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Molecular cloning and characterization of a novel human ribonuclease (RNase k6): increasing diversity in the enlarging ribonuclease gene family

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ABSTRACT

The discovery of Ribonuclease k6 (RNase k6) was an unexpected result of our ongoing efforts to trace the evolutionary history of the ribonuclease gene family. The open reading frame of RNase k6, amplified from human genomic DNA, encodes a 150 amino acid polypeptide with eight cysteines and histidine and lysine residues corresponding to those found in the active site of the prototype, ribonuclease A. The single-copy gene encoding RNase k6 maps to human chromosome 14 and orthologous sequences were detected in both primate and non-primate mammalian species. A single mRNA transcript (1.5 kb) was detected in all human tissues tested, with lung representing the most abundant source. At the cellular level, transcripts encoding RNase k6 were detected in normal human monocytes and neutrophils (but not in eosinophils) suggesting a role for this ribonuclease in host defense. Of the five previously identified human ribonucleases of this group, RNase k6 is most closely related to eosinophil-derived neurotoxin (EDN), with 47% amino acid sequence identity; slight cross-reactivity between RNase k6 and EDN was observed on Western blots probed with polyclonal anti-EDN antiserum. The catalytic constants determined, K_m = 5.0 μM and k_{cat} = 0.13 s^{-1}, indicate that recombinant RNase k6 has ~40-fold less ribonuclease activity than recombinant EDN. The identification and characterization of RNase k6 has extended the ribonuclease gene family and suggests the possibility that there are others awaiting discovery.

INTRODUCTION

The ribonuclease family is composed of proteins with similar primary structure and enzymatic activity that have diverged to support other, seemingly unrelated physiologic activities. The prototype of this family is ribonuclease A [bovine pancreatic ribonuclease (1,2)]. A human pancreatic ribonuclease with similar sequence has been identified (3–5) and pancreatic ribonucleases have been isolated from an extensive array of other mammalian species (6,7). Two related ribonucleases are the eosinophil-derived neurotoxin (EDN), also known as Rnase Us (8) and the eosinophil cationic protein (ECP) (reviewed in 9,10). ECP has been characterized as a cytotoxin, a neurotoxin and as an anti-parasitic and anti-bacterial agent; EDN, despite its sequence homology to ECP (67%) and 100-fold greater ribonuclease activity, shares only ECP's neurotoxicity. Angiogenin, a structurally atypical member of this gene family, is a tRNA-specific ribonuclease (11) that binds to actin on the surface of endothelial cells (12); bound angiogenin is endocytosed and translocated to the nucleus, thereby promoting the endothelial invasiveness necessary for blood vessel formation (13). Ribonuclease 4 (RNase 4) also has a unique substrate preference (14), although no specific physiologic functions of this protein have been identified. Others included in this family are bovine brain (15,16) and bovine seminal ribonucleases (17) and several ribonucleases isolated from species of the frog genus, *Rana* (18–20); no orthologous human sequences corresponding to these bovine or frog ribonucleases have been identified.

In our previous work, we traced the evolutionary history of the eosinophil ribonucleases, ECP and EDN (21). We found that the EDN/ECP gene pair arose from a gene duplication event that occurred relatively recently, sometime after the divergence of the Old World from the New World monkeys and that the genes encoding EDN and ECP are the most rapidly evolving functional coding sequences known among primate species. We have since determined that the unusual evolutionary constraints on these two proteins have promoted both enhanced toxicity (ECP) and increased ribonuclease activity (EDN) when the properties of these two proteins are compared with those of a representative single-sequence predecessor (22).

In hopes of tracing the molecular evolution of the single ECP/EDN gene through the non-primate mammalian Orders, we focussed on bovine kidney ribonuclease k2 (RNase k2), a protein originally isolated and described by Irie *et al.* (23). Specifically, we were interested in determining whether bovine RNase k2, whose amino acid sequence was more closely related to human EDN than it was to any of the other human ribonucleases, represented a direct evolutionary predecessor of the EDN/ECP genes, or instead represented the bovine ortholog of a unique human gene that had yet to be discovered. We found the latter to be the case. In this work, we have isolated unique genomic clones

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encoding both bovine Rnase k2 and a novel human ribonuclease, which we have named Ribonuclease k6 (RNase k6). We have provided a molecular characterization of RNase k6 and have examined the immunoreactivity and ribonucleolytic activity of the recombinant protein. Together, bovine RNase k2 and human RNase k6 represent a heretofore unrecognized arm of the apparently enlarging ribonuclease gene family and are likely to possess unique physiologic functions.

