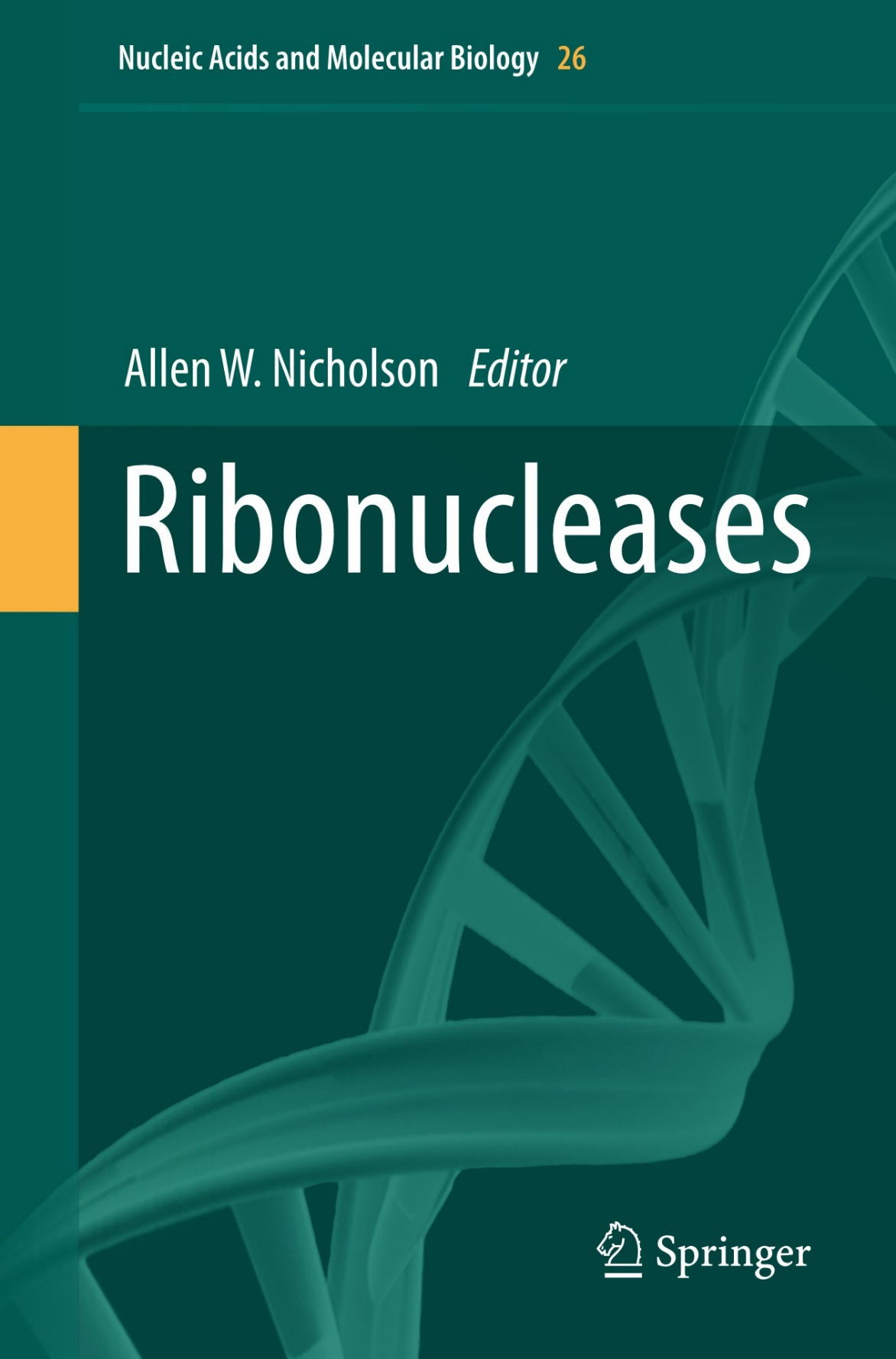
Nucleic Acids and Molecular Biology 26

Allen W. Nicholson *Editor*

Ribonucleases



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26

Series Editor
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Preface

Ribonucleases continue to attract much interest and investigation in the basic and translational science arenas. Our present understanding of ribonuclease structures, mechanisms, and functions emerged from a myriad of pioneering investigations that employed (as well as led to the development of) diverse experimental approaches. These studies have shed light on the fundamental aspects of biological catalysis and protein folding and ribonuclease function in post-transcriptional regulatory pathways. Indeed, multiple volumes would be needed to provide a comprehensive coverage of ribonucleases. It is instead the intent of this single volume to present a focused collection of reviews on the major groups of ribonucleases, and how their structures and mechanisms relate to biological function. The first three chapters by D'Alessio, Rosenberg, Vilanova, and coauthors focus on the fascinating family of vertebrate secreted ribonucleases, within which pancreatic ribonuclease A has served as the founding member. The extraordinary functional and evolutionary diversity of these enzymes is discussed along with their promise as anticancer agents. The chapters contributed by MacIntosh, Ivanov, Anderson, Meyers, and coauthors focus on the ribonuclease T2 family enzymes. Here, only recently has there been an appreciation gained of the central involvement of T2 family members in stress responses, host defense, and strategies of viral infection. The chapter by Tong and coauthors examines the structures and functions of 5′–3′ exoribonucleases, and the chapter by Arraiano and coauthors provides a comprehensive review of the diverse group of 3′–5′ exoribonucleases. The multisubunit RNA exosome, with its 3′–5′ exonuclease (and endonuclease) activity, is examined by Hopfner and Hartung, with a special focus on how specificity and regulation can be achieved in an otherwise nonselective manner of RNA breakdown. Condon and Gilet address the mechanistically and functionally intriguing metallo-β-lactamase family enzymes and their roles in processing tRNAs, mRNAs, and snRNAs. The structure, mechanism, and diverse functions of the double-strand-specific ribonuclease III is reviewed by Nicholson, and Hollis and Shaban next discuss the structures and functions of the ribonucleases H that cleave the RNA strand in RNA–DNA duplexes. Krasilnikov provides an in-depth examination of

