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Broadening the Mission of an RNA Enzyme

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ABSTRACT

The "RNA World" hypothesis suggests that life developed from RNA enzymes termed ribozymes, which carry out reactions without assistance from proteins. Ribonuclease (RNase) P is one ribozyme that appears to have adapted these origins to modern cellular life by adding protein to the RNA core in order to broaden the potential functions. This RNA-protein complex plays diverse roles in processing RNA, but its best-understood reaction is pre-tRNA maturation, resulting in mature 5' ends of tRNAs. The core catalytic activity resides in the RNA subunit of almost all RNase P enzymes but broader substrate tolerance is required for recognizing not only the diverse sequences of tRNAs, but also additional cellular RNA substrates. This broader substrate tolerance is provided by the addition of protein to the RNA core and allows RNase P to selectively recognize different RNAs, and possibly ribonucleoprotein (RNP) substrates. Thus, increased protein content correlated with evolution from bacteria to eukaryotes has further enhanced substrate potential enabling the enzyme to function in a complex cellular environment. J. Cell. Biochem. 108: 1244–1251, 2009.

KEY WORDS: RNase P; RNA PROCESSING; RNA WORLD; ANTISENSE RNA; NON-CODING RNA

Over 25 years ago the central dogma of biology was expanded with the discovery that, in addition to proteins, RNA can also have enzymatic activity, creating the "RNA World" hypothesis in which RNA-like macromolecules were thought to encode and catalyze their own duplication. Today these early discoveries have been extended and many more ribozymes have been found to play essential roles in cells, including the ribosome and RNase P. In modern organisms these ribozymes are virtually always ribonuclear protein (RNP) complexes with catalytic RNA cores, but the proteins act in various vital ways to ensure that the desired reaction is carried out and is localized correctly. These ribozyme catalyzed reactions carried out by RNase P and the ribosome are multiple turnover in vivo, defining them as true enzymes [Kazantsev and Pace, 2006]. It is worthwhile to note that in our drive to understand RNA catalysis the role of protein has usually been of secondary interest. Given the large protein content of these important RNP complexes in eukaryotes and some archaea, it is important to consider how the protein has played a role in enabling correct processing and possibly has enabled an expansion of processing functions.

One of the best-studied examples of a RNP complex has been RNase P. It was one of the first ribozymes discovered and is conserved in almost all organisms. RNase P has an RNA core that has adapted to complex cellular environments with the addition of protein subunits. RNase P is an essential RNP that is best known for catalyzing the 5′ endonucleolytic cleavage of pre-tRNA and this essential processing reaction is conserved throughout all forms of RNase P regardless of composition. The protein composition of the complex differs dramatically, from bacterial RNase P with one protein subunit, to archaeal with 4–5 proteins, to eukaryotes with 9–10 proteins (Fig. 1 and Table I). In addition to pre-tRNA cleavage, RNase P has been shown to cleave other RNA substrates both in vitro and in vivo (Table II). Understanding the functions of this dramatic increase in protein content of RNase P can provide insight into the molecular evolution of RNP complexes.

BACTERIAL RNase P

STRUCTURE

Bacterial RNase P contains a single protein subunit that combines in vivo with a catalytic RNA subunit. The catalyzed hydrolysis of a phosphodiester bond in the RNA substrate takes place within a conserved active site in the RNA subunit. At high salt in vitro the RNA can cleave substrate without protein, but the protein is required for activity in vivo [Smith et al., 2007]. There are two major groups of bacterial RNase P based on RNA secondary structure, ancestral type (A-type) and *Bacillus* type (B-type). These RNAs are very similar and were shown to be interchangeable in vivo [Smith et al., 2007]. A universal consensus RNA secondary structure for type A and type B is represented in Figure 1. In contrast to the RNA subunit, the bacterial protein in RNase P is ~14 kDa and is small

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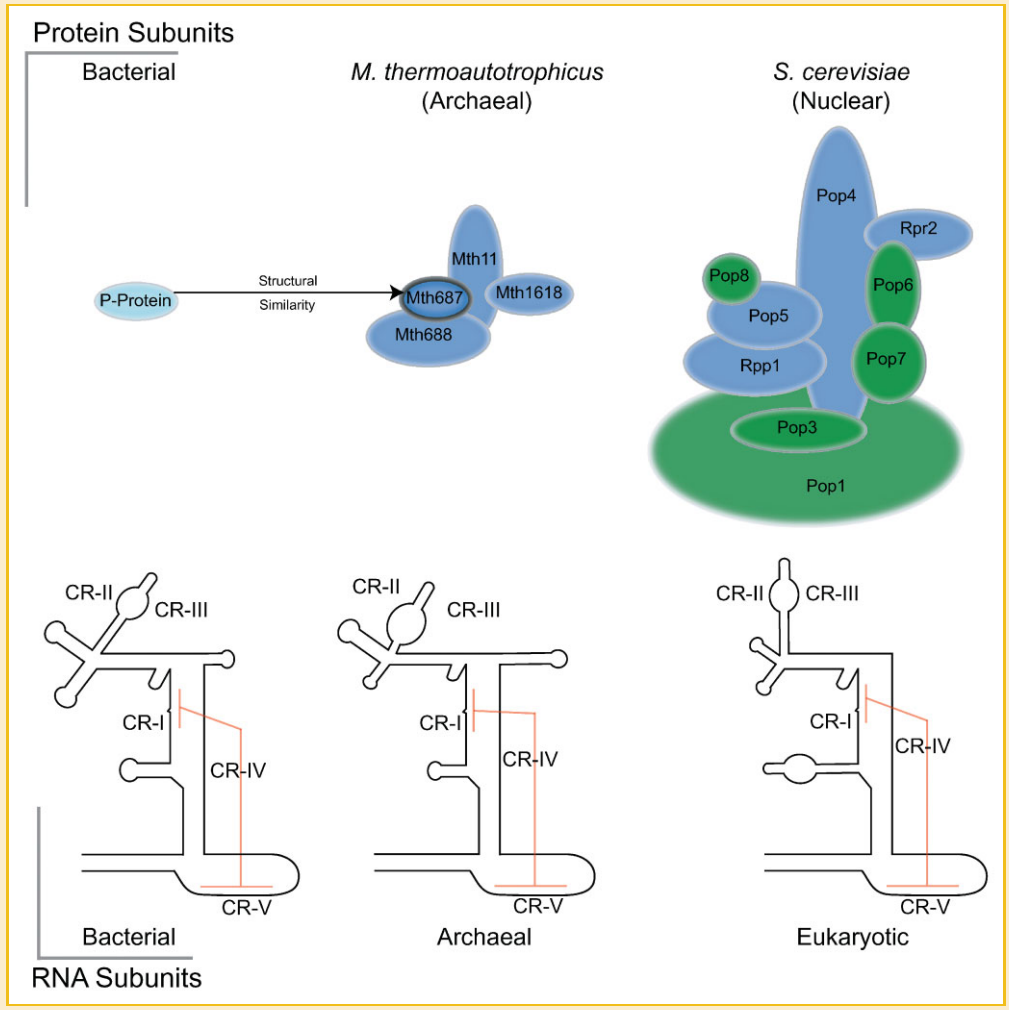

