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Evolution

Two highly homologous ribonuclease genes expressed in mouse eosinophils identify a larger subgroup of the mammalian ribonuclease superfamily

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ABSTRACT **Two putative ribonucleases have been isolated from the secondary granules of mouse eosinophils. Degenerate oligonucleotide primers inferred from peptide sequence data were used in reverse transcriptase–PCR reactions of bone marrow-derived cDNA. The resulting PCR product was used to screen a C57BL/6J bone marrow cDNA library, and comparisons of representative clones showed that these genes and encoded proteins are highly homologous (96% identity at the nucleotide level; 92/94% identical/similar at the amino acid level). The mouse proteins are only weakly homologous (≈50% amino acid identity) with the human eosinophil-associated ribonucleases (i.e., eosinophil-derived neurotoxin and eosinophil cationic protein) and show no sequence bias toward either human protein. Phylogenetic analyses established that the human and mouse loci shared an ancestral gene, but that independent duplication events have occurred since the divergence of primates and rodents. The duplication event generating the mouse genes was estimated to have occurred $<5 \times 10^6$ years ago (versus 30 to 40 $\times 10^6$ years ago in primates). The identification of independent duplication events in two extant mammalian orders suggests a selective advantage to having multiple eosinophil granule ribonucleases. Southern blot analyses in the mouse demonstrated the existence of three additional highly homologous genes (i.e., five genes total) as well as several more divergent family members. The potential significance of this observation is the implication of a larger gene subfamily in primates (i.e., humans).**

The mature eosinophil is predominantly a tissue-dwelling leukocyte implicated in mammalian defense mechanisms against large non-phagocytosable multicellular parasites (1, 2). Clinical studies have also correlated the production and recruitment of eosinophils in conditions and pathologies such as asthma (3–5) and allergic inflammatory disease (3, 6–9). Eosinophil effector functions result in part from the release of protein mediators stored in cytoplasmic lysosomal granules (10, 11). The components of the secondary granules of human eosinophils are well characterized and include four abundant proteins: (*i*) major basic protein, (*ii*) eosinophil peroxidase, and members of the ribonuclease superfamily (*iii*) eosinophil cationic protein (ECP) and (*iv*) eosinophil-derived neurotoxin (EDN).

ECP and EDN represent a unique branch of mammalian ribonucleases based on sequence similarities (12–15) and absolute enzymatic activities (16–18). These proteins are similar in size (≈150 amino acids) and amino acid identity (≈83%), although ECP is very cationic (pI 11.2) and EDN is less basic (pI 8.7). These granule proteins appear to provide effector functions independent of their associated ribonuclease activities. ECP is toxic to mammalian cells and has demonstrable bactericidal and anti-helminthic activities (19–23), yet its associated ribonuclease activity is relatively weak (15, 24). In contrast, EDN has little generalized toxicity (19, 20) but is an efficient ribonuclease having activity similar to pancreatic RNase A (24–26).

The taxonomic distribution of eosinophil granule ribonucleases is very limited. Low criterion hybridization screens using human cDNA probes and PCR-based experiments have been unsuccessful in the identification and cloning of eosinophil granule ribonuclease genes from non-primate mammalian species (27). In this study, we have purified and partially characterized two granule ribonucleases from mouse eosinophil secondary granules. Nucleotide inferences from amino acid sequence data were used to clone the genes encoding these eosinophil-associated ribonucleases (EARs). Evidence is also provided for the existence of several highly related genes in the mouse, as well as other genes of lower homology. The implication of these data is that humans may also have additional ribonucleases that could be active in the eosinophil or other cell types.

EXPERIMENTAL PROCEDURES

Eosinophil Secondary Granule Purification and Protein Isolation. Peritoneal cavity eosinophilia was induced using the helminth *Mesocestoides corti* (28). Extravascular eosinophils were collected from the peritoneal cavity by lavage and secondary granules were isolated as described in Larson *et al.* (29). The presence of only eosinophil secondary granules in the final preparations was determined by electron microscopy.

The abundant acid-soluble granule proteins were extracted as previously described (29), and peptides suitable for automated Edman degradation were generated from C_4 reverse-phase HPLC purified protein using either proteolytic digestion with trypsin or chemical cleavage with cyanogen bromide. Peptides resulting from either proteolytic or chemical cleavage were separated by HPLC using a C_{18} reverse-phase column and sequenced on an Applied Biosystems model 476A Protein Sequencer.