the ribonucleoprotein ribonucleases P and MRP, their central cellular roles in tRNA and rRNA processing, and the functions of the RNA and protein subunits in the catalytic mechanism. The chapter by Lönnberg addresses the inherent reactivity of RNA toward metal ions, and summarizes studies of small molecule ribonuclease mimics that exhibit diverse structures. Finally, Scheraga reviews pioneering experimental studies on protein folding that have employed pancreatic ribonuclease A as the primary model. What is evident from these chapters is the integral involvement of ribonucleases in a broad array of physiological processes, and that the simple act of cutting an RNA molecule, either internally or by removal of one or more nucleotides from either end, has profound effects on cell phenotype. Finally, detailed knowledge of ribonuclease structure, catalytic mechanism, and interacting partners are spurring new approaches to the treatment of disease. It is hoped that this volume will inform and stimulate further investigations of ribonucleases and their involvement in cellular pathways.

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I would like to thank Janusz Bujnicki for the invitation to edit a volume on ribonucleases as part of the Springer *Nucleic Acids and Molecular Biology* series, and Ursula Gramm of Springer for her assistance with the preparation of the volume.

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Chapter 1
The Superfamily of Vertebrate-Secreted Ribonucleases

Giuseppe D'Alessio

Contents

Abstract Recent investigations on vertebrate proteomes have revealed the presence of a single vertebrate-specific enzyme group: that of the RNases homologous to RNase A, the historical RNase archetype studied for more than a century. The genes encoding these RNases are all phylogenetically linked, and the gene products are all secreted proteins, thus forming an impressively large superfamily of vertebrate-secreted RNases, formerly called "RNase A superfamily." The vertebrate-secreted RNases display surprisingly different physiological functions, other than that of ribonucleolytic enzymes, including angiogenesis, host defense, immunosuppression, biogenesis of ribosomes, and stress response. Some of the RNases have antitumor activity, as they are capable of selectively killing malignant cells, and have inspired an intensely pursued research line of translational value.

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A particular attention has been given in this chapter to the family of mammalian RNases, especially to RNase 5 (angiogenin) and microbicidal RNases 2 and 3, to the RNase inhibitor, and the recently investigated family of fish RNases.

1.1 Premise

Among the great successes of biochemistry in the twentieth century were: the determination of the first complete amino acid composition of a protein; the first complete amino acid sequence of a protein much larger than the insulin peptides; the recognition that the three-dimensional structure of a protein is determined by its amino acid sequence; and the complete chemical synthesis of a protein enzyme. These results, which not only changed our understanding of protein chemistry but also significantly contributed to set the foundations of modern biology, all employed the same experimental model: an enzyme protein from bovine pancreas, a ribonuclease, RNase A. The authors of these achievements were all awarded Nobel Prizes: William Stein and Stanford Moore, Christian Anfinsen, and Bruce Merrifield.

Subsequently, RNase A became a convenient model for such innovative methodologies as protein X-ray crystallography, NMR, and calorimetry.

Phylogenetic studies soon revealed (Beintema et al. 1997) that many other RNases present in a variety of organisms, from amphibians to reptiles, birds, mammals, were structurally and functionally close to RNase A. An RNase superfamily was constructed, also called "the RNase A superfamily" from the historical, first described, archetypical ribonuclease. Recently, homologous RNases have also been found in fishes, thus allowing a vertebrate superfamily to be defined. Furthermore, the vertebrate RNases of this superfamily are all secreted, so it may be convenient, and appropriate, to name the superfamily as the Vertebrate-Secreted-RNase-Superfamily.

Interestingly, the sequencing of the human genome has unveiled an intriguing aspect. When vertebrate proteomes were explored, only a single vertebrate-specific enzyme group was found, that of RNases (Lander et al. 2001). Apparently, after the divergence of the vertebrate subphylum, about 500 million years ago, one or perhaps two new DNA sequences emerged (Cho and Zhang 2007), encoding a protein(s) absent in invertebrates, that rapidly evolved into many orthologs, following vertebrate speciation, to yield various numbers of paralogs within each evolving species.

There are several features that exclusively define vertebrate-secreted RNases: (1) They contain in their amino acid sequence a short stretch of residues (CKxxNTF) known as the vertebrate RNase "signature," at position 40–46 (the numbering of RNase A is used here and elsewhere in this chapter). (2) The reading frame of each protein is contained in a single exon. (3) The catalytic activity is carried out with the essential cooperation of His-12 and -119 and Lys-41. (4) Cleavage of the RNA P-diester bonds is initiated by the 3′-OH of a pyrimidine

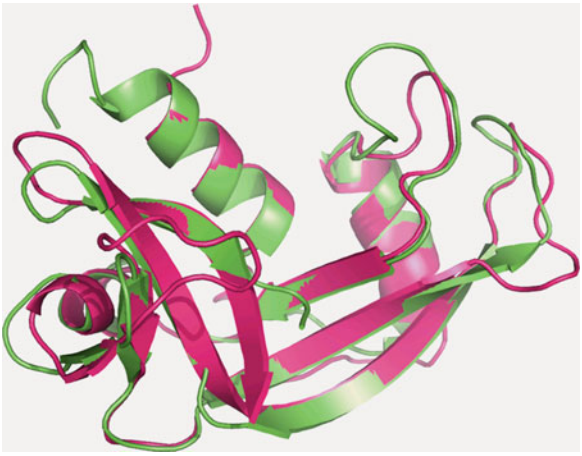

Fig. 1.1 Ribbon diagram of the superimposition of the structure of ZF-RNase 5 from *Danio rerio* or zebrafish (*green*, PDB code 3LJE) and that of RNase A from *Bos taurus* (*pink*, PDB code 1KF3)

nucleotide, which through transphosphorolysis leads to the formation of a 2′:3′-cyclic-phosphodiester, which (with exceptions) is subsequently hydrolyzed into a 3′-phosphate (Cuchillo and Vilanova 1997).

