Rabbit DNase I: purification from urine, immunological and proteochemical characterization, nucleotide sequence, expression in tissues, relationships with other mammalian DNases I and phylogenetic analysis

Toshihiro YASUDA*, Haruo TAKESHITA*, Tamiko NAKAJIMA, Osamu HOSOMI, Yoshimitsu NAKASHIMA and Koichiro KISHI†
Department of Legal Medicine, Gunma University School of Medicine, Maebashi, Gunma 371, Japan

DNase I from rabbit urine was purified approx. 3600-fold to apparent homogeneity with a 41% yield by affinity chromatography utilizing DNA–cellulose; the purity of the final preparation was assessed by SDS/PAGE, lack of contamination by other nucleases and production of a monospecific antibody against the enzyme. Although the proteochemical and enzymological properties of the purified enzyme resembled those of other mammalian DNases I, the enzymic activity of rabbit DNase I was less efficiently inhibited by monomeric actin than was that of human DNase I, probably due to substitution of an amino acid residue involved in actin binding (Tyr-65 to Phe). The effects of specific antibodies to human, rabbit and rat DNases I on the activities of the corresponding purified enzymes revealed that human DNase I lies between the rat and rabbit enzymes with regard to its immunological properties. An 1158 bp full-length cDNA encoding rabbit DNase I was constructed from the total RNA of rabbit pancreas using a combination of reverse transcriptase-PCR and rapid amplification of cDNA ends, followed by sequencing. This identified a 17- or 21-amino-acid signal sequence, with the mature enzyme containing 260 amino acids and a single N-glycosylation site at Asn-18. The amino acid sequence deduced from the cDNA sequence exactly matched that determined proteochemically from the purified enzyme up to residue 20. A systematic survey of DNase I distribution as measured by both enzymic activity and DNase I gene transcripts in 12 rabbit tissues showed the pancreas and parotid gland to produce equivalent levels, higher than those in other tissues. Enzymic activity and DNase I gene expression levels in each tissue correlated well. The results of phylogenetic and sequence identity analysis, immunological properties and tissue-distribution patterns of DNase I indicated a closer relationship between the rabbit and human enzymes than for other mammalian DNases I. Furthermore, differences between the enzymic activities expressed in mammalian parotid gland and pancreas suggest that the distribution of DNase I in mammalian tissue is species-specific.

INTRODUCTION

DNase I (EC 3.1.21.1) is an enzyme that preferentially attacks double-stranded DNA and produces oligonucleotides with 5′-phospho and 3′-hydroxy termini by Ca^{2+}- and Mg^{2+}-dependent endonucleolytic cleavage [1,2]. In view of the hydrolytic activity and presence of DNase I in both duodenal and pancreatic juices, DNase I has been assumed to be important for DNA metabolism *in vivo*. However, the demonstration of DNase I activity outside the exocrine organs of the alimentary tract, such as in the pancreas and parotid gland [3–7], raises doubts that its major role is merely a digestive function [8]. Peitsch et al. [9] showed that the nuclease extractable from isolated nuclei, which is responsible for internucleosomal DNA degradation during apoptosis (programmed cell death), is functionally and antigenically indistinguishable from DNase I. The finding [10] that overexpression of DNase I alone after transfection into cells was sufficient to induce the morphological and biochemical changes observed during apoptosis supports this hyphothesis. DNase I has also been reported to show high sequence similarity with the human urinary interleukin-1 inhibitor [11]. It is worth noting that DNase I interacts preferentially with monomeric actin (G-actin), which is present in many eukaryotic cells [12,13]. We have demonstrated previously that the isoform patterns of human DNase I in urine, serum, saliva, sweat and semen from different individuals were phenotypable by isoelectric focusing, showing the existence of genetic polymorphism controlled by four co-dominant alleles at chromosome 16p13.3 [14–17]. It has been suggested that changes in individual serum DNase I activity could be a reliable and useful marker for the therapeutic monitoring of various human malignant diseases [18], and a significant association between DNase I phenotype 2 and liver diseases has been found [19]. However, the intrinsic extra- and intra-cellular functions of DNase I remain to be elucidated.

Several DNases I have been isolated by conventional methods from bovine [20], ovine [21] and human [22] pancreatic tissues, human duodenal juice [23], serum [22], urine [2,24], semen [3] and kidney [25], bovine and rat parotid glands [26] and rat urine [27]. To date, cDNA sequences have been determined only for the human [28] and rat [29] enzymes. However, a full-length cDNA encoding DNase I has not yet been isolated from any mammal. With regard to the genomic structure of the DNase I gene, we have isolated and sequenced the structural gene encoding human DNase I [30] and elucidated the complete molecular basis for its

Abbreviations used: IEF/PAGE, isoelectric focusing in a thin layer of polyacrylamide gel; G-actin, monomeric actin; RACE, rapid amplification of cDNA ends; RT, reverse transcriptase; SRED, single radial enzyme diffusion.

* These authors have contributed equally to this study.
† To whom correspondence should be addressed.

The nucleotide sequence data reported will appear in GenBank/EMBL/DDBJ Nucleotide Sequence Databases under accession no. D82875.

genetic polymorphism [30–32]. The DNase I gene comprises at least nine exons, spanning approx. 3.2 kb of the genomic DNA. Recently, the structural gene encoding mouse DNase I has been reported to exhibit similar genomic organization to that of the human gene [33].

In the present study, we describe the purification of rabbit DNase I from urine, its proteochemical and immunological characterization, and its relationship with other mammalian enzymes. We also describe the determination of the complete cDNA sequence encoding rabbit DNase I, which allows comparison of this sequence with all known DNase I sequences and the construction of a phylogenetic tree for this protein family. In addition, we have elucidated the distribution of DNase I in rabbit tissues from the standpoint of both its enzymic activity and the levels of the DNase I gene transcript.