MATERIALS AND METHODS

Isolation of a genomic fragment encoding bovine RNase k2 by polymerase chain reaction (PCR)

Bovine genomic DNA was isolated from cells of the MDBK cell line (ATCC-CCL22). Degenerate oligonucleotide primers were designed from amino acids 10 to 15 (5′ to 3′ primer) and 98 to 102 (3′ to 5′ primer) of the published amino acid sequence of bovine RNase k2 (23), with sequences as follows: 5′ to 3′ primer: 5′-TGG TT(CT) GA(AG) AT(ACT) CA(AG) CA-3 and 3′ to 5′ primer: 5′-AT(GA) AA(GA) AA(TC) TT(GA) TA(TC) TG-3′. PCR reactions proceeded in a 100 µl volume with 10 µM of each primer, 1 µg template, 0.2 mM dNTPs and 2.5 U *Taq* polymerase and buffer (Boehringer Mannheim, Indianapolis, IN) in a 9600 thermocycler (Perkin-Elmer, Norwalk, CT) with the following parameters: 95°C for 2 min, followed by 35 cycles of 95°C for 30 s, 50°C for 30 s and 72°C for 30 s and completed with 72°C for 5 min. The product was identified by its mobility on a 6% TBE-acrylamide gel; the product remaining was then subjected to TBE–agarose gel electrophoresis and the band was excised, purified and ligated into a TA cloning vector (PCRII, Invitrogen, San Diego, CA) as described previously (24). The fragment was identified as encoding bovine RNase k2 by dideoxy sequencing (US Biochemical, Cleveland, OH). All sequence analyses (including homology and isoelectric point determinations) were performed with the assistance of the Wisconsin Genetics Computer Group software on-line at the National Institutes of Health.

Isolation of genomic fragments encoding the complete open reading frame (ORF) of a novel human ribonuclease

The degenerate oligonucleotides described above were used to isolate a human genomic fragment by polymerase chain reaction as described above using purified human genomic DNA as a template. An ~300 bp fragment was isolated and identified as encoding a novel ribonuclease by dideoxy-sequencing. The complete ORF of this intronless gene was obtained by extension in both the 5′ and 3′ directions with uni-directional PCR method with reagents included in the Promoter Finder kit (Clontech, Palo Alto, CA). The nested gene-specific primer pair designed to amplify genomic sequence located 5′ of the original isolate were as follows: 1a: (nt 364–338) 5′-ACT GGG CAG CAG CAC TAT AGC GGC ACT-3′; 2a: (nt 333–303) 5′-ATA CTT TCC TGA AGT GAG TCT GCA GTC-3′. The nested gene-specific primers for amplification of sequence 3′ of the original isolate were as follows: 1b: (nt 291–317) 5′-GCC TGT CAA CAT GAC TGA CTG CAG ACT-3′; 2b: (nt 325–351) 5′-GGA AAG TAT CCC CAG TGC CGC TAT AGT-3′; 1c: (nt 301–336) 5′-ATG ACT GAC TGC AGA CTC ACT TCA GGA AAG TAT CCC-3′; 2c: (nt 322–360) 5′-TCA GGA AAG TAT CCC CAG TGC CGC TAT AGT GCT GCT GCC-3′. The first round of amplification in a single direction included proceeded in a 25 µl reaction volume with 1× Tth buffer, 1.1 mM magnesium acetate, 0.2 mM dNTPs, 0.2 µM adaptor primer 1, 0.2 µM gene-specific primer 1 (a, b or c) and 0.5 µl Advantage Tth polymerase mix. The reaction parameters included 7 cycles at 94°C for 2 s followed by 68°C for 3 min, then 37 cycles at 94°C for 2 s and 63°C for 3 min, with a completion step of 67°C for 4 min. One µl of each primary amplification was reamplified in a 25 µl volume including buffer, magnesium acetate, dNTPs as described and including 0.2 µM adaptor primer 2, 0.2 µM gene-specific primer 2 (a, b or c) and 0.5 µl Advantage Tth polymerase mix, with parameters including 5 cycles of 94°C for 2 s and 68°C for 3 min, followed by 25 cycles at 94°C for 2 s and 63°C for 3 min, with the completion step as described above. Amplification products obtained were isolated, subcloned and sequenced (see Results). As a control for potential sequence mutations, the complete coding sequence was then re-amplified directly from another source of human genomic DNA using flanking primers; the sequence obtained from this isolate was identical to the original, suggesting the absence of PCR-induced mutations.

Genomic Southern blots

Nitrocellulose membranes with 20 µg restriction digested human or bovine genomic DNA were prepared, crosslinked and hybridized as described previously (24).

Chromosomal localization

Chromosomal localization was performed using a PCR based method as described (BIOS DNA, New Haven, CT) (24,25). Briefly, two oligonucleotide primers were tested in order to confirm that they amplified a specific fragment from only human (and not hamster) genomic DNA templates. The oligonucleotide primers selected (Fig. 2A, primers x and y, numbering as per Fig. 2B) 5′ to 3′: (nt 121–136) 5′-CCA AGT CCT CTC CAA T-3′ and 3′ to 5′: (nt 360–344) 5′-GGC AGC AGC ACT ATA GC-3′ amplified a single 240 bp fragment from human genomic DNA. Amplification of DNA isolated from the 25 characterized human:hamster somatic cell hybrids proceeded as described previously (24); products of each reaction were evaluated on TBE–agarose gels stained with ethidium bromide.