Vertebrate-secreted RNases are all structurally homologous, even when low identity scores are calculated between primary structures. They have an α/β structure arranged in a kidney shape by three alpha-helix stretches and a four-stranded antiparallel β-sheet. Figure 1.1 illustrates the 3D structures of archetypical RNase A, and that of a phylogenetically distant fish RNase, RNase-5 from zebrafish (Pizzo et al. 2010). Although the identity score is only 21%, the structural homology between the two proteins is strikingly apparent.

1.2 Mammalian RNases

1.2.1 Human RNases (Canonical, Noncanonical)

The complete identification of human RNase genes was obtained only few years ago when human chromosome 14q11.2 could be read and analyzed (Cho et al. 2005). The chromosome was found to contain all human RNases. However, only eight of them (RNases 1–8) are true RNases, called "canonical RNases"; the remaining "noncanonical" five, numbered 9–13 (Cho et al. 2005), lack one or more residues of the catalytic triad (see above); thus, they may not catalyze RNA degradation. As 3D structures of these proteins are not available, and no RNase assays have been performed or published, the unlikely possibility cannot be excluded that the missing residues are replaced by residues identical or conservative with respect to the characteristic RNase catalytic triad, even located at different sequence positions.

Most differences between noncanonical and canonical RNases are found at the C-terminal region of the proteins, but RNases 9 and 10 contain an insertion of 40–50 residues in their signal peptides (Castella et al. 2004; Penttinen et al. 2003). As for their function, RNases 9 and 10 appear to have a role in the reproductive tract, at least in some species, as they are expressed in mouse and porcine epididymis (Castella et al. 2004; Penttinen et al. 2003).

A compact review of human canonical RNases has been recently published (Sorrentino 2010).

1.2.1.1 Human RNase 1

Human RNase 1 was originally studied in pancreas (Beintema et al. 1984; Seno et al. 1994) so that it is also termed HP-RNase or hPR (for Human Pancreatic RNase). However, it may not be a digestive enzyme, as the single gene encoding the RNase (Breukelman et al. 1993) is expressed not only in pancreas, but in a variety of tissues and organs, and especially in endothelial cells (Landre et al. 2002). Thus, it may be proposed instead that the enzyme is involved in the control of the homeostasis of extracellular RNAs. This hypothesis is supported by the following: (1) RNase 1 is actively and directly secreted by endothelial cells into blood vessels, and (2) it displays a powerful activity both on single- and double-stranded RNA (Libonati and Sorrentino 2001), as well as on RNA in RNA:DNA hybrids (Potenza et al. 2006).

1.2.1.2 Human RNases 2 and 3, and the Fortunes of Microbicidal RNases

Human RNase 2 is also called EDN (Eosinophils-Derived Neurotoxin) because it was first isolated from eosinophils with an assay for neurotoxicity (Snyder and Gleich 1997). Also human RNase 3 was first isolated from eosinophils and labeled ECP (Eosinophils Cationic Protein) for its high content of cationic residues. ECP/RNase 3, however, is produced only in eosinophils, whereas the EDN/RNase 2 gene is expressed in a variety of organs and tissues (Beintema et al. 1988; Mizuta et al. 1990; Sorrentino et al. 1988).

The catalytic properties of RNases 2 and 3 are unusual for vertebrate RNases. The two RNases do not hydrolyze the 2′:3′-cyclic-phosphodiesters produced in the first transphosphorolytic event of catalysis. Moreover, their base preference at the 3′ side of the cleaved P-diester bond, like for human RNase 4 (see below), is for uracil rather than cytosine (Sorrentino and Libonati 1994).

RNases 2 and 3 evolved through gene duplication and share about 70% of their amino acid sequence. Both have a pronounced neurotoxic activity and an antiviral activity that are dependent on their RNase activity (Domachowske et al. 1998; Rosenberg and Domachowske 2001; Sorrentino et al. 1992).

EDN/RNase 2, but not ECP/RNase 3, has been found to activate human dendritic cells in response to pathogen stimulation (Yang et al. 2003, 2004). As this event

results in the production of an array of cytokines and growth factors from the immune system, EDN has been classified an alarmin (Oppenheim and Yang 2005).

ECP/RNase 3 is secreted by eosinophils activated by inflammation, and it was first identified as an antiparasitic agent (Hamann et al. 1990), then recognized as a powerful bactericidal protein (Lehrer et al. 1989), and later as an antiviral agent (Domachowske et al. 1998:532). These activities are not related to the RNase activity of the protein (Rosenberg 1995). M.V. Nogués, E. Boix and coworkers (Carreras et al. 2003; Torrent et al. 2007) have determined that the bactericidal activity of ECP is based on its ability to destabilize the bacterial membrane, an action in turn based on its high content of cationic and hydrophobic residues.

More recently, ECP has been found to be cytotoxic also for eukaryotic cells (Navarro et al. 2008). It does not enter the cells, but it aggregates on the cell surface, thus affecting membrane permeability. These interactions with the membrane bring about in the affected cells a series of impressive morphological and biochemical changes, such as chromatin condensation, membrane reversion, production of reactive oxygen species, and activation of caspase-3-like activity through eventual cell death. It has been confirmed that the RNase catalytic activity of ECP plays no role in these processes.

Two ECP polymorphic variants coexist in eosinophils, with Arg or Thr at position 97 of the protein sequence. Only the variant with Arg is cytotoxic for lung carcinoma cells (Rubin et al. 2009).

