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 Ca²⁺-binding site

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We purified four amphibian deoxyribonucleases I from the pancreases of one toad, two frog and one newt species, by using three different column chromatography methods in sequence. Each of the purified enzymes had a molecular mass of approx. 40 kDa and an optimal pH for activity of approx. 8.0. These values were significantly greater than those for other vertebrate DNases I. The full-length cDNA encoding each amphibian DNase I was constructed from the total RNA of the pancreas by using rapid amplification of cDNA ends. Nucleotide sequence analyses revealed two structural characteristics unique to amphibian DNases I: a stretch of approx. 70 amino acids with a high cysteine content (approx. 15%) in the C-terminal region, and the insertion of a serine residue at position 205 (in a domain containing an essential Ca²⁺-binding site). Expression analysis of

a series of mutant constructs indicated that both of these structures are essential in generating the active form of the enzyme. 'DNase I signature sequences', which are well conserved in other vertebrate DNases I, could not be found in any of the amphibian DNases I tested, whereas a 'somatomedin B motif' was identified in the Cys-rich stretches of all four. Although DNase I has so far been considered to be a secretory glycoprotein, amphibian DNase I seems to be non-glycosylated. These structural findings indicate strongly that amphibian DNases I are situated in a unique position on the phylogenetic tree of the DNase I family.

Key words: amphibia, cDNA cloning, deoxyribonuclease I, pancreas, protein purification.

INTRODUCTION

A recent report [1] suggesting that a lack of, or a decrease in, DNase I (EC 3.1.21.1) activity might be a critical factor in the initiation of human systemic lupus erythematosus has focused attention on the potential physiological role of this enzyme. Furthermore, DNase I or DNase I-like enzymes have now been postulated to be responsible for internucleosomal DNA degradation during apoptosis [2]. Although DNase I had previously been considered to be merely a digestive enzyme, the presence of its activity in mammalian tissues other than the digestive organs has suggested that it might have other functions *in vivo* [3,4]. With the use of isoelectric focusing, human DNase I has been shown to exhibit genetic polymorphism controlled by at least six alleles [5,6], of which the *DNASE1*2* allele might be a risk factor for gastric carcinoma [7]. Mammalian DNases I are classified into three types [pancreatic, parotid and parotid-pancreatic (mixed)], according to their main source, and their sensitivities to low pH are dependent on the type [4]. Because human and mouse DNases I are of the pancreatic and parotid types respectively, it remains to be seen whether the findings obtained from experiments with DNase I-deficient mice [1] can be extrapolated directly to explain the aetiology of human systemic lupus erythematosus.

In this context, to elucidate the intrinsic intracellular and extracellular functions of DNase I, as well as the phylogenetic origins of the DNase I family, comprehensive comparative studies of lower and higher animals are essential. Indeed, the DNase I of the South African clawed frog, *Xenopus laevis*, has been found to be distinctly different from those of other vertebrates, having a greater molecular mass, a higher optimal pH for activity and a lower stability at high temperatures [8,9]. These findings led us to believe that *X. laevis* DNase I might be situated in a unique position on the phylogenetic tree of the DNase I family. However, although the structural and catalytic properties of *X. laevis* DNase I might make it easily distinguishable from the DNases I of other vertebrates, it remains unknown whether these properties are shared by other amphibia. To address this question, a systematic survey of amphibian DNases I is required.

In the present study we purified DNases I from the pancreases of two species of frog, *Rana catesbeiana* and *X. laevis*, one toad, *Bufo vulgaris japonicus* (*B. vulgaris*), and one newt, *Cynopus pyrrhogaster*, and cloned the cDNA of each. The expression of these cDNA species, together with those of a series of mutant constructs, was examined in COS-7 cells; several common structural and functional characteristics of amphibian DNases I were confirmed.

Abbreviations used: C-stretch, stretch region at the C-terminus; PNGase F, recombinant peptide:N-glycosidase F; RACE, rapid amplification of cDNA ends; RT-PCR, reverse-transcriptase-mediated PCR; SRED, single radial enzyme diffusion.

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The nucleotide sequence data reported will appear in DDBJ, EMBL and GenBank® Nucleotide Sequence Databases under the accession numbers AB038776, AB041732, AB045037 and AB030958.

EXPERIMENTAL

Materials and biological samples

Four amphibians, *C. pyrrhogaster*, *B. vulgaris*, *R. catesbeiana* and *X. laevis*, weighing approx. 10, 130, 700 and 110 g respectively, were obtained from Shiraishi Animal Laboratories (Tokyo, Japan). Superscript II reverse transcriptase, LipofectaminPlus, all oligonucleotide primers and the 3' and 5' rapid amplification of cDNA ends (RACE) systems were obtained from Life Technologies (Gaithersburg, MD, U.S.A.). Phenyl-Sepharose CL-4B, Superdex 75 and DEAE-Sepharose CL-6B were purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). The Expanded High Fidelity PCR system and Ni²⁺-agarose column were purchased from Boehringer (Mannheim, Germany) and Qiagen (Chatsworth, CA, U.S.A.) respectively. Agarose beads conjugated with concanavalin A, lentil lectin, castor bean lectin and wheatgerm lectin were obtained from Seikagaku Kogyo (Tokyo, Japan); recombinant peptide:N-glycosidase F (PNGase F) was purchased from New England Biolabs (Beverly, MA, U.S.A.). Human and rat DNases I, and antibodies against each of these enzymes, were prepared by methods described previously [10,11]. All other chemicals used were of reagent grade and available commercially.

