Nucleic Acids Research, 1997, Vol. 25, No. 21 **4235–4239**

Molecular cloning of four novel murine ribonuclease genes: unusual expansion within the Ribonuclease A gene family

Dean Batten[+], Kimberly D. Dyer, Joseph B. Domachowske[§] and Helene F. Rosenberg*

The Laboratory of Host Defenses, Building 10, Room 11N104, National Institute of Allergy and Infectious Diseases, National Institutes of Health, 9000 Rockville Pike, Bethesda, MD 20892, USA

Received August 4, 1997; Revised and Accepted September 17, 1997

DDBJ/EMBL/GenBank accession numbers: U72031, U72032, AF017258–AF017261

ABSTRACT

We have characterized four novel murine ribonuclease genes that, together with the murine eosinophil-associated ribonucleases 1 and 2, form a distinct and unusual cluster within the RNase A gene superfamily. Three of these genes (mR-3, mR-4, mR-5) include complete open reading frames, encoding ribonucleases with eight cysteines and appropriately spaced histidines (His11 and His124) and lysine (Lys35) that are characteristic of this enlarging protein family; the fourth sequence encodes a non-functional pseudogene (mR-6P). Although the amino acid sequence similarities among these murine ribonucleases varies from 60 to 94%, they form a unique cluster, as each sequence is found to be more closely related to another of this group than to either murine angiogenin or to murine pancreatic ribonuclease. Interestingly, the relationship between the six genes in this 'mR cluster' and the defined lineages of the RNase A gene family could not be determined by amino acid sequence homology, suggesting the possibility that there are one or more additional ribonuclease lineages that have yet to be defined. Although the nature of the evolutionary constraints promoting this unusual expansion and diversification remain unclear, the implications with respect to function are intriguing.

INTRODUCTION

Ribonuclease A (RNase A, bovine pancreatic ribonuclease) is the prototype of an enlarging family of proteins defined by distinctive elements of conserved primary structure and enzymatic activity (1,2). The features shared by all members of the RNase A family include six to eight cysteines and specific histidine and lysine residues that form the ribonuclease active site. To date, several distinct lineages within the RNase A superfamily have been identified. Pancreatic ribonucleases (RNases 1) have been isolated from an extensive array of mammalian species (3,4), and mRNA encoding human pancreatic ribonuclease has been detected in numerous somatic tissues in addition to pancreas (5). RNases 2 and 3, eosinophil-derived neurotoxin (EDN) and eosinophil cationic protein (ECP), respectively, have been characterized primarily with respect to eosinophil function (6,7), although expression of EDN [also known as RNase Us (8) or human liver ribonuclease (9)] is also widespread (5). Angiogenin (RNase 5), a structurally atypical member of this family, was originally identified as an agent promoting blood vessel growth and development (10,11). RNase 4 (12,13) and RNase k6 (14) are also members of the RNase A superfamily, although their functions are currently obscure.

Recently, Larson and colleagues (15) described cDNAs encoding two highly homologous murine ribonucleases, the murine eosinophil-associated ribonucleases (mEAR) 1 and 2, which were cloned and identified via tryptic peptides isolated from murine eosinophil proteins. Although Southern analysis demonstrated that multiple copies of sequence homologous to mEAR-1 and mEAR-2 were present in murine genomic DNA, the precise nature of these copies—as pseudogenes, polymorphisms, or distinct functional genes—was unclear. In this work, we have identified four of these homologous sequences, three encoding novel ribonucleases, and one encoding a pseudogene. Together with mEAR-1 and mEAR-2, these six ribonuclease genes form an 'mR cluster,' whose members are more closely related to one another than they are to other murine ribonucleases, yet whose position with respect to the defined RNase A lineages remains unclear.