Interestingly, the aggregation of ECP into amyloid-like fibrils has been recently reported (Torrent et al. 2010:745). Also, it was found that the isolated N-terminal peptide segment of the protein (Arg1-Asn19) promotes the formation of a fibrillar network, even more extensive than that induced by the protein itself. Fibrils are formed only at acidic pH, but protein hydrophobicity is also important.

The most intriguing and elusive topic in a discussion on RNases 2 and 3 is the significance of the functionality of their ribonucleolytic active site for the various actions so far described for these proteins. As indicated above, the ribonucleolytic activity may be necessary (e.g., for the antiviral activity of EDN), or dispensable (e.g., for the bactericidal activity of ECP). In the former case, it is reasonable to imagine the damaging effects of an RNase on any cytosolic RNA, once the enzyme reaches the cell cytosol. In this case, the mechanism would be likely understood from the identification of the target RNA substrate. Here, research on the involvement of RNases with non-coding and interfering RNAs may provide new, unexpected clues.

Different and more intriguing is the latter case, in which the ribonucleolytic activity appears to be redundant, as the protein does not need it to perform its function. Here the crucial question is: Why in such a long evolutionary timeframe was the global 3D structure of a vertebrate RNase, and a precisely poised RNase catalytic site preserved with no apparent advantage?

Analysis of data from research on a bactericidal bird RNase (see also Sect. 1.3.2) has led Rosenberg and coauthors (Nitto et al. 2006) to consider the possibility that the RNase gene/protein structure is, for several RNases, merely a convenient source of biochemical, peptide material with toxic abilities. The hypothesis can be

expanded and read as follows: These RNases are microbicidal because they comprise in their sequences the sequence(s) of a microbicidal peptide(s). When the RNase reaches a cell membrane, or it enters a cell, it is fragmented by a membrane and/or cytosolic protease. The resultant free peptides would exert their toxic activities (bactericidal, antiparasitic, antiviral, cytotoxic) and provide the cells with valuable host-defense agents.

This scenario is especially convincing for RNases that conserve their bactericidal activity not only when the RNase activity is abolished, but also when they are completely and irreversibly denatured. There are several cases: (1) five zebrafish RNases, conserving their bactericidal activity after full denaturation (Pizzo et al. 2010), and (2) an active peptide contained within the sequence of bactericidal RNase 3 (Garcia-Mayoral et al. 2010); two Atlantic salmon RNases (Pizzo et al. 2008), and historical lysozyme as well (During et al. 1999; Ibrahim et al. 2001). The bactericidal activity of a zebrafish RNase (ZF-RNase-3) has been found to be due to a peptide fragment excised from the RNase by a membrane protease from the bacterium itself (Zanfardino et al. 2010).

It is widely recognized that cationic/hydrophobic antimicrobial peptides have an important role in the host innate defense mechanisms against invading microorganisms (Boix and Nogues 2007). However, it is difficult to envisage a biomolecule that has undergone any length of evolutionary process without possessing a properly folded structure. It is possible instead that evolution has indeed taken advantage of an RNase, perhaps both structurally and catalytically, but its physiological role is still unknown today.

Obviously, the artificially unfolded RNases described above have no relationships with the intrinsically destructured proteins that do not fold spontaneously into ordered and stable structures (Wright and Dyson 1999, 2009). These proteins, with key roles in the lives of cells, do acquire their structures when they interact and complex with their physiological partner(s) to perform their biological functions.

1.2.1.3 Human RNase 4

First isolated (Shapiro et al. 1986) from culture medium conditioned by an adenocarcinoma cell line, its characterization has been carried out in several laboratories (Seno et al. 1995; Vicentini et al. 1994, 1996; Zhou and Strydom 1993). The enzyme prefers uracil to cytosine as the base at the 3′ side of the cleaved P-diester bond, an unusual feature, as discussed above, for a vertebrate RNase. Surprisingly, the uridine-specific preference of the enzyme was found to be readily changed to cytidine-specific when the Asp residue at position 80 was replaced by Ala (Hofsteenge et al. 1998). The finding was intriguing because Asp-80 is a highly conserved residue in all orthologous sequences of RNase 1 and several other RNases. The possible conclusion drawn by the authors was that a residue, although conserved in evolution, may not have the same structural/functional role in all homologous enzymes.

An investigation (Rosenberg and Dyer 1995) on a genomic fragment from chromosome 14, where human RNase genes are located, has indicated that the mRNA encoding RNase 4 is much larger than those encoding closely related, homologous RNases. Surprisingly for a vertebrate RNase, two transcripts are identified in RNA from some organs (liver, kidney, pancreas). Furthermore, it has been established that RNase 4 is expressed in all tissues analyzed. However, the significance of these findings and the physiological role of RNase 4 remain to be investigated.

1.2.1.4 Human RNase 5

Human RNase 5 is most frequently known as "angiogenin," because it is an angiogenic RNase first isolated from the conditioned medium of HT-29 colon adenocarcinoma cells (Fett et al. 1985). Extensive reviews on angiogenin are available (Gao and Xu 2008; Riordan 1997; Strydom 1998).

The angiogenic activity of human angiogenin (ANG) depends on its activity as a ribonuclease, which is very low: With certain assays, it can be even one million-fold lower than that of RNase A, the archetype vertebrate RNase. The molecular basis for this low activity has been proposed to depend on the presence of a Gln at position 117 (substituting for Ala of RNase A), which hinders the access of the substrate to the pyrimidine binding site of the enzyme (Acharya et al. 1994; Russo et al. 1994). A recent kinetic analysis carried out with a rational series of substrates (Leland et al. 2002) has instead suggested that the low RNase activity of ANG is the result of a specific orientation of ANG catalytic residues, not favorable for cleavage of RNA.

ANG is a very versatile bio-effector, capable of exerting several biological actions, besides the angiogenic activity. Its interactions with the endothelial cell surface can be ascribed to a specific stretch of residues (positions 60–68 of the amino acid chain), as well as to the ability of ANG to bind to a smooth muscle type α-actin (Hu et al. 1991, 1993), and a receptor, although the latter has not yet been described at the molecular level.

