The RNase III Family: A Conserved Structure and Expanding Functions in Eukaryotic dsRNA Metabolism

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Abstract

The last few years have witnessed the appreciation of dsRNA as a regulator of gene expression, a potential antiviral agent, and a tumor suppressor. However, in spite of these clear effects on the cell function, the mechanism that controls dsRNA maturation and stability remains unknown. Recently, the discovery of eukaryotic orthologues of the bacterial dsRNA specific ribonuclease III (RNase III) suggested a central role for these enzymes in the regulation of dsRNA and eukaryotic RNA metabolism in general. This article reviews the structure-function features of the eukaryotic RNase III family and their roles in dsRNA metabolism with an emphasis on the yeast RNase III. Yeast RNase III is involved in the maturation of the majority of snRNAs, snoRNAs, and rRNA. In addition, perturbation of the expression level of yeast RNase III alters meiosis and causes sterility. These basic functions of the yeast RNase III appear to be widely conserved which makes it a good model to understand the importance of eukaryotic dsRNA metabolism.

The RNase III Family: A Confusion or Ordered Evolution?

The RNase III family include dsRNA specific ribonucleases that share loosely conserved structural and functional features (Figure 1). Members of the RNase III family are found in all species tested with the exception of archaeabacteria where the functions of RNase III are carried-out by the bulge-helix-bulge nuclease (BHB) (Lykke-Andersen et al., 1997). Membership in this family requires homology with the structural elements of the founding member, Escherichia coli RNase III (Court, 1993; Nicholson, 1999; Nicholson, 1996). These structural elements include a nuclease domain that exhibits a conserved signature motif and a dsRNA binding domain (dsRBD) that includes a motif specific to the dsRNA binding protein family (Kharrat et al., 1995; St. Johnston et al., 1992). Based on structural and evolutionary differences, the RNase III family could be divided into two major subfamilies, the bacterial RNase III family and the eukaryotic RNase III family (Figure 1). In bacteria, RNase III exists in one form characterized by a classical RNA binding domain and a nuclease domain (Nicholson, 1999). In contrast, eukaryotic RNase III exists in three isoforms that share the basic dsRBD but differ in the number of nuclease domains and in the composition of the N-terminal domain (Filippov et al., 2000; Jacobsen et al., 1999; Lamontagne et al., 2000). The first form contains three domains; the dsRBD, the nuclease domain, and an additional uniquely eukaryotic N-terminal domain required for correct protein conformation and efficient RNA cleavage (Lamontagne et al., 2000). The second form exhibits in addition to these three domains a second nuclease motif at the protein N-terminus (Wu et al., 2000). Finally, the third form of eukaryotic RNase III contains in addition to the three main eukaryotic domains a fourth distinct helicase domain (Jacobsen et al., 1999; Rotondo and Frendewey, 1996). These obvious variations in the structure of the eukaryotic members of the RNase III family may appear as a product of random evolution independent of the specific needs of the host organism. However, as discussed in this review, recent comparative and functional studies of the different RNase III isoforms suggest an evolutionary pathway that adapts RNase III functions to its eukaryotic environment.