MATERIALS AND METHODS

Isolation of genomic fragments encoding murine ribonucleases by polymerase chain reaction (PCR)

Genomic DNA was isolated from cells of the murine 3T3 fibroblast cell line. The mR-3, mR-5 and mR-6P sequences were amplified using oligonucleotide primers derived from the published coding sequence of mEAR-1 as follows: 5′-CAA ACC CTT TCC CAG AAG TTT GCC-3′ (amino acids 28–33); 5′-AAA TGT CCC ATC CAA GTG AAC TGG ACC-3′ (amino acids 156–148) (15). The PCR reactions were performed as described previously (14), and the multiple products present in the single ~400 bp product were identified by dideoxy-

*To whom correspondence should be addressed. Tel: +1 301 402 9131; Fax: +1 301 402 4369; Email: hr2k@nih.gov

Present addresses: [+]Duke University School of Medicine, Durham, NC 27701, USA and [§]Department of Pediatrics, State University of New York-Health Sciences Center at Syracuse, Syracuse, NY 13210, USA

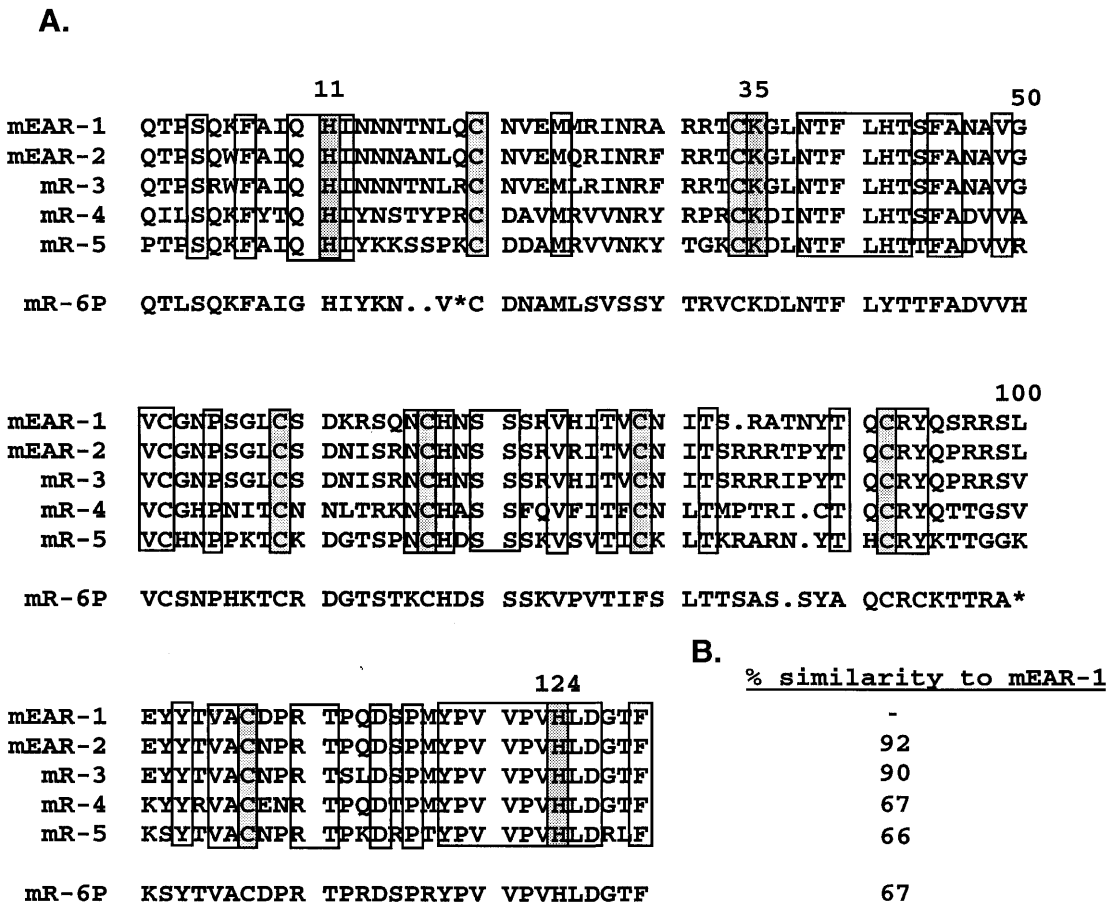

Figure 1. (**A**) Alignment of amino acid sequences encoded by six related murine ribonuclease genes. Regions of amino acid sequence identity among the five functional genes are enclosed in boxes; the boxes enclosing the active site residues (His11, Lys35, His124) and eight cysteines conserved in all members of the RNase A superfamily are shaded. (**B**) Percent similarities between amino acid sequences determined via the BESTFIT algorithm of the Wisconsin Genetics Computer Group (WGCG) program on-line at the National Institutes of Health. GenBank accession numbers: mEAR (murine eosinophil-associated ribonuclease)-1, U72032; mEAR-2, U72031; mR (murine ribonuclease)-3, AF017258; mR-4, AF017259; mR-5, AF017260; mR-6P (pseudogene), AF017261.