Northern blotting

A human multi-tissue Northern membrane was obtained from Clontech; the multi-leukocyte Northern membrane was a gift of Dr Philip Murphy. The membrane was pre-hybridized and hybridized as per manufacturer's instructions; the hybridization was performed with the radiolabelled human ribonuclease k6 probe described above. The membrane was then washed with 2× SSPE with 0.1% SDS for 1 h at 42°C followed by 0.2× SSPE with 0.1% SDS for 1 h at 50°C; autoradiograms were developed after 24 h exposure at –80°C. The hybridization with the oligonucleotide encoding human β-actin was as previously described (24).

Preparation of the human RNase k6 expression construct

The portion of the ORF encoding the mature RNase k6 peptide (base pairs 70–450; GenBank accession no. U64998) was re-amplified from human genomic DNA and ligated in-frame into the *Hin*dIII and *Eco*RI sites of the pFCTS bacterial expression vector (International Biotechnologies, Inc., New

Haven, CT) to create hK6#81 (Fig. 4A). The fidelity of the expression construct was confirmed by dideoxy sequencing.

Preparation and isolation of recombinant protein

Production and isolation of recombinant RNase k6 from bacterial transfectants was as described previously for recombinant EDN (22). The concentration of recombinant protein was determined by comparison to serial dilutions of a known concentration of a FLAG-conjugated protein standard on Western blots.

Western blotting

Protein samples were subjected to gel electrophoresis in 14% Tris-glycine gels (Novel Experimental Technologies, San Diego, CA). Proteins were transferred to nitrocellulose membranes and probed with antibodies as per published procedures (26).

Ribonuclease activity assay

The assay used was adapted from the procedure described by Slifman *et al*. (27) as described previously (22,28). Calculations included the following approximations: the average molecular weight (M_r) of tRNA as 28 100 (75–90 ribonucleotides/tRNA molecule × M_r 341/ribonucleotide), with A_{260} of 1.0 corresponding to 40 μg of RNA (29). All time points represented averages of triplicate samples. Equivalent volumes of sham isolations (M2-resin equilibration and glycine elution from equivalent volumes of pFCTS vector alone bacterial transfectants) had levels of ribonuclease activity that were insignificant compared with that of the recombinant RNase k6 (<0.1%).

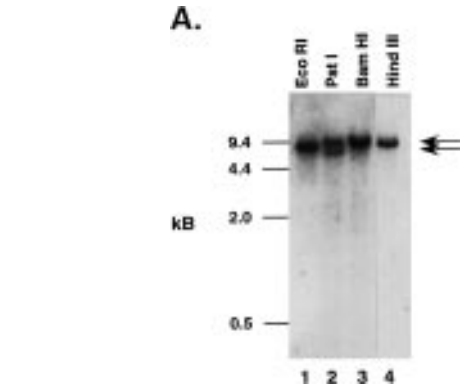
RESULTS

Molecular cloning of a genomic fragment encoding bovine RNase k2

The nucleotide sequence of a 281 bp genomic fragment encoding bovine ribonuclease k2 has been deposited in the GenBank database, accession number U64997. The fragment was amplified by PCR using degenerate oligonucleotide primers and bovine genomic DNA as template. The encoded amino acid sequence matches that determined from peptide fragments from the purified RNase k2 protein described by Irie *et al.* (23); included in this partial sequence are seven of the eight characteristic cysteines, the lysine and one of the histidines comprising the ribonuclease active site (2) and two potential sites for asparagine-linked glycosylation.

The bovine RNase k2 fragment was used to probe a Southern blot of restriction-digested bovine genomic DNA (Fig. 1A). Whereas single hybridizing bands were detected in the lanes containing DNA digested with *Eco*RI, *Bam*HI and *Hin*dIII, two bands were evident in the lane containing DNA digested with *Pst*I. As the RNase k2 probe contains no internal *Pst*I sites, these results suggest the existence of an as yet unidentified sequence similar to RNase k2 (either a novel gene or a polymorphism) within the bovine genome.

