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Human ribonuclease 4 (RNase 4): coding sequence, chromosomal localization and identification of two distinct transcripts in human somatic tissues

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ABSTRACT

We have isolated a unique genomic fragment encoding human ribonuclease 4 (RNase 4) of the mammalian ribonuclease gene family, whose members include pancreatic ribonuclease, eosinophil-derived neurotoxin, eosinophil cationic protein and angiogenin. We have determined that the coding sequence of RNase 4 resides on a single exon found on human chromosome 14. The mRNA encoding RNase 4 was detected by Northern analysis in a number of human somatic tissues, including pancreas, lung, skeletal muscle, heart, kidney and placenta, but not brain; liver represents the most abundant source. Interestingly, the mRNA encoding RNase 4 is ~2 kb in length, which is approximately twice as large as the mRNAs encoding other members of this gene family. A larger (~2.4 kb), second transcript was detected in hepatic, pancreatic and renal tissues. The ~2 kb RNase 4 mRNA was detected in cells of the human promyelocytic leukemia line, HL-60, that had been treated with dibutyryl-cAMP to promote neutrophilic differentiation. In contrast, no mRNA encoding RNase 4 could be detected in cells treated with phorbol myristic acid (PMA), an agent promoting differentiation toward monocyte/macrophages, suggesting the existence of elements regulating tissue specific expression of this gene.

INTRODUCTION

Ribonuclease 4 (RNase 4) is the fifth, and most recently discovered human member of the mammalian ribonuclease gene family, whose members include pancreatic ribonuclease (1), eosinophil-derived neurotoxin (2,3), eosinophil cationic protein (2,4) and angiogenin (5,6). Also included in this family are bovine brain and seminal ribonucleases (7–12), and bovine and porcine kidney ribonuclease (13,14); ribonucleases with similar primary structure have been isolated from frog oocytes of the genus *Rana* (15,16). Despite the high degree to which both structure and enzymatic activity have been conserved, individual members of this family have diverged to promote a variety of distinct and otherwise unrelated biological activities (2,5,6,8,11,17).

RNase 4 was first described by Shapiro and colleagues (18) who purified the 16 kDa protein from conditioned medium from the adenocarcinoma cell line HT-29. They went on to demonstrate the unique substrate specificity of this enzyme, a preference for cleavage of ribonucleic acid polymers at the 3′ side of uridine residues. Zhou and Strydom (19) determined the complete amino acid sequence of RNase 4, which included the characteristic eight spaced cysteines and histidines and lysine residues analogous to those found in the active site of bovine (pancreatic) ribonuclease A (1). Vicentini and colleagues (20) isolated a cDNA encoding porcine RNase 4 (RNase PL3) and provided evidence suggesting that residues 36–42 contributed significantly to its unique substrate specificity. Recently, Seno and colleagues (21) isolated a cDNA sequence encoding human RNase 4.

In this report, we provide an initial characterization of the gene and the transcript encoding human RNase 4. While some features of this gene resemble those encoding the other members of the ribonuclease gene family (chromosomal localization), other features (transcript size and complexity) are quite unique.

MATERIALS AND METHODS

Isolation and characterization of RNase 4 coding sequence by polymerase chain reaction (PCR)

Human genomic DNA was purified from leukocytes isolated by apheresis from normal volunteers (Department of Transfusion Medicine, Clinical Center, NIH). Degenerate oligonucleotide primers (14 base pairs each) were designed from the N- and C-termini of published amino acid sequence (19) as follows: (5′ to 3′) 5′-CA(AG) GA(CT) GG(ATCG) ATG TA-3′, and (3′ to 5′) 5′-TA(ATCG) CC(AG) TC(AG) AA(AG) 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 20 s, 55°C for 20 s, and 72°C for 30 s, and completed by 72°C for 5 min. The products were subjected to gel electrophoresis (TBE-agarose); the band of appropriate size (~300 bp, assuming an intronless coding sequence—see Results) was excised, purified (Gene Clean II, Bio 101, La Jolla, CA), ligated into a TA vector (pCR

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II, Stratagene, La Jolla, CA) and identified as the RNase 4 coding sequence by dideoxy sequencing (US Biochemicals, Cleveland, OH).