Rnt1p and RNA Processing in Yeasts

In yeast, four RNase III orthologues have been identified including Rnt1p from Saccharomyces cerevisiae (Abou Elela et al., 1996), Pac1 and Pac8 from Schizosaccharomyces pombe (Rotondo and Frendewey, 1996), and KLRNase III from Kluyveromyces lactis (Ozier-Kalogeropoulos et al., 1998). Rnt1p, Pac1 and KLRNase III belong to the classical RNase III family while Pac8 belongs to the helicase RNase III family. The enzymatic activities of Rnt1p and Pac1 were verified experimentally and were shown to affect pre-rRNA processing in vivo (Abou Elela et al., 1996; Kufel et al., 1999; Nagel and Ares, 2000; Rotondo et al., 1997; Zhou et al., 1999). In contrast, the in vivo existence and functions of both Pac8 and KLRNase III remain to be tested. Pac1 is the first eukaryotic orthologue to be identified based on sequence homology with the RNase III signature motif (ino et al., 1991; Xu et al., 1990). It was isolated as an essential gene that suppresses uncontrolled meiosis (ino et al., 1991; Xu et al., 1990), and suppresses a defect in snRNA metabolism (Rotondo et al., 1995). In addition, overexpression of PAC1 strongly inhibits sporulation in S. pombe. Recently, a temperature sensitive allele of PAC1 was isolated and used to demonstrate its role in the processing of U2 snRNA and pre-rRNA 3' ends (Zhou et al., 1999). Unprocessed U2 snRNA and pre-rRNA accumulate upon the inactivation of Pac1 in vivo. In vitro, purified recombinant Pac1 cleaves model substrates of both U2 and 25S rRNA 3' ends generating a product that is a few nucleotides longer than

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the mature form (Rotondo et al., 1997; Zhou et al., 1999). This product is believed to be trimmed by exonucleases to the mature site in vivo (Zhou et al., 1999). The relationship between the RNase III activity of Pac1 and the suppressor phenotype or the effect on sporulation is unclear, but the effects seem unlikely to be mediated through pre-rRNA processing. Unlike Pac1, the S. cerevisiae enzyme is not essential. However, deletion of Rnt1p alters the processing of pre-rRNA, snRNAs, and snoRNAs and results in severe growth defects (Abou Elela and Ares, 1998; Abou Elela et al., 1996; Chanfreau et al., 1998a; Kufel et al., 1999). Cells that lack Rnt1p are temperature sensitive capable of growing only at 26°C but not at temperatures higher than 30°C (Abou Elela and Ares, 1998; Lamontagne et al., 2000). These multiple severe effects of RNT1 deletion suggest an important role in RNA maturation and possibly RNA decay as well. The following section discusses in detail the biochemical and functional properties of Rnt1p and its impact on yeast RNA metabolism.

### Rnt1p Structure

*S. cerevisiae* Rnt1p is transcribed from a single gene located downstream of the spliceosome associated *CUS1* gene on chromosome XIII (Abou Elela et al., 1996). Rnt1p has a predicted sequence of 471 a.a with an estimated molecular weight of 54.5 kDa and calculated pI of 8.73 (Lamontagne et al., 2000). The canonical dsRBD motif is located at the C-terminus (positions 372-440) with 25% identity to the bacterial RNase III and 31% identity to fission yeast Pac1 (Figure 1 and Rotondo and Frendewey, 1996). However, unlike RNase III and Pac1 dsRBDs, Rnt1p has a highly basic 33 a.a. extension at the C-terminus (Figure 1). The Rnt1p 154 a.a. nuclease domain is similar in size to that of RNase III and Pac1 sharing the same charged amino acid clusters (Lamontagne et al., 2000; Mian, 1997).
It is likely that these stretches of acidic amino acids in Rnt1p binds divalent metal ions and directs RNA cleavage as suggested for the bacterial RNase III. In addition, Rnt1p possesses the characteristic eukaryotic N-terminal domain that spans 191 a.a. The N-terminal extension has neither an apparent functional motif nor significant homology to Pac1 N-terminal domain (Lamontagne et al., 2000). To determine the contribution of the various domains to Rnt1p function, the different domains were expressed individually and tested for function in vivo and in vitro (Lamontagne et al., 2000; Nagel and Ares, 2000). The dsRBD was shown to be required and sufficient for binding, while the nuclease domain is required for cleavage (Lamontagne et al., 2000; Nagel and Ares, 2000). On the other hand, the N-terminal domain was shown to play an auxiliary role ensuring efficient RNA cleavage (Lamontagne et al., 2000). Deletion of the N-terminal domain reduces the processing of the 25S rRNA 3' end by about 30% in vivo and slows growth by 35%-40%. Biochemical and genetic assays suggest that the N-terminal domain influences Rnt1p function by mediating both inter- and intramolecular interactions (Lamontagne et al., 2000). Since the N-terminal domain is unique for the eukaryotic members of the RNase III family, it may also serve in vivo as a nuclear localization signal.