Once in the cell, ANG is translocated to the nucleoli, where it eventually accumulates (Hu et al. 2000; Hu 1998; Moroianu and Riordan 1994b). This event requires the presence of a typical nuclear localization signal contained between Arg3I and Leu-35 in the ANG sequence (Moroianu and Riordan 1994a). In addition, or alternatively, the translocation of ANG to the nucleus may occur through passive diffusion, given the small size of the RNase (Lixin et al. 2001).

Once ANG has reached the nucleoli, the site of ribosomal RNA (rRNA) transcription, ANG binds to the promoter of ribosomal DNA (rDNA), and rRNA transcription is stimulated (Kishimoto et al. 2005; Xu et al. 2002).

The irony is that an RNA-degrading enzyme is implicated in RNA synthesis. In fact, the stimulation of rRNA biogenesis is essential for ANG angiogenic activity, and angiogenic factors, such as bFGF and VEGF, stimulate nuclear translocation of endogenous ANG (Hirukawa et al. 2005; Kishimoto et al. 2005). Silencing ANG

expression in endothelial cells inhibits bFGF- and VEGF-induced cell proliferation and leads to a decrease of rRNA transcription, an effect reversed by addition of exogenous ANG.

However, the way of ANG to angiogenesis described above poses a problem: How can ANG translocate to the nucleus moving through the cytosol in which there is a high concentration of RI, the RNase Inhibitor that binds ANG with exceedingly high affinity. Possibly, ANG travels in the cytosol fused to a carrier, which preserves, and obscures, the enzyme. However this carrier, if it exists, has not been found.

The RI/ANG interrelationship has been studied instead under para-physiological conditions. Under environmental stress, the cell economy requires the arrest of protein synthesis, as translation is energetically costly. It has been found that the arrest is determined by ANG, which under stress blocks translation through the inactivation of tRNAs. The inactivation is performed through cleavage by ANG (with its ribonucleolytic ability) of P-diester bonds at the anticodon loops of tRNAs (Emara et al. 2010; Fu et al. 2009; Yamasaki et al. 2009). Under normal conditions, ANG cannot exert this function because it is bound and neutralized by RI, the RNase inhibitor. We can surmise how under stress RI dissociates from the complex and ANG is free to act. This certainly happens during oxidative stress, when RI is damaged and knocked down (Blazquez et al. 1996).

In conclusion, ANG promotes translation through rRNA biosynthesis, although under stress conditions, it blocks translation. But there is no inconsistency between these events, as recent results (E. Pizzo, A. Furia and G. D'Alessio, unpublished data) have shown that under stress conditions, no ANG is detectable in nucleoli. Thus, while ANG arrests translation in the cytoplasm under stress, it simultaneously and coherently stops its nucleolar-based activation of rRNA synthesis.

The significance of angiogenin has been considered for a multiplicity of diseases which could be related to angiogenesis (see (Gao and Xu 2008)). Here we shall review the role(s) of ANG in two most relevant diseases: cancer and amyotrophic lateral sclerosis (ALS).

ALS is a disease that leads to a progressive degeneration of motor neurons. About 10% of the analyzed cases are familial, caused by alterations of a number of genes, including the ANG gene (Greenway et al. 2006; Wu et al. 2007). Furthermore, the ANG gene has been found to be strongly expressed in mouse CNS during development (Subramanian and Feng 2007), and in adult human spinal cord (Wu et al. 2007).

Angiogenin variants present in ALS patients have been characterized and shown to affect neurite extension and pathfinding, and survival of motor neurons (Crabtree et al. 2007). Furthermore, they display poor RNase activity and/or impaired nuclear translocation (Wu et al. 2007). When ANG is administered to cultured motoneurons, the cells are protected from hypoxic injury (Sebastia et al. 2009); on the other hand, silencing of ANG leads to an increase in hypoxia-induced cell death.

As for the involvement of ANG with cancer, its first role is that of providing tumors with the support needed for growth. Only a fraction of nascent tumors ever develop to detectable tumors unless they are provided with essential oxygen and

nutrients, carried by blood. Vascularization of a micro-tumor is induced by tumor angiogenesis factors, and angiogenin is one of these factors. Inhibition of angiogenesis has formed the rational basis for anticancer therapy with anti-angiogenic peptides and proteins (Boehm et al. 1997; O'Reilly et al. 1996). An additional approach to cancer therapy has been based on the performance of ANG in the presence of neomycin, an antibiotic that inhibits the translocation of ANG to nuclei (Hu 1998). It has been found that neomycin has also direct, inhibitory effects on cell proliferation (Tsuji et al. 2005).

1.2.1.5 Human RNase 6

First labeled RNase k6 as the sixth human RNase, with "k" denoting its relationship to the orthologous RNase k2 from bovine kidney, this RNase is expressed in most tissues, but not in eosinophils (Rosenberg and Dyer 1996), although its amino acid sequence is most closely related to those of RNases 2 and 3 (see Sect. 1.2.1.2). But, likely, also RNase 6 has a role in host defense, as it has been found in neutrophils and monocytes.

1.2.1.6 Human RNase 7

RNase 7 was found while searching for antimicrobial proteins of human skin (Harder and Schroder 2002), during screening the human genome (Zhang et al. 2003). This RNase is expressed not only in skin, but in several tissues, especially in liver. It is endowed with a bactericidal activity against several pathogenic microorganisms, both Gram-negative and Gram-positive.