Degenerate Oligonucleotide Primer Development and Reverse Transcription–PCR (RT-PCR). Degenerate oligonucleotide primers were synthesized using the amino acid sequences derived from peptide cleavage products. Complete degeneracy

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Abbreviations: ECP, eosinophil cationic protein; EDN, eosinophil-derived neurotoxin; EAR, eosinophil-associated ribonuclease; mEAR, mouse EAR; RT-PCR, reverse transcription–PCR.

Data deposition: The sequences reported in this paper have been deposited in the GenBank data base [accession nos. U72032 (MEAR-1) and U72031 (MEAR-2)].

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of nucleotide positions in the oligos did not extend throughout the sequence, but was restricted to the extreme 3′ nucleotide positions (i.e., eight or nine nucleotides). The degeneracy of nucleotide positions outside of this 3′-restricted region was determined by the nucleotide present at the homologous position in the sequences encoding the human EAR proteins, EDN and ECP. These oligonucleotides were augmented with restriction endonuclease recognition sites of either *NotI* or *SalI* (underlined sequences) for subsequent cloning steps: sense primer, 5′-TGGCTGACGCGGCCGCAATACTTTYYTNC-AY-3′; antisense primer, 5′-GATCGATCGTCGACAACTG-GAACCACMGGRTACAT-3′ (N = G, A, T, C; M = A, C; R = A, G; Y = T, C). PCR template cDNA was made from C57BL/6J mouse bone marrow total RNA, and single-stranded cDNA was synthesized using a standard reverse transcription protocol (30).

PCR reactions were performed with degenerate primers and bone marrow-derived cDNA in a volume of 50 μl using the Perkin–Elmer Gene Amp PCR System 9600. The final 50 μl reaction conditions included cDNA derived from 200 ng of total RNA, 10 mmol of each dNTPs (ATP, GTP, CTP, and TTP), 1 mM $MgCl_2$, 4% dimethyl sulfoxide, 10 pmol of each primer, 1× PCR Buffer II (Perkin–Elmer), and 2.5 units of *Taq* polymerase (Perkin–Elmer). The reaction program consisted of an initial denaturation step of 94°C for 5 min, followed by 30 cycles of 94°C for 1 min, 52°C for 2 min, and 72°C for 3 min. The reactions were completed with an extension period at 72°C for 10 min. The PCR products were digested with *SalI* and *NotI* prior to subcloning into the plasmid vector pBluescript KS(+) (Stratagene).

Isolation of cDNAs Representing Mouse EAR (mEAR) Encoding Transcripts. ^{32}P-radiolabeled hybridization probes were generated from cloned RT-PCR products by random priming and used to screen a plasmid bone marrow cDNA library (31). Plasmid DNAs for automated DNA sequencing were prepared using Qiagen (Chatsworth, CA) plasmid DNA isolation kits. Cycle-sequencing reactions were performed on an Applied Biosystems model 373A automated DNA sequencer.

DNA and Protein Sequence Analyses. Analyses of nucleotide and protein sequences, including calculated estimates of the pIs of reported proteins, were completed on MACVECTOR 4.5.3 software (IBI Sequence Analysis Software, Kodak Life Sciences Products, New Haven, CT) and the on-line Genetics Computer Group (Madison, WI) program. In particular, a progressive similarity alignment was completed using the methods of Feng and Doolittle (32) and the BLOSUM-62 matrix as tallied by Henikoff and Henikoff (33).

Genomic Southern Blot Analysis. Restriction enzyme digests of C57BL/6J mouse genomic DNA (15 μg per digest) were size-fractionated on a 1% TAE agarose gel and transferred to GeneScreen(+) as per the manufacturer's instructions (DuPont/NEN). The blots were prehybridized and hybridized at either 55°C (low criterion) or 65°C (high criterion) as described in Larson *et al.* (29).

RESULTS

Purification of Putative mEARs. Our earlier studies showed that the abundant acid-soluble secondary granule proteins from murine eosinophils fractionated on Sephadex G-50 columns into three molecular weight groups that were designated as regions 1, 2, and 3 (29). The near identity of the human/mouse G-50 column profiles (20, 34, 35), and preliminary assays showing ribonuclease activity in fractions composing region 2, suggested that this molecular weight group contained the murine protein homologues of the known human EARs. Proteins from the Sephadex G-50 fractions corresponding to Region 2 were pooled and fractionated on a C_4 reverse-phase HPLC column. This final HPLC purification of region 2 identified the existence of only two proteins that were present in approximately equal mass proportions (data not shown). The isolated proteins were each subjected either to proteolytic digestion with trypsin or to chemical cleavage with CNBr to generate multiple peptide fragments for amino acid sequence analysis. The alignment of the granule ribonuclease peptide sequences with the human EARs (Fig. 1) shows that these peptides display homology to both EDN (54% identity) and ECP (50% identity).