EXPERIMENTAL

Materials and biological samples

DEAE-Sepharose CL-6B, phenyl-Sepharose CL-4B, Superose 12 and a molecular mass calibration kit were purchased from Pharmacia Biotech (Uppsala, Sweden); rabbit muscular G-actin, salmon testicular DNA, PMSF and DNA–cellulose were from Sigma (St. Louis, MO, U.S.A.); Superscript II RNase H^- reverse transcriptase (RT), RNase H, *Taq* DNA polymerase, and 5′- and 3′-rapid amplification of cDNA ends (RACE) kits were from Gibco–BRL (Gaithersburg, MD, U.S.A.). Human and rat DNases I were purified from urine samples, and specific antibodies against each of these enzymes were prepared according to methods described previously [2,27]. All other chemicals used were of reagent grade or the purest grade available commercially.

Purification of DNase I from rabbit urine

Urine (about 15 litres) collected from five male Japanese white rabbits (average body weight approx. 3 kg; age between 9 and 12 months) was used for purification of the enzyme according to previously described methods [27,34]. The DNase I was partially purified from the lyophilized urine samples using successive DEAE-Sepharose CL-6B, phenyl-Sepharose CL-4B and DNA–cellulose columns, essentially under the same chromatographic conditions as for the rat enzyme [27]. The active fractions eluted from the DNA–cellulose column were concentrated, then subjected to gel filtration using the FPLC system (Pharmacia Biotech) equipped with a Superose 12 column and eluted with 50 mM Tris/HCl, pH 7.5, containing 0.25 M NaCl. The DNase I active fractions were pooled, concentrated and used as the purified enzyme for subsequent experiments.

Analytical methods

DNase I activity was determined by the test-tube method [2] or a single radial enzyme diffusion (SRED) method [4,35]. One unit of DNase I activity was defined as an increase of 1.0 unit in the absorbance at 260 nm. The activities of RNases and DNase II were assayed by the SRED method [36,37]. Proteins were determined using a protein assay kit (Bio-Rad, Richmond, CA, U.S.A.) with BSA as a standard. The position of the terminal phosphate in the products digested by the enzyme was determined mainly by the method of Doniger and Grossman [38]. The enzymological properties of the enzyme and the inhibitory effects of specific antibodies on its enzymic activity were examined as in our previous studies [2,39]. N-terminal amino acid sequence analysis and determination of the amino acid and carbohydrate compositions of the enzyme were carried out using reported methods [27,40].

Preparation of antiserum

Antiserum against rabbit DNase I was prepared by emulsifying the purified enzyme with an equal volume of Freund's complete adjuvant (Difco, Detroit, MI, U.S.A.) and injecting the emulsion, containing about 40 μg of the purified enzyme, intramuscularly into chickens, on five occasions at 2-week intervals. The IgG fraction was purified from the antiserum using DEAE Affi-Gel Blue (Bio-Rad) and was used as the relevant antibody.

Electrophoresis

SDS/PAGE was performed in 12.5 % (w/v) gels according to the method of Laemmli [41]. Separated proteins were stained with 0.2 % Coomassie Brilliant Blue R-250 (Bio-Rad), or with the chicken anti-(rabbit DNase I) as a primary antibody followed by peroxidase-labelled anti-chicken IgG (Zymed Lab. Co., San Francisco, CA, U.S.A.), after the proteins had been transferred on to a Durapore membrane (Millipore) by electroblotting according to a previous method [42]. Isoelectric focusing in a thin layer of polyacrylamide gel (IEF/PAGE) was performed using Ampholines 3.5–5.0 (Pharmacia Biotech) according to previous methods [34,43]. Visualization of focused DNase I was achieved by immunostaining as described above after capillary blotting, or by activity staining using a dried agarose film overlay method [34,43].

Determination of DNase I activity in tissue samples from rabbits

Twenty different kinds of tissue sample were obtained from six male Japanese white rabbits as soon as possible after they had been killed by exsanguination under general anaesthesia with diethyl ether. All the rabbits were acquired, maintained and used in accordance with the Guidelines for the Care and Use of Laboratory Animals (NIH, U.S.A.; revised 1985). The following procedures were carried out at 0–4 °C. The rabbit tissues were cut into small pieces, washed with cold saline to remove excess blood, homogenized in 0.5–1.0 ml of 50 mM Tris/HCl, pH 7.5, containing 1.0 mM PMSF, then centrifuged at 10000 ***g*** for 10 min. The supernatants obtained were used for subsequent analyses.

Construction of a rabbit full-length DNase I cDNA

Total RNA was extracted from the pancreas of a Japanese white rabbit by the acid guanidinium isothiocyanate/phenol/chloroform extraction method of Chomczynski and Sacchi [44]. First-strand synthesis of rabbit pancreas total RNA was performed using the RT reaction as follows. After denaturation at 65 °C for 10 min, total RNA (~ 2 μg) was incubated with 400 units of enzyme at 37 °C for 60 min in a reaction mixture (40 μl) comprising 50 mM Tris/HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 20 mM dithiothreitol, 0.5 μM random hexamer primers, 0.5 mM of each dNTP and 40 units of RNase inhibitor (TOYOBO, Tokyo, Japan). After heating at 70 °C for 10 min, the reactant was incubated with 4 units of RNase H at 37 °C for 20 min. The primers used to amplify the DNase I cDNA were based on the previously reported nucleotide sequence of rat DNase I cDNA [29], and were purchased from Toa-gosei Inc. (Tokyo, Japan).

Table 1 Primers used in this study and their sequences

Ra-U1, -U2, -D1 and -D2 were designed based on rat DNase I cDNA sequence data [29]. Other primers were based on the sequence data obtained in the present study.