Shown in Figure 1B are the amino acid sequence homologies between pairs of bovine ribonucleases. Bovine brain, seminal and pancreatic ribonucleases are very closely related to one another (78–81% amino acid sequence identity); RNase 4 is clearly a more distant relative (47–49% identical). Both RNase k2 and angiogenin display even less sequence similarity to the brain, seminal and pancreatic group than does RNase 4 (38–40% and



B.

	p	br	se	4	ag	k2
pancreatic (p)	100					
brain (br)	78	100				
seminal (se)	81	78	100			
rnase 4	47	49	48	100		
angiogenin (ag)	37	38	36	41	100	
kidney (k2)	40	38	38	34	26	100

Figure 1. (**A**) Bovine genomic DNA restriction-digested with enzymes shown and probed with the ^{32}P-radiolabeled fragment encoding bovine RNase k2 (GenBank accession no. U64997). Arrows indicate the positions of the two distinct bands found in lane 2 (*Pst*I-digested DNA) which cannot both be accounted for by the restriction map of the RNase k2 probe. (**B**) A comparison of the amino acid sequence identities among the known bovine ribonucleases. Identities determined using the GAP algorithm of the Wisconsin Genetics Computer Group program on-line at the National Institutes of Health; sequences were obtained from the SWISSPROT database, with accession names as follows: bovine pancreatic ribonuclease (p), rnp_bovin.sw; brain ribonuclease (br), rnbr_bovin.sw; seminal ribonuclease (se), rns_bovin.sw; ribonuclease 4 (4), rnl4_bovin.sw; angiogenin (ag), angi_bovin.sw; kidney (k2), rnkd_bovin.sw.

36–38% sequence identity, respectively) and display no more sequence similarity than this to one another (26%). These results indicate that RNase k2 has diverged significantly from the prototype pancreatic ribonucleases and suggest that RNase k2, like angiogenin, may support a biologic activity that distinguishes it from the group as a whole (see Discussion).

Molecular cloning of a genomic fragment encoding a novel human ribonuclease (RNase k6)

Using the degenerate primers designed to amplify the bovine RNase k2 genomic fragment (primers p and q, Fig. 2A), we were successful in isolating a 281 bp fragment encoding a novel human ribonuclease. This novel isolate shared 77% nucleotide sequence identity and 70% amino acid sequence identity with the previously isolated bovine fragment. The 5′- and 3′-ends of the complete coding sequence were isolated using a uni-directional PCR method with adaptor ligated ‘genomic libraries’ and adaptor primers supplied in the Promoter Finder kit from Clontech