Analytical methods

DNase I activity was assayed by the single radial enzyme diffusion (SRED) method [10] or the test tube method [9], except that 50 mM Tris/HCl buffer, pH 8.0, containing 20 mM MgCl₂ and 2 mM CaCl₂ was substituted for the previously described reaction buffer. One unit of activity was defined as an increase of 1.0 in *A*₂₆₀. The enzymological and chemical properties of the enzymes, the inhibitory effects of specific antibodies on their activities and their tissue distributions were examined as described previously [4,12,13]. Proteins were determined with a protein assay kit (Bio-Rad, Richmond, CA, U.S.A.) with BSA as a standard. The N-terminal amino acid sequences of the purified enzymes were analysed by Edman degradation [11]. SDS/PAGE was performed in 12.5% (w/v) gels [14]; the proteins thus separated were detected by staining with silver or Western blotting with specific antisera. Activity staining for DNase I was performed with a DNA casting-PAGE method [15]. The β -galactosidase assay was performed by the conventional method [16]. The presence of DNase I-specific mRNA was verified by reverse-transcriptase-mediated PCR (RT-PCR) amplification of the total RNA extracted from each of the amphibian tissues with sets of primers corresponding to the N-terminal and C-terminal amino acid sequences of the respective enzymes [17].

Purification of amphibian pancreatic DNases I

All procedures were performed at 0–4 °C. Pancreases obtained from *X. laevis*, *R. catesbeiana*, *B. vulgaris* and *C. pyrrhogaster*, weighing approx. 0.88, 2.5, 0.77 and 0.03 g respectively, were minced and homogenized in approx. 2.5–5 ml of 25 mM Tris/HCl, pH 7.5 (buffer I), containing 1 M (NH₄)₂SO₄ and 1 mM PMSF. After centrifugation (10000 g, 20 min), the supernatant (crude extract) was applied to a phenyl-Sepharose CL-4B column (1.6 cm × 15 cm) pre-equilibrated with the same buffer. The adsorbed materials were eluted with a 300 ml linear reverse gradient of 1–0 M (NH₄)₂SO₄ in buffer I; the fractions exhibiting DNase I activity were eluted with approx. 0.2 M (NH₄)₂SO₄. After the addition of solid (NH₄)₂SO₄ to a final concentration of 1 M, these fractions were applied to a second phenyl-Sepharose CL-4B column under the same conditions, except that the column

size was 1.0 cm × 10 cm. This second phenyl-Sepharose CL-4B separation was omitted for *C. pyrrhogaster* DNase I. After elution, the active fractions were applied to a DEAE-Sepharose CL-6B column (1.0 cm × 15 cm) pre-equilibrated with buffer I. The adsorbed materials were eluted with a 100 ml linear NaCl gradient (0–1 M) in buffer I. The active fractions were eluted at 0.25–0.3 M NaCl, concentrated, then subjected to gel filtration with the ÄKTAFLC system (Amersham Pharmacia Biotech) equipped with a Superdex 75 column and eluted with buffer I containing 0.15 M NaCl. The active fractions were collected and used as the purified enzyme for subsequent experiments. Antisera specific for the purified *R. catesbeiana* and *X. laevis* enzymes were prepared as described previously [10,11].

Construction of the cDNA species encoding *C. pyrrhogaster*, *B. vulgaris* and *R. catesbeiana* DNases I

Total RNA was isolated from the pancreas of each amphibian by the acid guanidinium thiocyanate/phenol/chloroform extraction method [18]. Possible DNA contamination was removed by treatment with RNase-free DNase I (Stratagene, La Jolla, CA, U.S.A.). The 3' RACE for all the amphibians was performed by using nested PCR amplification with two degenerate primers based on amino acid sequences that are highly conserved in mammalian DNases I, 5'-GGAGACTTCAATGCTAATGCTGA(C/T)TGC-3' and 5'-TGG(T/C)T(A/G)T(A/C/T)(C/G)-(C/G)IGA(C/T)AA(A/G)G(A/C)IGA(T/C)ACIACIG-3', corresponding to the Gly¹⁶⁷-Cys¹⁷³ and Trp¹⁹⁴-Thr²⁰³ sequences of human DNase I [19] respectively. Next, the 5'-end region of the cDNA was amplified by the 5' RACE method with gene-specific primers based on the nucleotide sequences determined in this study. These RACEs were performed with 3' and 5' RACE systems in accordance with the manufacturer's instructions. The RACE products were subcloned into the pCR II TA cloning vector (Invitrogen, San Diego, CA, U.S.A.) and sequenced. The nucleotide sequences were determined by the dideoxy chain-termination method with a Dye Terminator Cycle Sequencing kit (FS; Applied Biosystems, Urayasu, Japan). The sequencing run was performed on a Genetic Analyzer (model 310; Applied Biosystems). All DNA sequences were confirmed by reading both strands.