Designation	Sense/antisense	Sequence	Corresponding region in DNase I	
			From	To
Ra-U1	Sense	5′-CTGAGAATTGCAGCCTTCAAC-3′	Leu-1	Asn-7
Ra-U2	Sense	5′-CCATACACTGAGGTCCGAAGA-3′	Phe-121	Glu-127
Ra-D1	Antisense	5′-GGTCTTTCTGAGTGTCACCTC-3′	Glu-256	Thr-262
Ra-D2	Antisense	5′-TGAGTGCAAGGGCACAATCGC-3′	Ser-135	Ala-129
5RACE-1	Antisense	5′-CTTCTCATTGAGCTTGTCCA-3′	Leu-52	Lys-58
5RACE-2	Antisense	5′-ATGTCGTAGCGTTGCAGGAT-3′	Ile-28	Ile-34
3RACE	Sense	5′-CTCGCTTGCAGGACGCTGTT-3′	Pro-219	Val-225
U3	Sense	5′-CTGAAGATTGCAGCCTTCAAC-3′	Leu-1	Val-7
D3	Antisense	5′-GGCGAGCGTCACCTCCACAGG-3′	Pro-254	Ala-260

Table 1 shows the primers used in the present study. A 5 μl aliquot of the first-strand cDNA mixture was subjected to PCR using a Minicycler (model PTC-150; MJ Research, Watertown, MA, U.S.A.). The PCR reaction mixture (50 μl) comprised 20 mM Tris/HCl, pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01 mM dithiothreitol, 0.2 mM of each dNTP, 4 μM of each primer and 2.5 units of *Taq* DNA polymerase. After denaturation at 94 °C for 3 min, amplification was carried out for 30 cycles, each of which involved denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 2 min, followed by a further 10 min extension at 72 °C. Two pairs of primers, Ra-U1/Ra-D2 and Ra-U2/Ra-D1, yielded two partially overlapping fragments of 409 bp and 420 bp respectively. The 5′- and 3′-end regions of the rabbit DNase I cDNA were obtained separately by the RACE method [45], using 5′-RACE and 3′-RACE systems (Gibco-BRL) respectively. Total RNA ($\sim$ 1 μg) from rabbit pancreas was subjected to 5′- and 3′-RACE according to the manufacturer's instructions. The primers used for RACE were based on the nucleotide sequence data obtained in the present study: two rabbit DNase I-specific primers, 5RACE-1 and -2, and one primer, 3RACE, were employed for 5′- and 3′-RACE respectively. These RACE methods allowed the 5′- and 3′-RACE products (approx. 350 bp and 300 bp respectively) to be amplified separately and identified. Each PCR and RACE product was directly subcloned into TA cloning vector pCR II (Invitrogen, San Diego, CA, U.S.A.) and sequenced [31,32]. Sequence analysis of each fragment was performed at least three times.

Detection of the DNase I gene transcript in rabbit tissues by PCR

Total RNA was extracted from rabbit tissues, including pancreas, kidney, liver, spleen, heart, lung, stomach, small intestine, cerebrum, cerebellum, parotid gland and thymus, by the method of Chomczynski and Sacchi [44], then 1 μg of each RNA was transcribed using RT as described above. After heat denaturation at 90 °C for 5 min, a 1 μl aliquot of each sample was subjected to PCR analysis. A set of primers, U3 and D3 (Table 1), corresponding to the N-terminal and C-terminal portions respectively of rabbit DNase I protein, was synthesized and used. The DNase I gene transcript was amplified by PCR as described above, except that the annealing temperature used was 60 °C, and the amplified products were subjected to electrophoresis on a 2% (w/v) agarose gel. The resulting bands were visualized by ethidium bromide staining.

Semi-quantification of DNase I transcript levels expressed in rabbit tissues

A competitive PCR method based on a non-homologous internal standard approach [46] was employed. A non-homologous DNA fragment with a primer template corresponding to the U3/D3 set, tentatively named PCR-MIMIC, was used as a competitive internal standard during PCR amplification, and was prepared using a PCR MIMIC Construction kit (Clontech, Palo Alto, CA, U.S.A.) according to the manufacturer's instructions. A set of two oligonucleotides, 5′-CTGAAGATTTGCAGCCTTCACG-CAAGTGAAATCTCCTCCG-3′ and 5′-GGCGAGCGTCAC-CTCCACAGATTTGATTCTGGACCATGGC-3′, containing the nucleotide sequences of U3 and D3 respectively, was used for construction of PCR-MIMIC, yielding a 546 bp fragment on co-amplification with a target DNase I cDNA using the U3/D3 primer set. Therefore there is about a 200 bp difference in size between the PCR-MIMIC and the target DNA, which makes them easily distinguishable after separation on an agarose gel. After estimating the molar quantity of the PCR-MIMIC by spectrophotometry, a 10-fold dilution series (from 100 to 10^{-6} amol/μl) of the PCR-MIMIC was prepared for use in a competitive PCR. A constant amount of the first-strand cDNA derived from the total RNA of each rabbit tissue was subjected to PCR amplification along with each of the PCR-MIMIC dilutions. Series of competitive PCRs using the U3/D3 primer set in the co-presence of PCR-MIMIC at different concentrations were performed separately under the same conditions as described above. These products were then separated by 2% agarose gel electrophoresis and visualized by ethidium bromide staining. The levels of the DNase I gene transcript derived from each rabbit tissue were estimated by visual inspection of the appropriate ethidium bromide-stained agarose gel.

RESULTS

Purification of rabbit DNase I from urine samples

Typical values for the purification of DNase I from rabbit urine are summarized in Table 2. The four-step procedure resulted in approx. 3600-fold purification with a 41% yield, and consequently about 800 μg of DNase I could be obtained from 15 litres of urine.