![Figure 3. A general model for in vivo Rnt1p dependent RNA processing pathway.](image)

Figure 3. A general model for in vivo Rnt1p dependent RNA processing pathway. The gray box represents the mature RNA sequence and the lines represent the 5’ and 3’ end nascent extensions. Soon after RNA transcription, Rnt1p binds its cleavage site, cleaves the RNA, and initiates the downstream processing and modification machinery. Rnt1p cleavage site is normally 29-94 nucleotides away from the end of the mature RNA. After Rnt1p cleavage, a helicase activity is required to unwind the lower RNA helix resulting from cleavage reaction. Once unfolded, the RNA is trimmed by exocleavases (e.g. the exosome complex) up to the mature end which is specified by a chaperon or assembly protein (e.g. Lhp1p). The chaperon is normally replaced during the assembly of the mature RNP particles by members of the RNP complex (Allmang et al., 1999; Kufel et al., 2000; van Hoof et al., 2000; van Hoof et al., 2000).
Expression of Rnt1p mutants that lack the N-terminal domain does not complement RNT1 deletion (Nagel and Ares, 2000). However, similar N-terminal deletion expressed in fusion with nuclear localization signal or from strong promoters support cellular growth albeit less efficiently than wild type enzymes (Lamontagne et al., 2000). Deletion of S. pombe Pac1 N-terminal domain inhibits RNA cleavage in crude extracts suggesting a similar role in RNA cleavage (Iino et al., 1991). It is tempting to suggest that, despite the lack of sequence conservation, the basic functions of the N-terminal domain are preserved among eukaryotic RNase III.

Rnt1p Substrate

Comparison of Rnt1p natural substrates (Abou Elela and Ares, 1998; Chanfreau et al., 2000; Nagel and Ares, 2000) reveals the presence of a terminal tetraloop 14 to 16 bp from the cleavage site with the conserved sequence AGNN (Figures 2 and 3). Deletion of this sequence abolishes cleavage while deletion of sequences close to the cleavage site does not affect the substrate recognition or cleavage (Abou Elela and Ares, 1998; Chanfreau et al., 2000; Nagel and Ares, 2000). Mutations that change the tetraloop conserved AGNN sequence to GUNN slow RNA cleavage in low monovalent salt concentrations and block it in concentrations higher than 100 mM (Nagel and Ares, 2000). Changing the tetraloop sequence to GANN or GAAA also slows cleavage indicating that the AG sequence plays an important role in substrate recognition (Chanfreau et al., 2000). Indeed, gel mobility shift assays demonstrate that the dsRBD of Rnt1p which contains the 74 a.a. canonical RNA binding motif and an additional non-conserved 54 a.a. is capable of recognizing the AGNN tetraloop (Nagel and Ares, 2000). These experiments prompted the surprising conclusion that Rnt1p uses the apparently single stranded tetraloop to determine the cleavage site. On the other hand, Rnt1p may not recognize the AGNN sequence itself but instead it may recognize a particular structure that forms through that sequence and the adjacent RNA helix. It is also unclear whether the canonical dsRNA binding motif itself recognizes the tetraloop or this particular recognition is derived by the adjacent non-conserved amino acid within the C-terminus of Rnt1p (Lamontagne et al., 2000; Nagel and Ares, 2000). A role for the non-conserved amino acids in the recognition of the tetraloop would explain the inability of Pac1 and RNase III to recognize the tetraloop (Nicholson, 1999; Rotondo et al., 1997). In addition to its proposed role as a substrate identity element, the tetraloop appears to assist in determining the site of cleavage. It was shown that Rnt1p cleavage always takes place at a consistent distance from the terminal tetraloop (Chanfreau et al., 2000). This suggests that Rnt1p effectively acts as a “helical ruler” that measures the distance from the tetraloop to the cleavage site (Chanfreau et al., 2000). Alternatively, the distance between the tetraloop and the cleavage site may not be measured but instead imposed by the enzyme structure that can only extend a certain distance once it binds to the terminal tetraloop. A tetraloop independent mechanism of substrate recognition must also exist because Rnt1p is capable of cleaving long duplex RNAs that lack the conserved tetraloop (Abou Elela et al., 1996). Additional experiments are required to decipher the mechanism of substrate recognition and to determine the ability of Rnt1p to select the tetraloop containing substrate when in competition with a long RNA duplex. Regardless of the exact mechanism, it is clear that Rnt1p selects the substrate in a different way than E. coli RNase III, which is influenced by antideterminant nucleotides near the cleavage site (Zhang and Nicholson, 1997).