Construction of expression vectors

A DNA fragment containing the entire coding sequence of *R. catesbeiana* DNase I cDNA was prepared from the total RNA derived from the pancreas by RT-PCR amplification with an Expanded High Fidelity PCR system with a set of two primers, 5'-GAATTCGAGGAAATGAAGTCCCTC-3' and 5'-CTCG-AGAATTAAATTTGCAGACTGCTGCTGC-3', corresponding to the nucleotide sequences of the cDNA from positions 63–84 and 1110–1136 respectively. The amplified fragment was ligated into the pcDNA3.1(+) vector (Invitrogen) to construct wild-type expression vectors. Five other wild-type expression vectors with cDNA inserts encoding *X. laevis*, *C. pyrrhogaster*, *B. vulgaris*, human and rat DNases I were prepared in the same manner. Two further expression vectors, *R. catesbeiana*-(6 × His) and *X. laevis*-(6 × His), in which a hexahistidine tag was attached to the C-terminus of the *R. catesbeiana* and *X. laevis* wild-type DNases I respectively, were prepared similarly, except that the nucleotide sequence coding for His₆ was introduced into the corresponding anti-sense primers. Two insertion mutants, human-ins(S205) and rat-ins(S205), in which a Ser residue was inserted between the Ala²⁰⁴ and Thr²⁰⁵ residues of human and rat DNases I, and two deletion mutants, *X. laevis*-del(S205) and

R. catesbeiana-del(S205), in which the Ser residue at position 205 was deleted from the *X. laevis* and *R. catesbeiana* DNases I, were constructed with the method of splicing by overlap extension [20], with the appropriate wild-type construct as a template. Two further deletion mutants, *X. laevis*-del(C-stretch) and *R. catesbeiana*-del(C-stretch), in which an additional stretch in the C-terminal region was deleted from the *X. laevis* and *R. catesbeiana* enzymes to produce a coding region of the same length as that in human DNase I, were prepared by a method described previously [21]. All constructs were sequence-confirmed and purified with the Plasmid Midi kit (Qiagen) for transfection.

Transient expression of the constructs in COS-7 cells

COS-7 cells were maintained in Dulbecco's modified Eagle's medium containing 1 mM L-glutamine, 50 i.u./ml penicillin, 50 µg/ml streptomycin and 10% (v/v) fetal calf serum (Life Technologies) at 37 °C under air/CO₂ (19:1). The cells were transiently transfected with LipofectaminPlus reagent [22]. A mixture containing 2 µg of the expression vector and 0.6 µg of the pSV-β-galactosidase vector (Promega, Madison, WI, U.S.A.) was introduced into the cells to estimate transfection efficiency. After 2 days the medium and cells were recovered for analysis. The DNase I activity in the medium was determined by the SRED method and the cell lysates were used for the assay of β-D-galactosidase. After electrophoresis the expressed DNase I was analysed by Western blotting with each of the specific antibodies or activity staining [15].

RESULTS AND DISCUSSION

Purification and characterization of amphibian pancreatic DNases I

The degree of purification of each of the amphibian pancreatic DNases I after each stage of the four-step procedure is summarized in Table 1. This procedure, using three different kinds of column chromatography, allowed all four amphibian DNases I

Figure 1 Electrophoretic patterns of the purified wild-type amphibian DNases I (A) and recombinant enzymes (B)

(A) Purified DNases I (approx. 1 µg) from humans (lane 1), *B. vulgaris* (lane 2), *C. pyrrhogaster* (lane 3), *R. catesbeiana* (lane 4) and *X. laevis* (lane 5) were dissolved in 10 mM Tris/HCl, pH 6.8, containing glycerol (10%, v/v), SDS (2%, w/v) and 25 mM dithiothreitol, heated at 100 °C for 5 min and subjected to SDS/PAGE, followed by staining with silver. (B) Recombinant vectors containing *R. catesbeiana* (lane 2) and *C. pyrrhogaster* (lane 4) DNase I cDNA inserts were separately transfected into COS-7 cells by the lipofection method. The recombinant DNase I secreted into the medium was subjected to DNA-casting PAGE, followed by activity staining [15]. The purified wild-type DNases I from *R. catesbeiana* (lane 1) and *C. pyrrhogaster* (lane 3) were analysed in the same manner. The recombinant DNases I of *B. vulgaris* and *X. laevis* exhibited the same electrophoretic mobility as the corresponding purified wild-type enzymes (results not shown). The cathode is at the top. The positions of molecular mass markers are indicated at the left.

to be easily and reproducibly isolated to electrophoretic homogeneity (Figure 1A), representing approx. 1500-fold purification. The purified amphibian DNases I had a molecular mass of approx. 40 kDa as determined by both SDS/PAGE and gel filtration; thus the amphibian enzymes are significantly larger than those of other vertebrates, which average approx. 34 kDa. The catalytic properties of the four purified enzymes closely resembled those of the other vertebrate DNases I [23]. However, it is worth noting that the pH-activity profile of all the amphibian

Table 1 Summary of the purification of DNase I from the pancreases of four kinds of amphibian

The results of the enzyme purification sequence, with pancreases obtained from five animals of each species as starting material, are summarized. The second separation with phenyl-Sepharose CL-4B was omitted for *C. pyrrhogaster*.