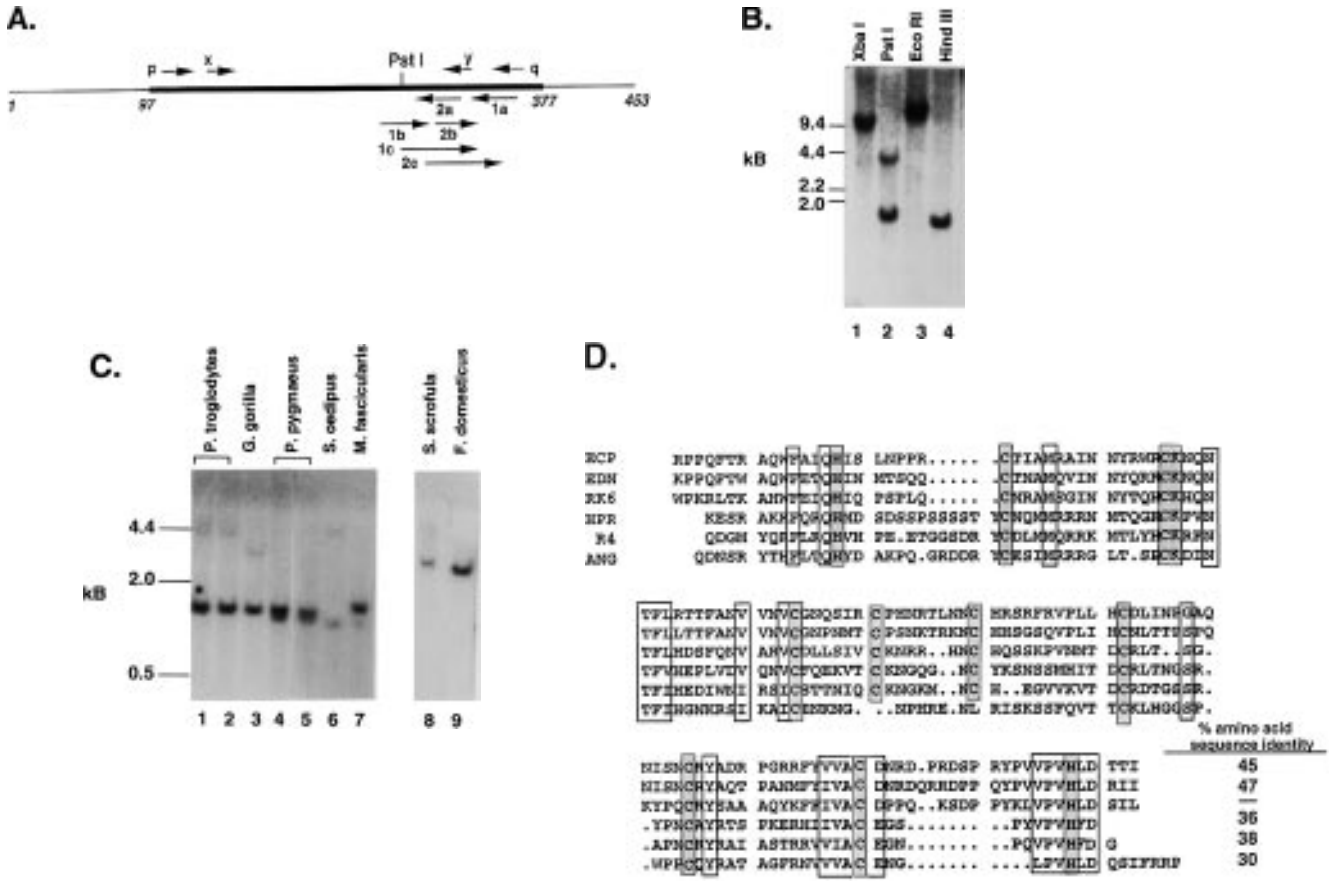


Figure 2. (**A**) Schematic of the placement of the oligonucleotide primers within the sequence of the complete ORF of human RNase k6 (nt 1–453). The bold line (nt 97–377) represents the original fragment isolated with degenerate oligonucleotide primers p and q. Primers x and y were used for chromosomal localization. Primer pairs 1a and 2a were used to extend the ORF in the 5′ direction by uni-directional PCR; primer pairs 1b and 2b and 1c and 2c were used to extend the ORF in the 3′ direction. (**B**) Human genomic DNA restriction-digested with the enzymes shown and probed with a ^{32}P-radiolabeled fragment (nt 97–377) encoding human RNase k6 (GenBank accession no. U64998). (**C**) Genomic DNA from several primate and non-primate mammals restriction-digested with *Pst*I and probed as in (B). Common names include: chimpanzee, (lanes 1 and 2, *P.troglodytes*); gorilla (lane 3, *G.gorilla*); orangutan (lanes 4 and 5, *P.pygmaeus*); marmoset/New World monkey (lane 6, *S.oedipus*); macaque/Old World monkey (lane 7, *M.fascicularis*); pig (lane 8, *S.scrofula*); cat (lane 9, *F.domesticus*). (**D**) Alignment of the amino acid sequences of the six human members of the ribonuclease gene family. Shaded boxes enclose eight cysteines and catalytic histidines and lysine; open boxes enclose additional conserved residues. Percentages amino acid sequence identity to RNase k6 are as listed in the final column. Amino acid sequences were translated from nucleotide sequences as reported to GenBank: pancreatic ribonuclease (HPR), X79235; eosinophil-derived neurotoxin (EDN), M24157; eosinophil cationic protein (ECP), X15161; RNase 4 (R4), U36775; angiogenin (ANG), M11567; RNase k6 (RK6), U64998.

Laboratories, Inc., Palo Alto, CA. The locations of the nested gene-specific primers directed to the 5′ (1a and 2a) and 3′ (1b and 2b, 1c and 2c) ends of the gene are shown in Figure 2A (for sequences, see Materials and Methods). For extension in the 3′ direction, a 130 bp fragment was amplified from the *Eco*RV-restricted library using nested gene-specific primers 1b and 1c, which provided ~25 bp overlap with the original isolate and an additional 60 bp of ORF at the 3′-end. The 3′-end of the ORF was completed using primers 1c and 2c which amplified an 800 bp fragment from the *Dra*I-restricted library. Similarly, 5′-end extension resulted in the amplification of a 500 bp fragment from the *Sca*I-restricted library which provided an ~200 bp overlap with the original isolate and an additional 90 bp completing the ORF at the 5′-end. The complete ORF was then re-amplified using flanking primers from a separate source of human genomic DNA to confirm the original sequence.

The complete coding sequence has been deposited in the GenBank database, accession number U64998. Highlights of this 14.7 kDa protein (calculated molecular weight), which we have named RNase k6 (see Discussion), include eight characteristic cysteines, the ribonuclease active site residues [histidines and a lysine residue within a conserved sequence motif (30)], two potential sites for asparagine-linked glycosylation and a 23 amino acid hydrophobic leader sequence. Overall, the amino acid sequence of human RNase k6 is 72% identical to the complete amino acid sequence of its bovine RNase k2 ortholog (23). Interestingly, the calculated isoelectric point (pI) of human RNase k6 (pI = 9.49) is significantly higher than that of the bovine protein (pI = 7.65); a similar increase in pI was noted for human ECP (pI = 11.4) and its evolutionary predecessor from the New World monkey, mEDN (pI = 8.25) (21).

