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Ancient expansion of the ribonuclease A superfamily revealed by genomic analysis of placental and marsupial mammals

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Abstract

Members of the ribonuclease (RNase) A superfamily participate in a diverse array of biological processes, including digestion, angiogenesis, innate immunity, and possibly male reproduction. The superfamily is vertebrate-specific, with 13–20 highly divergent members in primates and rodents, but only a few members in chicken and fish. This has led to the proposal that the superfamily started off from a progenitor with structural similarities to angiogenin and that the suerfamily underwent a dramatic expansion during mammalian evolution. To date this evolutionary expansion and understand the functional diversification of the superfamily, we here determine its entire repertoire in the sequenced genomes of dog, cow, and opossum. We identified 7, 20, and 21 putatively functional RNase genes from these three species, respectively. Many of the identified genes are highly divergent from all previously known RNase genes, thus representing new lineages within the superfamily. Phylogenetic analysis indicates that the superfamily expansion predated the separation of placental and marsupial mammals and that differential gene loss and duplication occurred in different species, generating a great variation in gene number and content among extant mammals.

Keywords: RNase; Gene family; Eosinophile-associated ribonuclease; Angiogenin; Marsupial; Gene sorting; *Canis familiaris*; *Bos taurus*; *Monodelphis domestica*

1. Introduction

Represented by the famous prototype, bovine pancreatic ribonuclease (RNase A), the RNase A superfamily is one of the best studied protein families. Numerous fundamental discoveries have been made in biochemistry, molecular biology, structural biology, enzymology, and molecular evolution by studying various members of the superfamily (Anfinsen, 1973; Blackburn and Moore, 1982; Jermann et al., 1995; Rosenberg et al., 1995; D'Alessio and Riordan, 1997; Beintema and Kleineidam, 1998; Zhang et al., 1998, 2002b; Cho et al., 2005). Note that some unrelated proteins, such as RNases H, L, P, and T, and also ribozymes, have the ribonucleolytic activity, because this activity originated multiple times in evolution. However, for simplicity, we use "RNase" to refer to the RNase A superfamily or its members in this article. RNases are known to have a diverse array of physiological functions. For example, pancreatic ribonuclease (RNase 1) degrades dietary RNA molecules in the digestive gut (Barnard, 1969), angiogenin (RNase 5) stimulates blood vessel formation (Fett et al., 1985; Strydom et al., 1985), and RNases 2, 3, and 7 have antibacterial or antiviral activities that are believed to be used in innate immunity (Young et al., 1986; Domachowske et al., 1998a,b; Harder and Schroder, 2002; Zhang and Rosenberg, 2002a; Zhang et al., 2003). RNases share a number of common sequence features. The entire open reading frame (ORF) of ~130 codons is encoded in a single exon. RNases have a signal peptide at the N-terminus and six to eight conserved cysteines that form disulfide bridges. They have three distinct catalytic residues, called the catalytic triad, and several other conserved motifs, although the level of conservation varies among proteins (Beintema and Kleineidam, 1998; Cho et al., 2005). To date, 13 human RNase genes have been reported (Cho et al., 2005). Eight of them have been subject to functional characterizations and are known to have

Abbreviations: RNase, ribonuclease; EAR, eosinophil-associated ribonuclease; EDN, eosinophil-derived neurotoxin; ECP, eosinophil cation protein; Ang, angiogenin; Mb, mega base pairs.

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the ribonucleolytic activity. These RNases are the pancreatic ribonuclease (RNase 1), eosinophil-derived neutotoxin (EDN or RNase 2), eosinophil cationic protein (ECP or RNase 3), RNase 4, angiogenin (RNase 5), RNase 6 (or k6), RNase 7, and RNase 8. RNases 9–13 were identified only recently and RNases 9 and 10 have no detectable RNase activities, while RNase activities have not been examined for RNases 11–13 (Penttinen et al., 2003; Castella et al., 2004a,b; Devor et al., 2004; Cho et al., 2005). Except for RNases 2 and 3 and RNases 7 and 8, which emerged by gene duplication within primates (Rosenberg et al., 1995; Zhang et al., 2002a, 2003), all human RNase genes are represented (in one or multiple copies) in the mouse and rat (Cho et al., 2005). In the chicken and zebrafish genomes, however, only a limited number of closely related RNase genes are found (Cho et al., 2005). In the bullfrogs, although several divergent RNases have been reported, they are more closely related to each other than to any mammalian RNases, thus representing an amphibian-specific cluster (Rosenberg et al., 2001). RNases have never been found in any invertebrates, including the genomes of two completely sequenced urochordates, *Ciona intestinalis* and *Ciona savignyi* (Cho et al., 2005). Evolutionary analysis and structural features indicate that angiogenins are the first diverging group in mammalian RNases (Cho et al., 2005) with all non-mammalian vertebrate RNases having angionenin-like structures, although the angiogenic activity has yet to be examined or detected in these RNases. The phylogenetic patterns suggest that the superfamily arose in early vertebrate evolution as an angiogenin-like molecule and it underwent independent expansions during amphibian evolution and mammalian evolution. There are several interesting questions with regard to the mammalian expansion of the RNase superfamily. First, a previous study (Cho et al., 2005) showed that the expansion occurred after the bird–mammal divergence [~310 My ago; (Hedges et al., 1996)], but before the primate–rodent separation [~85 My ago; (Murphy et al., 2004)], leaving the precise date of the expansion uncertain. Second, previous studies were limited to primates and rodents, with the size and diversity of the RNase superfamily unknown in any other orders of placental mammals or any non-placental mammals. The draft genome sequences of the dog (*Canis familiaris*), cow (*Bos taurus*), and opossum (*Monodelphis domestica*) became available recently, providing an opportunity to address the above questions. As a marsupial, the opossum is particularly valuable for unraveling the timing of the mammalian RNases superfamily expansion. In this work, we identify all RNase genes from the above three genome sequences and report that the mammalian RNase superfamily expanded before the split between placental and marsupial mammals [~180 My ago; (Murphy et al., 2004)]. We further show that this expansion was followed by differential gene retention and duplication among different orders of placental mammals, generating a great variation in the RNase gene repertoire among species.

2. Materials and methods

In this paper, we use "dog" for *C. familiaris* (Cf for short), "cow" for *B. taurus* (Bt), and "opossum" for the gray short-tailed opossum *M. domestica* (Md). A "functional gene" refers to an RNase gene that contains an uninterrupted open reading frame (ORF), whereas a "pseudogene" refers to a gene whose ORF is interrupted by a premature stop codon anywhere in the ORF or by frame-shifting insertions/deletions. Pseudogenes are distinguished from functional genes by having "ps" after the gene name (e.g., RNase13ps). Genes and pseudogenes are named according to the order in which they were identified.

Using currently available human RNase sequences (RNases 1–13) as queries, TBLASTN and BLASTN searches (http://www.ncbi.nlm.nih.gov/BLAST/) were carried out on the dog and cow genome sequences available at the National Center for Biotechnology Information (NCBI) and the opossum genome sequence available at Ensembl (heep://www.ensembl.org). The dog genome sequence used was version NCBI 2.1, with 7.6× coverage (http://www.ncbi.nih.gov/genome/guide/dog/). The cow genome sequence was version Btau_2.0, with 6.2× coverage. The opossum genome sequence was version 0.5, with 7.19× coverage. We used 10^{-10} as the *E*-value cutoff in all TBLASTN and BLASTN searches.