sequencing of individual plasmids after subcloning into the pCR II TA cloning vector (Invitrogen, San Diego, CA). The complete open reading frames were obtained by extension in both 5′ and 3′ directions by uni-directional PCR (Genome Walker kit, Clontech, Palo Alto, CA), involving two amplifications with nested primers and Tth polymerase as described previously (14). The nested gene-specific primer pairs were as follows: for 5′ extension of mR-3, 5′-CTG GAA CCA CTG GAT ACG TGG GAC TGT CCT-3′ and 5′-ACG TGG GAC TGT CCT GTG GAG TTC TGG GTT-3′; for 3′ extension of mR-3, 5′-ATG CTG TTG GTG TGT GTG GAA ATC CAA GTG-3′ and 5′-GCT TGT GCA GTG ACA ATA TAA GTC AAA ACT-3′; for 5′ extension of mR-5, 5′-GGT GGG ACG GTC CTT TGG AGT TCT GGG GTT ACA-3′ and 5′-GGG GTT ACA GGC AAC TGT GTA GGA CTT CTT TCC-3′; for 3′ extension of mR-5, 5′-GAT GTT GTC CGT GTG TGT CAC AAT CCA CCC-3′ and 5′-AAG ACT TGC AAA GAC GGG ACA AGT CCA AAT-3′; for 5′ extension of mR-6P, 5′-TTG TTG TTT TGC ATC TGC ATT GTG CAT AAC TGC-3′ and 5′-TTC TGC ATA ACT GCT TGC TGA ACT TGT GAG TGA-3′; for 3′ extension of mR-6P, 5′-ATG TGA TAA TGC AAT GCT GTC TCT TAG CAG TTA-3′ and 5′-TAC AAG AGT ATG TAA GCC ATT GAA TCA TTT TCT-3′. The products of these reactions were likewise subcloned into the PCR II TA vector and evaluated by dideoxy-sequencing. The mR-4 sequence was amplified with a 36 bp primer derived from sequence encoding the amino-terminal signal sequence of mEAR-1; 5′-ATG GGT CCG AAG CTG CTT GAG TCC CGA CTT TGT CTC-3′ with the carboxy-primer described above.

Northern analysis

The murine multi-tissue Northern blot was purchased from Clontech, and pre-hybridized and hybridized as per manufacturer's instructions with radiolabelled oligonucleotide probes. The membrane was washed with 5× SSPE with 0.1% SDS for 1 h at 37°C and the autoradiogram developed after exposure to film at –80°C. The mEAR-1/mEAR-2/mR-3 probe: 5′-CTC TTG TCA CTG CAC AAG CCA CTT GGA TTT CC-3′, the mR-5 probe: 5′-GTC CCG TCT TTG CAA GTC TTG GGT GGA TTG TG-3′, and the actin probe: 5′-GCA CAT GCC GGA GCC GTT GTC GAC GAC GAG CGC GGC GAT ATC ATC ATC-3′ (16).