Chromosomal localization

Chromosomal localization was performed using a PCR based method as described (22; BIOS DNA, New Haven, CT). Briefly, 18 bp oligonucleotide pairs were tested in order to identify a pair that amplified the specific fragment only from the human (and not from the hamster) DNA template. The 18 bp oligonucleotides selected (5′-CTG CGG CAA CAC GTG CAC-3′, 5′-GGC AAT GAC AAC ACG TCT-3′, as shown in Fig. 1A) amplified a single 283 bp fragment from human genomic DNA; no bands were amplified from hamster genomic DNA (data not shown). Amplification of DNA isolated from the characterized human:hamster somatic cell hybrids proceeded as follows: reactions included 0.2 mM dNTPs, 0.1 μM of each oligonucleotide primer, 1× PCR buffer without magnesium (Perkin Elmer), 2 μM magnesium sulfate, 50 ng DNA template and 2.5 U *Taq* polymerase (Boehringer Mannheim) in a 50 μl final reaction volume. After an initial denaturation step (2 min at 95°C), the reactions continued for 30 cycles of 20 s at 95°C, 20 s at 55°C, and 30 s at 72°C. Products of each reaction were evaluated on 6% TBE-acrylamide gels (Novel Experimental Technologies, San Diego, CA) stained with ethidium bromide.

Southern blotting

Purified human genomic DNA (20 μg) was digested for 3 h at 37°C with the restriction enzymes indicated prior to gel electrophoresis and transfer to nylon membranes (Hybond-N, Amersham). Membranes were UV crosslinked (Stratalinker, Stratagene), and pre-hybridized and hybridized overnight at 37°C in formamide-based buffer [6× SSPE, 50% formamide, 0.5% sodium dodecyl sulfate (SDS) and 50 μg/ml sheared ssDNA] with 2×10^6 c.p.m./ml ^{32}P-radiolabelled RNase 4 probe (Random priming kit, Boehringer Mannheim). Membranes were washed with 2× SSPE with 0.1% SDS for 1 h at 50°C; autoradiograms were developed after overnight exposure at –80°C.

Northern blotting

The human multi-tissue Northern blot was obtained from Clontech (Palo Alto, CA). The membrane was pre-hybridized and hybridized as per manufacturer's instructions with radiolabelled probe as described above. The membrane was 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 4 and again after 24 h exposure at –80°C. To assess relative loading, the blots were stripped and reprobed with a ^{32}P-radiolabelled (T4 polynucleotide kinase, Boehringer Mannheim) 42 bp human beta actin-specific oligonucleotide (5′-GCA CAT GCC GGA GCC GTT GTC GAC GAC GAG CGC GGC CAT ATC ATC ATC ATC-3′) (23).

Cell culture, differentiation and preparation of RNA

The human promyelocytic cell line, HL-60 was obtained from American Type Tissue Culture Collection, Rockville, MD. Cells were grown in RPMI medium (Biofluids, Rockville, MD) with 10% heat inactivated fetal calf serum (Gibco-BRL, Gaithersburg, MD), supplemented with 2 mM glutamine and 100 U/ml

