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The ribonuclease A superfamily: general discussion

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Abstract. Enzymic properties of members of the ribonuclease A superfamily, like the activity on RNA, the preference for either cytosine or uracil in the primary binding site B_1, the preference for the other side of the cleaved phosphodiester bond, the B_2 site, and features of the two noncatalytic phosphate-binding sites P_0 and P_2 are discussed in several articles in this multi-author review, and are summarized in this closing article. A special feature of members of the ribonucleases 1 family is their destabilizing action on double-stranded nucleic acid structures. A feature of the ribonuclease A superfamily is the frequent occurrence of gene duplications, both in ancestral vertebrate lineages and in recently evolved taxa. Three different bovine ribonucleases 1 have been identified in pancreas, semen and brain, respectively, which are the result of two gene duplications in an ancestral ruminant. Similar gene duplications have been identified in other ribonuclease families in several mammalian and other vertebrate taxa. The ribonuclease family, of which the human members have been assigned numbers 2, 3 and 6, underwent a still mysterious pattern of gene duplications and functional expression as proteins with ribonuclease activity and other physiological properties.

Key words. Ribonuclease A; pyrimidine base specificity; eosinophil-derived neurotoxin; eosinophil cationic protein; angiogenin; gene duplication.

Bovine ribonuclease A (RNase 1) has been and is still being intensively studied as a chemical entity, irrespective of its proposed digestive biological role in ruminants [1]. As far as we know, this role has never yet been investigated by comparative physiologists studying digestive systems of mammals. At the other end of the spectrum are many current studies on the role ribonucleases play in many biological processes, with emphasis both on several human ribonucleases and on others with potential application in human medicine. Human ribonuclease 1 occupies a kind of orphan position in between. But it may be used as a carrier which can be modified by introducing novel biological activities as derived from studies of nonhuman ribonucleases, for use as a less antigenic molecule in human medicine [2].

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The ribonuclease 1 content of human pancreas is less than 1% of that of bovine pancreas, and is even less than that of human kidney and spleen, for example [3, 4]. However, Northern analysis indicates a relatively higher messenger RNA (mRNA) content for ribonuclease 1 in pancreas than in other investigated human organs [5], which may be explained by the secretory function of the pancreas.

The amino acid sequences of ribonucleases 1 from humans and langur monkey (a primate species with ruminant-like digestion) differ at 14 (11%) of the positions [6]. A comparison of these two orthologous ribonucleases is interesting, as we have here an example of two species within one mammalian order, one of which has a ribonuclease 1 which shows adaptations to ruminant-like digestion. We advanced the hypothesis that species with ruminant-like digestion not only have a higher ribonuclease content in their pancreas [1] but also have less excess of positive charge and fewer attached carbo-

hydrates [7–9], which is in agreement with data presented in table 1.

Enzymic properties

Generally the first property of a new member of the superfamily to be investigated is its depolymerizing activity on RNA, which usually is measured around pH 7.5 with commercially available yeast RNA. A relatively high activity on this substrate, like that observed with bovine ribonuclease A, may indicate that the enzyme is adapted to digest foreign RNA, and this property has been demonstrated for several, evolutionarily weakly related members of the superfamily. All mammalian ribonucleases 1 (except bovine brain ribonuclease), human ribonuclease 2 [10], bovine ribonuclease 6 [11] and the reptilian turtle and iguana pancreatic ribonucleases [12, 13] have similar specific activities on RNA, while a four times higher value has been reported for porcine ribonuclease 6 [14]. Specific activities on RNA relative to bovine pancreatic ribonuclease of bovine brain ribonuclease, chicken liver ribonuclease and frog ribonucleases are 20 [15], 5 [16] and 10% [17], respectively. Human ribonuclease 3 (ECP), New World monkey ribonucleases 2/3 [18], human ribonuclease 6 [18] and the angiogenins (ribonucleases 5) [19] have very low depolymerizing activities on RNA. However, ribonucleases with a low specific activity in depolymerizing unspecific RNA may be more active on specific substrate molecules, as has been demonstrated for angiogenin [19]. For human ribonuclease 4, 2.5–4 times lower specific activities than for bovine pancreatic ribonuclease 1 have been reported, depending on the assay conditions [10, 20]. A rather high specific activity was found for the bovine ribonuclease 4 [21], but much lower ones for the porcine [22] and rat [23] enzymes. These discrepancies, which may be caused by differences in experimental protocols, need reinvestigation, as they do not concur with the very similar primary structures of mammalian ribonucleases 4 [24].