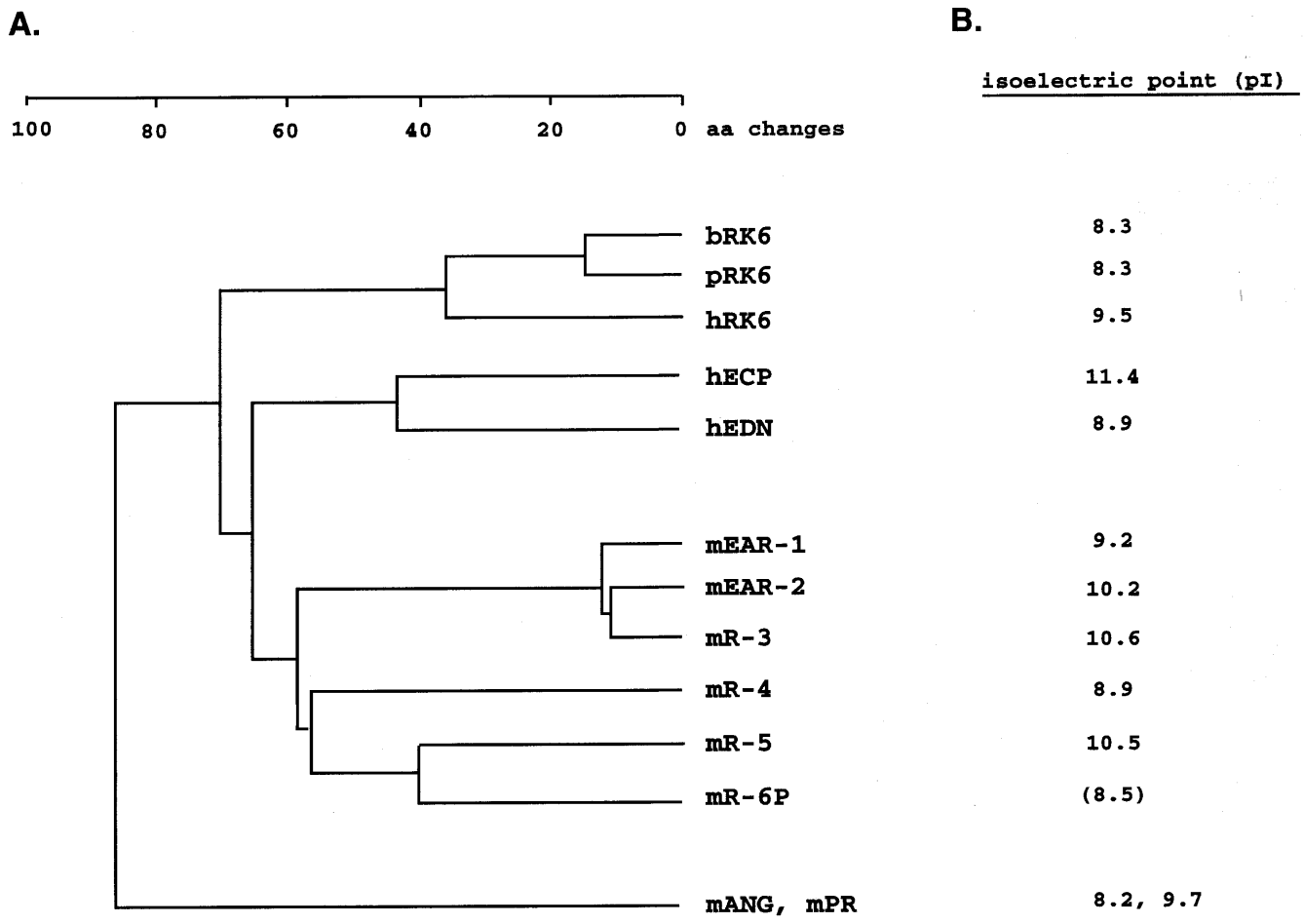

Figure 2. (**A**) Dendrogram depicting relationships among the eight characterized murine ribonucleases as determined by a modified UPGMA method (17). Abbreviations are as in Figure 1A, also mPR (murine pancreatic ribonuclease, sw:rnp_mouse), mANG (murine angiogenin, U72672), hEDN (human eosinophil-derived neurotoxin, M24157), hECP (human eosinophil cationic protein, X15161), hRK6 (human ribonuclease k6, U64998), pRK6 (porcine kidney ribonuclease (24), and bRK6 (bovine kidney ribonuclease, sw:rnkd_bovin). (**B**) Calculated isoelectric points from amino acid sequences in (A) as determined via the PEPTIDESORT algorithm of WGCG.

Table 1. Amino acid sequence comparisons of murine and human ribonucleases

	hEDN	hECP	hRK6	hPR	hR4	hANG
mEAR-1	56	**59**	58	47	50	49
mEAR-2	56	**59**	**59**	48	53	48
mR-3	54	57	**59**	49	51	51
mR-4	**60**	59	53	48	52	47
mR-5	**60**	58	56	57	54	47
mANG	43	49	49	55	57	**80**
mPR	53	52	59	**79**	66	60

Values are expressed as percent similarity between pairs of amino acid sequences as determined by the BESTFIT algorithm of the Wisconsin Genetics Computer Group program on-line at the National Institutes of Health. Value representing the highest degree of sequence homology in each row is indicated in boldface. Abbreviations are as defined in Figures 1 and 2, and also include hR4 (human ribonuclease 4, sw:rnl4_human), hANG (human angiogenin, M11567), and hPR (human pancreatic ribonuclease, X62946).