Species	Purification step	Protein (mg)	Total activity (units)	Specific activity (units/mg)	Purification (fold)	Yield (%)
<i>X. laevis</i>	Crude extract	560	490	0.9	1.0	100
	Phenyl-Sepharose CL-4B	60	450	7.5	8.3	92
	Phenyl-Sepharose CL-4B	38	380	10	11	78
	DEAE-Sepharose CL-6B	5.1	250	49	54	51
	Superdex 75	0.14	230	1600	1800	46
<i>R. catesbeiana</i>	Crude extract	1200	160	0.13	1.0	100
	Phenyl-Sepharose CL-4B	96	120	1.3	10	75
	Phenyl-Sepharose CL-4B	1.9	80	42	320	50
	DEAE-Sepharose CL-6B	0.8	43	54	410	27
	Superdex 75	0.16	37	230	1800	23
<i>B. vulgaris</i>	Crude extract	66	17	0.25	1	100
	Phenyl-Sepharose CL-4B	2.6	8.1	3.1	13	49
	Phenyl-Sepharose CL-4B	0.30	7.5	25	100	45
	DEAE-Sepharose CL-6B	0.10	4.5	45	180	27
	Superdex 75	0.010	3.3	330	1300	20
<i>C. pyrrhogaster</i>	Crude extract	4.7	4.0	6.0	7.1	60
	Phenyl-Sepharose CL-4B	0.060	1.8	30	35	45
	DEAE-Sepharose CL-6B	0.0050	1.4	280	330	35
	Superdex 75	0.0010	1.2	1200	1400	30

Figure 2 Inhibitory effects of species-specific antibodies on the activities of amphibian DNases I

Each purified DNase I (1.0 unit) was incubated with antibodies specific for DNases I from *X. laevis* (A), *R. catesbeiana* (B) and humans (C). The residual activities were measured as described previously [10,11]. The effects of each antibody on the activities of DNases I purified from humans (▲), *B. vulgaris* (○), *C. pyrrhogaster* (□), *R. catesbeiana* (●) and *X. laevis* (■) are shown.

enzymes was bell-shaped with an optimum at pH 8.0. Because the additional amino acid residues in amphibian DNases I might be involved in their catalytic activities, this shift to a higher optimal pH might reflect conformational alterations around the active site compared with the other vertebrate enzymes, which have optimal pH values of 6.5–7.0 [10–12,24]. Although G-actin is known to be a potent inhibitor of bovine [24], human [10], mouse [13] and rabbit [12] DNase I, the activities of the amphibian enzymes were completely unaffected by G-actin. In addition, Tyr⁶⁵, Val⁶⁷ and Ala¹¹⁴, which have been assumed to be responsible for actin binding in human DNase I [25], were rarely conserved in the amphibian enzymes. Therefore the lack of actin affinity might be attributable to the replacement of these amino acids in amphibian DNases I.

Three different antibodies (specific for human, *R. catesbeiana* and *X. laevis* DNase I respectively), were examined for mutual cross-inhibition of human, *R. catesbeiana*, *B. vulgaris*, *C. pyrrhogaster* and *X. laevis* enzymes (Figure 2). The anti-(human DNase I) antibody completely blocked the activity of human DNase I, but was ineffective against all of the amphibian DNases I. Anti-(rat DNase I) antibody had the same effects on rat and amphibian enzymes (results not shown). Similarly, the anti-

bodies against the *R. catesbeiana* and *X. laevis* DNases I completely inhibited the activity of the corresponding DNase I but not that of the human enzyme. These findings confirm that none of the amphibian DNases I tested is immunologically close to mammalian DNase I. Of the two anuran-specific anti-(DNase I) antibodies, the anti-(*R. catesbeiana*) and anti-(*X. laevis*) antibodies exhibited moderate and slight cross-reactivity with the other anuran (*B. vulgaris*) and urodele (*C. pyrrhogaster*) enzymes respectively, indicating that *C. pyrrhogaster* DNase I is distinct from the three anuran enzymes. These immunological relationships are consistent with the results of the nucleotide-sequence-identity study, supporting the hypothesis that many of the evolutionary substitutions of amino acid residues that have occurred in globular proteins are immunologically detectable [26].

Tissue distribution of amphibian DNases I

The DNase I activity in 14 different tissue extracts from each amphibian was determined separately (Table 2). The highest activity was observed in the pancreas, with low activity in the small intestine; however, the other tissues tested exhibited no activity under our assay conditions. These activities were completely abolished by 10 mM EDTA, 1 mM EGTA and the respective specific antisera, confirming the activities to be those of DNase I [10–12,23]. The presence of DNase I-specific mRNA was verified by RT-PCR of the total RNA extracted from the amphibian tissues. Specific PCR products were amplified from the total RNA species of both the pancreases and small intestines of the four amphibians, whereas no amplified products could be obtained from the RNA species of the other tissues. Thus, the expression of gene transcripts of DNase I was well correlated with the DNase I activity levels measured in these tissues. The activity of DNase I is known to reflect DNase I gene expression in the tissues [12]. The restriction of DNase I gene expression to only two tissues in amphibians contrasts with the situation in mammals, in which expression has been observed in various tissues including the kidney, liver and stomach as well as the pancreas and small intestine [4]. Pancreatic-type DNases I (human and pig) are more sensitive to low pH than the parotid (rat and mouse) and pancreatic-parotid (bovine and rabbit) types [4]. Because of their sensitivity to low pH (results not shown), the four amphibian DNases I were classified as being of pancreatic type.