The Molecular Cloning and Characterization of Two Genes Encoding Eosinophil Granule Ribonucleases. Primary amino acid sequence data from two regions of the putative mEAR proteins were used to generate partially degenerate oligonucleotide primers for RT-PCR reactions with bone marrow-derived cDNA. The RT-PCR reactions produced a 285-bp product that was used as a hybridization probe in a low criterion screen [55°C (29)] of a random-primed plasmid cDNA library of C57BL/6J bone marrow (31). Analyses of these hybridizing cDNAs resulted in the identification of sequences encoding two different proteins (Fig. 2). These two genes (mEAR-1 and mEAR-2) are unique and encode proteins whose amino acid sequences match the identified peptides from purified eosinophil granule protein (see Fig. 1). mEAR-1 and mEAR-2 code for putative ribonucleases (ref. 25; see below) corresponding to 155 and 156 amino acids, respectively. mEAR-1 displays 96% identity at the nucleotide level and 92%/94% (identical/similar) at the protein level to mEAR-2.

Molecular Evolution of mEAR-1 and mEAR-2. The proposed amino acid sequences for mEAR-1 and mEAR-2 were aligned to the known human EARs and their primate orthologues as well as several representative sequences of other

FIG. 1. Peptide sequence data from putative murine EAR proteins. The amino acid sequences of peptides derived from tryptic and CNBr cleavage of HPLC purified putative mEAR proteins were aligned to maximize homology with the known human ECP and EDN protein sequences. Two consensus peptides, mEAR peptide A and mEAR peptide B, were used to create degenerate oligonucleotide primers. The amino acids corresponding to these peptides are identified as white letters on a black background. A question mark (?) represents ambiguous amino acid identifications. The asterisk (*) denotes sequence termination.

mEAR-1

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1   ATG GGT CCG AAG CTG CTT GAG TCC CGA CTT TGT CTC CTG CTG CTG CTA GGA CTT GTC CTA ATG CTT GCC TCA TGC CTG GGT CAA ACC CCT
      M   G   P   K   L   L   E   S   R   L   C   L   L   L   L   L   G   L   V   L   M   L   A   S   C   L   G   Q   T   P   30

91  TCC CAG AAG TTT GCC ATC CAG CAT ATC AAT AAT AAT ACC AAC CTC CAA TGT AAT GTT GAA ATG ATG CGT ATT AAC AGG GCT AGA AGA ACA
      S   Q   K   F   A   I   Q   H   I   N   N   N   T   N   L   Q   C   N   V   E   M   M   R   I   N   R   A   R   R   T   60

181 TGT AAG GGC TTA AAT ACT TTT CTT CAT ACA AGT TTT GCT AAT GCT GTT GGT GTG TGT GGA AAT CCA AGT GGC TTG TGC AGT GAC AAG AGA
      C   K   G   L   N   T   F   L   H   T   S   F   A   N   A   V   G   V   C   G   N   P   S   G   L   C   S   D   K   R   90

271 AGT CAA AAC TGT CAT AAT AGT TCA TCT CGG GTA CAT ATA ACT GTC TGT AAC ATC ACC AGT CGG GCA ACA AAT TAT ACC CAA TGC AGA TAC
      S   Q   N   C   H   N   S   S   S   R   V   H   I   T   V   C   N   I   T   S   R   A   T   N   Y   T   Q   C   R   Y   120

361 CAA TCA AGA AGA TCA TTG GAG TAC TAC ACA GTT GCC TGT GAC CCC AGA ACT CCA CAG GAC AGT CCC ATG TAT CCA GTG GTT CCA GTT CAC
      Q   S   R   R   S   L   E   Y   Y   T   V   A   C   D   P   R   T   P   Q   D   S   P   M   Y   P   V   V   P   V   H   150