Sequence analysis

All DNA sequence analysis and comparisons were performed with the assistance of the Wisconsin Genetics Computer Group and Sequencher (Gene Codes Corporation) programs available at the National Institutes of Health. The dendrogram in Figure 2A was constructed by a modified version of the unweighted pair group method with arithmetic mean (UPGMA) (17), constructed using four initial pairings, mEAR-2 to mR-3, mR-5 to mR-6P, hEDN to hECP and bRK6 to pRK6.

RESULTS

Isolation of genomic fragments encoding novel murine ribonucleases

The alignment in Figure 1A displays the amino acid sequences encoded by the six related ribonuclease genes. Murine eosinophil-associated ribonucleases-1 and -2 (mEAR-1 and mEAR-2) are the predicted amino acid sequences from two genes described previously by Larson and colleagues (15); murine ribonucleases 3, 4, 5 and 6-pseudogene (mR-3, mR-4, mR-5 and mR-6P), are the predicted amino acid sequences encoded by novel DNA

fragments amplified from murine genomic DNA. All five functional genes encode amino acid sequences with eight cysteines and appropriately spaced catalytic histidines and lysine that are conserved among the members of the RNase A superfamily. The predicted coding sequence of the non-functional pseudogene (mR-6P) includes two aberrant stop codons (positions 19 and 100) as well as a point mutation resulting in the destruction of the cysteine at position 78. Each genomic fragment also encodes a 28 residue amino terminal signal sequence (not shown). The amino acid sequences are displayed in order of decreasing similarity to mEAR-1 (Fig. 1B). Genomic fragments encoding mEAR-1 and mEAR-2 were also detected.

Relationships among the murine ribonucleases

A dendrogram depicting the relationships among several of the murine and human ribonucleases is shown in Figure 2A. The mEAR-1, mEAR-2, and mR-3 sequences form a closely related sub-group, with only nine amino acid sequence differences noted between mEAR-2 and mR-3, and 12 and 17 between these two and mEAR-1, respectively. The mR-4, mR-5, and mR-6P sequences diverge from this subgroup and from one another as well. These six sequences are more closely related to one another than they are to either murine pancreatic ribonuclease (mPR) or to murine angiogenin (mANG) or to any of the three human ribonucleases shown (hEDN, hECP or hRK6).

Isoelectric points

The isoelectric points of all six related ribonucleases range from ~8–11, as is typical for members of the RNase A superfamily (Fig. 2B). Interestingly, the isoelectric points within the mEAR-1/mEAR-2/mR-3 subgroup vary by a full 1.0 unit (mEAR-1 at 9.2 to mEAR-2 at 10.2) in response to only 12 discrepancies in amino acid sequence. As increased charge has been associated with increased toxicity in other branches of the RNase A gene family (18), this finding has potential consequences with respect to the function of these proteins (see Discussion).

Northern analysis

Tissue-specific expression of mRNA encoding these murine ribonucleases is shown in Figure 3. The probe complementary to all three sequences of the mEAR-1/mEAR-2/mR-3 subgroup hybridizes to an ~1.4 kb mRNA isolated from renal tissue (Fig. 3A). As the probe cannot distinguish among the three genes, it is not possible to conclude whether the band detected represents mRNA encoding one, two, or all three of the ribonucleases of this subgroup. In contrast, the probe complementary to the mR-5 genomic fragment hybridizes to two prominent mRNA species in hepatic tissue, one at 1.4 kb, and a second at ~2.0 kb. A prominent mRNA of ~1.8 kb was also detected in mRNA isolated from murine testicular tissue; fainter hybridizing mRNAs of varying size were detected in lung and skeletal muscle. The size variation may represent differential splicing of the mR-5-encoding mRNA, or may represent mRNAs encoding one or more as yet undiscovered murine ribonucleases that are more closely related to mR-5 than those whose sequences are reported here.