The human RNase k6 gene fragment was used to probe a Southern blot of restriction-digested human DNA (Fig. 2B). Single hybridizing bands were detected in the lanes containing DNA digested with *Eco*RI, *Bam*HI and *Hin*dIII and two bands were detected in the lane containing DNA digested with *Pst*I, as anticipated from the internal *Pst*I site shown in the map in Figure 2A. In contrast to our findings with the bovine RNase k2 probe,

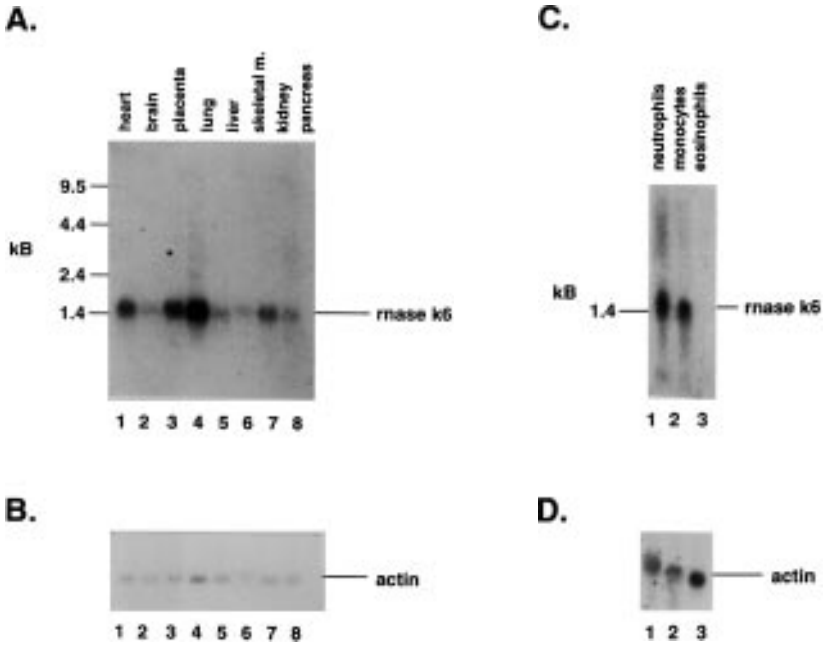


Figure 3. (**A**) Total RNA from the normal human tissues indicated (lanes 1–8) probed with the ^{32}P-radiolabeled fragment encoding human Rnase k6 as described in Figure 2C. (**B**) As in (A), probed with a human β-actin-specific oligonucleotide (24). (**C**) Total RNA from normal human neutrophils (lane 1), monocytes (lane 2) and eosinophils (lane 3) probed as described in (A). (**D**) As in (C), probed with the human β-actin-specific oligonucleotide (24).

we were unable to detect any additional, related sequences with these restriction digestions.

As shown in Figure 2C, we detected orthologs of RNase k6 in a wide variety of primate species, including chimpanzee (*P.troglodytes*), gorilla (*G.gorilla*), orangutan (*P.pygmaeus*), Old World monkey (macaque, *M.fascicularis*) and New World monkey (marmoset, *S.oedipus*). Orthologous sequences were also detected in non-primate mammals, including (in addition to the bovine sequence) pig (*S.scrofula*) and cat (*F.domesticus*).

In Figure 1D, we present an alignment of the amino acid sequences of human RNase k6 and the five previously identified human members of the ribonuclease gene family, which include pancreatic ribonuclease, EDN, ECP, RNase 4 and angiogenin. Interestingly, the amino acid sequence homologies suggest that RNase k6 is most closely related to the eosinophil ribonucleases, EDN and ECP.

Chromosomal localization of human ribonuclease k6

The chromosomal mapping of RNase k6 was achieved using a PCR-based technique (24). Genomic DNAs derived from a characterized panel of human:hamster somatic cell lines served as templates and a pair of gene-specific primers (Fig. 2A, arrows x and y) were used to amplify a specific fragment of human RNase k6. The anticipated 240 bp fragment was amplified from DNA templates from hybrids numbered 423, 507, 750, 756, 867, 909 and 937 (see reference 24), consistent with assignment to human chromosome 14.

Size and somatic distribution of RNase k6 transcripts

As shown in Figure 3A, mRNA encoding human RNase k6 was detected in all human tissues tested, with lung representing most abundant source. A single transcript of ~1.5 kb was detected. In Figure 3C, the 1.5 kb mRNA transcript encoding RNase k6 was detected in total RNA isolated from normal human monocytes and neutrophils, but could not be detected in total RNA isolated from eosinophils.