It has been proposed (Huang et al. 2007) that a key role in the antibacterial mechanism of RNase 7 is played by Lys residues from flexible N- and C-terminal cationic clusters. Its bactericidal activity would be due to its ability to permeate the bacterial membrane, whereas its ribonucleolytic activity has no role. In contrast with RNase 3 (or ECP, see Sect. 1.2.1.2), RNase 7 does not initiate its toxic action through agglutination of the bacteria (Torrent et al. 2010). This suggests that beside disruption of the bacterial plasma membrane, a key factor in the mechanism are interactions of the protein with the bacterial cell wall. It has been found that the bactericidal activity of RNase 7 on *P. aeruginosa* is based first on its binding to a cell wall lipopolysaccharide, then to the oligomeric membrane lipoprotein OprI (Lin et al. 2010).

RNase 7 is not only constitutively expressed in keratinocytes, but also induced by pro-inflammatory cytokines, such as interleukin-1β, interferon-γ, and bacterial challenge (Harder and Schroder 2002).

Abtin and coworkers (Abtin et al. 2009) recently reported that RNase 7, RNase 5 (angiogenin), and RI, the RNase inhibitor, are coordinated in a complex "system" with a role in the innate antimicrobial defense of the skin. In the differentiating keratinocytes of epidermis, the RNases are complexed to RI and inhibited, but when

the keratinocytes mature into the stratum corneum, the RI is dislocated and then degraded by serine proteases. Thus, the released RNases are free to exert their antimicrobial activity. The authors have reported that the latter is dependent upon the ribonucleolytic activity of RNase 5, as this is inhibited also by small nonprotein inhibitors, such as diethylpyrocarbonate. This conclusion, and that proposed by other authors (Huang et al. 2007) on the irrelevance of the RNase activity for RNase 7 bactericidal activity, are surprisingly in contrast. Further experiments will clarify this issue.

1.2.1.7 Human RNase 8

RNase 8 (Harder and Schroder 2002; Zhang et al. 2002) has a high sequence identity with RNase 7 (78%), but the two evolutionarily related RNases are in several ways different: (1) RNase 7 is present in several tissues, whereas RNase 8 is produced only in placenta and (2) the disulfide bridge that links Cys residues at positions 84 and 26, conserved in all mammalian RNases, cannot form in RNase 8, because Cys-84 is not in the sequence. As a Cys residue, absent in all other RNases, is found at position 69, one should presume that disulfide 84/26 is replaced by a 69/26 disulfide. It would be interesting to examine the 3D structure of RNase 8.

RNase 8 is active as an antibacterial agent against several Gram-positive and Gram-negative bacteria (Rudolph et al. 2006); its apparent function is to protect placenta from infections.

1.2.2 Rodent RNases

The pancreatic RNase from rat (*Rattus norvegicus*) was the next vertebrate RNase to be isolated (Beintema and Gruber 1965) following RNase A, the bovine pancreatic enzyme, by a quarter of a century (Kunitz 1940). The 3D structure of the protein, obtained by X-ray crystallography, was determined more recently (Gupta et al. 1999).

Also recent is a detailed study on the characterization of mouse RNase 6 (Dyer et al. 2004), and mouse eosinophil-associated RNases (EAR). They have received a special attention for their rapid evolutionary expansion, characterized by gene duplication or deactivation (Cho et al. 2005; Zhang et al. 2000).

As for murine angiogenins, the family includes six members (Brown et al. 1995; Strydom 1998), with angiogenin 1 from mouse (mAng-1) identified as the murine orthologue of human angiogenin (Bond and Vallee 1990; Holloway et al. 2005). mAng-1 is expressed in a wide variety of tissues during and after embryogenesis, whereas mAng-4 is expressed only in gut and pancreas (Crabtree et al. 2007). As mAng-4 is upregulated in the Paneth cells of the gut by bacteria (Hooper et al. 2003), it has been proposed that it is implicated not only in gut angiogenesis, but also in gut innate immunity.

Site-directed mutagenesis studies and the 3D structure of mAng-4 have shown that Glu115 has a role in the low enzymatic activity of mAng-4 similar to that of Gln117 in human angiogenin (see above, Sect. 1.2.1.4) (Crabtree et al. 2007).

1.2.3 Bovine RNases

1.2.3.1 RNase A

The prehistory of the superfamily of secreted vertebrate RNases started with an investigation of Walter Jones from Johns Hopkins Medical School (Jones 1920) aimed at defining the nature of the internucleotide linkage in "yeast nucleic acid," the predecessor of RNA in biochemical nomenclature. The enzyme that degraded RNA entered the stage of the scientific literature mainly for its high heat resistance: Surprisingly, an aqueous extract of pig pancreas after boiling could still degrade RNA into nucleotides. There was substrate specificity, because thymus nucleic acid (i.e., DNA) was not degraded.

Many years later, René Dubos isolated the enzyme, called it ribonuclease, and confirmed that it was a protein very resistant to heat (Dubos and Thompson 1938). Almost simultaneously, Moses Kunitz crystallized the enzyme from bovine pancreas (Kunitz 1940). Then in the 1950s, the enzyme was further purified using the new, sophisticated chromatographic procedures offered to biochemists, mostly based on ion-exchange and affinity chromatography. The preparation of fully homogeneous enzyme led also to the identification of two RNases in bovine pancreas: RNase A and RNase B, the latter a glycosylated form of RNase A (Plummer and Hirs 1964).

RNase A however, as illustrated in the Premise to this chapter, has become over the years a synonym of ribonuclease, after its use as a model protein/enzyme, and the discoveries that these studies produced. In fact, RNase A has been used as a name for the whole superfamily of tetrapod, later of all vertebrate RNases, and it is not unusual to read in scientific journals the expression "RNase A RNase" to denote an RNase from the so-called RNase A superfamily. Here, as indicated in the Premise, we use the more straightforward "vertebrate-secreted RNase superfamily".