Rnt1p Mechanism of Action

Rnt1p functions as a 108 kDa homodimer (Lamontagne et al., 2000; Nagel and Ares, 2000) that forms through dimerization signals located at the N-terminal domain and the dsRBD. Rnt1p cleaves RNA by binding to a cleavage site followed by hydrolysis and product release. RNA binding may take place in the absence of divalent metals and is largely insensitive to monovalent salt concentrations. RNA recognition and binding are performed by the 114 a.a. C-terminus that harbors the canonical dsRNA binding motif (Lamontagne et al., 2000; Nagel and Ares, 2000). To date, all RNA substrates found to bind Rnt1p could also be cleaved, suggesting that the dsRBD is the major determinant of RNA recognition (Chanfreau et al., 2000; Nagel and Ares, 2000). However, not all the Rnt1p/RNA complexes display the same cleavage efficiency. Also gel-shift assays revealed that recombinant Rnt1p could form at least two protein/RNA complexes of differing molecular weight (Lamontagne et al., 2000). The larger complex readily cleaves its associated RNA upon the addition of Mg$^{2+}$, while the smaller complex is less efficient and does not result in full RNA cleavage (Lamontagne et al., 2000). This suggests that the protein may bind RNA without cleaving, possibly due to miss-association or as a step towards an active RNA/protein complex.

Rnt1p requires Mg$^{2+}$ for efficient RNA cleavage but may function with Mn$^{2+}$. It is believed that Rnt1p like E. coli RNase III is a two metal-binding enzyme (Steitz and Steitz, 1993) because of the conserved stretches of acidic amino acids observed in its nucleic domain. However, the actual association of these conserved amino acid residues with divalent metal ions remains to be determined experimentally. In vitro, Rnt1p conducts two coordinated cleavage events and single cleavage is a rare event. In contrast, the product of a single cleavage event is more visible in vivo or in cell extracts (Chanfreau et al., 1997; Kufel et al., 2000; Kufel et al., 1999; Nagel and Ares, 2000). This difference may be a factor of the in vitro cleavage conditions or because elements that dissociate the enzyme after single cleavage exist in vivo. The kinetics of Rnt1p dependent RNA cleavage is similar to that of E. coli RNase III with a $K_m$ of 1.2 M and $k_{cat}$ of 5.5 min$^{-1}$ (Lamontagne et al., 2000). The chemical composition of the cleavage products and its affinity to Rnt1p have not been determined. Nevertheless, it is believed that Rnt1p functions similarly to both S. pombe Pac1 (Rotondo and Frendewey, 1996; Rotondo et al., 1997) and the E. coli enzyme (Court, 1993; Nicholson, 1992; Nicholson et al., 1988) in leaving 3' hydroxyls and 5' phosphates.
Rnt1p Cellular Functions