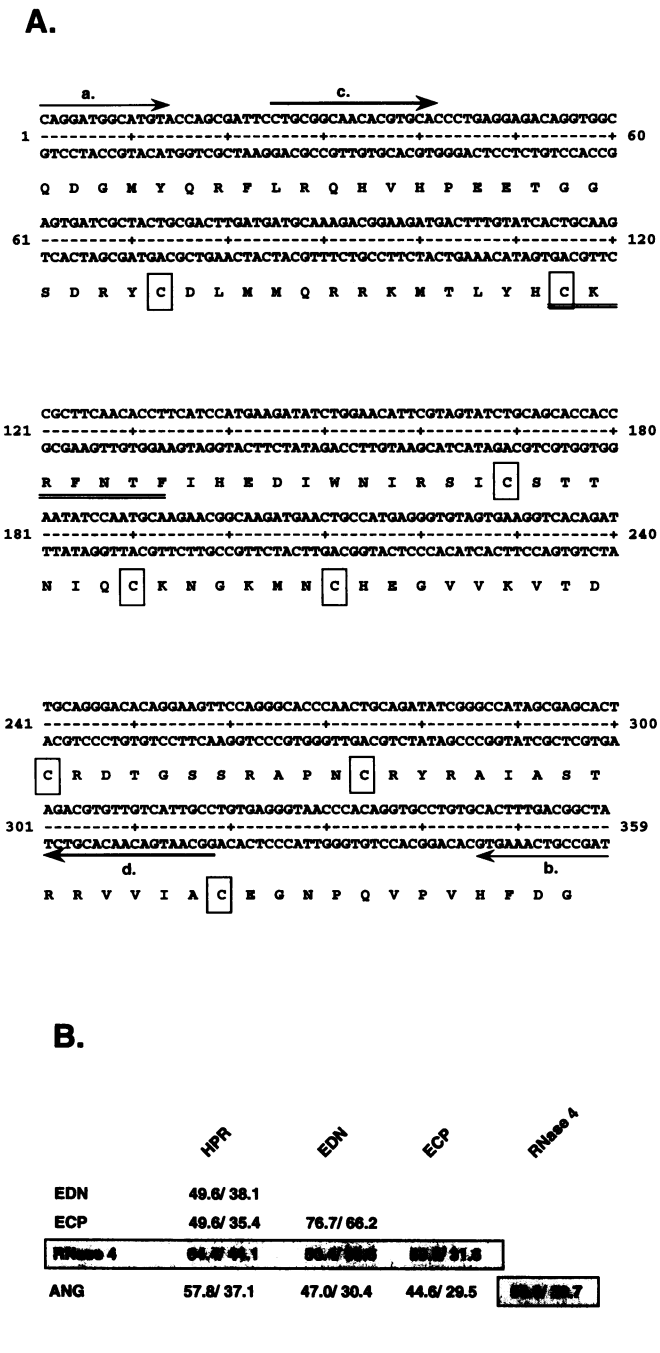

Figure 1. (**A**) Coding sequence of human ribonuclease 4 (RNase 4) isolated directly from genomic DNA by polymerase chain reaction (PCR) using degenerate 14 bp oligonucleotide primers (arrows a. and b.; see Methods). The eight cysteines characteristic of the mammalian ribonuclease gene family (1) are enclosed in boxes; the CKXXNTF motif (37) is double-underlined. The arrows labelled c. and d. indicate 18 bp oligonucleotide primers used for the chromosomal localization study (Table 1). This sequence has been assigned the GenBank accession number U36775. (**B**) Relationships among the human ribonucleases. Percentages indicate amino acid similarity/identity to human pancreatic ribonuclease (HPR) as determined by the BESTFIT algorithm of the Wisconsin Genetics Computer Group Program on-line at the National Institutes of Health. Amino acid sequences were as encoded by DNA sequences reported to GenBank; accession numbers: HPR; X62946, ANG; M11567, EDN; M24157, ECP; X15161.

Table 1. Chromosomal localization of human ribonuclease 4 (RNase 4)

human: hamster hybrid line																								
chr #	212	324	423	507	683	734	750	756	803	811	852	860	862	867	904	909	937	940	967	968	983	1006	1049	1079	1099
1														60%											65%
2											+														
3			+	+								30%												+	
4									+													55%			
5	D			+	+	+	D	D	+			+	+	+	D	D	+	+	+	+	+	+	+	+	D
6								65%				+			+	+									
7								+														+			
8									+	+						+			+			+			
9						+							+							+					
10												15%									15%				
11									15%														+		
12				+	45%			+							+										
13							+	65%						+						+		+			+
14				65%	+		+	45%						+		+	+								
15							+															+		10%	
16															5%					+				+	
17										65%							+								
18		+				+				65%				+											
19					+		+	+				35%		+								+			40%
20				40%				+										+							
21					+			+				+			+		+					+			+
22				25%	+				+																+
X									+							+				65%					
Y	+			+				+							+										
RNase 4				x	x			x	x					(x)		x	(x)								
EDNp				x	x			x	x					(x)		x	(x)								

Gene-specific primers as indicated in Figure 1A and genomic DNA templates purified from 25 characterized hybrid cell lines were subjected to PCR and analyzed by TBE-acrylamide gel electrophoresis (see Methods). The characterization of the templates replicated here was as described in the product literature (BIOS DNA); '+' indicates that >75% of the cells contained the human chromosome indicated, with numerical percentages reflecting lower percentages. 'D' indicates known chromosomal deletions. The 'X's in the RNase 4 row indicate DNAs which yielded the appropriate (283 bp) PCR product; the '(X)s' are those DNAs which required additional cycles of amplification to yield the anticipated band. The identical pattern was obtained using gene-specific primers for a human EDN pseudogene (EDNp) which has been previously mapped by direct methods to human chromosome 14 (25).