When the purified rabbit DNase I was subjected to SDS/PAGE followed by protein staining and immunostaining with the anti-(rabbit DNase I) antibody, only a single band was detected, and

Table 2 Summary of the purification of DNase I from rabbit urine

Step	Protein (mg)	Total activity (units)	Specific activity (units/mg of protein)	Purification (fold)	Yield (%)
Urine (~ 15 litres)	7000	16100	2.30	1	100
DEAE-Sepharose CL-6B	766	12500	16.3	7	78
Phenyl-Sepharose CL-4B	56.7	9050	160	70	56
DNA–cellulose	3.18	8040	2530	1100	50
Superose 12	0.79	6580	8320	3600	41

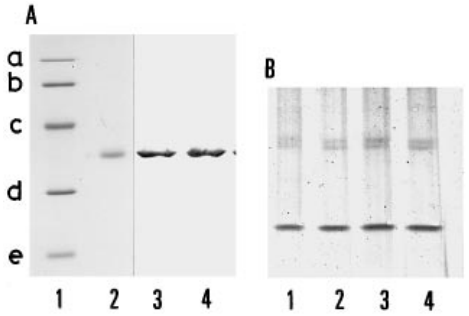

Figure 1 SDS/PAGE (A) and IEF/PAGE (B) patterns of purified rabbit DNase I

(**A**) The purified enzyme (~ 5 μg; lane 2) eluted from the Superose 12 column was dissolved in 10 mM Tris/HCl, pH 6.8, containing glycerol (10%, v/v), SDS (2%, w/v), 25 mM dithiothreitol and a trace of Bromophenol Blue, heated at 100 °C for 5 min and electrophoresed, after which the protein was stained with Coomassie Brilliant Blue R-250. Purified enzyme (~ 1 μg; lane 3) and rabbit urine concentrate (~ 10 μg; lane 4) were subjected to SDS/PAGE in the same manner, after which the proteins were transferred to a Durapore membrane (Millipore) and visualized by immunostaining with anti-(rabbit DNase I) antibody as described previously [41]. Lane 1 contains molecular-mass markers: a, phosphorylase *b* (94 kDa); b, BSA (67 kDa); c, ovalbumin (43 kDa); d, carbonic anhydrase (30 kDa); e, trypsin inhibitor (20 kDa). (**B**) Samples before and after sialidase digestion were applied to an IEF/PAGE gel (pH 3.5–5.0), electrophoresed and immunostained with anti-(rabbit DNase I) antibody as described in the text. Sialidase treatment of the samples was performed according to a previous method [34]. Lanes 1 and 2, purified enzyme (~ 1 μg) eluted from the Superose 12 column; lanes 3 and 4, rabbit urine concentrate (~ 10 μg). Lanes 1 and 3, samples before sialidase digestion; lanes 2 and 4, samples after sialidase digestion. The anode is at the top.

this occurred at a corresponding position on the two gels (Figure 1A). Contamination with other nucleases was investigated in the enzyme preparation, but no DNase II [35], RNases [37,42], phosphodiesterase [47] or protease could be detected. On a double-immunodiffusion plate, the anti-(rabbit DNase I) antibody gave a single, completely fused line with the purified enzyme, rabbit urine and rabbit pancreas extract. On SDS/PAGE of rabbit urine followed by immunostaining with this antibody, only one band, corresponding to that of the purified enzyme, could be observed. These findings confirmed the purity of the final rabbit DNase I preparation.

Characterization of the purified enzyme

The molecular mass of rabbit DNase I was estimated to be 35 kDa by SDS/PAGE under reducing conditions, which corresponded to that for the native enzyme obtained by gel filtration on Superose 12. The amino acid composition of the rabbit enzyme resembled not only those of other mammalian DNases I, but also that deduced from its cDNA data (results not shown). Furthermore, Edman degradation of the enzyme allowed the N-terminal amino acids sequence up to residue 20 to be identified as follows: Leu^{1}-Lys-Ile-Ala-Ala^{5}-Phe-Asn-Ile-Arg-Ser^{10}-Phe-Gly-Glu-Thr-Lys^{15}-Met-Ser-(Xaa)-Ala-Thr^{20}.

Table 3 Carbohydrate composition of DNase I from rabbit urine and comparison with that of other urinary DNases I

Values are averages of triplicate determinations and are expressed as 'nearest integer'. The data for rat and human enzymes are taken from refs. [27] and [2] respectively.

Carbohydrate component	Composition (residues/molecule)		
	Rabbit DNase I	Human DNase I	Rat DNase I
Fucose	5	1	1
Galactose	6	7	10
Mannose	12	10	17
Glucosamine	6	6	12
Sialic acid	0	2	3

By carbohydrate analysis, rabbit DNase I was shown to be composed of about five fucose, six galactose, 12 mannose and six glucosamine residues/molecule (Table 3). The enzyme was found to contain a relatively higher content of fucose than other mammalian urinary DNases I, but sialic acid was not detected, in contrast with the rat and human enzymes. Since the rabbit enzyme contains no galactosamine, its carbohydrate moieties might be attached to the amide group of an Asn residue. Nucleotide sequence analysis of the rabbit DNase I cDNA revealed that rabbit DNase I contains one potential glycosylation site, located in the N-terminal region at position 18, as indicated by the presence of the tripeptide code sequence Asn-Xaa-Thr/Ser for N-glycosidically linked glycans. It is plausible that this Asn residue could not be identified during proteochemical analysis because residue 18 may present a potential N-linked glycosylation site.

The catalytic properties of the purified rabbit DNase I resembled those of other mammalian DNases I [2,27]. G-actin is known to be a potent inhibitor of mammalian pancreatic DNase I [12,13]. Although the activity of the rabbit enzyme was inhibited by G-actin (in contrast with the rat enzyme [27]), the amount of G-actin required for 50% inhibition was about 10 times greater than that needed to produce similar inhibition of human enzyme.

On IEF/PAGE analysis, both the purified rabbit DNase I and crude rabbit urine yielded one major band, accompanied by a few minor ones which had different pI values migrating into the region corresponding to pH 4.0–4.5 (Figure 1B). Desialylation of the enzyme caused little shift in any of the isoforms. Therefore,

Table 4 DNase I activities detected and levels of DNase I gene transcripts expressed in tissues from male rabbits

DNase I activity in each tissue extract was measured by the SRED assay method. Values are means ± S.D. of triplicate determinations on each tissue derived from five different rabbits [2,4]. Levels of DNase I gene transcripts were semi-quantified using RT-competitive PCR of total RNA extracted from rabbit tissues as described in the text. –, not measured.