Similar to the E. coli RNase III, Rnt1p is not essential but its deletion in yeast causes severe growth defects and temperature sensitivity (Abou Elela and Ares, 1998). The most visible function of Rnt1p is on pre-rRNA processing (Abou Elela et al., 1996; Kufel et al., 1999). Inactivation of Rnt1p blocks the maturation of the 25S pre-rRNA 3' end and slows the formation of mature 5' ends (Abou Elela et al., 1996; Kufel et al., 1999). In addition, Rnt1p initiates the 3' end processing of all Pol II transcribed small nuclear RNAs (snRNAs) including U1 (Seipelt et al., 1999), U2 (Abou Elela and Ares, 1998), U4 (Allmang et al., 1999), and U5 (Chanfreau et al., 1997). Deletion of RNT1 blocks 3' end formation of U2 and the large form of U5 (USL). In contrast, the steady-state levels of mature U1, U4, and U5 small form (USS) are unchanged in A.RNT1 cells because of an alternative processing pathway. Rnt1p also processes yeast polycistronic small nuclear RNAs (snRNAs) to separate them into individual RNAs (Chanfreau et al., 1998b). Unlike the majority of snRNAs, snoRNAs processing is almost completely blocked in the absence of Rnt1p. As in the case with U2 snRNA, essential snoRNAs like U14 must remain partially functional without processing because RNT1 deletion is not lethal.

Rnt1p dependent processing of 25S pre-rRNA, snRNAs, and snoRNAs seems to follow a general pathway that leads to mature RNA (see Figure 3). The first step is normally conducted by Rnt1p soon after RNA transcription followed by a helicase dependent unwinding of the cleaved helix, then trimming to the mature site by members of the exosome family (Kufel et al., 2000). In most cases, the mature site appears to be determined by an assembly protein (e.g. ribosomal protein) or a chaperon (e.g. yeast La protein) that binds and protects the mature site from further trimming by exonucleases (Allmang et al., 1999; Kufel et al., 2000; van Hooft et al., 2000; van Hooft et al., 2000).

A function of Rnt1p in the degradation or processing of mRNA similar to E. coli RNase III remains unconfirmed. However, the demonstrated effects of other eukaryotic RNase III orthologues like the fission yeast Pac1 and plant Caf on cell division and meiosis (Iino et al., 1991; Jacobsen et al., 1999; Xu et al., 1990) suggest that roles other than the processing of stable untranslated RNAs also exist.

Plant RNase III

In plant, five potential RNase III orthologues were identified based on sequence homologies (See Figure 1) but only one (Caf) was identified experimentally (Jacobsen et al., 1999). The five orthologues originate from a single plant (Arabidopsis thaliana) and exhibits a N-terminal domain, a nuclease domain, a helicase domain, and a dsRBD. All A. thaliana orthologues possess two nuclease domains and a single dsRBD with the exceptions of Tal3 that possesses 3 nuclease domains and Caf that possesses two dsRBDS (Jacobsen et al., 1999). The presence of multiple nuclease domains suggests the capacity of these enzymes to produce multiple cleavages with a single binding event. This is in contrast to the bacterial and yeast RNase III which function as homodimers to introduce a coordinated double cleavage. The in vivo function and biochemical activities of plant RNase III remain unclear. However, the isolation of one A. thaliana RNase III (Caf) as a suppressor of a recessive mutation that causes unregulated cell division in floral meristems suggests a possible function in cell cycle or mitosis (Jacobsen et al., 1999).

Metazoan RNase III

In metazoan cells, both simple and helicase associated RNase III orthologues have been identified. In fly (Filippov et al., 2000), worm (Filippov et al., 2000), and human (Matsuda et al., 2000; Wu et al., 2000) all orthologues possess two nuclease domains like their plant counterpart. However, the human (hRNase III) and fly (drosha) simple version of RNase III appear to possess additional proline rich domain and serine/arginine rich domain of yet to be identified function (Filippov et al., 2000; Wu et al., 2000).