Protein and DNA sequences were aligned by CLUSTAL_X (Thompson et al., 1997) with manual adjustments. Phylogenetic trees were reconstructed using the neighbor-joining method (Saitou and Nei, 1987) with 2000 bootstrap replications (Felsenstein, 1985). MEGA3 (Kumar et al., 2004) was used for evolutionary analyses. Note that the complete deletion option in MEGA3 was enforced. That is, any site with a gap in one or more sequences was removed from the analysis.

3. Results and discussion

3.1. Identification of RNase A genes in dog, cow, and opossum

From whole-genome searches based on TBLASTN, we identified RNase genes and pseudogenes from the genome sequences of the dog, cow, and opossum. We also conducted BLASTN-based nucleotide sequence searches to identify pseudogenes that had not been found by TBLASTN. The entire catalogs of the RNase genes identified in this study are listed in Table 1 and Supplementary Tables S1–S3, and the DNA sequences of the RNase genes are provided in Supplementary Data Sets 1–3. The chromosomal locations of the genes are drawn to scale in Fig. 1, except for cow, because the chromosomal locations of many cow genes are not available in the current genome assembly. For pseudogenes, start and stop codons were determined according to their closest functional homologs.

3.1.1. Dog RNase genes

Seven functional RNase genes were identified from the dog genome sequence (Table 1, Supplementary Table S1), which is a relatively small number compared with those in human (13), mouse (20), and rat (17) (Cho et al., 2005). A single dog ortholog was identified for each of human RNases 1, 4, 9, 10, 12, and 13. However, the orthologous relationship of the other dog gene to human RNases was unclear; it was equally closely related to human RNases 2, 3, 6, 7, and 8, a cluster of

Table 1
Numbers of RNase genes and pseudogenes identified from complete or nearly complete genome sequences of mammals

Species	R1	EARs	R4	Ang	R6–8	R9–13	New[a]	EAR group[b]	Total	Classification	Reference
Human (*H. sapiens*)	1	2 (1)	1	1	3	5	0	5 (1)	13 (1)	Primates	(Cho et al., 2005)
Mouse (*M. musculus*)	2	6 (12)	1	5 (3)	1	5	0	7 (12)	20 (15)	Rodentia	(Cho et al., 2005)
Rat (*R. norvegicus*)	3	5 (1)	1	2	1	5	0	6 (1)	17 (1)	Rodentia	(Cho et al., 2005)
Dog (*C. familiaris*)	1	0	1	0 (1)	1 (1)	4 (1)	0	1 (1)	7 (3)	Carnivora	This study
Cow (*B. taurus*)	3	2	2	3	2 (2)	6 (1)	2	4 (2)	20 (3)	Artiodactyla	This study
Opossum (*M. domestica*)	1	0	1	1	1	2 (1)	15 (2)	4 (0)	21 (3)	Marsupial	This study

Numbers of pseudogenes are in parentheses.
[a] Note that this group does not represent an established monophyletic group, but it simply includes all the novel genes that do not belong to any of the previously identified groups.
[b] The EAR group includes genes corresponding to human RNases 2, 3, 6, 7, and 8 and opossum RNases 19, 20, and 21.

evolutionary related RNases with similar structures like a short S-peptide loop and an extension after the final cysteine residue. We tentatively named this gene Cf-RNase 6 based on its chromosomal location (Fig. 1). Noticeably, the dog does not have a functional ortholog of human angiogenin (RNase 5), a situation previously known to occur in a colobine monkey (Zhang and Zhang, 2003). Dog does not have RNase 11 either. The seven functional RNase genes in dog are clustered in a 300-kb region on chromosome 15. The gene order and transcriptional direction of the dog genes are identical to those of human, mouse, and rat RNase genes, but the size of the dog cluster is considerably smaller than that in the other mammals (Fig. 1). In human, angiogenin and RNase 11 genes are located next to RNase 4 and RNase 12, respectively (Cho et al., 2005; Dyer and Rosenberg, 2005). Using this synteny information, we carefully searched for any pseudogene relics of angiogenin and RNase 11 in the corresponding regions of the dog genome. Interestingly, we found dog sequences that are 65% and 58% identical to human angiogenin and RNase 11 genes, respectively. However, the start codons of the dog sequences are not found at the expected locations and the ORFs are disrupted by multiple insertions/deletions, suggesting that the pseudogenization events were not recent.

3.1.2. Cow RNase genes

Twenty functional RNase genes and three pseudogenes (Table 1; Supplementary Table S2) were identified from the cow genome. Although the coverage of the cow genome sequence is not low, the assembly is incomplete. Due to this reason, three of the cow RNase sequences are incomplete (RNase 4, Ang3 and

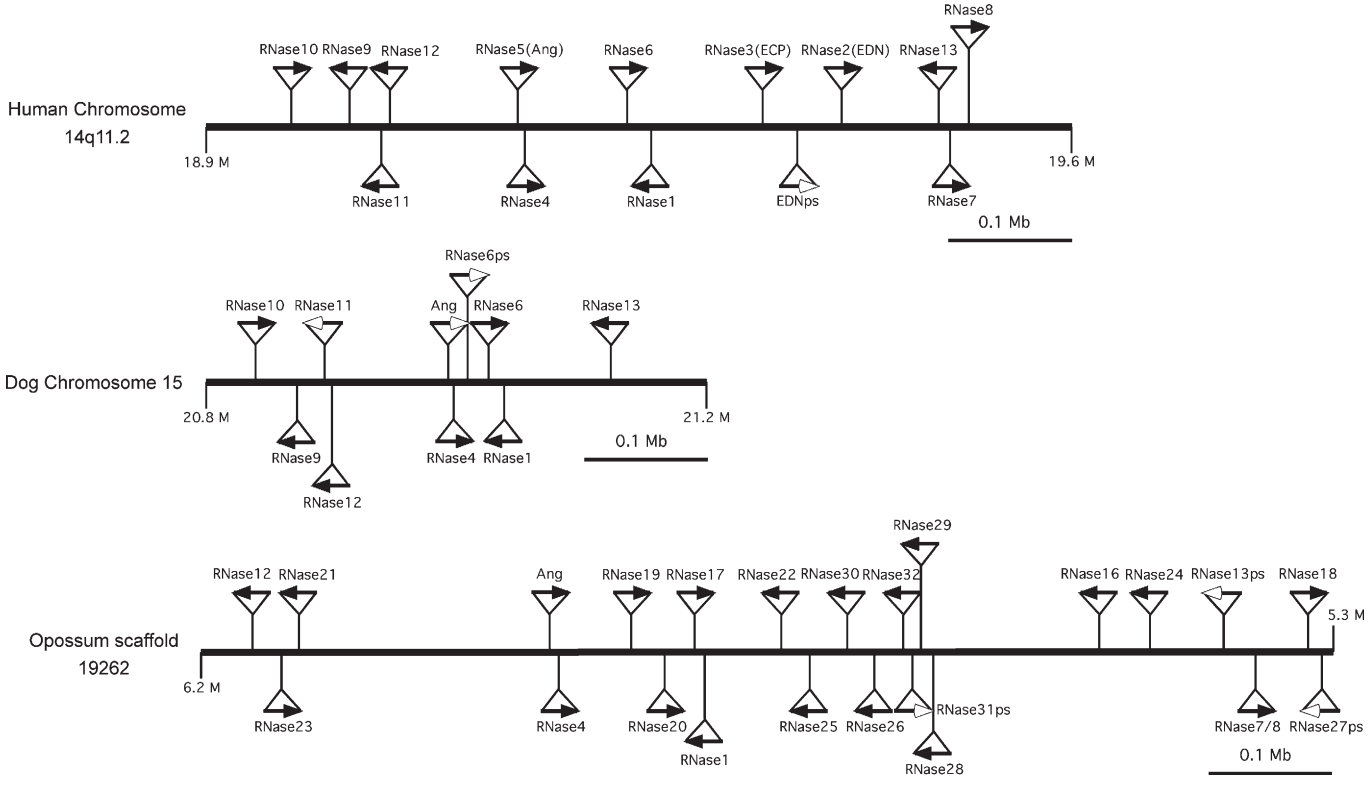

Fig. 1. Chromosomal locations of human, dog, and opossum RNase genes. Gene locations are drawn to scale. Arrows indicate transcriptional directions. Filled arrows show functional genes and open arrows show pseudogenes. In human chromosome 14, RNase 10 is closer to the centromere, whereas RNase 8 is closer to the telomere. For dog and opossum, the location of the centromere is not known. The human map was adapted from Cho et al. (2005).