cDNA structures encoding the four amphibian DNases I

The total RNA species isolated from the four amphibian pancreases were amplified separately by RACE methods to construct cDNA species encoding DNase I. The use of two primers based on amino acid sequences that are highly conserved in vertebrate DNases I [9] permitted the successful amplification of specific RACE products from the total RNA of each species. The full-length cDNA encoding *R. catesbeiana* DNase I (accession number AB038776) was composed of 1206 bp, including an open reading frame of 1062 bp coding for 352 residues, a 72 bp 5' untranslated region and a 72 bp 3' untranslated region. The sequence flanking the first ATG (positions 73–75) was in accordance with the Kozak consensus for a translation start site [27]. The putative upstream signal sequence containing the first ATG initiation codon is 19 residues long. We cloned and sequenced the cDNA species encoding the three other amphibian DNases I, *C. pyrrhogaster* (accession number AB041732), *B. vulgaris* (AB045037) and *X. laevis* (AB030958) [9], and found them to have full-length sequences of 1280, 1170 and 1200 bp

Table 2 Distribution of DNase I activities in tissues from four species of amphibian

The DNase I activity in each tissue extract was measured by the SRED method. Values are means $\pm$ S.D. for triplicate determinations on tissues derived from five different animals. Under our assay conditions, no DNase I activity was observed in the cerebrum, heart, lung, liver, kidney, spleen, skin, muscle, oesophagus, stomach, duodenum or large intestine.

Tissue	DNase I activity (m-units per g wet weight)			
	<i>X. laevis</i>	<i>R. catesbeiana</i>	<i>B. vulgaris</i>	<i>C. pyrrhogaster</i>
Pancreas	810 000 $\pm$ 290 000	145 000 $\pm$ 43 000	68 900 $\pm$ 3800	390 000 $\pm$ 240 000
Small intestine	37 $\pm$ 27	410 $\pm$ 303	77 $\pm$ 19	170 $\pm$ 86

Human	-22	MR GKLLGALLA LAALLQGAVS		
<i>X. laevis</i>	-19	MRFLVLVAM TACFLQAGFA		
<i>R. catesbeiana</i>	-19	MKS·L·TL A···H··S		
<i>B. vulgaris</i>	-19	MKSVI··L V·····FS		
<i>C. pyrrhogaster</i>	-22	MT PATFW··LLV ATS·YCTSH·		

Human	1	LKIAAFNIQT FGETKMSNAT LVSYIVQILS RYDIALVQEV RDSHLTAVGK LLDNLNQDAP DTYHYVVSSEP LGRNSYKERY
<i>X. laevis</i>	1	PKIASFNIQR FSKTKVDDPV VLELLIRILS RYGIATIEEV MNADNTAIS LVKELSLATK LMYNVLISDH LGRSSTREKY
<i>R. catesbeiana</i>	1	L···G···E···A········NR··Q··R··EL··VQ···K·D····R··R··NK··G··S··L·····AY····
<i>B. vulgaris</i>	1	L·······E···AVG·····T··S···Q··R··EL··VQ···SK······R··Q··NA··G··H··L·····Y····
<i>C. pyrrhogaster</i>	1	··V·····A·····S··T·T·A··RDR·VQ··R··EL·S·Q···S·E·E·····R··NQ··G··P··V·V·····TY····

Human	81	LFVYRPDQVS AVDSYYDDG CEPNGNDTFN REPAIVRFFS RFEVREFAI VPLHAAPGDA VAEIDALYDV YLDVQEKWGL
<i>X. laevis</i>	81	AYVYIEEIVK PTEWYHFDG CENCGTDSFI REPFVARFTS LTTVVKDFAL ISIHTSPDYA IMEVDALYDA WVDKQRLKM
<i>R. catesbeiana</i>	81	V···R·D·L·····F·Y········V·M·······S···E·····AVV······VR·····F·V··E·····L··
<i>B. vulgaris</i>	81	V·I·R·D·L··S····Y········V········S···EL·····V········VR····G··V··E·····LL
<i>C. pyrrhogaster</i>	81	····R·D·L······Y········V·M········S········L··V········VR·····F·V··E·VQ···LT

Human	161	EDVMLMGDFN AGCSYVRPSQ WSSIRLWTSP TFQWLIPDSA DTTA-TPTHCA YDRIVVAGML LGAVVPDSPA LFFNFQAAYG
<i>X. laevis</i>	161	ENILILGDYN AACSYGASRN WPIIRLHVE ELVWLIGDKE DTTVSTNTNCA YDRMVAGGEE LQRGIVPDTA KAFNYHVAID
<i>R. catesbeiana</i>	161	·D·F·····G···VK·A···T····QEA S·Q····NA····S····P···I·V··SR··DSV··GS·····QE··G
<i>B. vulgaris</i>	161	·D·······G···VKTS···H····QES S·Q····T··S··S··H·P··L·V··AR F·DIVI·G········
<i>C. pyrrhogaster</i>	161	QN·······D·K·VTNK··S····EP Q·H··S·D····S··YH····K··S·V··MLNA·F·E··T····E··G