Timely and exhaustive reviews have been produced on RNase A in a chronological order by: Frederic Richards and Harold Wyckoff; Peter Blackburn and Stanford Moore; Ronald T. Raines (Blackburn and Moore 1982; Raines 1998; Richards and Wyckoff 1971). In a book on ribonucleases (D'Alessio and Riordan 1997), several chapters were dedicated to various aspects of RNase A as an enzyme and as a protein.

The identification of key residues in the mechanism of action and stability of RNase A has been successfully carried out for decades, first by modifying through chemistry the side-chains of the appropriate residues, then by heterologous production of recombinant variants.

Yet, quite recently, an original methodology has been proposed (Smith and Raines 2006), in which RNase A and human angiogenin (RNase 5, see Sect. 1.2.1.4) were used as model proteins in a methodical search for residues important for structure and function of the two proteins. Libraries of mutated RNase A and angiogenin genes were obtained by using error-prone polymerase chain reactions. When a gene encoding an active RNase was expressed in a bacterial cell, engineered to allow for disulfide bond formation in the cytosol, the cell was killed by the active RNase. Thus, inactive variants could be readily selected. Many residues (about 10–15% of the total) were found to be not amenable to substitution in either RNase A or homologous angiogenin, and only a few were sensitive in both proteins. Although the importance of many among these residues was not clear, the identified genes may be suggestive targets for future studies.

Investigations aimed at producing dimeric variants of RNase A have been favored in the last decades. The rational basis of this approach was twofold. It was prompted: (1) by the results with homologous, but dimeric, seminal RNase (see Sect. 1.2.3.2), endowed with many surprising, interesting bioactivities and (2) by the startling discovery that lyophilization of RNase A from a solution of acetic acid led to the formation of dimers (Crestfield et al. 1962). Furthermore, these RNase A dimers were found to be constructed by the exchange or swap of the N-terminal segments of the two protomers (Crestfield and Fruchter 1967).

Years later, Mazzarella and coworkers determined the three-dimensional structure of seminal RNase (BS-RNase), and found that this dimeric protein was organized with the swap of N-terminal segments between subunits (Mazzarella et al. 1993).

These data motivated Eisenberg and coworkers, working at that time on a similar exchange of parts between the subunits of diphtheria toxin (Bennett et al. 1994), to study the "Crestfield-type" dimers. They crystallized and determined the structure by X-ray crystallography of an RNase A dimer with N-terminal swap (Liu et al. 1998), and later of a dimer in which the swap involved segments from the C-terminal region of the protein (Liu et al. 2001). Eisenberg proposed the name of "three-dimensional domain swap" for the exchange of parts between subunits in an oligomeric protein, and demonstrated that the swap of structural domains between oligomers is a general solution to oligomer stability and function (Bennett et al. 1995).

Libonati and coworkers later reported that when RNase A is lyophilized from dilute acetic acid, it not only forms dimers, but also trimers, tetramers, and pentamers (Gotte et al. 1999; Gotte and Libonati 2004).

The ordered assemblage of RNase A into oligomeric structures upon treatment with acetic acid and lyophilization is surprising, as both the acid itself (Lopez-Alonso et al. 2010), and lyophilization (Griebenow and Klibanov 1995), induce a profound perturbation of the protein structure, especially its secondary structure.

An alternative method to make dimers of RNase A consisted using bifunctional reagents to cross-link two RNase A molecules (Bartholeyns and Baudhuin 1976). Later, site-directed mutagenesis was used to demonstrate that RNase A could be transformed into a stable and active dimeric RNase when key residues were

replaced by the corresponding residues present at the intersubunit surface of naturally dimeric seminal RNase (Di Donato et al. 1994).

Another approach to make dimers of RNase A was based on the use of the poorly active enzyme variants H12A and H119A (Park and Raines 2000). When a 1:1 mixture of the two variants was incubated at pH 6.5 and 65°C, a large increase in ribonucleolytic activity resulted, suggesting that dimers were constructed with swap of parts between two enzyme units. Furthermore, when the mixture was lyophilized, active dimers were obtained. The results suggested the hypothesis of a monomer–dimer equilibrium, with a K_d 20-fold greater than the concentration of RNase A in the cow pancreas. Furthermore, these results, and physicochemical considerations, led the authors to two provocative conclusions: (1) There are RNase A dimers also in vivo even at 37°C, and (2) RNase A has an intrinsic, pre-evolved ability to form domain-swapped dimers.

More recently, dimeric RNase A was constructed as a tandem protein (Leich et al. 2006) made up of two RNase A units fused through a peptide linker connecting the C terminus of one unit to the N terminus of another unit. Even though one of the dimer protomers was bound to and neutralized by the RNase inhibitor (see below), the tandem dimer was found to be active and strongly cytotoxic toward malignant cells.

An even more radical solution toward protein dimerization has appeared (Simons et al. 2002), and applied to RNase A as a model protein (Simons et al. 2007). The protocol consists in linking covalently two RNase A molecules by creating an amide bond between the side-chain of Lys66 in one unit and that of Glu9 in a partner unit. The bond was generated by heating in vacuo at 85°C lyophilized preparations of RNase A. The linkage was dubbed "zero-length amide cross-link."

Later, the product of the heating reaction was found to be heterogeneous, with various amino and carboxyl groups forming amide bonds (Vottariello et al. 2010). Furthermore, the "zero-length" dimers of RNase A were found to have no cytotoxic activity toward tumor cells, unless "cationized" as described by J. Futami and coworkers (see below). However, the "cationized" dimers were as cytotoxic toward nonmalignant cells as toward malignant cells.

A different approach to investigate the structure and the function of RNase A by X-ray crystallography and molecular dynamics simulations provided evidence that subtle β-sheet motions are essential in RNase A for substrate binding and product release (Berisio et al. 2002; Vitagliano et al. 2000, 2002), and that these motions corresponded to intrinsic dynamic properties of the native enzyme (Merlino et al. 2002; Merlino et al. 2003).