Tissue	10^{-4} × Activity (units) per g wet weight	10^{-4} × Activity (units) per mg of protein	Levels of DNase I gene transcript (amol per μg of total RNA)
Pancreas	22000 ± 880	18900 ± 470	1.0
Kidney	910 ± 6.0	44 ± 17	2×10^{-3}
Liver	470 ± 150	11 ± 4.3	2×10^{-3}
Spleen	< 0.05	< 0.01	$< 1 \times 10^{-4}$
Heart	< 0.05	< 0.01	$< 1 \times 10^{-4}$
Lung	< 0.05	< 0.01	$< 1 \times 10^{-4}$
Stomach	2.6 ± 1.6	1.3 ± 0.5	2×10^{-4}
Small intestine	120 ± 95	13 ± 6.6	2×10^{-3}
Large intestine	3.4 ± 2.5	0.5 ± 0.0	–
Cerebrum	< 0.05	< 0.01	$< 1 \times 10^{-4}$
Cerebellum	< 0.05	< 0.01	$< 1 \times 10^{-4}$
Adrenal gland	4.0 ± 2.7	0.3 ± 0.3	–
Parotid gland	36000 ± 6200	59000 ± 1700	2×10^{-1}
Submaxillary gland	< 0.05	< 0.01	–
Thymus	31 ± 4.6	2.8 ± 1.4	1×10^{-3}
Skeletal muscle	6.4 ± 4.0	3.9 ± 1.7	–
Testis	5.3 ± 1.3	1.5 ± 0.2	–
Epididymis	9.5 ± 3.9	8.4 ± 0.3	–
Ampulla	0.8 ± 0.2	0.5 ± 0.1	–
Prostate	0.6 ± 0.4	0.2 ± 0.1	–

when also taking into account the results shown in Table 3, it seems reasonable to assume that the sialic acid content makes very little contribution to the multiplicity of rabbit DNase I isoforms, unlike with the human and rat enzymes [2,27].

Distribution of DNase I activity in rabbit tissues

Under the optimal assay conditions established on the basis of the catalytic properties of the purified enzyme, the DNase I activity of each of 20 different tissue extracts was determined separately by the SRED method [4]. As shown in Table 4, high activity was observed in the pancreas and parotid gland, moderate activity in the kidney and low activity in the small intestine and liver. Several tissues, including the spleen, heart, lung, cerebrum, cerebellum and submaxillary gland, exhibited no DNase I activity under our assay conditions. The enzyme activity detectable in the tissues was inhibited by 1 mM EDTA, 1 mM EGTA and G-actin. Most notably, the activities were completely abolished by anti-(rabbit DNase I) antibody. Therefore this activity was confirmed to be that of DNase I.

Construction of a full-length cDNA encoding rabbit DNase I and determination of its nucleotide sequence

Total RNA was extracted from the rabbit pancreas (in which a particularly high level of DNase I activity was expressed compared with other rabbit tissues), amplified by RT-PCR and used to construct a rabbit DNase I cDNA. Using a PCR primer based on the nucleotide sequence of rat DNase I cDNA [29], we succeeded in amplifying two overlapping PCR products, which contained the majority of the open reading frame of rabbit DNase I cDNA. Two pairs of primers, Ra-U1/Ra-D2 and Ra-U2/Ra-D1, yielded unique 409 bp and 420 bp fragments respectively, which shared a nucleotide sequence corresponding to the region between Phe-121 and Ser-135. In order to obtain the 5′- and 3′-end regions of the cDNA separately, two RACE methods were designed using corresponding primers designed from the nucleotide sequence data obtained. These RACE methods allowed fragments of approx. 350 bp and 300 bp respectively to be amplified separately. Sequencing analysis of these PCR and RACE products revealed that the full-length 1158 bp cDNA contained a 166 bp 5′-untranslated region, an 846 bp coding region and a 3′-untranslated region of more than 146 bp (Figure 2). This cDNA has an overall open reading frame of 281 amino acid residues. From our N-terminal amino acid sequence analysis of the rabbit enzyme, the first amino acid residue of the mature enzyme was identified as Leu. The putative upstream signal sequence containing the ATG initation codon is 17 or 21 amino acids long. The amino acid sequence deduced from the cDNA data exactly matched that determined proteochemically from the purified enzyme up to residue 20. These results established that the mature rabbit DNase I protein is composed of 260 amino acids. Furthermore, the poly(A) addition signal sequence is located at positions 1137–1142.

Nucleotide identities were determined by comparing the parts corresponding to the mature rabbit DNase I enzyme with sequences in the GenBank/EMBL databases (accession nos.: human, M55983; mouse, U00478; rat, X56060) using GENETYX-MAX (Software Development Co., Tokyo, Japan). Rabbit DNase I exhibits a higher degree of identity (82.7 %) with human DNase I than with the mouse or rat enzymes (77.9 % and 78.8 % respectively).

Expression of the DNase I gene transcript in rabbit tissues

The presence of DNase I-specific mRNA was verified by RT-PCR of total RNA extracted from several rabbit tissues. A set of primers, U3 and D3, specific to the rabbit DNase I cDNA was designed based on the nucleotide sequence of the cDNA determined in the present study. Using these primers, a unique

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   1 tcccgggatcctgagacccaacaggtctaaccggggccttggggtaagttcttcagcggc   60

  61 ctccccctttgtaaactaacatttttccttaaagcagcacaaggagaagaaattaacaatc  120

 121 cgaatattccacaattggagaattactgcactctcattgtcttggcATGAGGAGCGAGAT  180
                                                  M  R  S  E  M   -17

 181 GCTGACGGCGCTGCTGACCCTGGCTGTCCTGCTGCAGGTGGCCGGGTCTCTGAAGATTGC  240
 -16   L  T  A  L  L  T  L  A  V  L  L  Q  V  A  G  S  L  K  I  A    4

 241 AGCCTTCAACATCCGGTCTTTTGGGGAGACCAAGATGTCTAATGCCACCCTCACCAGCTA  300
   5   A  F  N  I  R  S  F  G  E  T  K  M  S  N  A  T  L  T  S  Y   24

 301 CATCGTGCGGATCCTGCAACGCTACGACATCGCCCTCATCCAGGAGGTCAGAGACAGCCA  360
  25   I  V  R  I  L  Q  R  Y  D  I  A  L  I  Q  E  V  R  D  S  H   44