Human	241	LSDQLAQAIS DHYPVEVMLK
<i>X. laevis</i>	242	LTYEMAKAVY DHYPVEVELY DDVFTSGQCF EPSASTGISF GLSINGPCTC EGWDFSSCRG RCGASGKTFP <u>CNCNASCTN-</u>
<i>R. catesbeiana</i>	242	··TEGKAAS·······R N·P·T···K· EV···I··G·····V·D· L·V··T··V····NSSS·····T·SSS
<i>B. vulgaris</i>	242	········S·····M·IR D·SL·NN·KL PF·G·I··G·····V·D· V·V··T··V····NSSS·····SSS
<i>C. pyrrhogaster</i>	242	···E·D·S·····MQ·R L·SD·N·ER· QT·PTL··NG··P·T··A·S· A·V·VT··Q····TS··L······GQT

<i>X. laevis</i>	321	<u>--CCVDYTSS CKL</u>
<i>R. catesbeiana</i>	322	<u>KK··A··AAV··I</u>
<i>B. vulgaris</i>	322	<u>NK··A··GAS··V</u>
<i>C. pyrrhogaster</i>	322	<u>GS··A··KAH··</u>

Figure 3 Alignment of the amino acid sequences of the four amphibian DNase I molecules with that of the human enzyme

The amino acid sequences of the amphibian DNases I were deduced from their respective cDNA species and compared with the published sequence for the human enzyme [19]. Amino acid position 1 was assigned by comparison with the N-terminal amino acid sequences determined chemically from the purified enzymes. Only residues differing from the *X. laevis* sequences are shown for the *R. catesbeiana*, *B. vulgaris* and *C. pyrrhogaster* sequences. The numbers on the left relate to the first residue in each line. Two unique structural features of amphibian DNases I, the C-stretch region and the insertion of a Ser residue at position 205, are shown in bold. The 'DNase I signature' and 'somatomedin B motif' sequences are singly and doubly underlined respectively.

respectively. The N-terminal amino acid sequences deduced from their cDNA data exactly matched those determined chemically from the purified enzymes. The amphibian DNase I cDNA species were similar in structural organization; however, they were characterized by shorter 3' untranslated regions (average 89 $\pm$ 44 bp) than those (201 $\pm$ 89 bp) of other cloned vertebrate DNase I cDNA species including human [19], rabbit [12], mouse [13], rat [28], bovine [29], hen [30] and pig (S. Mori, T. Yasuda, H. Takeshita, T. Nakajima, E. Nakazato, K. Mogi, Y. Kaneko and K. Kishi, unpublished work).

Each of the expression vectors containing the entire coding regions of the *B. vulgaris*, *C. pyrrhogaster*, *R. catesbeiana* and *X. laevis* DNase I cDNA species was transiently transfected into

COS-7 cells. The mean DNase I activities (means $\pm$ S.E.M., $n = 6$) secreted into the medium were 0.21 $\pm$ 0.011, 0.110 $\pm$ 0.021, 0.35 $\pm$ 0.10 and 0.17 $\pm$ 0.034 unit/ml respectively. These activities were completely abolished by 10 mM EDTA or 1 mM EGTA and the corresponding antisera. Furthermore, the enzyme activities expressed in the COS-7 cells migrated to the positions corresponding to the purified DNases I on the DNA-casting PAGE gels [18] (Figure 1B), confirming that the isolated cDNA species did indeed encode the expected amphibian DNases I. In contrast, the human and rat wild-type DNase I expression vectors yielded activities of 2.2 $\pm$ 0.24 and 3.0 $\pm$ 0.58 units/ml respectively under the same transfection conditions. These values were approx. 10–20-fold higher than those for the amphibian

Figure 4 Electrophoretic patterns of the *X. laevis* and *R. catesbeiana* DNases I before (lanes 1 and 3) and after (lanes 2 and 4) digestion with PNGase F

Purified DNases I from *X. laevis* (lane 2) and *R. catesbeiana* (lane 4) were subjected to digestion with PNGase F [35]. Both the digested and undigested enzymes were then subjected to DNA-casting PAGE followed by activity staining. As a control, bovine DNase I (Sigma, St Louis, MO, U.S.A.) was subjected to electrophoresis before (lane 5) and after (lane 6) digestion. Digestion with PNGase F had no effect on the electrophoretic mobilities of the *B. vulgaris* and *C. pyrrhogaster* DNases I (results not shown).

wild-type constructs. Thus when wild-type amphibian DNase I cDNA is transfected into cells, the resulting levels of DNase I activity are significantly lower than those yielded by other vertebrate DNase I cDNA species.