The enzymic activities of members of the ribonuclease A superfamily on nonnatural, but chemically well-defined substrates have been summarized in previous contributions of this multi-author review. Although these enzymes are pyrimidine-preferential, mammalian ribonucleases 1 like the enzyme from bovine pancreas also cleave polyadenylate (poly(A)) [10].

Polycytidylate (poly(C)), polyuridylate (poly(U)), dinucleotides and nucleoside 2′,3′-cyclic phosphates ($C > p$ and $U > p$) have been used to investigate the relative preference for cytosine or uracil in the primary substrate binding site B_1 (the code for the binding sites is explained in Fig. 1 in [25]). Mammalian ribonucleases 1 have a strong preference for poly(C) over poly(U), ribonucleases 2 and 3 prefer poly(U) slightly over poly(C), while ribonucleases 4 have a high preference for poly(U) [10, 24]. (Hosaya et al. [21] report a higher preference of bovine ribonuclease 4 for poly(C) over poly(U), but this was not found by Zhao et al. [23]). Other poly(U) preferential ribonucleases are the frog ribonucleases [17], and the enzyme from iguana pancreas [16], while the enzymes from turtle pancreas [13] and chicken liver [12] have a preference for cytosine. Several mammalian ribonucleases 1 are three times less active on $C > p$ relative to $U > p$ than the bovine pancreatic enzyme [26], for which amino acid replacements in the C-terminal region of the sequence may be responsible.

There are no unique amino acid replacements, which may explain a shift from cytosine to uracil preference. Hofsteenge et al. [24] summarized several experiments performed on porcine ribonuclease 4, which point to an important role of the replacement of Val-43 in ribonuclease 1 by Phe in porcine ribonuclease 4. Furthermore Asp-83 and probably also the shortened C-terminal tail of ribonucleases 4 may play a role. However, following a suggestion by Mosimann et al. [27], it may also be that the presence of a basic residue at either position 42 or 43 as observed in frog ribonucleases [28], and in the enzyme from iguana pancreas [16], may explain a preference for uracil.

Table 1. Several characteristics of human and langur pancreatic ribonucleases.

	Human	Langur
μg/g Tissue	5	280
Net charge	+6	+1
Number of possible glycosylation sites (Asn-X-Ser/Thr sequences)	3	2
Extent of glycosylation	all molecules; extensive glycosylation of one site in all molecules and of another site in about half of the molecules	part of the molecules; one simple chain

There is less specificity for the base at the other side of the cleaved phosphodiester bond, the B_2 site. However, bovine pancreatic ribonuclease has a strong preference for adenine at that position. Several residues in a loop structure, connected by the Cys-65–Cys-72 disulphide bridge, contribute to this site. Irie et al. [28] summarized evidence that members of the superfamily in which this loop is lacking, like the frog ribonucleases, and the enzymes from chicken liver [12] and turtle pancreas [13] have a preference for cleaving next to guanine. Human angiogenin, which also lacks the loop with the B_2 site, still favours A over G by a factor of about 3, which is, however, much less than the 17-fold preference observed for bovine pancreatic ribonuclease [29]. The activity of an angiogenin mutant in which the bovine ribonuclease loop with residues 58–70 has been introduced increased 5–10 times on CpG and UpG, but more than 200 times on CpA and UpA, which also supports the role assigned to this loop in the B_2 site specificity [29].