451 TTG GAT GGG ACA TTT TAG
      L   D   G   T   F   ter
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mEAR-2

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1   ATG GGT CCG AAG CTG CTT GAG TCT CGA CTT TGT CTC CTG CTG CTG CTA GGA CTT GTC CTA ATG CTT GCC TCA TGC CTG GGA CAA ACC CCT
      M   G   P   K   L   L   E   S   R   L   C   L   L   L   L   L   G   L   V   L   M   L   A   S   C   L   G   Q   T   P   30

91  TCC CAG TGG TTT GCC ATC CAG CAT ATC AAT AAT AAT GCC AAC CTC CAA TGT AAT GTT GAA ATG CAG CGT ATT AAC AGG TTT AGA AGA ACA
      S   Q   W   F   A   I   Q   H   I   N   N   N   A   N   L   Q   C   N   V   E   M   Q   R   I   N   R   F   R   R   T   60

181 TGT AAG GGC TTA AAT ACT TTT CTT CAT ACA AGT TTT GCT AAT GCT GTT GGT GTG TGT GGA AAT CCA AGT GGC TTG TGC AGT GAC AAT ATA
      C   K   G   L   N   T   F   L   H   T   S   F   A   N   A   V   G   V   C   G   N   P   S   G   L   C   S   D   N   I   90

271 AGT AGA AAC TGT CAT AAT AGT TCA TCT CGG GTA CGT ATA ACT GTC TGT AAC ATC ACC AGT CGG AGG AGA ACA CCT TAT ACC CAA TGC AGA
      S   R   N   C   H   N   S   S   S   R   V   R   I   T   V   C   N   I   T   S   R   R   R   T   P   Y   T   Q   C   R   120

361 TAC CAA CCA AGA AGA TCA TTG GAG TAC TAC ACA GTT GCC TGT AAC CCC AGA ACT CCA CAG GAC AGT CCC ATG TAT CCA GTG GTT CCA GTT
      Y   Q   P   R   R   S   L   E   Y   Y   T   V   A   C   N   P   R   T   P   Q   D   S   P   M   Y   P   V   V   P   V   150

451 CAC TTG GAT GGG ACA TTT TAG
      H   L   D   G   T   F   ter
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FIG. 2. Nucleotide and deduced amino acid sequences of murine EAR-1 and EAR-2. The sequences shown are derived from single random-primed bone marrow cDNA clones and are segregated as triplet codons with the inferred amino acid sequence below. Amino acid residues in bold correspond to sequences identified in cleavage peptides recovered from the purified granule proteins. Nucleotides are numbered on the left and amino acids are numbered on the right.

vertebrate ribonucleases (Fig. 3). These sequences were chosen to represent the other subgroupings of the ribonuclease superfamily (32, 37, 38). In addition, the amphibian (*R. pipiens*) protein, onconase (39) was also included in the alignment because it shared conserved structural features and a demonstrable neurotoxicity similar to both EDN and ECP (39). The alignment demonstrates that mEAR-1 and mEAR-2 have maintained the identity of the amino acid residues necessary for ribonuclease activity and secondary/tertiary structure. This alignment also shows that mEAR-1 and mEAR-2 display a low degree of sequence homology with the human eosinophil-associated ribonucleases EDN and ECP. Since both mEAR-1 and mEAR-2 are only slightly cationic (pIs 9.0 and 9.7, respectively), these proteins are more similar in charge to the slightly basic human granule ribonuclease EDN (pI 8.7). However, despite the greater similarity of the mEARs to EDN based on calculated pIs, the overall sequence homology of the mEARs to either of the human EARs is the same at both the DNA (64–65% identity) and amino acid (48–52%/58-61% identity/similarity) levels. In addition, the mEARs share an even lower degree of primary amino acid conservation to the expanded family of vertebrate ribonucleases represented by the sequences in Fig. 3.

The alignment of the expanded family of ribonuclease sequences identifies sequence motifs specific for a subset of proteins that also include the EARs. These sequence motifs are the absence of 5 or 6 amino acids near the N terminus of the secreted ribonucleases (Fig. 3, ‡), a higher degree of conservation of amino acids comprising the signal peptides associated with protein secretion (data not shown), and the presence of an 8–10 amino acid insertion near the C termini of the eosinophil-associated ribonucleases (Fig. 3, §). Conservation among these ribonucleases is displayed graphically in the unrooted phylogenetic tree derived from the alignment of the representative vertebrate ribonuclease sequences (Fig. 4). This molecular cladogram demonstrates that mEAR-1 and mEAR-2 are conserved members of a larger mammalian EAR gene family that includes the human proteins ECP and EDN and their primate orthologues. Fig. 4 also shows that the bovine kidney ribonuclease (*B. taurus* RNase K2) is a divergent member of the primate/rodent EARs. The primary node of divergence among the ribonucleases appears to divide RNase K2 and the EARs from the remaining ribonucleases such as pancreatic ribonucleases and the angiogenins.