RNase 7/8psB) (Supplementary Table S2). Notwithstanding, the cow genome has all the members of the RNase superfamily that were previously known. As previously reported (Confalone et al., 1995), three RNase 1 genes (pancreatic, brain, and seminal RNases) are found in the cow. Being reminiscent of EDN and ECP, two eosinophil-associated ribonucleases (EARs) specific to catarrhine primates, there are also two cow EAR genes, named Ear1 and Ear2. These two cow genes have only one nucleotide difference (nonsynonymous), suggesting recent duplication. There are also two cow sequences similar to human RNase 4. One of them is identical to the previously identified bovine RNase 4 gene (Hosoya et al., 1990) but its N-terminal part is not sequenced in the genome. The other one has a premature stop codon at the C-terminus, which would remove 15 amino acids including the catalytic H_{119}. We therefore consider it as a pseudogene. Another interesting feature of this pseudogene is that its sequence after amino acid residue 102 appears to be unrelated to other RNase 4 genes. In addition to the two previously identified cow angiogenin genes (Ang1 and Ang2) (Maes et al., 1988; Strydom, 1998; Zhang and Rosenberg, 2002b), we identified a third angiogenin gene (Ang3), although its sequence in the database is incomplete. There is one functional gene and two pseudogenes in the cow genome that are related to human RNases 7 and 8. These three bovine sequences are more similar to each other than to human RNase 7 or RNase 8 (see below for details). Two bovine genes and one pseudogene related to human RNase 9 were identified. One bovine ortholog for each of human RNases 10–13 was also found. Noticeably, we also identified two bovine genes whose relationships to any of these known genes are ambiguous. Phylogenetic analysis revealed that these two genes represent newly identified lineages of the superfamily (see below for details). We named them Bt-RNases 14 and 15 (Supplementary Table S2). Bt-RNase 15 has no angiogenin-like structural features, although its phylogenetic position is close to angiogenins and chicken RNases (Fig. 2). It is noteworthy that the position of H_{12}, a residue in the catalytic triad, is shifted by one amino acid toward the N-terminus in Bt-RNase 15.

3.1.3. Opossum RNase genes

The RNase A superfamily is larger in opossum than in any other species studied (human, mouse, rat, dog, cow, chicken, and zebrafish), with 21 functional genes and three pseudogenes (Table 1; Supplementary Table S3). A single functional ortholog for RNase 1 (69% identical to the kangaroo RNase 1), RNase 4, angiogenin, RNase 7/8, RNase 12, and RNase 13, and a pseudogene related to RNase 13 were identified. To our surprise, 15 additional genes and two pseudogenes that do not belong to any of the previously described groups of RNases were found. We named them Md-RNases 16–32 (Supplementary Table S3). We will provide detailed descriptions of these novel genes later in the paper. All opossum RNase genes and pseudogenes except for the functional copy of RNase 13 are located in a cluster of ~850 kb on the scaffold 19262 of the partially assembled opossum genome, indicating that they are on the same chromosome. We found that a pseudogene related to RNase 13 was located in the region syntenic to RNase 13 in other species, indicating that after the duplication of RNase 13 the original copy was pseudogenized whereas the duplicated copy survived. This functional RNase 13 is located in the middle of a different scaffold of >6.3 Mb, suggesting that the duplication did not generate tandem copies. Human RNase 13 is located next to a non-RNase gene, NDRG2. We found that the opossum ortholog of human NDRG2 is located next to Md-RNase 13ps in the opossum RNase cluster (from 5,399,311 to 5,406,206), confirming that Md-RNase 13ps is the original RNase 13 copy that was pseudogenized. Although the opossum genome has the largest number of RNase genes (Table 1), many known members of the superfamily are missing, including EARs, RNase 9, RNase 10, and RNase 11. It is unlikely that these missing genes actually exist in opossum but were not sequenced, because the current opossum genome sequence has a relatively high coverage (7.19×). Our attempts to find any pseudogene relics for these missing genes using syntenic information were not successful. In spite of the antiquity (~180 My ago) of the split between placental and marsupial mammals (Murphy et al., 2004), the order of the RNase genes in the chromosome and the transcription directions are well conserved between placentals and marsupials (Fig. 1).

3.2. Evolution of the mammalian RNase A superfamily

Our previous genomic surveys in the human, mouse, rat, and chicken (Cho et al., 2005) indicated that the major groups (RNases 1, EARs, RNase 4, angiogenins, RNases 6–8, and RNase 9–13) of the RNase A superfamily were formed before the divergence between primates and rodents, but after the bird–mammal separation. Although the gene number for a few groups varies among the three mammals, every species has all of the major groups, giving the impression that all major groups are important and probably conserved in all mammals. But, our present expanded surveys in the dog, cow, and opossum show that the above view is incorrect. For example, in dogs, many RNase groups are missing or pseudogenized, including angiogenin, EARs, and RNase 11, leaving only seven functional RNase genes in the genome (Table 1). In the opossum, EARs, RNases 6, 9, and 11 are also missing. These findings indicate that some RNase groups may be dispensable and that the RNase gene repertoire has changed substantively during mammalian evolution.

It has been proposed that the RNase A superfamily started off as an angiogenin-like gene in early vertebrates and that it experienced a dramatic expansion in mammalian evolution (Strydom, 1998; Cho et al., 2005). When and how did this mammal-specific expansion take place? To address this question, we reconstructed a phylogenetic tree using all RNases identified from the dog, cow, and opossum along with human and chicken RNases (Fig. 2). As previously reported (Cho et al., 2005) and aforementioned, several previously known major groups of RNases are found in the tree, such as the RNase 1 group, the RNase 4 group, the group of EARs and RNases 6–8, the angiogenin group, and each of RNases 9–13. Each of these