The phosphate-binding site p_0 involves Lys-66 in bovine pancreatic ribonuclease [25], which also is located in the deleted loop in members of the superfamily mentioned above. It is possible that the role of this residue is taken over by a positive group at position 122 in active enzymes like human ribonuclease 2 [18] and turtle ribonuclease [12]. However, the assignment of a residue with this role in the frog ribonucleases is not yet possible [28].

Nogués et al. [25] summarized evidence for the role of Lys-7 and Arg-10 in the phosphate-binding site p_2. The k_{cat} values on $C > p$ of bovine pancreatic ribunuclease mutants in which these two residues have been replaced by Gln are about 10 times smaller than those of the unmodified enzyme, although this substrate binds only to the catalytic B_1p_1 site [25]. It is tempting to correlate this observation with the lack of hydrolytic activity towards 2′,3′-cyclic phosphodiesters of human ribonuclease 2, an active transphosphorylating ribonuclease in which Lys-7 and Arg-10 are also replaced by neutral residues [10].

A special feature of mammalian ribonucleases 1 is their DNA-helix-destabilizing action and double-stranded (ds) RNA degrading activity [10]. These activities are a function of the number of positive charges present on the enzyme protein surface. However, specific interactions are also involved, as basic members of other families of the superfamily exhibit these activities to a much lower extent or not at all [10]. It is difficult to assign basic residues in mammalian ribonucleases 1, which may have the highest influence on their double-helix-destabilizing properties. Sorrentino [10] proposes several likely ones, of which Arg-39 may have a predominant role by interacting with the negatively charged phosphates of the double-helical substrate. This interaction could be disturbed by the presence of an acidic residue at position 38, as it occurs in bovine ribonuclease A [10]. This proposal is in agreement with observations by Jermann et al. [30] with a mutant of ribonuclease A concerning the influence that substitution at a single position (D39G) has on the extent of the enzymatic efficiency towards ds RNA. However, the interplay of several charged residues may be more complex considering the sequences of mammalian ribonucleases 1, their glycosylation characteristics and their capabilities to attack dsRNA [31].

Multiple gene products

A striking feature of the ribonuclease A superfamily is the multiplicity of representatives in investigated species, indicating a frequent occurrence of gene duplications, both in ancestral vertebrate lines and in recently evolved taxa. Table 1 in the Introduction of this multi-author review [32] presents four separate families recognized in mammals. The evolutionary origin of these families will be discussed in the last section of this Discussion paper.

We summarize below observations made in separate families:

Mammalian ribonucleases 1. Three different bovine ribonucleases 1 from pancreas, semen or seminal vesicles and brain, respectively, have been identified by protein and DNA sequencing. Evolutionary analyses of the sequences indicated that these proteins are the product of two gene duplications in an ancestral ruminant [9]. An indication from protein studies of a rather recent gene duplication of another ribonuclease 1 has been the finding of two different ribonuclease sequences in enzyme preparations from guinea pig pancreas [9].

Genomic DNA coding for ribonucleases 1 from several ruminant species has been amplified by polymerase chain reaction (PCR), cloned, and sequenced. Sequences orthologous with bovine pancreatic, brain and seminal vesicle ribonuclease have been identified in giraffe, two deer species and several bovid species [33–35]. The pancreatic-type sequences were generally in agreement with those previously determined at the protein level [9]. The brain-type sequences have several interesting features, but there are no indications that they will not have the same expression pattern as the bovine brain enzyme [33, 35]. However, the semen-type sequences have many typical characteristics of pseudogenes, like deletions and insertions leading to change of reading frames, premature stop codons, replacement of active-site residues and so on. These features were demonstrated in semen-type sequences of giraffe and sheep [33], kudu and cape buffalo [34], and two deer species [35]. Only in water buffalo were a normal open reading frame for seminal ribonuclease and an active enzyme in semen found [33].