Demonstration of common structures unique to amphibian DNase I proteins

The amino acid sequences of the four amphibian DNases I predicted by each of the nucleotide sequences are shown in Figure 3. The mature enzymes from *B. vulgaris*, *C. pyrrhogaster*, *R. catesbeiana* and *X. laevis* were determined as consisting of 334, 332, 334 and 331 residues respectively. Comparison of the primary structures of the four amphibian DNases I with those published for other vertebrate enzymes [12,13,19,24,28–30] allowed us to demonstrate several common structural features that were unique to the amphibian DNases I. The four residues responsible for the catalytic activity of other vertebrate DNases I, Glu⁷⁸, His¹³⁴, Asp²¹² and His²⁵² [12,13,19,24,28–30], were conserved in the amphibian enzymes, although the last two were shifted to Asp²¹³ and His²⁵³ by the insertion of a serine residue at position 205 in the amphibian DNases I. Two residues, Cys¹⁷³ and Cys²⁰⁹, which form the disulphide bond responsible for the stability of the enzyme [31], were also found in all the amphibian enzymes. The essential sites that mediate DNase I–DNA contact in other vertebrate DNases I include two well-conserved residues, Arg⁴¹ and Tyr⁷⁶, which orientate the scissile phosphate relative to the enzyme [32]. Met was substituted for Arg⁴¹ in all four of the amphibian enzymes, whereas Thr was present instead of Tyr⁷⁶ in *X. laevis*. A mutant human DNase I in which an alanine residue was substituted for Arg⁴¹ was reported to exhibit 65-fold lower activity than the wild-type enzyme [33]. This structural alteration might therefore be one of the factors responsible for the lower activity of the enzyme expressed in the transfected cells compared with other vertebrate enzymes.

Amphibian DNases I had two main, unique structural alterations. First, they all possessed an additional stretch region containing 70 (*X. laevis*), 71 (*C. pyrrhogaster*) or 73 (*B. vulgaris* and *R. catesbeiana*) residues in their C-terminal regions and were therefore considerably longer (331–334 residues) than the enzymes from other vertebrates (258–262 residues). A particular feature of this C-stretch region was its high Cys content, which is approx. 10-fold that in the remainder of the enzyme (about 15%, compared with 1.5%). The similarity between the C-stretches was low (average, 60.7±8.9%) compared with that (73.0±4.3%) between the remaining parts of amphibian enzymes and the corresponding parts of other vertebrate ones, indicating

that the C-stretch is less well conserved than the rest of the enzyme structure in amphibians. Secondly, all the amphibian DNases I tested had an additional Ser²⁰⁵ residue in their mid-region, located in an area that has been postulated to form an essential Ca²⁺-binding site in other vertebrate enzymes [34] and corresponding to a position between Ala²⁰⁴ and Thr²⁰⁵ in the human enzyme. In addition, although two potential N-glycosylation sites, Asn¹⁸ and Asn¹⁰⁶, are well conserved in other vertebrate DNases I and are attached to carbohydrate chains [23], the corresponding amphibian residues were replaced by Asp¹⁸ and Thr¹⁰⁶ respectively. Another N-linked carbohydrate chain-binding motif was found at position Asn³¹⁵ in the C-stretch. The four purified amphibian DNases I migrated to the same positions on the SDS/PAGE gel before and after digestion with PNGase F [35] (Figure 4) and showed no affinity when passed through four different lectin-conjugated columns, in contrast with the human enzyme [36]. These findings indicate that amphibian DNases I have no carbohydrate moieties; this might therefore be the first report of a carbohydrate-free DNase I.

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)₂PX₅E(L/I/V/M)(D/M)XLX-(D/E)V and GDFNAXC(S/A), in which arbitrary residues are indicated by an X, have been identified at positions 130–150 and 167–174 respectively in all of the vertebrate DNase I sequences determined so far. Both of these sequences were poorly conserved in the amphibian enzymes. However, a 'somatomedin B motif' sequence, CXCX₃CX₅CCX(D/N)(F/Y)X₃C, was found at positions 313–331 in the C-stretch of the amphibian DNases I, although three residues were deleted in *X. laevis* DNase I. This motif has also been identified in the N-terminal region of vitronectin [37]. Somatomedin B has been reported to be a naturally occurring growth-hormone-dependent serum factor with protease-inhibiting activity [38]. It has been suggested that somatomedin B might be involved in the regulation of the fibrinolytic system through an interaction with type 1 plasminogen activator–inhibitor, and its high Cys content predicts an intricate, compact structure that probably anchors a type 1 plasminogen-activator–inhibitor-binding motif in its active configuration [39]. Nevertheless, any physiological similarities between the amphibian C-stretch region and somatomedin B remain to be elucidated.

These structural characteristics were found in all of the amphibian DNases I, irrespective of any other differences between the *Urodela* (newt) and *Anura* (two frogs and a toad) enzymes.

Biochemical significance of the insertion of a serine residue at position 205 and the addition of a cysteine-rich C-stretch in amphibian DNases I

The heat stabilities of the amphibian DNases I were examined by preincubating the wild-type enzyme and several of the mutants at 50 °C for up to 60 min (Figure 5). The activities of all the wild-type amphibian DNases I were decreased to approx. 50% of the initial levels after only 5 min of incubation, whereas those of the human and rat DNases I remained unchanged. Thus amphibian DNases I are more heat-labile than other vertebrate enzymes such as the bovine, mouse, rabbit, pig and hen forms (results not shown), as well as the human and rat enzymes. This fact could also account for the observation that, under the same transfection conditions, expression vectors containing amphibian wild-type DNase I cDNA produced a smaller amount of enzyme

Figure 5 Heat stability of amphibian (A), human (B) and rat (C) DNases I and their insertion mutants

COS-7 cells transfected with *R. catesbeiana* (○) and *X. laevis* (●) wild-type constructs (A), human wild-type DNase I (▲) and its insertion mutant human-ins(S205) (■) (B) and rat wild-type DNase I (△) and its insertion mutant rat-ins(S205) (□) (C) were incubated at 50 °C for the durations indicated; the residual DNase I activities in the medium were determined by the SRED method.

activity than the human and rat enzymes, possibly owing to a shorter half-life of the amphibian DNase I.