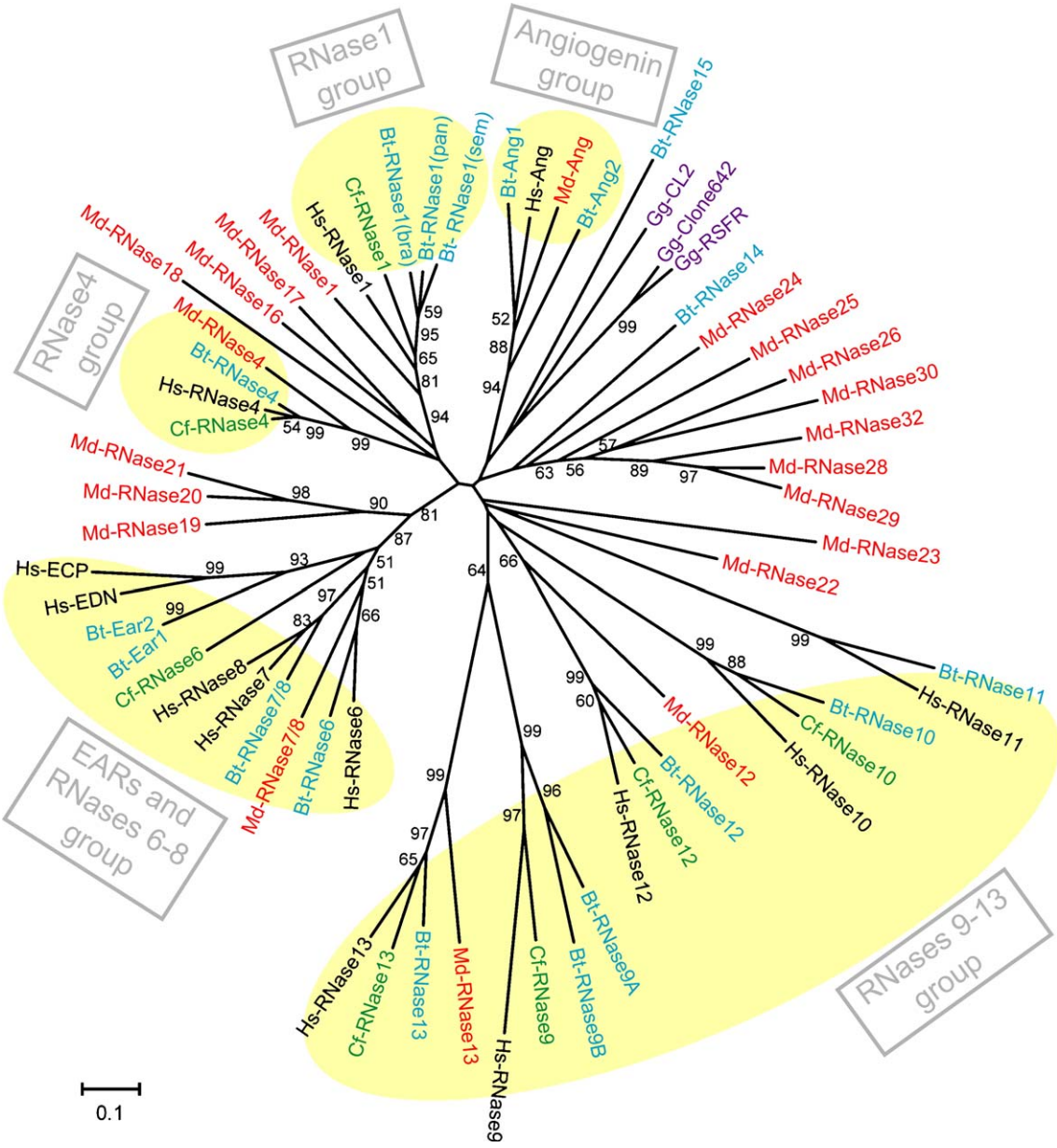

Fig. 2. Phylogenetic relationships of all RNases identified from the human, dog, cow, opossum, and chicken genomes. A total of 80 amino acid sites are used in tree-making after the removal of alignment gaps. The neighbor-joining method with protein Poisson-corrected distances is used to make the tree. Bootstrap percentages greater than 50 (out of 2000 replications) are shown on interior branches.

groups has an opossum gene except for RNases 9–11, indicating that the divergences of these groups predated the split between placental and marsupial mammals.

Remarkably, opossum RNases 16–32 do not belong to any of the major groups, thus representing newly identified lineages in the superfamily. However, the evolutionary relationships of these new opossum genes to the previously known groups of RNases are ambiguous due to low bootstrap percentages, except for Md-RNases 19–21, which form a relatively highly supported clade that is sister to the group of EARs plus RNase 6–8 (Fig. 2). The low bootstrap percentages for most of the deep nodes in the tree are probably due to the great sequence divergence among the major groups and the decrease of the number of informative sites caused by many gaps in the alignment. Noticeably, six of the new opossum genes (Md-RNases 25, 26, 28, 29, 30, and 32) form a monophyletic group, which is supported by a moderate bootstrap value (63%) and also by their clustering on the chromosome (Fig. 1). For the rest of the novel opossum genes (Md-RNase 22–24), their relationships to other genes cannot be unambiguously determined. Two newly identified cow genes, Bt-RNases 14 and 15, are also divergent from any other genes, and they do not appear to be sister to each other.

Our phylogenetic analysis (Fig. 2) provides three significant clues for understanding the evolution of the RNase superfamily. First, the extant placental and marsupial mammals share multiple RNase genes, indicating that the major expansion of the mammalian RNase superfamily predated the split between

placental and marsupial mammals. Second, it also appears that the common ancestor of placentals and marsupials had several RNase genes that were subsequently lost in either placentals or marsupials. For example, the presence of two ancient RNase genes in cows (Bt-RNases 14 and 15) but not in any other placental mammals suggests that different mammalian lineages have suffered different degrees of gene loss. The extreme case is the dog, which has lost many RNase genes (Table 1) presumably after the separation between carnivores and artiodactyls. Finally, there were at least two cases of within-group expansions in the opossum lineage: Md-RNases 19–21 and Md-RNases 25–32. Similar expansions have been reported in rodent EARs and angiogenins (Singhania et al., 1999; Zhang et al., 2000; Cho et al., 2005).

3.3. Evolution of EARs and RNases 6–8

In Fig. 2, the positions of some genes, such as Cf-RNase 6 and Md-RNase 7/8, are not well resolved, indicated by low bootstrap percentages (51% or lower). In order to determine the evolutionary relationship of these genes to other RNases, we made another tree using the nucleotide sequences of all the genes and pseudogenes from this group (Fig. 3). By limiting our analysis to the sequences within this group, we could enhance the reliability of the tree because the number of alignment gaps became smaller. In addition, clarifying the relationship of each pseudogene to its functional ortholog in other species would help us understand the evolutionary path of each gene. We found that the dog pseudogene Cf-RNase6ps is closely related to Cf-RNase 6, whereas two cow pseudogenes are most closely related to Bt-RNase 7/8 (Fig. 3; the sequence of Bt-RNase7/8psB is incomplete thus not included in the analysis). The tree elucidates the time and order of the gene duplication events that shaped this group of RNases. The duplication giving rise to RNases 7 and 8 is most recent, and it is specific to the primate lineage. Previously, it was estimated that this duplication occurred shortly before the separation between New World and Old World monkeys (Zhang et al., 2003). Our study in the mouse and rat (Cho et al., 2005) showed that the common ancestor of RNases 7 and 8 (RNase 7/8) emerged from duplication of RNase 6, which predated the separation of primates from rodents. It can be seen clearly from Fig. 3 that the opossum gene Md-RNase 7/8 is orthologous to the human and cow RNases 7/8 genes, but not with RNase 6. This result strongly suggests that the duplication of RNase 6 and RNases 7/8 predated the split between placental and marsupial mammals. Consequently, it is apparent from the tree that the dog lost RNases 7/8, the opossum lost RNase 6, but the human and cow retained both. The phylogenetic position of the sole canine gene in this group (Cf-RNase 6) is unexpected (Fig. 3), because if it is orthologous to the human and cow RNase 6, it should have diverged from cow RNase 6 more recently than from human RNase 6, as carnivores and artiodactyls are closer to each other than they are to primates (Murphy et al., 2004). Thus, the tree suggests that dog RNase 6 is not the ortholog of human and cow RNase 6. It is likely that the RNase 6 gene was duplicated before the radiation of placental mammals and different genes were retained in different lineages. The most ancient duplication in the group is at node C of the tree (Fig. 3), which separated EARs from RNases 6, 7, and 8. EAR genes had many duplications during rodent evolution (Singhania et al., 1999; Zhang et al., 2000; Cho et al., 2005), but the present analysis suggests that this EAR lineage expansion is unique to rodents and unfound in other mammals.