The pseudogene character of the seminal ribonuclease gene in most investigated ruminant species confronts us with a problem concerning its evolutionary history. Two scenarios are possible. One is that, after its origin by gene duplication, it was an active gene all the time to the origin of the taxon with ox and water buffalo, which have active seminal enzymes, and that in the lines to deer, giraffe and sheep the capacity to express active seminal ribonuclease was lost independently. The other possibility is that, after the gene duplication in an ancestral ruminant, an inactive pseudogene was been conserved, which has been reactivated only in the ancestor of ox and water buffalo. Both scenarios are rather illogical [36].

Five sequences coding for ribonucleases 1 have been found in the DNA of mouse deer, a representative of the earliest diverged taxon of the ruminants (H. Warmels and H. J. Breukelman, unpublished observations). One is expressed in the pancreas. Two others probably are orthologues of the seminal- and brain-type ribonucleases from other ruminants, of which the former may be again a pseudogene. However, we also identified a gene which is a hybrid with rather high sequence similarities with the N-terminal region of the pancreatic, and with the C-terminal region of the brain-type sequence, respectively. The fifth gene is a pseudogene, rather similar in sequence to that of the gene coding for the pancreatic enzyme.

Southern blotting experiments of DNA of several mammalian species indicated the presence of one ribonuclease 1 gene, except for the investigated ruminant species [37]. However, evidently we had overlooked a second ribonuclease 1 gene in camel, which was found by PCR and could be sequenced. In pig, hippopotamus and two cetacean species (porpoise and false killer whale) one ribonuclease sequence was identified after PCR, cloning and sequencing. The pig and hippopotamus sequences were in reasonable agreement with the earlier determined protein sequences [9]. Evolutionary analyses of sequences indicated that gene duplications of ribonucleases 1 in ancestral ruminants and in a camel ancestor have been independent events, and that the whales may share an ancestor with hippopotamus (fig. 1), for which there is also evidence from sequence studies of other biomacromolecules [38].

In mouse only one ribonuclease 1 gene could be identified [9, 37]. However, results presented by Rosenberg et al. [39] indicate that in the genome of rat more than one ribonuclease gene is present. PCR amplification experiments of genomic DNA of more than 20 murid rodent species indicate the presence of only one ribonuclease 1 gene in most of them, except for species belonging to the genus *Rattus*, in which two to three ribonuclease 1 genes were found (J.-Y. Dubois, unpublished observations). One of these is expressed in the pancreas of *R. norvegicus* and *R. rattus*, but two are expressed in that of *R. exulans* (P. A. Jekel, unpublished observations).

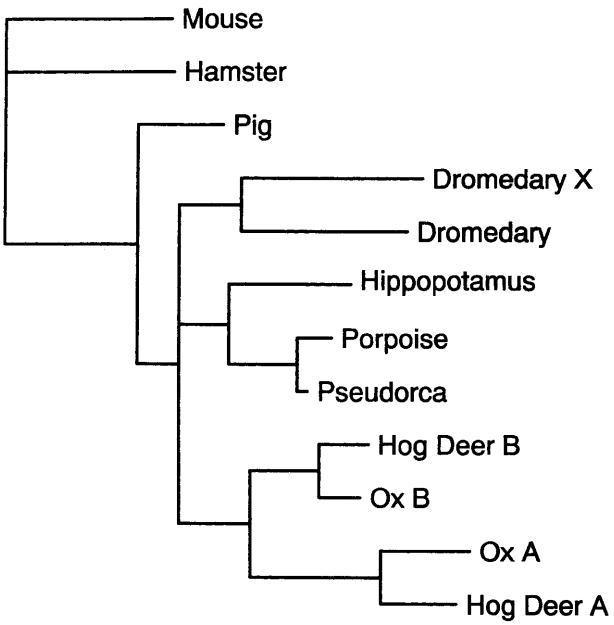

Figure 1. Unrooted tree of ribonuclease 1 sequences of several artiodactyls and cetaceans determined using the maximum likelihood procedure (R. G. Kleineidam et al., unpublished observations). A, pancreatic ribonucleases; B, brain-type ribonucleases [35]. A most parsimonious tree has the same topology. Branch lengths are proportional to evolutionary distances.