The phylogenetic analysis shows that two independent gene duplication events have occurred since the divergence of the orders Primata and Rodentia. We have estimated the divergence time of the mEAR-1/mEAR-2 duplication on the basis of accumulated synonymous (i.e., silent) nucleotide substitutions and estimated rates of synonymous nucleotide divergence in rodents (40):

$$\text{Rate of synonymous nucleotide substitution } (R_{Ks}) = \frac{\text{Ks (synonymous substitutions/total synonymous sites)}}{2\text{ (time of duplication)}}.$$

A comparison of mEAR-1 and mEAR-2 yields a Ks value of 0.0193 (i.e., two synonymous mutations in 471 total nucleotides of coding sequence). If R_{Ks} for rodent gene-pairs is assumed to be 0.0066 per 10^6 years (41, 42), then these calculations show that the mouse EARs have diverged $\approx 1.5 \times 10^6$ years ago [0.0193/2(0.0066)]. However, since the assumed R_{Ks} is based on a mean Ks value of rodent gene pairs that vary by a factor of 3 (42), the actual duplication event generating mEAR-1 and mEAR-2 may have occurred anywhere within the time frame of $0.5–4.5 \times 10^6$ years ago.

Preliminary Identification of Additional EAR Genes in the Mouse. The possibility that additional gene duplications may have occurred in this ribonuclease subfamily was assessed by estimating gene copy number in the mouse using genomic