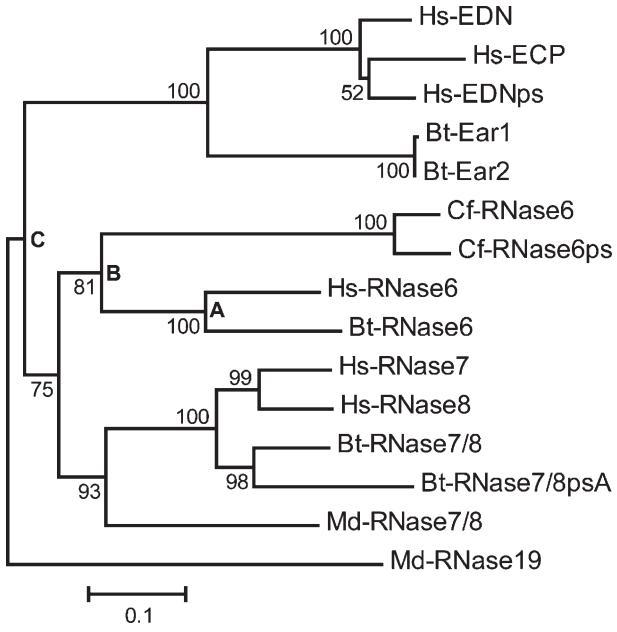

Fig. 3. Phylogenetic relationships of functional genes and pseudogenes of EARs and RNases 6–8 of the human, dog, cow, and opossum. A total of 419 nucleotide sites are used in tree-making after the removal of alignment gaps. The neighbor-joining method with Kimura's two-parameter distances is used to make the tree. The DNA sequences rather than the protein sequences are used because of the inclusion of pseudogenes in the phylogenetic analysis. Bootstrap percentages higher than 50 (out of 2000 replications) are shown on interior branches. Note that Bt-RNase 7/8psB was not included in the tree because its incomplete sequence would compromise the reliability of the phylogeny.

Overall, the group of EARs and RNases 6–8 has experienced a dynamic repertoire change during mammalian evolution. Our analysis suggests that the common ancestor of all placental mammals had at least three genes in this group (EARs, RNase 6, and RNase 7/8) and possibly four genes if we consider the direct ancestor of Cf-RNase 6. These genes were generated through a series of gene duplications that likely predated the divergence between placentals and marsupials. Subsequently, differential retention of ancestral gene lineages, or gene sorting (Zhang et al., 2000), has shaped the current profiles of these genes in extant mammals. The presence of recently duplicated genes and multiple new pseudogenes suggests that this process is still ongoing (Fig. 3).

3.4. Novel members of the RNase A superfamily

One of the most intriguing findings of this study is the novel RNase genes from the cow and opossum. These genes are quite divergent from the previously known members of the superfamily, as their amino acid sequences are only ~25% identical to human RNase 1, for instance (Fig. 4). Nonetheless, three lines of evidence indicate that they are new members of the RNase A

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                                                                                   *           ↓
Hs-RNase1    MALEKSLVRL LLLVLILLVL GWVQPS---- ---------- LGKESRAKKF QRQHMDSDSS PSSSS--TYC NQMMRRRNMT 80
Bt-RNase14   ---------M N.TWTL..L. LLELTV---- ---------- ---FASGLP. S.R.I.NPR. WVPGGQHR.. DV.....WLI
Bt-RNase15   ---------M ..TWTL..F. LMD...---- ---------- ---F.E.QDL LHHQT.RPRT KI.ANHKY.. DL..KA.GLA
Md-RNase16   ----MIPE.S -..LSL.TLM VLGLSHH--- ---------- SLA..QED.. K...L..GTK GNPGK--K.. D...AK.G..
Md-RNase17   ----MA.D.A A..FPL.AMV VVGLV.---- ---------- --S..A.E.. ....V..K.L AFNDN--... .H..KI.G..
Md-RNase18   ---------- ---------- ---MTR---- ---------- -----.EEN. WQ..YNDQR. DEAIQRQMRL IS.VK-----
Md-RNase19   .IHTLKGTFP ..QLTLWVTM FL.TAEL--- ---------- PSNFTP.QW. NK..VQHPKT KA.ND-DI.. .NE..QI.NY
Md-RNase20   ..QNVMGNSQ F..LRLS.MV LL.YSQP--- ---------- PPGFNW.QW. NE..VQYPK. NAPND-NQ.. .AE.Y.V.NH
Md-RNase21   ..QNVMGNSQ ...LGLS.LV LL.YSQT--- ---------- RPGWE.FQR. KN..VRYPKH LA.NN-DQ.. MME.KWV.DY
Md-RNase22   ....TIHLPQ S..L.L..L. SLLL.PG--- ------VCDI Y...T.IQR. W.F.V.NPK. LIPGGNTK.. TV.I...QIS
Md-RNase23   .VYMALKMAF S.MI.LMAQP IAGTIEYVEV TEEQHMGFGI SWDQNP.RN. .IK.IT---D GYFTN----. TV..N.--VN
Md-RNase24   --------MY AGMWTFF.A. FLDLAN---- ---------- ---F.YTND. W.R.V.YPR. -TPKEEDN.. .W..E..G.V
Md-RNase25   -------MVH AGMKTLF.L. LLDLTN---- ---------- ---F.Q.FH. WNY.V.YPKT QV.GTPDQ.. .VI.Q..G.I
Md-RNase26   ---------- --MWTVF.LI FLDLIH---- ---------- ---F.QTQD. WTH.V.FPKT HVFDNPSQ.. DL.IMNKGLV
Md-RNase28   -------MAC AVIWIFF..S FMHLAN---- ---------- ---V.QSQD. WTR.V.YPRT NI.RIPSK.. .GI.KQ.GLE
Md-RNase29   ---------- --MWILF..S FMHLAN---- ---------- ---V.QSQS. WTH.V.YPRT NI.G.PSK.. KM.I.Q.ELK
Md-RNase30   -------MSS AVKWTVF.LI ILNLTH---- ---------- ---FYQ..D. WTL.V.YPKT KA.GKPPE.. .NI..Q.GLI
Md-RNase32   -------MDC AVMWILF..S FMHLAN---- ---------- ---V.QSQD. WK..V.YPRT EV.GGPSK.. .A.VKQ.GLE
                ↓*                   ↓          ↓         ↓           ↓             ↓
Hs-RNase1    -QGRCKPVNT FVHEPLVDVQ NVCFQEKVT- --CKNGQ--G NCYKSNSSMH ITDCRLTNGS RYPN---CAY RTSPKE-RHI 160
Bt-RNase14   HR....QI.. .I..D.ATIA DF.TTPA.P- --.TSSGSLL S.HN.SHDVS V...F-AKAG TR.P--Y.H. QKKDSI-.P.
Bt-RNase15   TDQS..KNK. ...SVSPGTR GL.EGPA..- --.RKMSEVY ..H--LIIFK V.Q.N-LYPE AV.P--H.Y. EGVTFQ-MDV
Md-RNase16   -KRK...... .L..SYQNIE .I.HEPN.P- --.A.KNM-H ..H...HP.K ..E.H.SG.. KPGK---.R. .MNNVK-KNV
Md-RNase17   -K.N..VF.. .IL.SIWKIR AI.WNK.T-- -A..QKF--F ..HM.REPLK V.E.QIKGNP KDSP---.K. Q.INDK-KCV
Md-RNase18   --E....K.. II.DSPDKIR DI.TNPNS.N VR.R..S--D ...EGPNPFS V.V.ED.GSI YSVYP-N.Q. NCHKE.PVKV
Md-RNase19   -TH...SF.. .LDYM.E.II .A..TPNI.- --...L.--K ..H..TLKVP ..N.V..SDH YPN----.R. HGIS.M-AFF
Md-RNase20   -TNV..AF.S .L.NQTQNII ...LNPNTP- --...SR--T ..HN.TFRND ..E.T..R.Q FPN----.Q. .GRAVM-KYF
Md-RNase21   -NNA..GF.S .L.NQTQNII ....HPNIF- --.R.NE--T ..HR.IFRNS ..E..SAG.Q YPD----.Y. NGTAVR-KYF
Md-RNase22   ER.K..RE.. .I.AMAGAIL D..KAQS.K- -A..V.R--K ..HT.DH.FK Q.L.V.QGFG Y...----.. VSF..F-KK.
Md-RNase23   RK.S..SI.. .I.SHKNE.N K..KEKGTF- ID..QNRNYK C.FI.KIKFN L.I.E--EKE SRKS---.E. HALK..-A.V
Md-RNase24   WGNK.V.G.. .I..KNSTIE .M.NRTPRP- --...HRN-Q F.HT.VR.FS VS..F-LE.G SQ.P--Y.K. WVLVQS-.K.
Md-RNase25   TRNH.FQI.A .I..TNSTL. .I.KTSPK.- --.GGPS-WL L.HE.SKPLK L.Y.S-.QSY AR.P--N.G. KAQAFS-EK.
Md-RNase26   NNSD...F.S .I..SNKTIF .I.ADSYGS- FQDP.E---L ...E.TYPLR ....FARCI. NCYP--R.S. VEIFHL-AK.
Md-RNase28   IHNS.VYFT. .I..MNQTIL .I.ANSN.H- HQ.P..---L T.HI.PYPIM V...F-MKLD ANFP--D.R. .GKIYF-TR.
Md-RNase29   MHDS.LYFH. .I..TNQTLL .I.ANSN.H- HE.L.R---L T.HI.HYPLM V...F-KIAH PNVP--D.R. .GKIYF-TR.
Md-RNase30   NNTYLR.FH. .I..SNSTIL .I.ANSTNP- HYASFA---P .S.E.QNPL. L.E.---CIK TCSPNFV.T. KEVIRM-SK.
Md-RNase32   M.NS.LYS.I .I..TNHSIV .I.ENTNHC- FPSFK.---L P.HR.LYPVL V.H.F-IRLH SVFP--H.R. .GEIYF-AR.