 361 CCTGACGGCCGTGGGCAAACTGCTGGACAAGCTCAATGAGAAGGCCGCAGACACCTACCG  420
  45   L  T  A  V  G  K  L  L  D  K  L  N  E  K  A  A  D  T  Y  R   64

 421 CTTTGTGGCCAGTGAGCCCCTGGGGCGCAGCACCTACAAGGAGCGGTACCTCTTCGTGTA  480
  65   F  V  A  S  E  P  L  G  R  S  T  Y  K  E  R  Y  L  F  V  Y   84

 481 CAGGCCTGACCAGGTGTCTGTCCTGGACAGCTACTACTACGATGACGGCTGTGAGCCCTG  540
  85   R  P  D  Q  V  S  V  L  D  S  Y  Y  Y  D  D  G  C  E  P  C  104

 541 TGGGACTGACACCTTCAGCCGAGAGCCAGCCGTTGTCAGGTTCTCCTCCCCGTCCACGAA  600
 105   G  T  D  T  F  S  R  E  P  A  V  V  R  F  S  S  P  S  T  K  124

 601 GGTCCGCGAGTTCGCCATCGTGCCCCTGCACTCAGCCCCCGAGGACGCGGTAGCGGAGAT  660
 125   V  R  E  F  A  I  V  P  L  H  S  A  P  E  D  A  V  A  E  I  144

 661 CGATGCTCTCTACGACGTCTACCTGGATGTCCAGAAGAAGTGGGGCCTGCAGGATGTCAT  720
 145   D  A  L  Y  D  V  Y  L  D  V  Q  K  K  W  G  L  Q  D  V  M  164

 721 GCTCATGGGGGATTTCAACGCGGACTACAGCTACGTCACCTCCTCCCAGTGGTCGTCCAT  780
 165   L  M  G  D  F  N  A  D  Y  S  Y  V  T  S  S  Q  W  S  S  I  184

 781 CCGCCTGCGCACGAACCCTGCCTTCAAGTGGCTGATTCCTGACACGGCGGACACCACGGC  840
 185   R  L  R  T  N  P  A  F  K  W  L  I  P  D  T  A  D  T  T  A  204

 841 CACCTCCACCAACTGTGCCTATGACAGAATCGTGGTCGCCGGACCTCTGCTGCAGGACGC  900
 205   T  S  T  N  C  A  Y  D  R  I  V  V  A  G  P  L  L  Q  D  A  224

 901 TGTTGTCCCCAACTCGGCCGCTCCTTTCAACTTCCAGGCTGCGTACGGACTGAGCAACCA  960
 225   V  V  P  N  S  A  A  P  F  N  F  Q  A  A  Y  G  L  S  N  Q  244

 961 ACTGGCCCAGGCCATCAGTGACCACTACCCTGTGGAGGTGACGCTCGCCTGAtgcggtgg 1020
 245   L  A  Q  A  I  S  D  H  Y  P  V  E  V  T  L  A  *           260