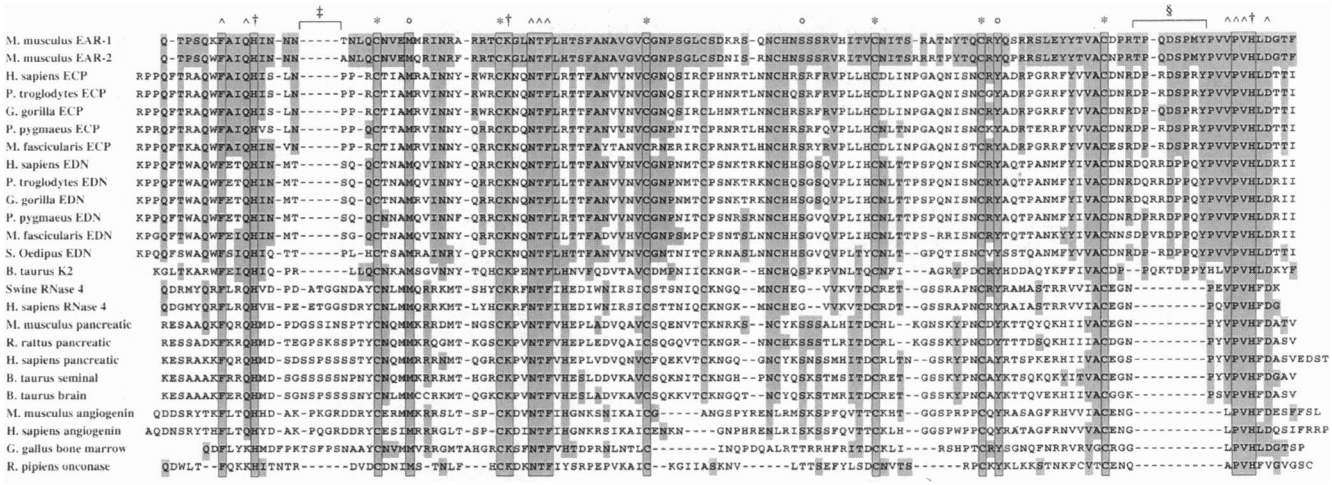

FIG. 3. Primary amino acid sequence alignments of mEAR-1 and mEAR-2 and representative members of the vertebrate ribonuclease superfamily. The signal peptides of each protein were removed prior to amino acid alignment. Conserved amino acid residues between all of the proteins are noted by a solid box, while those conserved with mEAR-1 and mEAR-2 are shaded. Conserved structural features of these proteins (25, 36) are identified as follows: *, cysteines proposed to be involved in intramolecular disulfide bonds; †, amino acids composing the ribonuclease catalytic domain; ∧, residues involved in RNA-substrate binding; °, residues associated with tertiary structure. Consensus deletion and insertion events unique to EAR related proteins are identified by ‡ and §, respectively. Dashes represent gaps introduced into the alignments. The sequence alignments result from the application of methods outlined in Feng and Doolittle (32) and the BLOSUM-62 matrix as applied by Henikoff and Henikoff (33), with a gap penalty score of 6. The order of the sequences presented has been changed from that which was entered into the program. The order of sequence input was as follows: *Homo sapiens* EDN (X16546), *Pan troglodytes* EDN (U24102), *Gorilla gorilla* EDN (U24100), *Pongo pygmaeus* EDN (U24104), *Macaca fascicularis* EDN (U24096), *Saguinus oedipus* EDN (U24099), *H. sapiens* ECP (X15161), *P. troglodytes* ECP (U24103), *G. gorilla* ECP (U24097), *M. fascicularis* ECP (U24098), *P. pygmaeus* ECP (U24101), *Mus musculus* EAR-1, *M. musculus* EAR-2, *Bos taurus* K2 (P08904), *H. sapiens* RNase4 (D37931), swine (species unknown) RNase 4 (S73478), *Rattus rattus* pancreatic (J00771), *M. musculus* pancreatic (M27814), *H. sapiens* pancreatic (D26129), *B. taurus* seminal ribonuclease (X51337), *B. taurus* brain ribonuclease (X59767), *H. sapiens* angiogenin (M11567), *M. musculus* angiogenin (U22516), *Gallus gallus* RNase superfamily related gene (X64743), and *Rana pipiens* onconase (P22069).

Southern blot hybridization. Since the mEAR-1 and mEAR-2 genes in the mouse are nearly identical, probes derived from either of these genes cross-react even at a high criterion [65°C (29)] of hybridization (data not shown). The original 285bp RT-PCR product representing the mEAR-2 gene was used in the genomic Southern blot experiments that are shown in Fig. 5. This figure shows two identical Southern blots of different restriction enzyme digests of mouse (C57BL/6J) genomic DNA hybridized with the mEAR-2 probe. The 65°C (high criterion) blot reveals the presence of three to five mEAR hybridizing bands in any one lane (i.e., restriction enzyme digest). The pattern and intensity of the bands observed are particularly informative. Since the genomic DNA used was derived from an inbred strain, it is likely that only a single genomic allele (i.e., a single restriction fragment length polymorphism) of each gene is present. Moreover, the probe used in this Southern blot is contiguous in the genome (determined by primer-specific PCR with genomic DNA; data not shown) and none of the restriction enzymes used in the Southern blot cut within the probe sequence. As a result, the number of bands and their intensity represent estimates of copy number and because a maximum of five equally intense bands appears in any one restriction enzyme digest, these data suggest the existence of as many as five highly homologous EAR genes in the mouse (mEAR-1, mEAR-2, and three others). Fig. 5 also contains an identical genomic Southern blot performed at a low criterion of hybridization (55°C). This blot shows additional hybridizing bands in each genomic digest and thus reveals the existence of other genes with a lower degree of homology to the highly conserved EAR genes thus augmenting the size of this gene subfamily in the mouse.

DISCUSSION

Two putative mouse eosinophil ribonucleases stored in the secondary granules of this cell type were identified through the purification and partial characterization of acid soluble granule proteins. Amino acid sequencing data provided the necessary information for the development of degenerate oligonucleotide primers specific for the isolated mouse proteins and the subsequent isolation of cDNAs from a mouse bone marrow cDNA library. The two mouse genes are very homologous (96% identity at the nucleotide level) and encode proteins of similar size and physical characteristics. The mouse genes are also only weakly homologous (48–52% amino acid identity) with the human EARs EDN and ECP, and do not show substantive sequence bias toward either human protein/gene. The putative mouse proteins encoded by the cDNAs maintain the necessary amino acids for ribonuclease activity although an assessment of the ribonuclease activity of each granule protein awaits the purification of enough protein to perform enzyme activity measurements.

Primate/Rodent EAR Genes Result from Multiple Gene Duplication Events and Have Diverged To Create a Subfamily of Ribonuclease Genes. The isolation and characterization of eosinophil granule ribonuclease proteins in the mouse suggest that these proteins have important (i.e., conserved) mammalian eosinophil effector function(s) not necessarily unique to primates. The identification of EARs in two divergent orders of Mammalia further suggests that these genes may be widespread, possibly accompanying the emergence of the eosinophil blood cell-type.