                ↓                 *
Hs-RNase1    IVACEG---- ----SPYVPV HFDASVEDST ---------- ---------- ------ 216
Bt-RNase14   C.G.KN---- ------GA.A .L.S------ ---------- ---------- ------
Bt-RNase15   RIV.V.---- ------KR.I .L.E------ ---------- ---------- ------
Md-RNase16   T...V.---- ----KTLK.. .L.PPK---- ---------- ---------- ------
Md-RNase17   T...N.---- ----W.PL.. ...S.KD.IC QRLPTT---- ---------- ------
Md-RNase18   T....N---- ------GK.. .....----- ---------- ---------- ------
Md-RNase19   VI..NPPLLA DHSK.KLL.. .L.S.T.GPG PILLSS---- ---------- ------
Md-RNase20   VI..NS--IV --PRG.LL.. .L.NTF---- ---------- ---------- ------
Md-RNase21   V...IP--IS --PSGRLL.. .L.STFWNPG QVFLLPPP-- ---------- ------
Md-RNase22   SL..T.---- ----L.R... YV.P------ ---------- ---------- ------
Md-RNase23   VLG.NN---- -----NNQ.. ..QGYFCETK PYRVYMEKRN LNDHLPSTYM SGAQEE
Md-RNase24   S.F.QY---- ------EL.F .LED------ ---------- ---------- ------
Md-RNase25   L.V.VR---- ------GS.I YLFQT----- ---------- ---------- ------
Md-RNase26   R...VN---- ------GR.Q RIIRQIYCPP FWIRYWVPYG YYTYP----- ------
Md-RNase28   K.I.VN---- ------GR.K YVFSQAYLPP F--------- ---------- ------
Md-RNase29   K.I.VK---- ------GR.E YLIHFPDLKE ---------- ---------- ------
Md-RNase30   RL..LN---- ------GH.D .LITYINY.P TKILINLLFG GPRR------ ------
Md-RNase32   KII.MN---- ------GK.G FII------- ---------- ---------- ------
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Fig. 4. Amino acid sequence alignment of the novel RNases identified in this study. Dashes show alignment gaps and dots represent the same amino acids as in human RNase 1. The eight structural cysteines are indicated by arrows, whereas the three catalytic residues are indicated by "*". The conserved CKXXNTF motif is highlighted in a box. The predicted first amino acid residue of the mature peptide is underlined. We used the SignalP 3.0 server (http://www.cbs.dtu.dk/services/SignalP) to infer the signal peptide cleavage sites.

superfamily. First, they form a cluster on the chromosome with the known RNase genes (Fig 1), suggesting that they are produced by tandem gene duplications. Second, the ORFs of these genes are contained in a single exon, as in all other RNase genes. Third, except for a single gene, Md-RNase 18 (see below), all of them have a distinct signal peptide sequence (Fig. 4), suggesting that they function in the extracellular environment. Fourth, they possess the signatures of the superfamily, such as the catalytic triad [note that some of the novel ribonucleases (Md-RNases 22–32) do not have the catalytic triad like RNase 9–13], the "CKXXNTF" motif, the "PVHXD/E" motif, and the conserved cysteines important for forming disulfide bonds, although the degree of conservation varies among the genes (Table 2).

Table 2
Surveys of the important amino acid residues in the novel RNase genes identified from the cow and opossum

Gene name	H_{12}	$CK_{41}XXNTF$	$PVH_{119}XD/E$	No. of conserved cysteines
Bt-RNase14	+	**CK**QI**NTF**	**P**A**H**L**D**	8
Bt-RNase15	+[a]	**CK**KN**NTF**	**P**I**H**L**D**	8
Md-RNase16	+	**CK**PV**NTF**	**PVH**L**D**	8
Md-RNase17	+	**CK**VF**NTF**	**PVH**F**D**	8
Md-RNase18	+	**CK**PK**NT**I	**PVH**F**D**	7
Md-RNase19	+	**CK**SF**NTF**	**PVH**L**D**	8
Md-RNase20	+	**CK**AF**NSF**	**PVH**L**D**	8
Md-RNase21	+	**CK**GF**NSF**	**PVH**L**D**	8
Md-RNase22	+	**CK**RE**NTF**	**PV**YV**D**	8
Md-RNase23	+	**CK**SI**NTF**	**PVH**FQ	8
Md-RNase24	+	**C**VPG**NTF**	**P**F**H**L**E**	8
Md-RNase25	+	**C**FQI**NAF**	**P**IYLF	8
Md-RNase26	+	**CK**PF**NSF**	**P**QRII	7
Md-RNase28	+	**C**VYFT**TF**	**P**KYVF	8
Md-RNase29	+	**C**LYFH**TF**	**P**EYLI	8
Md-RNase30	+	LRPFH**TF**	**PD**H**L**I	5
Md-RNase32	+	**C**LY**SNIF**	**P**GFII	7

[a] H_{12} shifted upstream by one amino acid.