Mammalian ribonucleases 2, 3 and 6. This mammalian ribonuclease family is the most enigmatic one. Recent studies by the group of Rosenberg [18] and others have solved several puzzles, but at the same time have raised new questions. Sequence features clearly indicate a monophyletic protein family. However, the dendrogram presented in ref. 18 also suggests that there has been an ancestral gene duplication with one line leading to human, bovine and porcine ribonucleases 6, which have evolved with rates similar to those of the ribonucleases 1, The other line leads to rapidly evolving eosinophil proteins, identified so far in primates (with another gene duplication leading to the genes for ribonucleases 2 and 3 in a primate ancestor), mouse (six identified sequences, of which one pseudogene) and rat [40]. But the differences between these primate, mouse and rat sequences are so large that at this moment evolutionary hypotheses can only be very tentative. Zhao et al. [23] have isolated and characterized, but not yet sequenced, a ribonuclease from rat liver which is different from the rat eosinophil protein [40], and may be a ribonuclease 6.

The most peculiar fact about this ribonuclease family is that bovine, porcine and rat ribonucleases 6 have high activities on RNA, while that of human ribonucleases 6 is very low [18]. However, in humans eosinophil ribonuclease 2 (EDN) is a ribonuclease with high enzymic activity and a rather ubiquitous distribution in human organs and body fluids [3]. On the other hand, ribonucleases 2/3 in New World monkeys have very low activity. It is as if in an ancestral primate the (still unknown) function of the ribonuclease 6 gene product has been taken over by ribonuclease 2.

No mammals with more than one *ribonuclease 4* have been found yet.

Angiogenins: ribonucleases 5. In most investigated mammalian species, only one angiogenin sequence has been found, except in mouse (six identified sequences, including two pseudogenes) [19]. The evolutionary analysis presented by Strydom [19] indicates that the mouse genes duplicated after divergence from the other investigated mammalian species. However, a second bovine angiogenin sequence diverges from the other mammalian ones before species divergences. With this tree topology one would expect the occurrence of orthologous sequences of this second bovine angiogenin in other mammalian species. These have not been found yet.

The multiplicity of mouse ribonucleases 2 and 3 and angiogenins contrasts with the situation in the ribonuclease 1 family, where there is only one gene for the mouse enzyme, but several genes for rat. If a gene duplication has taken place, other duplications are facilitated by processes like unequal crossing-over as long as the duplicated sequences are still very similar. This may explain the fact that in the ribonuclease superfamily