penicillin and streptomycin. Chemical differentiation agent [either 0.75 μM dibutyryl cyclic AMP (cAMP, Sigma Chemical Co., St Louis, MO) or 100 nM phorbol myristic acid (PMA, Sigma)] were added to freshly passed cells (0.5×10^6 cells/ml) at time = 0. Appropriate response to differentiating agents was monitored by growth rates, and, in the case of PMA, by adherence to the culture flask. Cells were harvested at time points indicated, washed once in phosphate buffered saline (without calcium or magnesium), and total RNA isolated with the RNazol reagent (Teltest, Friendship, TX). Total RNA (20 μg) was subjected to formaldehyde–agarose electrophoresis and transferred to a nylon membrane. Hybridization and washing conditions were as described above.

RESULTS

The genomic fragment containing the coding sequence of human ribonuclease 4 (RNase 4) is shown in Figure 1A. This sequence was obtained by PCR using degenerate 14 bp oligonucleotide primers (arrows a. and b., see Methods) with purified human genomic DNA as template. The predicted amino acid sequence confirms that reported by Zhou and Strydom (19) save for the presence of aspartate (D) at amino acid position #26 in place of asparagine (N) reported in their sequence.

Our results also indicate that the sequence encoding the mature protein is located on a single exon. Using non-degenerate oligonucleotide primers corresponding to base pairs 28 to 45 (5′ to 3′) and base pairs 468 to 451 (3′ to 5′) of the human cDNA sequence reported by Seno and colleagues (21), we determined by PCR followed by dideoxy-sequencing that the entire open reading frame of RNase 4 (including signal sequence) resides on a single exon (data not shown). Intronless coding sequences have been reported for human pancreatic ribonuclease (HPR) (24), eosinophil-derived neurotoxin (EDN) and eosinophil cationic protein (ECP) (25), and angiogenin (ANG) (6).

The calculations in Figure 1B describe the relationships among the five ribonucleases. As shown, RNase 4 exhibits greatest amino acid sequence similarity (and identity) to HPR, followed by ANG. EDN and ECP, which are closely related to each other (2,4,26), are more distantly related to RNase 4.

Shown in Figure 2A is a genomic Southern blot probed with the RNase 4 coding sequence shown in Figure 1A. A single hybridizing band was detected in each of the DNA samples digested with *Pst*I (P), *Eco*RI (RI) and *Xba*I (X); the two bands

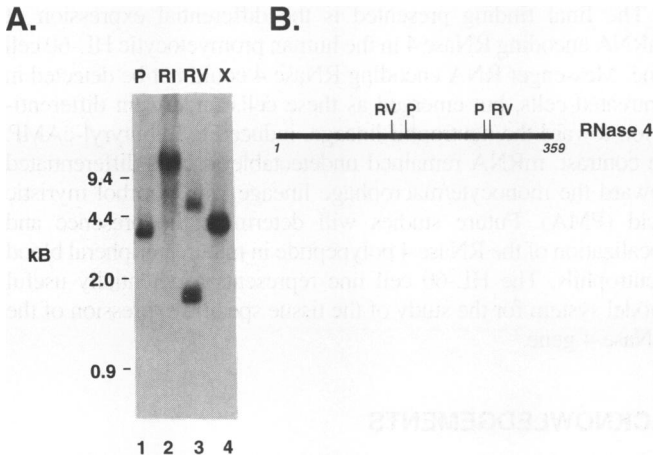

Figure 2. (**A**) Human genomic DNA (20 μg/lane) digested with restriction enzymes *Pst*I (P, lane 1), *Eco*RI (RI, lane 2), *Eco*RV (RV, lane 3), or *Xba*I (X, lane 4) and probed with the RNase 4 coding sequence shown in Figure 1A. (**B**) Restriction map of the genomic isolated shown in Figure 1A. There are no internal *Xba*I or *Eco*RI sites in this sequence.

detected in the sample digested with *Eco*RV (RV) can be accounted for by the presence of *Eco*RV sites within the probe (Fig. 2B). These results suggest that RNase 4 represents a unique sequence; there appear to be no closely related sequence homologs within the human genome.