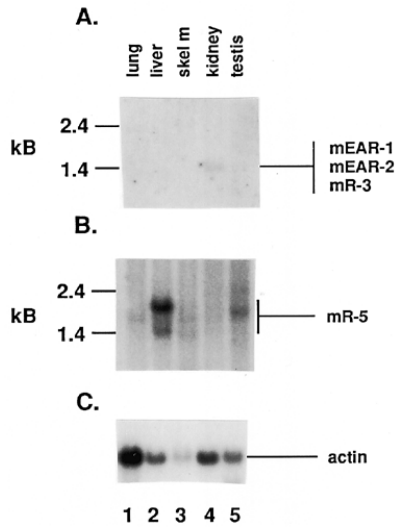

Figure 3. (**A**) Murine RNA probed with a 32 bp oligonucleotide complementary to mEAR-1, mEAR-2 and mR-3 coding sequences. Tissue source of each RNA is indicated above each lane. (**B**) Same as in (A), probed with a 32 bp oligonucleotide complementary to mR-5. (**C**) Same as in (A), probed with a 48 bp oligonucleotide complementary to human actin (16), demonstrating relative loading of each lane.

Relationship to human ribonucleases

The data in Table 1 denote the amino acid sequence similarity between pairs of murine and human ribonucleases of the RNase A superfamily. Overall, the similarities within the identified cluster range from 47 to 60%, with remarkably little variation among the pairs. Although the sequences of the mEAR-1/mEAR-2/mR-3 subgroup are slightly more similar to ECP and RNase k6, and mR-4 and mR-5, to EDN and ECP, the orthologous relationships of these murine ribonucleases cannot be discerned from these data. This stands in direct contrast to mPR and mANG, whose human orthologs can be clearly distinguished on the basis of their amino acid sequence homologies.

DISCUSSION

In this work, we have identified four novel murine ribonuclease genes that, together with mEAR-1 and mEAR-2 defined by Larson and colleagues (15), form an unusual cluster within the RNase A gene superfamily. The six genes within this cluster have varying amino acid sequence homologies to one another, but are clearly more closely related to one another than to either murine pancreatic ribonuclease (mPR) or angiogenin (mANG), or to any of the human ribonucleases of the RNase A family. These results suggest that the ‘mR cluster’ emerged via multiple duplications of a gene that had already diverged from those encoding the other murine ribonucleases. Although this type of expansion is unusual in this gene family, it is actually not unique; similar expansion has occurred in bovine species, with gene duplication resulting in the three closely-related bovine pancreatic, bovine brain (19,20) and bovine seminal (21) ribonucleases.

One interesting feature of the mR cluster is that its relationship to any of to the six known human RNase A-type genes cannot be

determined from their respective amino acid sequence homologies. Although the amino acid sequences encoded by mEAR-1 and mEAR-2 match those of tryptic peptides derived from murine eosinophil proteins (15), the homology data do not stand in overwhelming support of a unique relationship between any of the mR cluster ribonucleases and the human eosinophil ribonucleases EDN and ECP. The existence of an additional, as yet unidentified human ribonuclease (or ribonucleases) more closely related to those of the mR cluster cannot be ruled out.

Perhaps the most important issue raised by this work is the question of why so much evolutionary energy has been devoted to enlarging and diversifying the RNase A gene superfamily. To date, this superfamily now includes several distinct lineages, two species-limited clusters, and the two most rapidly evolving functional coding sequences known among primates (22). The question of evolutionary energy takes on particular significance here, where five functional ribonucleases have emerged in what appears to be a remarkably short period of evolutionary time (15). It is conceivable that each of these ribonucleases might serve a unique but related function. On this point, a number of studies have suggested a distinct role for the human ribonuclease ECP in eosinophil-mediated host defense (6,7); in addition, we have recently shown that eosinophil ribonucleases can inhibit retroviral transduction of human target cells (23). The possibility that certain ribonucleases have diverged to promote distinct and specific host defense-related activities remains an intriguing hypothesis.