1021 ctcccgggccccaccccagcacggggtgggggcttccttctccagtatccctgtctcc 1080

1081 cagcgcccacacagaagtctgaagtgtaggtgcagagacacctttaatgcaggtgaata 1140

1141 aagagggtgcttggccttaaa                                        1161
```

Figure 2 Full-length cDNA sequence of rabbit DNase I and its deduced amino acid sequence

Untranslated regions are shown in lower case and the coding sequence in upper case. The initiation and termination codons and the poly(A) addition signal sequence are underlined. The amino acid sequence of the putative signal peptide is indicated by negative numbers and underlined with a wavy line. The potential N-linked glycosylation site is double-underlined.

780 bp fragment corresponding to the region encoding the mature enzyme could be amplified from the total RNA of rabbit pancreas, parotid gland, kidney, liver, stomach, small intestine and thymus (Figure 3); no amplified product was obtained from the total RNA of spleen, heart, lung, cerebellum or cerebrum under our amplification conditions.

Semi-quantification of the levels of the DNase I gene transcript in the rabbit tissues was achieved by competitive PCR using a non-homologous internal standard approach. The results are summarized in Table 4. Large amounts of the DNase I gene transcript were expressed in both the pancreas and the parotid gland. Rabbit kidney, liver, small intestine, stomach and thymus contained low amounts of DNase I cDNA. However, expression levels of the DNase I gene transcript in spleen, heart, lung, cerebellum and cerebrum were found to be below the minimum detection limit (1×10^{-4} amol/μg of total RNA). These levels of

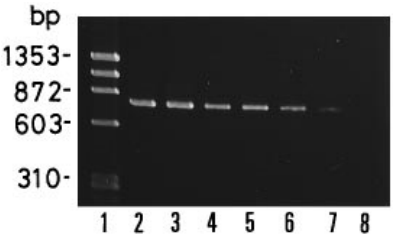

Figure 3 PCR analysis of reverse-transcribed rabbit tissue total RNA

Total RNA isolated from each rabbit tissue was transcribed and PCR-amplified using a set of specific primers (D3 and U3, as shown in Table 1) according to the method described in the text. After the products had been separated on a 2% agarose gel, each band was visualized by ethidium bromide staining. A unique 780 bp fragment was amplified from total RNA isolated from pancreas (lane 2), parotid gland (lane 3), kidney (lane 4), small intestine (lane 5), stomach (lane 6) and thymus (lane 7); no amplified product was obtained from heart (lane 8). Lane 1 contains ϕX174 DNA/*Hae*III digestion DNA markers (Gibco-BRL). The cathode is at the top.

DNase I-specific mRNA expressed in each tissue correlated well with DNase I activity levels measured in the corresponding tissue extract.

Comparative studies of the immunological properties of mammalian DNases I using several specific antisera

The SDS/PAGE pattern of DNase I in the crude urine sample detected by immunostaining with the chicken antiserum to rabbit DNase I exhibited only a single band, corresponding to the purified enzyme (Figure 1A). IEF/PAGE patterns of the purified DNase I were the same as those of the original urine sample when detected by immunostaining (Figure 1B) and activity staining (results not shown). In addition to the results of the double-immunodiffusion tests, this antibody was demonstrated not to react with any other urinary proteins separated by either IEF/PAGE or SDS/PAGE. The antibody to rabbit DNase I completely blocked the activity of the purified DNase I, as well as that in the original urine. These results demonstrate all the activity present in urine to be that of DNase I that is cross-reactive with the anti-(rabbit DNase I) antibody.

Next, three different antibodies specific to human [2], rat [27] and rabbit DNases I were tested for cross-inhibition of the enzyme activities of the human, rat and rabbit enzymes (Figure 4). Anti-(rabbit DNase I) completely blocked the rabbit enzyme activity, whereas it was ineffective against the rat enzyme activity. Conversely, the anti-(rat DNase I) completely blocked the rat enzyme activity, but not the rabbit enzyme activity. The human enzyme exhibited relatively low cross-reactivity with the antibodies against rat and rabbit DNases I. Although the amount of antibody required to inhibit activity by 50% was about 15-fold greater, the anti-(human DNase I) antibody also exerted an inhibitory effect on the activities of both the rat and rabbit DNases I, as well as that of the human DNase I. The results of these inhibition tests using specific antibodies were consistent with each other. These findings confirm that, from the immunological standpoint, the rabbit enzyme is closer to the human enzyme than to the rat enzyme, and the human enzyme is situated in a position between the rabbit and rat enzymes. This immunological relationship between different mammalian DNases I is consistent with the results of the nucleotide sequence identity analysis. The data obtained for DNase I correlating immunological cross-reactivity with sequence differences are consistent with the hypothesis, previously proposed for mammalian pancreatic RNase [48], that a large fraction of the

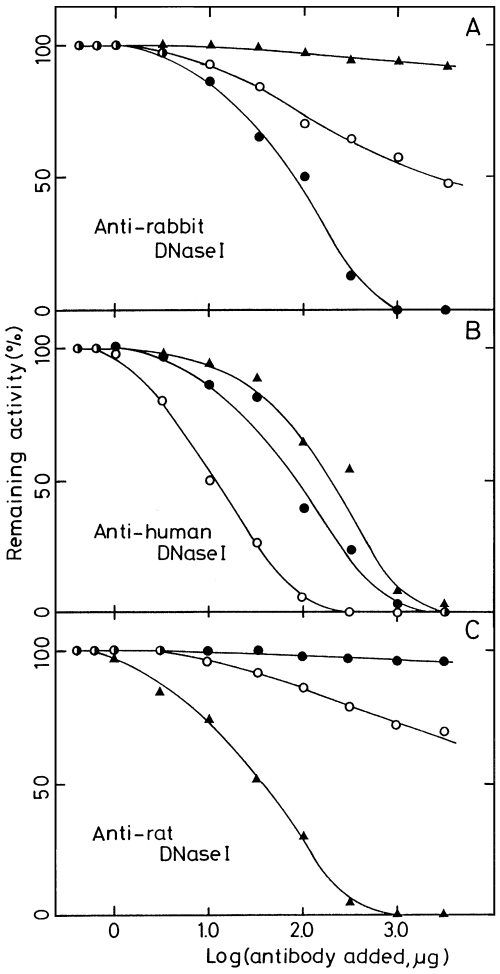

Figure 4 Inhibitory effects of species-specific antibodies on the activities of purified DNases I

Each purified DNase I (0.5 unit) was incubated with antibody specific to the urinary DNases I from rabbit (**A**), human (**B**) and rat (**C**), and the residual activities were measured as described in a previous paper [2]. The effects of each antibody on the activities of urinary DNases I purified from rabbit (●), human (○) and rat (▲) are shown.

evolutionary substitutions of amino acids in globular proteins are immunologically detectable.

DISCUSSION

In this paper we describe an affinity chromatographic procedure for the purification of rabbit urine DNase I to apparent homogeneity. The purity of the final preparation could be assessed on the basis of several criteria, including SDS/PAGE, assays for other nucleases and the production of a monospecific antibody against the purified enzyme. Our preliminary surveys found 8-(6-aminohexyl)amino-5′-AMP [49] and G-actin [50] to be ineffective as affinity resins in the purification of rabbit urinary DNase I. Recently, we succeeded in purifying the DNase I from rat urine by means of a two-step affinity chromatographic procedure involving both DNA–cellulose and poly(G)–agarose [27]. In the purification of rabbit urinary DNase I, use of DNA–cellulose allowed the enzyme to be separated from other urinary materials to a considerable extent, with little loss of activity (Table 2). As shown in Figure 1(B), all the DNase I isoforms present in rabbit urine were recovered in the final preparation, showing the usefulness of this purification procedure for isolating all isoforms of the enzyme. The fact that 0.8 mg of the purified DNase I could be obtained from about 15 litres of urine collected from five rabbits over a few weeks indicates that urine is one of the best source materials for purification of rabbit DNase I. We have already demonstrated that human urine is a good source of several nucleases, such as DNase I [2], DNase II [35] and secretory-type and non-secretory-type RNases [41,51]. Urine has a further advantage, in that its collection is non-invasive.