The two novel cow genes, Bt-RNases 14 and 15, have an intact set of catalytic triad, a well-conserved "CKXXNTF" signature motif, and a complete set of eight conserved cysteines, suggesting that they have the ribonuclease activity. Similarly, these signatures are well-conserved in eight of the novel opossum genes (Md-RNases 16–23). Noticeably, two of these genes, Md-RNases 22 and 23, have an insertion of 5–15 amino acids between the signal peptide and the catalytic H_{12}, as in RNases 9–11 (Devor et al., 2004; Cho et al., 2005). Although with low bootstrap support, these two genes are indeed remotely related to RNases 9–11 (Fig. 2), suggesting that these new opossum genes may share ancestry with RNases 9–11. Interestingly, Md-RNase 18 does not have a signal peptide, an unprecedented exception to the RNase A superfamily. The absence of a signal peptide in Md-RNase 18 suggests that it may have acquired a new intracellular function. Its isoelectric point (p*I*) (6.46) is much lower than all other opossum RNases, which also suggests functional modifications. For the rest of the novel opossum genes (Md-RNases 24–32), the signature motifs are poorly conserved and some genes miss one to three of the structural cysteines (Table 2). Moreover, none of them has an intact catalytic triad that is required for the ribonuclease activity. Therefore, this latter group (Md-RNases 24–32) probably has lost the ribonuclease activity. It would be interesting to examine if these genes have acquired other functions.

3.5. Final remarks

In this paper, we report the identification of the entire RNase A superfamily from the genome sequences of the dog, cow, and opossum. We discovered 17 novel RNase genes, 2 from the cow and 15 from the opossum. Phylogenetic analysis of these genes and previously identified RNase genes elucidates how the repertoires of the superfamily changed during mammalian evolution (Fig. 5). Briefly, the superfamily started off as an RNase with the structural features of angiogenin in early vertebrate evolution. After the bird–mammal separation, but before the split between placental and marsupial mammals, an extensive expansion occurred in ancestral mammals that resulted in most of the extant members of the superfamily. After this split, marsupials underwent further expansions resulting in RNases 19–21 and 24–32. The common ancestor of all placental mammals lost at least two genes (orthologous to Md-RNases 16, 17 and 19–23), and subsequently two other genes (orthologous to Bt-RNases 14 and 15) were lost in the common ancestor of primates and rodents, after it diverged from carnivores and artiodactyls. There was a massive gene loss in the dog RNase superfamily, further reducing the size of the repertoire. In several individual lineages of RNase genes, gene duplication and gene loss occurred in various orders of placental mammals. For example, rodent EARs and angiogenins underwent expansions and gene sorting. In addition, similar processes

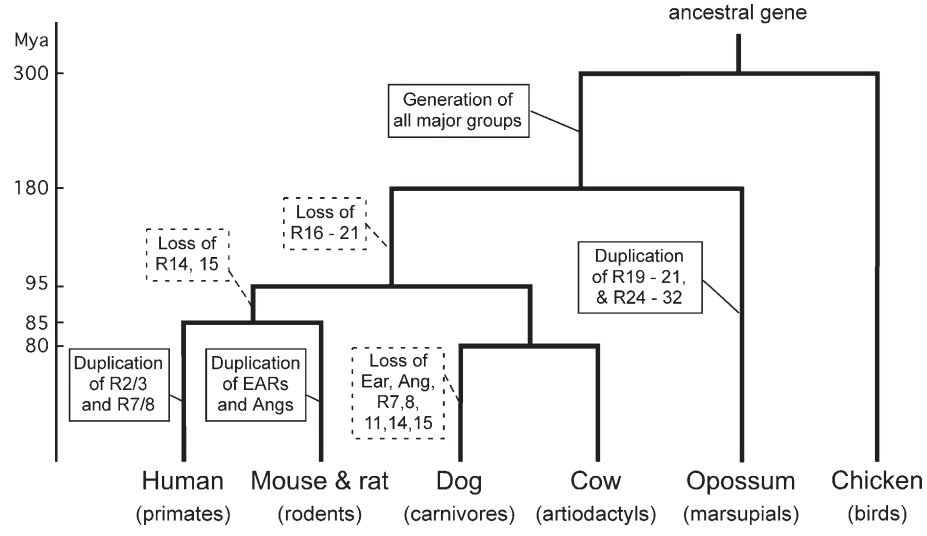

Fig. 5. Evolutionary history of the RNase A superfamily shown on the mammalian phylogeny. The phylogeny was adopted from Murphy et al. (2004). Major changes in the superfamily are labeled on the branches.

may have also occurred for the RNase 6 genes of placental mammals.

Before this study, the genome-scale survey of RNase genes was conducted only in the human, mouse, rat, and chicken, with the impression that the major lineages of the superfamily should be conserved across placental mammals. Our present survey in the dog, cow, and opossum not only identified many novel RNase genes, but also revealed a clear pattern that the superfamily underwent dynamic evolutionary changes within mammals. The reasons underlying these dynamic changes are unclear. We previously conjectured that the superfamily was initially used for innate immunity (Cho et al., 2005). Being in need of responding to ever-changing pathogens, the superfamily might have been under an evolutionary pressure for diversification through recurrent expansions and gene sorting. Other gene families involved in host-defense systems are also known to have undergone such gene birth-and-death or gene sorting processes (Hughes and Nei, 1988; Cadavid et al., 1997; Nei et al., 1997; Sitnikova and Nei, 1998; Su et al., 1999).

In spite of the analysis of several completely sequenced mammalian genomes, our understanding of the evolution of the RNase A superfamily requires more species (Fig. 5). One of the unsolved mysteries is how the major groups of the superfamily were generated during early mammalian evolution. Because non-mammalian vertebrates have only a limited number of closely related RNases, the major expansion must have taken place after the bird–mammal separation. Further studies in more primitive mammals, such as monotremes, are required to determine the time of the mammalian expansion with better resolution. Another interesting question is why the evolutionary paths that marsupial RNase genes took are different from those of placental mammals. The genome of tammar wallaby (*Macropus eugenii*), another marsupial mammal, is currently being sequenced, and this will allow further investigations toward answering the above question.

The dog and cow genome sequences we analyzed are based on domesticated animals. Would this factor bias our interpretation of RNase evolution? For three reasons, we think domestication should not affect our results. First, domestication is extremely short (~0.01 My) compared to the evolutionary time we consider in this work (~200 My). Thus, the impact of domestication on RNase evolution is expected to be minimal. Second, it is difficult to imagine any traits controlled by RNases that would have been the targets of artificial selection. Third, it is also difficult to imagine that the animal species chosen to be domesticated by our ancestors would be a biased set with regard to RNase genes.

Our discovery of the novel RNase genes opens the door for future investigations on their functions. The high divergence from other members of the superfamily (Fig. 2) and the non-conventional patterns of functional motifs (Table 2) suggest that they have functions different from the previously studied RNases. In particular, the signal-peptide-lacking Md-RNase 18 is worth investigation. The RNase A superfamily has served as an excellent experimental model for studying the evolution of new gene functions. Studies on these novel RNases will not only broaden our understanding of the functional diversity of the superfamily, but also provide general insights into the processes by which new functions emerge after gene duplication (Zhang, 2003).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.gene.2006.01.018.

References

Anfinsen, C.B., 1973. Principles that govern folding of protein chains. Science 181, 223–230.

Barnard, E.A., 1969. Biological function of pancreatic ribonuclease. Nature 221, 340–344.

Beintema, J.J., Kleineidam, R.G., 1998. The ribonuclease A superfamily: general discussion. Cell. Mol. Life Sci. 54, 825–832.

Blackburn, P., Moore, S., 1982. Pancreatic Ribonuclease. Academic Press, New York.

Cadavid, L.F., Shufflebotham, C., Ruiz, F.J., Yeager, M., Hughes, A.L., Watkins, D.I., 1997. Evolutionary instability of the major histocompatibility complex class I loci in New World primates. Proc. Natl. Acad. Sci. U. S. A. 94, 14536–14541.