The high quality of the electron density maps obtained for RNase A structures at six distinct pH values (Berisio et al. 1999, 2002) has allowed direct detection of the deprotonation of the catalytic His12 residue, corroborating the reaction mechanism proposed by kinetic and structural studies (Cuchillo and Vilanova 1997). Furthermore, this approach led to an accurate picture of the active site as well as to the observation of concerted structural changes in regions even remote from the active site.

It has been pointed out above that the rational basis for the construction of RNase A dimers was largely due to the unusual dimeric structure of BS-RNase. But it was the antitumor action of dimeric BS-RNase, absent in its monomer (Vescia et al. 1980), that effectively stimulated the research on dimerized RNase A, especially for its translational value in innovative cancer therapies.

RNase A had long been tested as an antitumor agent, but eventually, this research track was abandoned when it became clear that RNase A was antitumoric only when administered in very large amounts (Ledoux 1955). It has been hypothesized (Youle and D'Alessio 1997) that prolonged storage and/or repeated lyophilizations could have induced the production of dimeric forms of the enzyme, as recorded in the literature (Richards and Wyckoff 1971). Likely, these dimeric derivatives, present in old preparations of RNase A as minor protein contaminants, were the actual responsible determinants of the antitumor action of the RNase A preparations tested.

All dimeric RNase A derivatives described above have been found to possess various degrees of cytotoxic activity, although it was not always determined that such cytotoxic activity was selective for tumor cells, with no adverse effects on nonmalignant cells. However, monomeric variants of seminal RNase have been found to have cytotoxic activity (Lee and Raines 2005). They will be discussed in Sect. 1.2.3.2.

Beside dimerization of the protein, other experimental approaches were tested for providing RNase A with a cytotoxic activity. One approach has been to render the protein much more cationic through chemical modifications. This conferred a pronounced cytotoxic activity on RNase A toward a malignant fibroblast cell line (Futami et al. 2001). The observed cytotoxicity could be readily correlated with the net positive charge of the derivatized protein. On the other hand, the enzyme activity decreased and was only partially inhibited by the RNase inhibitor. Yet, the authors concluded that the cytotoxic activity of the "cationized" RNase was mainly due to its lower affinity for the acidic RNase inhibitor, and possibly also to its more efficient adsorption by the cells. No tests were carried out on nonmalignant cells, and the possibility was not verified that the derivatives were as toxic to cells as any super-cationic protein material might be, including polylysine and other cationic, nonbiological substances (Kornguth et al. 1961).

Another approach to the construction of RNase A derivatives with antitumor activity has been proposed recently (Rutkoski et al. 2010), based on the production of RNase A multimers generated with thiol-reactive cross-linking reagents. Derivatives with various degrees of cytotoxic activity were obtained, depending on the affinity of the derivatives for RI, which in turn depended on the site of conjugation and the propinquity of the monomers within the conjugate. While in vitro the antitumor activity was hindered by the increased hydrodynamic radius of the derivatives, tests in vivo on laboratory mice gave more favorable results, likely for the higher levels of large derivatives in circulation.

1.2.3.2 Seminal RNase and the Roles of the RNase Inhibitor

In the 1950s, the most prominent textbook of Biochemistry (Fruton and Simonds 1958) described RNase, obviously RNase A, as a very small and very heat-resistant protein. Now, given that heat-resistant, small proteins would be, in principle, more practical to study than large, heat-labile proteins, it is not surprising that an RNase was chosen for a new research line. The source selected for the study was bovine seminal plasma, a secretion of seminal vesicles, very rich in many enzyme activities, and very rich, it was soon discovered, in RNase activity (D'Alessio 1963). The enzyme was called bovine seminal RNase (BS-RNase).

Seminal RNase was much larger and more cationic than RNase A (D'Alessio et al. 1997, 1991), as reported also by others who co-discovered the RNase (Hosokawa and Irie 1971). Furthermore, BS-RNase was not monomeric, but a homodimer with two disulfide bridges linking the two subunits (Di Donato and D'Alessio 1973). To date, BS-RNase is still the only dimeric RNase within the whole superfamily of secreted vertebrate RNases.

Seminal RNase is less active as an RNase than many other RNases of the superfamily – e.g., it is about 50% as active as RNase A – but it displays several special, i.e., non-catalytic, biological actions: it is aspermatogenic, immunosuppressive, cytotoxic for tumor cells, and antiviral.

This extraordinary multiplicity and variety of biological activities (D'Alessio 1993), and the protein dimeric structure, unique for an RNase and the structural basis for most of these bioactions, have directed the research on this RNase. It should be noted, however, that most of these special activities of BS-RNase are not physiologically significant. They are merely reflections off the mirrors proposed by the assay systems with which the RNase is confronted (D'Alessio 1993).

A seminal-type gene evolved about 35 million years ago, at the time of the divergence of ruminants, likely through a duplication of the gene encoding bovine pancreatic RNase A. However, it remained a pseudogene in all evolved ruminants until about 5–10 million years ago, when the pseudogene was repaired (D'Alessio 1999; Sassi et al. 2007; Trabesinger Ruef et al. 1996), likely through gene conversion (Sassi et al. 2007; Trabesinger Ruef et al. 1996). Interestingly, in water buffalo, a seminal-type gene is expressed (Kleinedam et al. 1999), but the protein is not produced, apparently because one of the Cys residues engaged in the intersubunit disulfide bridges is replaced by a Phe. Thus, not only one of the two intersubunit disulfides cannot form in buffalo seminal RNase, but also the presence of a free thiol (the surviving Cys residue) can severely impair RNase survival in an extracellular, oxidizing environment.

Considering the co-presence in seminal RNase of two structural arrangements (see below), and the ability of only one of them to exert a special bioaction(s), it has been surmised that the case of the rapid BS-RNase evolution is that of an "evolution in progress" toward a protein with a new physiological role (D'Alessio 1995, 1999; Trabesinger Ruef et al. 1996).