Although various cDNA sequences are available for human [28] and rat [29] DNases I, a full-length cDNA encoding DNase I has not yet been isolated from any mammal. A combination of RT-PCR and 3′- and 5′-RACE methods enabled a full-length DNase I cDNA to be constructed from the total RNA of rabbit pancreas, the tissue in which the highest enzyme activity was observed. It was notable that the 5′-RACE method allowed only a 350 bp fragment to be amplified, suggesting that the rabbit DNase I gene has a single transcriptional initiation site. Similarly, RNAs from mouse kidney and parotid gland provided only a single 5′-RACE product (T. Yasuda and K. Kishi, unpublished work). The coding region of the rabbit DNase I gene was translated into the corresponding amino acid sequence and compared with all other mammalian DNase I sequences available [28,29,33,52]. All four residues (Glu-78, His-134, Asp-212 and His-252) postulated to be involved in the active site [53] were conserved. G-actin has been identified as a potent inhibitor of DNase I [12,13]; amino acid residues at positions 13 (Glu), 65 (Tyr), 67 (Val) and 114 (Ala) of the enzyme have been assumed to be responsible for actin binding [29,52]. Rabbit urinary DNase I was inhibited by G-actin to a smaller extent (about 10-fold) than the human enzyme, probably owing to a weaker interaction of G-actin with the former DNase I than with the latter. Among the residues involved in actin binding, Val-67 and Tyr-65 were replaced by Ala and Phe in the rabbit enzyme when compared with the human enzyme (its closest homologue). On the other hand, engineering of actin-resistant human DNase I found substitution of Val-67 by Ala to exert little effect on actin binding [53]. Therefore the change in affinity might be attributable to the amino acid replacement Tyr-65 to Phe in rabbit DNase I. Two potential N-linked glycosylation sites, Asn-18 (Asn-Ala-Thr) and Asn-106 (Asn-Asp-Thr/Ser), are both conserved in all mammalian DNases I so far determined. However, the amino acid residue corresponding to the Asn-106 site was identified as a Thr in rabbit DNase I. On the other hand, since the corresponding Asn residue could not be identified during proteochemical analysis, the carbohydrate moieties of the enzyme might be attached to the amido group of the Asn at position 18. It has been reported that bovine pancreatic DNase I has only one carbohydrate side chain, whereas the parotid gland enzyme has two [54], suggesting tissue-specific glycosylation of DNase I [3].

Systematic examination of the tissue distributions of DNase I in mammals have so far been limited to rats [6,7,55], mice [56] and humans [4]. Previously, we measured DNase I and II activities in the reproductive organs and accessory glands of male rabbits. In addition to comparison of the IEF/PAGE patterns, the results demonstrated that the high DNase I activity detected in rabbit semen was derived from the epididymis and seminal vesicle, whereas DNase II activity originated from the epididymis and prostate [5]. In the present study, we determined the DNase I activity of 20 different rabbit tissues by the SRED method, and estimated the levels of the DNase I gene transcript expressed in these tissues by a semi-quantitative method involving a competitive PCR (Table 4). The assay conditions were corrected for the catalytic properties of the purified enzyme, and all activities detected in each tissue extract were abolished by EDTA, EGTA,

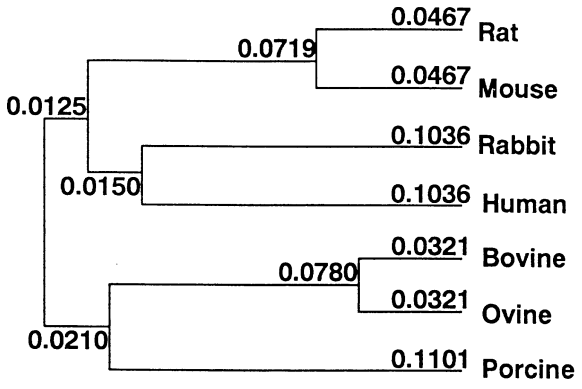

Figure 5 Phylogenetic tree of the DNase I family

The tree was constructed by the unweighted pair grouping method with arithmetic mean on the basis of amino acid sequences corresponding to the mature enzyme from each mammal shown. The numbers represent evolutionary distance [57]. The sequences of the DNases I were obtained from various databases and have the following accession numbers: mouse, GenBank U00478; rat, EMBL X56060; human, GenBank M55983; bovine, Swiss-Prot drn1-bovine; ovine, Swiss-Prot drn1-sheep; porcine, Swiss-Prot drn1-pig.

G-actin and the anti-(rabbit urinary DNase I) antibody. Although another distinct type of DNase, DNase II, occurs widely in mammalian body fluids and tissues [35], this enzyme was confirmed not to be detectable under our assay conditions. Therefore, from these findings, it seems reasonable to conclude that the activity detected by the SRED method was indeed that of DNase I. Elucidation of the cDNA sequences encoding rabbit DNase I enabled a set of PCR primers, corresponding to the N- and C-terminal regions of the mature enzyme and specific to rabbit DNase I, to be designed for RT-PCR analysis and for semi-quantification of the DNase I gene transcript levels expressed in each tissue. The pancreas and parotid gland, which exhibited the highest enzyme activity, also expressed the largest amounts of mRNA specific for DNase I, whereas several tissues, such as the spleen, heart, lung, cerebellum and cerebrum, which had undetectable activities, provided no amplified products under our PCR conditions (Table 4). Thus DNase I enzyme activities and levels of the DNase I gene transcript correlated well in each rabbit tissue tested. It is plausible to assume that enzyme activity reflects DNase I gene expression in the tissues tested. The finding that detectable levels of the DNase I gene were expressed in non-digestive tissues, such as the kidney, liver and thymus, contradicts the previous assumption that DNase I serves a solely digestive role. The highest enzyme activities in both the rat and mouse were found in the parotid gland [55,56], whereas little activity could be detected in the pancreas. In contrast with the rodents, the human pancreas exhibits a 100-fold higher enzyme activity than the human parotid gland (T. Yasuda and K. Kishi, unpublished work). In terms of the distribution of DNase I in the parotid gland and pancreas, the rabbit lies between rodents and humans. Thus DNase I gene expression in mammalian tissues was confirmed to be species-specific.

Complete nucleotide and amino acid sequences for several mammalian DNases I were obtained from the GenBank/Swiss-Prot Database, and the amino acid sequences of the mature enzymes were aligned in order to construct a phylogenetic tree for the DNase I family. The tree was tentatively obtained by the unweighted pair grouping method with arithmetic mean [57]. Mammalian DNases I can be classified into three branches on the phylogenetic tree (Figure 5). The phylogenetic tree reflects a closer relationship between rabbit and human DNases I than between other mammalian DNases I. The relationships between the immunological properties and tissue distributions of human, rat and rabbit DNases I were found to be consistent with the results of the phylogenetic analysis.

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