The data in Table 1 describe the chromosomal mapping of RNase 4 using a polymerase chain reaction technique (22). Genomic DNA templates derived from a characterized panel of human:hamster hybrid somatic cell lines served as templates for PCR reactions using a pair of gene-specific primers (arrows c. and d. as shown in Figure 1A). Under the conditions described in Methods, this primer pair amplified a single 283 bp fragment from human genomic DNA; no product was detected from hamster genomic DNA (data not shown). The lanes marked 'X' in the RNase 4 row indicate those DNAs from which the 283 bp band was amplified; those marked '(X)' required an additional 10 cycles of amplification to yield (faint) bands. No bands were detected from DNAs from any of the other hybrid cell lines indicated even with the additional 10 cycles of amplification. The pattern obtained is consistent with an assignment to human chromosome 14. The PCR analysis was repeated with a primer pair specifying a human EDN pseudogene (EDNp; Rosenberg and colleagues, manuscript in preparation). Hamann and colleagues (25) showed that EDN, ECP (and thus by default, the 93% identical EDNp) coding sequences mapped to indistinguishable loci on human chromosome 14 (14q24 q31). The pattern obtained with the EDNp primer pair was identical to that of RNase 4.

The Northern blots shown in Figure 3 demonstrate the size and somatic distribution of RNase 4 mRNA. Messenger RNA encoding RNase 4 was detected in all human tissues examined, save for brain (Fig. 3A and B), with liver representing the most abundant source. The predominant mRNA species in all tissues examined is ~2 kb. This is about twice the size of the mRNAs encoding any of the other members of this gene family (see Discussion). In addition, a second transcript of ~2.4 kb is apparent in RNA derived from liver, kidney and pancreas (Fig. 3A and B).

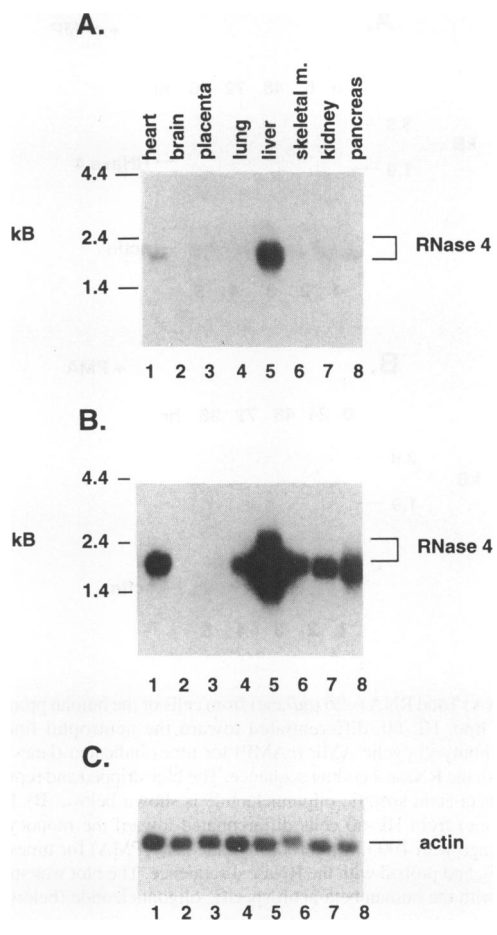

Figure 3. (**A**) Total RNA from normal human tissues as indicated (lanes 1–8) probed with the RNase 4 coding sequence. Autoradiogram developed after a 4 h exposure. (**B**) Same as in (A), 24 h exposure. (**C**) Same blot, stripped and reprobed with a human beta-actin-specific oligonucleotide (see Methods).

Messenger RNA encoding RNase 4 (the ~2 kb form) was also detected in the HL-60 promyelocytic leukemia cell line grown under conditions promoting differentiation toward neutrophils (27,28; Fig. 4A). The ~2 kb transcript was initially detected after 48 h of growth in the presence of 0.75 μM cAMP, and remained detectable to 96 h. In contrast, neither transcript could be detected in these cells when grown under conditions promoting differentiation toward the macrophage/monocyte lineage (with 100 nM PMA, Fig. 4B).