The two forms of human RNase III, hRNase III (Wu et al., 2000) and HERNA (Matsuda et al., 2000) have been cloned but only the enzymatic activity of hRNase III has been examined. The predicted protein sequence of hRNase III reveals a 1374 a.a. long protein that possesses five different domains. A C-terminal dsRBD followed by two nuclease domains, a proline rich domain, and an arginine/serine rich domain. The hRNase III shares 37% of its amino acids sequence composition with the fly (drosha) and worm (Ce-drosha) RNase III (Filippov et al., 2000). The amino acid conservation extends well beyond the classical RNase III domain suggesting new conserved functions for the metazoan RNase III. In vitro, a recombinant GST fusion with the C-terminal domain of the human protein which includes both the dsRBD and the 2 nuclease domains cleaves dsRNA, but not ssRNA in presence of Mg2+ (Wu et al., 2000). This suggests that the human RNase III cleavage activity is carried by the classical RNase III domains. It is not clear, if the human RNase III functions as dimer like its prokaryotic orthologues or if the inclusion of a second nuclease domain lift the need for dimerization. In vivo, antisense mediated depletion of hRNase III does not affect the level of nascent pre-rRNA transcript (45S) but results in a slight accumulation of the 32S and 12S rRNA precursor (Wu et al., 2000). This suggests a role in rRNA processing as shown for other RNase III orthologues. However, unlike yeast and E. coli RNase III, the human enzyme appears not to affect the early cleavage event of pre-rRNA processing. Thus, hRNase III may participate in pre-rRNA processing either indirectly or at a step other than that suggested for yeast and bacterial RNase III. In addition to its potential function in pre-rRNA processing, the hRNase III may serve different nuclear functions as implied by its cell cycle dependent shutting between the nucleus and the nucleolus. Indeed, hRNase III may affect cell division like the plant and fission yeast orthologues (Iino et al., 1991; Jacobsen et al., 1999; Xu et al., 1990). Antibodies against different peptides of hRNase III show that the protein is predominantly nucleolar in the S phase of the cell cycle but exits to the nucleus in the G2/M phase (Wu et al., 2000). This is consistent with a role in pre-rRNA
processing which is normally transcribed in the S phase. Finally, the universal distribution of the hRNase III in human tissues suggests a general function such as its role in pre-rRNA processing.

Eukaryotic RNase III is a Potential Regulator of dsRNA Metabolism

The discovery of eukaryotic RNase III will undoubtedly revolutionize the understanding of dsRNA metabolism in eukaryotic cells. Several intriguing dsRNAs related phenomena in eukaryotic cells await an explanation that can be easily provided by an RNase III-like enzyme. One fascinating example is the phenomena of RNA interference (RNAI) or dsRNA mediated post-transcriptional gene silencing (PTGS). PTGS occurs when an RNA duplex corresponding to a sense and antisense sequences of endogeneous gene is introduced in the cell (Fagard and Vaucheret, 2000; lyer et al., 2000; Marathe et al., 2000; Meins, 2000; Morel and Vaucheret, 2000; Wassmann and Pelissier, 1998). Once in the cell, the RNA duplex appears to be converted into small RNA oligomers that range from 21 to 25 nucleotides in length (Caplen et al., 2000; Yang et al., 2000; Zamore et al., 2000).

The newly generated RNA oligomers were proposed to interact with the target mRNA that displaces the sense strand of the duplex through an ATP-dependent helicase activity leading to mRNA degradation (Zamore et al., 2000). These PTGS associated activities strikingly fit the predicted profile of the human RNase III orthologues (Bass, 2000). The helicase associated RNase III are the only known enzymes that by themselves could convert long duplex RNA into short oligomers, then unwind the short oligonucleotides in an ATP-dependent manner.

RNase III orthologues are also predicted to participate in dsRNA mediated interferon response that combat viral infection and control the proliferation of malignant cells (Nicholson, 1996). In mammalian cells, dsRNA introduced ectopically or via viral infection binds to PKR (RNA dependent protein kinase) and triggers its autophosphorylation. Upon phosphorylation, PKR acquires a protein serine/therionine kinase activity that inhibits cellular immunity in mammalian cells.

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