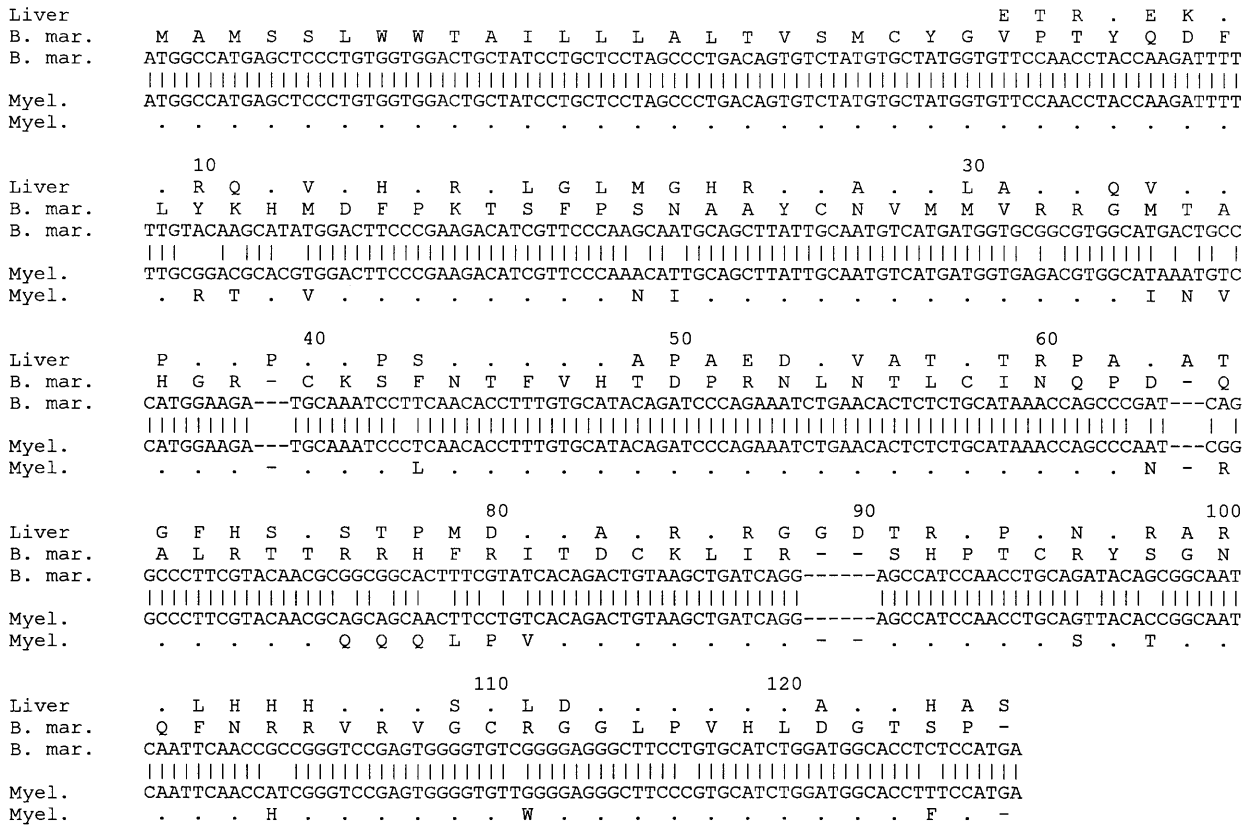

Figure 2. Amino acid and nucleotide sequence alignments of three members of the ribonuclease A superfamily identified in chicken: liver ribonuclease, sequenced as protein [16], and two ribonucleases from transformed bone marrow cells: B. mar. [42] and Myel. [41]. Identical nucleotides are indicated with vertical bars. Amino acid residues, identical to the ones in the B. mar. sequence, are indicated with dots. Dashes indicate deletions. Several residue numbers of the bovine pancreatic ribonuclease sequence are given on top of the alignments. The similarities between the two blood cell sequences extend outside the coding region: about 100 nucleotides are identical in the 5′ noncoding regions (except for 4 gaps involving 6 nucleotides in the B. mar. sequence) and about 140 nucleotides are identical in the 3′ noncoding regions (except for one substitution). (Although the Myel. sequence received the label 'angiogenin' in databanks, this property has not been demonstrated for the protein, and sequence comparisons do not indicate that it is an orthologue of mammalian angiogenins.)

frequently repeated gene duplications have occurred in separate branches of the evolutionary tree.

Bird ribonucleases. Sequences of three members of the superfamily in birds are known (fig. 2). All three are from chicken, indicating that they are paralogous gene products. The sequence similarities between chicken liver [16] and the other two ribonucleases [41, 42] (about 38%) are higher than those between any of the three and other members of the superfamily, indicating that the gene duplications occurred after divergence of the birds from their reptilian stock. The amino acid sequences of the enzymes from myelomonocytic cells [41] and from bone marrow cells of similar origin [42] are 85% identical. Figure 2 shows that also at noncoding positions of the genes there are many identities, and the pattern of long stretches of identities interrupted by less conserved sequence parts indicates that the genes coding for the two proteins may be closely linked and have undergone recent gene conversion(s).

Only two *reptilian ribonuclease* sequences are known. These are the pancreatic enzymes from turtle [12] and iguana [13], which differ at 54% of the amino acid positions.