DISCUSSION

We have isolated a unique genomic fragment encoding human RNase 4. Our sequence differs from the reported cDNA sequence by a single nucleotide (G instead of A at position 76), encoding aspartate (D) in place of asparagine (N). Additional work will be necessary to determine whether this single nucleotide discrepancy represents a PCR-based artefact or a true polymorphism. The predicted amino acid sequence is most closely related to human pancreatic ribonuclease (HPR), suggesting that duplication of a single shared predecessor led to the formation of this gene pair.

Along with the other mammalian ribonucleases whose localizations have been characterized (25,29), the coding sequence of

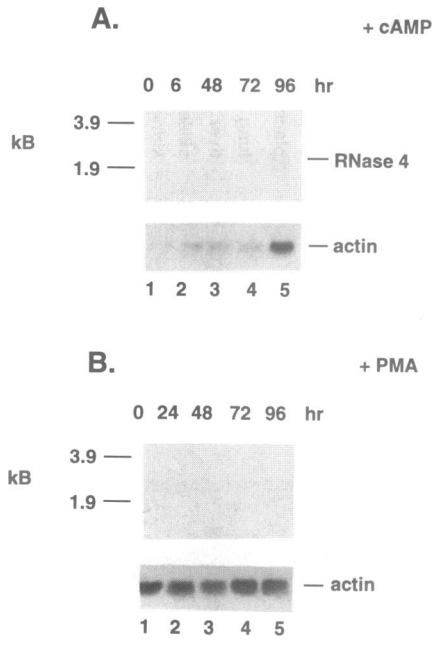

Figure 4. (**A**) Total RNA (~20 μg/lane) from cells of the human promyelocytic leukemia line, HL-60, differentiated toward the neutrophil lineage with 0.75 μM dibutyryl-cyclic AMP (cAMP) for times indicated (lanes 1–5), and probed with the RNase 4 coding sequence. The blot stripped and reprobed with a human beta-actin-specific oligonucleotide is shown below. (**B**) Total RNA (~10 μg/lane) from HL-60 cells differentiated toward the monocyte/macrophage lineage with 100 nM phorbol myristic acid (PMA) for times indicated (lanes 1–5), and probed with the RNase 4 sequence. The blot was stripped and reprobed with the human beta-actin-specific oligonucleotide (below).

RNase 4 maps to human chromosome 14. These results suggest that it might be interesting to 'walk' along specific regions of human chromosome 14 in anticipation of discovering additional, heretofore unidentified ribonuclease coding sequences.

RNase 4 has a widespread tissue distribution, with liver representing the most abundant source of mRNA among those human tissues tested. Interestingly, the mRNA transcript encoding RNase 4 is ~2 kb, which is more than twice as large as the individual transcripts encoding EDN (900 bp) (3), ECP (900 bp) (4) or ANG (800 bp)(30). Seno and colleagues (21) described a cDNA sequence containing 987 bp, including 28 bp of 5′ and 509 bp of 3′ untranslated sequence, in addition to the 450 bp coding sequence. The remaining ~1000 bp remains to be isolated and evaluated.

Another unique feature of the mRNA encoding RNase 4 is the presence of a second transcript that is both larger (~2.4 kb) and less abundant than the primary (~2 kb) transcript; both the ~2 and ~2.4 kb transcripts were detected in human hepatic, pancreatic and renal tissues by Northern analysis. In contrast, only single transcripts have been reported to encode EDN and ECP (3,4). Rybak and colleagues (31) reported that several hybridizing bands could be found in RNA isolated from the HT-29 adenocarcinoma cell line probed with angiogenin cDNA; in contrast, Weiner and colleagues (30) described a single (~800 bp) transcript in this cell line as well as in non-malignant rat somatic tissues. The relationship between the two RNase 4 transcripts remains to be elucidated; multiple transcripts have been reported for a number of unrelated gene sequences, often the result of differential splicing and assembly of non-coding exons (32–36).

The final finding presented is the differential expression of mRNA encoding RNase 4 in the human promyelocytic HL-60 cell line. Messenger RNA encoding RNase 4 could not be detected in untreated cells, but emerged as these cells underwent differentiation toward the neutrophil lineage, induced by dibutyryl-cAMP. In contrast, mRNA remained undetectable in cells differentiated toward the monocyte/macrophage lineage with phorbol myristic acid (PMA). Future studies will determine the presence and localization of the RNase 4 polypeptide in mature peripheral blood neutrophils. The HL-60 cell line represents a potentially useful model system for the study of the tissue specific expression of the RNase 4 gene.

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