ACKNOWLEDGEMENTS

We would like to thank Dr Jianzhi Zhang for helpful discussions, and Drs Harry L. Malech and John I. Gallin for their ongoing suppport of the work in our laboratory. D.B. is a Howard Hughes Medical Institute-National Institutes of Health Research Scholar

REFERENCES

1 D'Alessio, G. and Riordan, J.F. (1997) In D'Alessio, G. and Riordan, J.F. (eds) *Ribonucleases: structure and function*. Academic Press, Inc., New York, NY.
2 Sorrentino, S. and Libonati, M. (1997) *FEBS Lett.* **404**, 1–5.
3 Beintema, J.J., Breukelman, H.J, Carsana, A., and Furia, A. (1997) In D'Alessio, G. and Riordan, J.F. (eds) *Ribonucleases: structure and function*. Academic Press, Inc., New York, NY.
4 Confalone, E., Beintema, J.J., Sasso, M.P. Carsana, A., Palmieria, M., Vento, M.T. and Furia A. (1995) *J. Mol. Evol.* **41**, 850–858.
5 Futami, J., Tsushima, Y., Murato, U, Tada, H., Sasaki, J., Seno, M., and Yamada, H. (1997) *DNA Cell Biol.* **16**, 413–419.
6 Ackerman, S.J. (1993) In Makino, S. and Fukuda, T. (eds) *Eosinophils: biological and clinical aspects.* CRC Press, Inc., Boca Raton, FL
7 Spry, C.J.F. (1988) *Eosinophils: a comprehensive review and guide to the scientific and medical literature.* Oxford University Press, Oxford, UK.
8 Beintema, J.J., Hofsteenge, J., Iwama, M., Morita, T., Ohgi, K., Irie, M., Sugiyama, R.H., Schieven G.L., Dekker, C.A., and Glitz, D.G. (1988) *Biochemistry* **27**, 4530–4538.
9 Sorrentino, S., Tucker, G.K., and Glitz, D.G. (1988) *J. Biol. Chem.* **263**, 16125–16131.
10 Vallee, B.L. and Riordan, J.F. (1988) *Adv. Exp. Med. Biol.* **234**, 41–53.
11 Moroianu, J. and Riordan, J.F. (1994) *Proc. Natl. Acad. Sci. USA* **91**, 1677–1681.
12 Seno, M., Futami, J., Tsushima, Y., Akutagawa, K., Kosaka, M., Tada, H. and Yamada, H. (1995) *Bioch. Biophys. Acta* **1261**, 424–426.
13 Rosenberg, H.F. and Dyer, K.D. (1995) *Nucleic Acids Res.* **23**, 4290–4295.
14 Rosenberg, H.F. and Dyer, K.D. (1996) *Nucleic Acids Res.* **24**, 3507–3513.
15 Larson, K.A., Olson, E.V., Madden, B.J., Gleich, G.J., Lee, N.A. and Lee, J.J. (1997) *Proc. Natl. Acad. Sci. USA* **93**, 12370–12375.
16 Nakajima-Iyima, S., Hamada, H., Reddy, P. and Kakanaga, T. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 6133–6137.
17 Li, W.-H. and Graur, D. (1991) *Fundamentals of Molecular Evolution*. Sinauer Associates, Sunderland, MA.
18 Rosenberg, H.F. and Dyer, K.D. (1995) *J. Biol. Chem.* **270**, 21539–21544.
19 Watanabe, H., Katoh, H, Ishii, M., Komoda, Y., Sanda, A., Takizawa, Y., Ohgi, K. and Irie, M. (1988) *J. Biochem.* **104**, 939–945.
20 Sasso, M.P., Carsana, A., Confalone, E., Cosi, C., Sorrentino, S., Viola, M., Palmieri, M., Russo, E. and Furia, A. (1991) *Nucleic Acids Res.* **19**, 6469–6474.
21 Palmieri, M., Carsana, A., Furia, A. and Libonati, M. (1985) *Eur. J. Biochem.* **152**, 275–277.
22 Rosenberg, H.F., Dyer, K.D., Tiffany, H.L. and Gonzalez, M. (1995) *Nature Genet.* **10**, 219–223.
23 Domachowske, J.B. and Rosenberg, H.F. (1997) *J. Leukoc. Biol.*, **62**, 363–368.
24 Iwama, M., Sanda, A., Ohgi, K., Hofsteenge, J., and Irie, M. (1993) *Biosci. Biotech. Biochem.* **57**, 2133–2138.