Expression, activity and cross-reactivity of recombinant human RNase k6

The sequence encoding the mature protein (without leader sequence) was used to create plasmid construct hK6#81 (Fig. 4A). This construct was prepared using the prokaryotic expression vector pFCTS, which we have used previously to prepare and isolate recombinant ribonucleases directly from the bacterial periplasm in a biologically active state (22,28). Features of this expression system include the inducible *tac* promoter, a bacterial secretion piece and a C-terminal sequence tag (FLAG), which does not interfere with proper folding or activity of recombinant ribonucleases (22,28). Shown in Figure 4B are Western blots containing extracts of bacteria transfected with either hEDNS#1 [induced to produce human EDN, (22)] or hK6#81 (producing human RNase k6) probed with the M2 anti-FLAG monoclonal antibody or with polyclonal anti-EDN, respectively. Slight cross-reactivity is detected between human EDN and RNase k6. In Figure 4C, the ribonuclease activities of these two proteins are compared. As determined from double reciprocal plots of initial rates, the Michaelis constant (K_m) of RNase k6 is 5.0 μM and the catalytic constant, k_{cat} is 0.13 s^{-1}. A comparison to the constants determined for human EDN (K_m = 0.70 μM, k_{cat} = 0.91 s^{-1}) (22) indicates that RNase k6 is ~40-fold less active than EDN when tested against a standard substrate (yeast tRNA).

DISCUSSION

We have isolated a genomic fragment encoding a previously unrecognized member of the ribonuclease gene family, which is perhaps not the last of these structurally related proteins to be

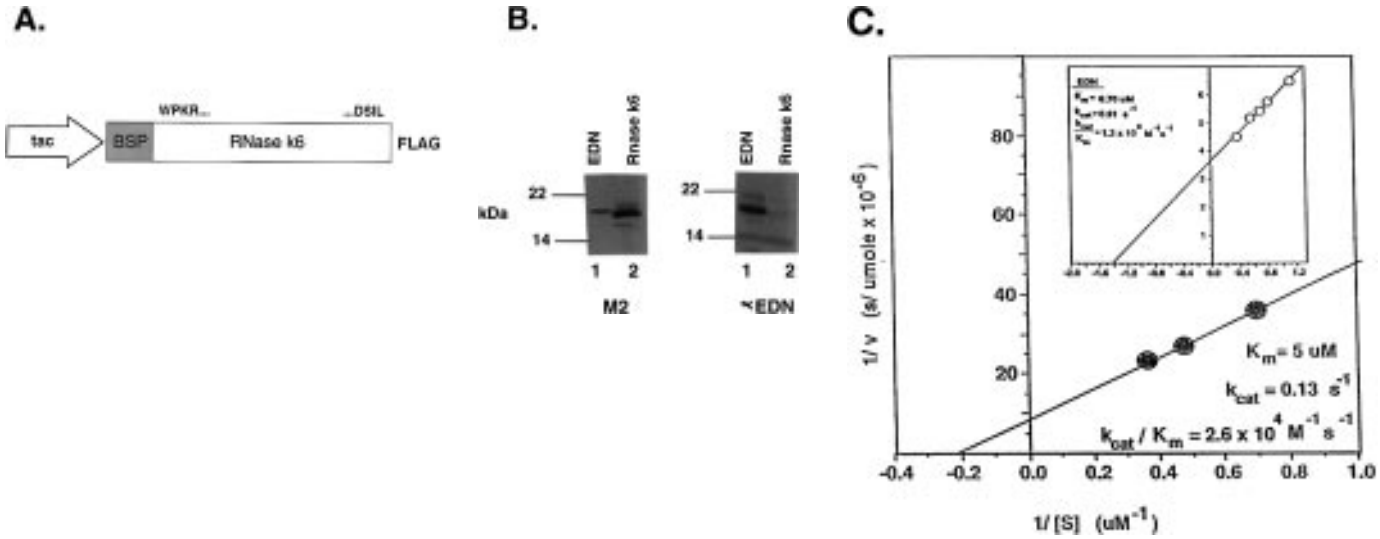


Figure 4. (**A**) Schematic of the prokaryotic expression construct hK6#81. The IPTG-inducible promoter (tac), bacterial secretion piece (BSP), sequence encoding human RNase k6 (without leader, N- and C-terminal amino acids of RNase k6 as shown) and the C-terminal sequence tag (FLAG) are indicated. (**B**) Western blots containing extracts of transfected bacteria induced with IPTG to produce EDN (lane 1) or RNase k6 (lane 2) probed with the M2 monoclonal antibody detecting the C-terminal FLAG sequence tag or with rabbit polyclonal anti-EDN antiserum. (**C**) Lineweaver–Burk plots (1/*v* versus 1/[*S*]) derived from initial rates of reactions containing 1 pmol recombinant RNase k6 with varying concentrations of a yeast tRNA substrate (1.4–2.7μM). Values for K_m (μM) and k_{cat} (s^{-1}) are as shown. (Inset) Lineweaver–Burk plot and catalytic constants determined for 0.3 pmol recombinant human EDN (22).

