acid sequence of each vertebrate enzyme is listed in the legend to Fig. 5.

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