Castella, S., Benedetti, H., de Llorens, R., Dacheux, J.L., Dacheux, F., 2004a. Train A, an RNase a-like protein without RNase activity, is secreted and reabsorbed by the same epididymal cells under testicular control. Biol. Reprod. 71, 1677–1687.

Castella, S., et al., 2004b. Identification of a member of a new RNase a family specifically secreted by epididymal caput epithelium. Biol. Reprod. 70, 319–328.

Cho, S., Beintema, J.J., Zhang, J., 2005. The ribonuclease A superfamily of mammals and birds: identifying new members and tracing evolutionary histories. Genomics 85, 208–220.

Confalone, E., et al., 1995. Molecular evolution of genes encoding ribonucleases in ruminant species. J. Mol. Evol. 41, 850–858.

D'Alessio, J., Riordan, J.F., 1997. Ribonucleases: Structures and Functions. Academic Press, New York.

Devor, E.J., Moffat-Wilson, K.A., Galbraith, J.J., 2004. LOC 390443 (RNase 9) on chromosome 14q11.2 is related to the RNase a superfamily and contains a unique amino-terminal preproteinlike sequence. Hum. Biol. 76, 921–935.

Domachowske, J.B., Dyer, K.D., Adams, A.G., Leto, T.L., Rosenberg, H.F., 1998a. Eosinophil cationic protein RNase 3 is another RNaseA-family ribonuclease with direct antiviral activity. Nucleic Acids Res. 26, 3358–3363.

Domachowske, J.B., Dyer, K.D., Bonville, C.A., Rosenberg, H.F., 1998b. Recombinant human eosinophil-derived neurotoxin/RNase 2 functions as an effective antiviral agent against respiratory syncytial virus. J. Infect. Dis. 177, 1458–1464.

Dyer, K.D., Rosenberg, H.F., 2005. The mouse RNase 4 and RNase 5/ang 1 locus utilizes dual promoters for tissue-specific expression. Nucleic Acids Res. 33, 1077–1086.

Felsenstein, J., 1985. Confidence-limits on phylogenies—an approach using the bootstrap. Evolution 39, 783–791.

Fett, J.W., et al., 1985. Isolation and characterization of angiogenin, an angiogenic protein from human carcinoma-cells. Biochemistry 24, 5480–5486.

Harder, J., Schroder, J.M., 2002. RNase 7, a novel innate immune defense antimicrobial protein of healthy human skin. J. Biol. Chem. 277, 46779–46784.

Hedges, S.B., Parker, P.H., Sibley, C.G., Kumar, S., 1996. Continental breakup and the ordinal diversification of birds and mammals. Nature 381, 226–229.

Hosoya, K., et al., 1990. Primary structure of an alkaline ribonuclease from bovine liver. J. Biochem. 107, 613–618.

Hughes, A.L., Nei, M., 1988. Pattern of nucleotide substitution at major histocompatibility complex class-I loci reveals overdominant selection. Nature 335, 167–170.

Jermann, T.M., Opitz, J.G., Stackhouse, J., Benner, S.A., 1995. Reconstructing the evolutionary history of the artiodactyl ribonuclease superfamily. Nature 374, 57–59.

Kumar, S., Tamura, K., Nei, M., 2004. MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. Brief. Bioinform. 5, 150–163.

Maes, P., Damart, D., Rommens, C., Montreuil, J., Spik, G., Tartar, A., 1988. The complete amino-acid sequence of bovine-milk angiogenin. FEBS Lett. 241, 41–45.

Murphy, W.J., Pevzner, P.A., O'Brien, S.J., 2004. Mammalian phylogenomics comes of age. Trends Genet. 20, 631–639.

Nei, M., Gu, X., Sitnikova, T., 1997. Evolution by the birth-and-death process in multigene families of the vertebrate immune system. Proc. Natl. Acad. Sci. U. S. A. 94, 7799–7806.

Penttinen, J., Pujianto, D.A., Sipila, P., Huhtaniemi, I., Poutanen, M., 2003. Discovery in silico and characterization in vitro of novel genes exclusively expressed in the mouse epididymis. Mol. Endocrinol. 17, 2138–2151.

Rosenberg, H.F., Dyer, K.D., Tiffany, H.L., Gonzalez, M., 1995. Rapid evolution of a unique family of primate ribonuclease genes. Nat. Genet. 10, 219–223.

Rosenberg, H.F., Zhang, J., Liao, Y.D., Dyer, K.D., 2001. Rapid diversification of RNase a superfamily ribonucleases from the bullfrog, *Rana catesbeiana*. J. Mol. Evol. 53, 31–38.

Saitou, N., Nei, M., 1987. The neighbor-joining method—a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4, 406–425.

Singhania, N.A., et al., 1999. Rapid evolution of the ribonuclease A superfamily: adaptive expansion of independent gene clusters in rats and mice. J. Mol. Evol. 49, 721–728.

Sitnikova, T., Nei, M., 1998. Evolution of immunoglobulin kappa chain variable region genes in vertebrates. Mol. Biol. Evol. 15, 50–60.

Strydom, D.J., 1998. The angiogenins. Cell. Mol. Life Sci. 54, 811–824.

Strydom, D.J., et al., 1985. Amino-acid sequence of human-tumor derived angiogenin. Biochemistry 24, 5486–5494.

Su, C., Jakobsen, I., Gu, X., Nei, M., 1999. Diversity and evolution of T-cell receptor variable region genes in mammals and birds. Immunogenetics 50, 301–308.

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., Higgins, D.G., 1997. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 25, 4876–4882.

Young, J.D., Peterson, C.G.B., Venge, P., Cohn, Z.A., 1986. Mechanism of membrane damage mediated by human eosinophil cationic protein. Nature 321, 613–616.

Zhang, J., 2003. Evolution by gene duplication: an update. Trends Ecol. Evol. 18, 292–298.

Zhang, J., Rosenberg, H.F., 2002a. Complementary advantageous substitutions in the evolution of an antiviral RNase of higher primates. Proc. Natl. Acad. Sci. U. S. A. 99, 5486–5491.

Zhang, J., Rosenberg, H.F., 2002b. Diversifying selection of the tumor-growth promoter angiogenin in primate evolution. Mol. Biol. Evol. 19, 438–445.

Zhang, J., Zhang, Y., 2003. Pseudogenization of the tumor-growth promoter angiogenin in a leaf-eating monkey. Gene 308, 95–101.

Zhang, J., Rosenberg, H.F., Nei, M., 1998. Positive Darwinian selection after gene duplication in primate ribonuclease genes. Proc. Natl. Acad. Sci. U. S. A. 95, 3708–3713.

Zhang, J., Dyer, K.D., Rosenberg, H.F., 2000. Evolution of the rodent eosinophil-associated RNase gene family by rapid gene sorting and positive selection. Proc. Natl. Acad. Sci. U. S. A. 97, 4701–4706.

Zhang, J., Dyer, K.D., Rosenberg, H.F., 2002a. RNase 8, a novel RNase A superfamily ribonuclease expressed uniquely in placenta. Nucleic Acids Res. 30, 1169–1175.

Zhang, J., Zhang, Y., Rosenberg, H.F., 2002b. Adaptive evolution of a duplicated pancreatic ribonuclease gene in a leaf-eating monkey. Nat. Genet. 30, 411–415.

Zhang, J., Dyer, K.D., Rosenberg, H.F., 2003. Human RNase 7: a new cationic ribonuclease of the RNase A superfamily. Nucleic Acids Res. 31, 602–607.