discovered. We have named this protein 'Ribonuclease k6' (RNase k6): '6' indicates that this protein is the sixth human ribonuclease of this family to be characterized and 'k' reflects its orthologous relationship with bovine RNase k2 (23). RNase k6 has features that are fairly typical for this gene family (Fig. 2D). As a group, the members of this family maintain a set of distinctly spaced cysteine residues which facilitate formation of specific intrachain disulfide bonds, as well as conserved lysine and histidine residues analogous to those originally found in the catalytic site of Ribonuclease A (2). All members of this family maintain some degree of ribonuclease activity, although at different levels of efficiency and with some degree of substrate preference. There are several amino acids other than those mentioned above that appear to be conserved in all the human members of this family; some of these are also conserved in the other mammalian and amphibian ribonucleases whose sequences have been reported (6,21). Although the significance of these additional conserved residues has not been determined, it is interesting to note that interaction between human EDN and the chaperonin, groEL includes the conserved residue M^{36}, suggesting a role for this residue in the folding and subcellular sorting of nascent ribonucleases within the cells of origin (31).

There are several additional features of the gene encoding RNase k6 that are shared with other members of the ribonuclease gene family, including the intronless coding sequence (24,32–34) and localization to human chromosome 14 (24,32–35). The mRNA transcript encoding RNase k6 has a near ubiquitous distribution, similar to that determined for angiogenin (36,37), human RNase 4 (24) and for human pancreatic ribonuclease (Handen and Rosenberg, unpublished data). The 1.5 kb transcript is slightly larger than the mRNAs encoding EDN, ECP or angiogenin (36,38,39), but much smaller and less complex than those encoding RNase 4 (24). As anticipated from amino acid sequence homology, we demonstrated slight cross-reactivity between RNase k6 and EDN when both recombinant proteins were probed with a polyclonal anti-EDN antiserum. In addition, we determined that recombinant RNase k6 was somewhat less enzymatically active than recombinant EDN (~40 fold), but had more activity than recombinant mEDN, the representative single-sequence predecessor of the EDN/ECP gene pair isolated from a New World monkey (22).

From an evolutionary perspective, it would seem unlikely that so many distinct and divergent versions of ribonuclease would have evolved to support the simple enzymatic activity alone. Indeed, certain members of this family were originally identified on the basis of their ability to support other biologic activities. For example, angiogenin was originally identified on the basis of its ability to promote blood vessel formation (40) and ECP and EDN were known to be toxins (9,10). Since its discovery, the anti-neoplastic activity of bovine seminal ribonuclease has also become appreciated (41–43). Similarly, other, related ribonucleases from the frog genus *Rana* have displayed both anti-viral and anti-neoplastic activity (44–46).

Evolutionary principles would also suggest that these proteins have maintained ribonuclease structure and activity for some crucial reason; the relationship between ribonuclease activity and biologic function still remains unclear. Angiogenin is a most interesting example of this point, with many experiments clearly documenting a direct relationship between angiogenic and ribonuclease activities (47,48). Yet, most recent evidence suggests that angiogenin binds to an actin monomer on the surface of endothelial cells, where it is endocytosed and then translocated to the nucleus (12,13,49); Moroianu and Riordan (13) have established that the nuclear translocation step is crucial for angiogenesis and that enzymatically inactive angiogenins are capable of translocation. The relationship between these two apparently crucial phenomena—ribonuclease activity and nuclear translocation—is not immediately clear. Similarly, the tumoricidal and immunosuppressive activities of bovine seminal ribonuclease have been shown to be directly dependent on its

ribonuclease activity (50) and, after internalization, bovine seminal ribonuclease appears to function by degrading rRNA and thus inhibiting protein synthesis (51). It is not yet clear how one achieves specificity, so that somatic cells, including those producing this ribonuclease, are not damaged excessively in the process. The two eosinophil ribonucleases, EDN and ECP, likewise present their own series of questions. Sorrentino *et al.* (52) and Newton *et al.* (53) presented evidence suggesting that the (non-physiologic) neurotoxic activity of EDN required active ribonuclease activity. However, both Molina and colleagues (54) and Rosenberg (28) showed that the anti-parasitic and anti-bacterial activities of ECP, respectively, did not require active ribonuclease activity. ECP is the only ribonuclease identified with an apparent separation of enzymatic and physiologic activities; the possibility that there are physiologic functions of ECP and EDN that have not yet been identified remains an intriguing possibility.

Thus, RNase k6 is currently a protein with enzymatic activity in search of a physiologic function (as are RNase 4 and EDN). The detection of mRNA encoding this protein in both neutrophils and monocytes suggests a role for this ribonuclease in human host defense. We anticipate that the further elucidation of the biology of this newly discovered ribonuclease will provide more information on the range and scope of activities promoted by the enlarging ribonuclease gene family.

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