To examine the biochemical significance of the Ser²⁰⁵ insertion in amphibian DNase I, we constructed a series of insertion mutants [human-ins(S205) and rat-ins(S205)] and deletion mutants [*X. laevis*-del(S205) and *R. catesbeiana*-del(S205)] and compared their heat-stability with those of the corresponding wild-type enzymes. The insertion of a Ser residue between Ala²⁰⁴ and Thr²⁰⁵ of the human and rat DNases I rendered these enzymes heat-labile, with a heat tolerance similar to that of amphibian wild-type DNase I (Figure 5). An X-ray structural analysis of bovine DNase I [34] has indicated that a Ca²⁺-binding site co-ordinated with the main-chain oxygens of Thr²⁰³, Thr²⁰⁵ and Thr²⁰⁷ and the two oxygens of the Asp²⁰¹ side chain seems to be responsible for conformational changes and protection of the enzyme from inactivation. Both the human-ins(S205) and rat-ins(S205) mutants acquired similar heat instabilities to those of the amphibian enzymes; it is therefore plausible that the structural alteration caused by the insertion of a serine residue into this Ca²⁺-binding site might have induced the instability. In contrast, although the *R. catesbeiana*-del(S205) and *X. laevis*-del(S205) mutants yielded immunoreactive bands at the same positions as the wild-type enzymes on SDS/PAGE Western blot analysis, they showed no enzyme activity. These findings strongly suggest that Ser²⁰⁵ in amphibian DNases I is essential for the generation of the active enzyme conformation, irrespective of whether this induces instability of the enzyme protein.

Next, the biochemical significance of the C-stretch in amphibian DNases I was examined with the use of several mutants. The mutants secreted from cells transfected with *R. catesbeiana*-(6 × His) and *X. laevis*-(6 × His) vectors had similar enzyme activities to those of the corresponding wild-type enzymes and were completely retained on a Ni²⁺-agarose column, from which they could be selectively eluted with 200 mM imidazole. Because the His₆ tag was covalently attached to the C-terminus of the mature amphibian wild-type DNase I, the C-stretch was not eliminated during proteolytic processing of the precursor form, confirming that the presence of the C-stretch contributes to the creation of large mature forms of the amphibian enzymes (Figure 1). The

Figure 6 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. A phylogenetic tree was constructed by aligning the sequences with the use of the neighbour-joining algorithm of the CLUSTAL W program [40]. The sequences of the DNase I enzymes were obtained from various databases: mouse, GenBank D83038; rat, EMBL X56060; human, GenBank M55983; bovine, Swiss-Prot dm1-bovine; sheep, Swiss-Prot der1-sheep; pig, Swiss-Prot drn1-pig; hen, GenBank AB013751; rabbit, EMBL D82875; tilapia (fish), EMBL AJ001305.

two deletion mutants, *R. catesbeiana*-del(C-stretch) and *X. laevis*-del(C-stretch), yielded no immunoreactive bands on SDS/PAGE Western blot analysis, suggesting that the resulting mutant proteins might be rapidly degraded in the transfected cells. Although all the residues responsible for catalytic activity were situated outside the C-stretch region, the C-stretch might be required for the formation and/or protection of the active form. Thus, both the additional Ser²⁰⁵ and the Cys-rich stretch in the C-terminal region might be essential in generating the active forms of the amphibian enzymes; however, further studies will be necessary to gain a deeper understanding of their real functions.

Phylogenetic analysis of the amphibian DNase I family

The entire amino acid sequences of the four amphibian DNases I characterized during this study were aligned with those of other vertebrate enzymes available from various genetic databases to construct a phylogenetic tree for the vertebrate DNase I family, with the use of the unweighted pair grouping method and the arithmetic mean (Figure 6). The amphibian DNase I group forms a relatively tight cluster quite distinct from the mammalian, fish (tilapia) and avian (hen) DNases I. This grouping seems reasonable in the light of the various unique structural characteristics of amphibian DNases I. Furthermore, the slight distance between *Urodela* (*C. pyrrhogaster*) and *Anura* (*B. vulgaris*, *R. catesbeiana* and *X. laevis*) in the amphibian DNase I cluster indicates a classical phylogenetic relationship. When determined separately, the degree of intraclass similarity for the amphibian DNase I group (average 69.9 ± 5.5%) was lower than that for the mammalian DNase I group (79.8 ± 1.4%), suggesting that the former group exhibits greater diversity than the latter from the standpoint of molecular evolution. More systematic surveys, not only of amphibian DNases I but also of other animal DNases I, might help to elucidate inherent but as yet unknown physiological role(s) of the DNase I family.

We thank Mrs M. Itoi and Miss E. Nakazato for their excellent technical assistance. This work was supported in part by Grants-in-Aid from Japan Society for the Promotion of Science (12357003 to T. Y.; 12770216 to H. T.; 09357004 and 12307011 to K. K.).

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Received 26 February 2001/30 April 2001; accepted 11 May 2001