The mammalian EAR genes appear to have diverged more quickly than the other members of the ribonuclease superfamily. The low amino acid identity displayed between mEAR-1/mEAR-2 and EDN/ECP is in contrast to murine versus human comparisons of the pancreatic ribonucleases and angiogenins that share 70% and 74% amino acid identity, respectively. Since the amino acid residues necessary for ribonuclease activity (i.e., amino acids associated with the catalytic domain, substrate binding, and protein tertiary structure) compose only a fraction of the total residues (25, 36), the

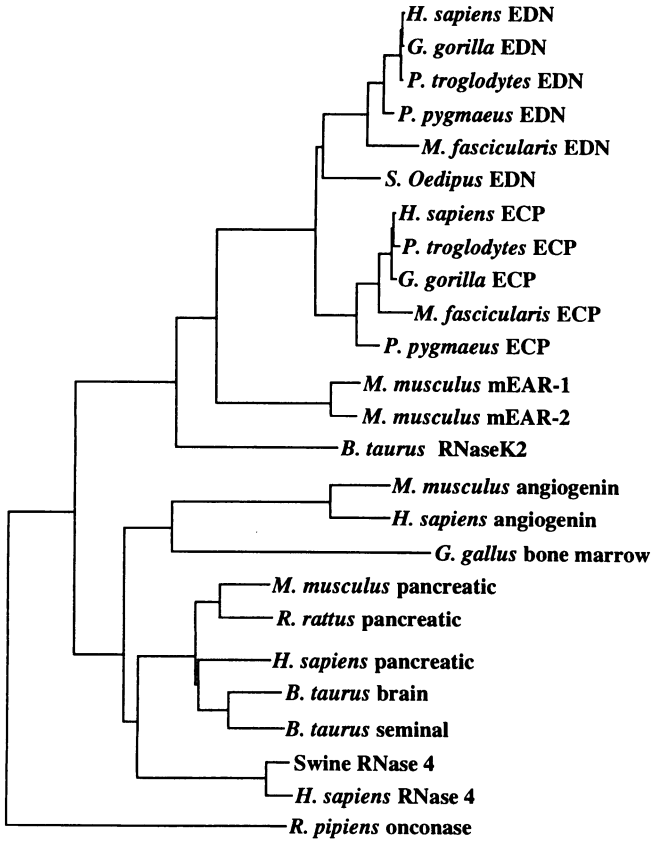

FIG. 4. An unrooted phylogenetic tree of representative vertebrate ribonuclease genes. Construction of this tree was based upon the progressive similarity alignment presented in Fig. 3. Horizontal distances correspond to evolutionary divergence.

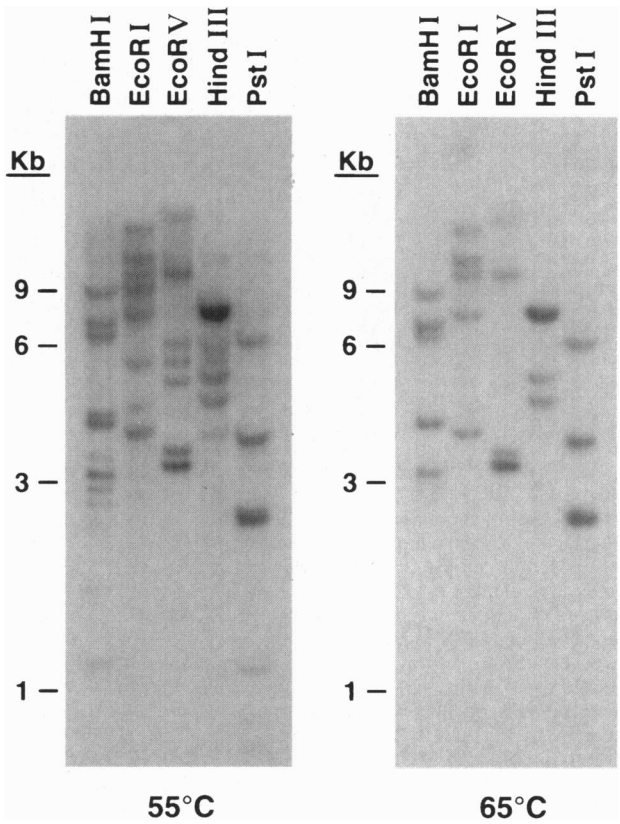

FIG. 5. Genomic Southern blots of mouse DNA hybridized with an mEAR-1/mEAR-2 probe. Mouse genomic DNA (15 μg) was digested by a series of restriction enzymes, and each sample was electrophoresed on a 1% TAE agarose gel. The DNA was blotted in duplicate onto GeneScreen (+) and each blot hybridized (and subsequently washed) at either high (hybridization: 5× SSPE, 65°C; final wash: 0.1× SSC, 65°C) or low (hybridization: 5× SSPE, 55°C; final wash: 1× SSC, 55°C) criteria.