Amphibian ribonucleases. Irie et al. [28] present a comprehensive discussion of structures and properties of four frog ribonucleases. Recently, a fifth amphibian member of the superfamily has been discovered (also mentioned by Strydom [19]). This is one of two secreted proteins in toad (*Xenopus*) which activate the fibroblast growth factor (FGF) receptor [43]. The amino acid sequence of this protein translated from complementary DNA (cDNA) is about 35% identical to those of the frog ribonuclease sequences (not 27% [43]). This value is higher than the sequence similarities of toad and frog ribonucleases with other members of the superfamily (20–25%) and suggests that the amphibian ribonucleases are monophyletic and that the ligand for the FGF receptor and the four frog proteins are the result of a gene duplication which occurred not long after divergence of the amphibians and the amniotes (reptiles, birds and mammals). An interesting feature of this *Xenopus* protein is that it has an extension at its C-terminus, similar to those observed in ruminant brain ribonucleases 1 [33, 35].

The two sialic acid-binding lectins (cSBL and jSBL [28]) from two different frog species are probably orthologous proteins, but the other frog ribonucleases discussed in [28] are paralogous ones.

Evolution of the families: concluding remarks

Strydom [19] and Irie and co-workers [14, 16] have derived evolutionary relationships between members of separate families of the ribonuclease A superfamily and present phylogenetic trees in their publications. If the most divergent family of the frog ribonucleases is positioned at the root of the tree, the following bifurcation is a branch leading to the other nonmammalian ribonucleases and mammalian angiogenins, and one to mammalian ribonucleases 1, 4, 2/3 and 6. Irie et al. also grouped mammalian angiogenins with the nonmammalian members of the superfamily because of the absence of a loop structure, connected by the Cys-65–Cys-72 disulphide bridge [28]. (The recent gain of this bridge in evolutionary time argues against the hypothesis that it may play a role as folding nucleus in bovine pancreatic ribonuclease, as suggested by in vitro studies of regeneration of ribonuclease A from the reduced protein [44].)

Other unambiguous conclusions about evolutionary relationships between the ribonuclease A families are difficult to make, except for the already discussed monophyly of mammalian ribonucleases 2/3 and 6, and a possible monophyly of the reptilian pancreatic ribonucleases from turtle and iguana [19], although they differ at more than 50% of the amino acid positions [13].

Members of the ribonuclease A superfamily bind cytoplasmic protein ribonuclease inhibitors (RI) with very high affinities [45]. In a previous review [9] we pointed to the paradox that these cytoplasmic inhibitors bind to noncytoplasmic ribonucleases. However, since then much evidence has been obtained that many of these ribonucleases are internalized in cells to exert specific biological actions (several chapters in [46]). Within mammalian orders there is little species specificity in ribonuclease-inhibitor interactions. But ribonucleases and inhibitors from different vertebrate classes (mammals, birds, amphibians) do not bind to each other [28, 47]. This means that if the angiogenins and the other mammalian ribonuclease families are not monophyletic, the ability to react with mammalian ribonuclease inhibitors may have developed independently. Also, X-ray studies show that residues in the binding interfaces of angiogenin and ribonuclease 1 with the inhibitor are quite different (except for the active site residue 41) [19].

By inspection of recently collected expressed sequence tag (EST) data, Strydom [19] discovered that human and mouse angiogenins and ribonucleases 4 mRNAs may share identical 5′ untranslated regions as a result of deletion of extensive parts of the genes during splicing. This should be the result of a more recent development 'linking' the angiogenins again closer to the other mammalian ribonuclease familes.

If many ribonuclease lineages evolved rather recently during vertebrate evolution, developing a large array of specific biological functions, what kind of molecules exercize these functions in more distantly related taxa? A major question therefore is: When and how will

members of the ribonuclease A superfamily in fishes or invertebrates be discovered? Will they still be ribonucleases? Will there still be some sequence similarity, or will homology be suggested only by a common polypeptide fold?

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