utilitarian function of large segments of these proteins remains obscure. If these non-enzymatic residues are important for relative effector activities (e.g., the maintenance of specific protein–protein interactions), the greater amino acid divergence displayed by the EAR gene subfamilies (and/or individual EAR gene family members) may reflect different selective constraints associated with these ribonucleases.

The homology data presented here demonstrates the existence of an emerging subgroup of genes in the ribonuclease superfamily that have now been identified in three orders of Mammalia (Primata, Rodentia, and Artiodactyla). Members of this gene family are distinguished from other members of the superfamily by the deletion of a 5 or 6 amino acid contig near the N termini of the secreted proteins, an 8 or 9 amino acid insertional event near their C terminal ends of the proteins, and elevated rates of sequence divergence relative to other mammalian ribonucleases. The genes encoding these proteins appear to have similar, but not necessarily identical, expression patterns. Most of the identified gene family members are expressed in the eosinophil leukocyte; however, some genes are expressed in the liver, kidney, spleen, or urine depending on the species examined (43–46). The conserved characteristics of these proteins indicate that this subgroup of the ribonuclease superfamily probably have some common effector functions in different orders of mammals. The characterization of a non-mammalian member of this gene family (e.g., an avian EAR gene) may help establish these effector functions.

The EAR Gene Family in the Mouse: Implications for Other Mammalian Genomes. Hamann *et al.* (47) used the nucleotide divergence between the human ECP and EDN introns to calculate that the duplication event generating these two genes occurred approximately 30–40 million years ago, just after the divergence of Old World and New World primates (47). Since this event is 50 million years after the divergence of the mammalian orders of Primata and Rodentia (48), the identified eosinophil-associated genes in the mouse cannot be orthologues of the human genes. Calculations based on synonymous nucleotide substitutions between mEAR-1 and-2 show that the duplication event generating these genes is an evolutionarily recent event, probably occurring within the last 5 million years. It seems serendipitous that representatives from two extant mammalian orders would have independently duplicated a primordial ribonuclease gene expressed in eosinophils. Recognizing that until the relative ribonuclease activities of the mouse proteins are assessed and the identification of effector functions can be specifically associated with the granule ribonucleases, we suggest that there may be an underlying significance to eosinophil effector function by having two or more granule ribonuclease genes, i.e., both primate and rodent species have independently responded to similar selective pressures on eosinophil effector function in an evolutionarily convergent fashion.

The high criteria Southern blot analysis presented demonstrates that, in addition to mEAR-1 and mEAR-2, the mouse may have as many as three additional (five total) highly homologous EAR genes. The intensities of the hybridizing genomic fragments at high criteria with an mEAR-1/mEAR-2 probe suggest that the additional mEAR genes in the mouse genome are very homologous and probably arose by duplication events occurring about the same time (or perhaps more recently) as the duplication of the primordial gene creating mEAR-1 and mEAR-2. These additional genes remain yet to

be identified as our purification efforts thus far have failed to identify other ribonucleases stored in the eosinophil secondary granule. An identical Southern blot hybridized/washed at low criteria showed that additional hybridizing bands appeared, thus suggesting the existence of an even larger family of related EAR genes in the mouse. Interestingly, these genes probably do not represent other known members of the murine ribonuclease family. The probe used for the Southern blot has very limited nucleotide homology to both murine angiogenin (43%) or murine pancreatic ribonuclease (52%), and would not hybridize to fragments derived from either gene under the low criteria conditions used here. There is no reason *a priori* to assume that the existence of a larger EAR gene family is unique to the mouse. We suggest that other mammals are likely to contain additional EAR genes. The implication in the case of humans is the possibility of other ribonucleases with significant homology to ECP and/or EDN. Additional genes homologous to EDN are of particular interest because of the apparent expression of an EDN-like protein in many non-eosinophil cell types (43, 44, 46, 49).

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