Fig. 1. The evolution of RNase P from bacteria to eukaryotes. Archaeal proteins indicated for *M. thermoautotrophicus* (Mth). Color coding indicates protein homology between eukaryotic and archaeal RNase P. Bacterial protein shown with structural homology to Mth687 in archaeal RNase P. Protein subunit interactions are shown from two-hybrid data [Houser-Scott et al., 2002; Hall and Brown, 2004]. The RNA structures illustrate conserved regions (CR), with red lines indicating tertiary interactions, and estimate general structural characteristics of the indicated consensus structures. Refer to RNase P database for more details: http://www.mbio.ncsu.edu/RNaseP/home.html.

TABLE I. Subunit Composition of RNase P: Bacterial, Archaeal, Eukaryal

Bacteria[a]		Archaea[b]		Eukarya				
	RNase P		RNase P			Yeast Nuclear RNase P[c]	Yeast RNase MRP[c]	Human Nuclear RNase P
Name	Mass (kDa)	Name	Mass (kDa)	Yeast Name	Human Name	Mass (kDa)	Mass (kDa)	Mass (kDa)
PROTEIN								
P-Protein	~14	MTH687p	14.6	POP5	hPOP5	19.6	19.6	19
		MTH11p	10.7	POP4	RPP29	32.9	32.9	29
		MTH688p	27.7	RPP1	RPP30	32.2	32.2	30
		MTH1618p	17	RPR2	RPP21	16.3		21
				POP1	hPOP1	100.5	100.5	115
				POP7	RPP20	15.8	15.8	20
				POP3	RPP38	22.6	22.6	38
				POP6		18.2	18.2	
				POP8		15.5	15.5	
					RPP40			40
					RPP25			25
					RPP14			14
				SNM1			22.5	
				RMP1			23.6	
RNA								
P-RNA	~95-150	*RNPB*	95	*RPR1*		120		
				NME1			112	
					H1			109

Solid box indicates sequence homology.
Dashed box represents structural similarity.
[a]Masses are shown with an approximate range of sizes for type A and type B RNase P.
[b]Representative of type A RNase P from *M. thermoautotrophicus* is shown.
[c]*S. cerevisiae.*

TABLE II. RNase P and RNase MRP in vitro and in vivo Substrates/Inhibitors

Bacteria	Eukarya	
RNase P	Nuclear RNase P	RNase MRP
Pre-tRNA	Pre-tRNA	Pre-rRNA (A3 site)
Mitochondrial RNA primers for DNA replication	HRA1 antisense RNA	Mitochondrial RNA primers for DNA replication
Pre-4.5S RNA	Box C/D intron encoded snoRNA	CLB2 mRNA
C4 RNA	Long non-coding RNA (lncRNA)	
tmRNA (10Sa RNA & *Cyanelle*)	Pre-rRNA (multiple sites)	
TYMV Virus RNA		
ColE1 RNA	Inhibitors	
Polycistronic mRNA	Poly-nucleotides (G>U>A>>C)	
Riboswitches (transient structures)	ssRNA (mixed sequences)	
Small RNA (>5 nt)		
Φ-80 induces RNA		
Long non-coding RNA (lncRNA)		

Not all substrates or inhibitors have been shown to be physiologically relevant, illustrating the promiscuity of RNase P in vitro.

compared to the RNA subunit, which differs in size based on the bacteria (~95–150 kDa) (Fig. 1 and Table I) [Brown, 1999; Evans et al., 2006]. The protein adopts an α–β sandwich fold and is structurally related to other RNA-binding proteins [Smith et al., 2007]. The primary sequence of the bacterial protein is not tightly conserved, but the crystal structure shows relative conservation of tertiary structure [Smith et al., 2007]. Even though the bacterial RNA is catalytic in vitro at high salt, the protein makes vital contacts with both substrate and the catalytic RNA subunit. Protein contacts with the RNA subunit help fold and stabilize RNA tertiary structure enabling more efficient cleavage [Smith et al., 2007]. In addition, the protein subunit appears to normalize the rates of pre-tRNA cleavage between different tRNAs by expanding the active site, thus enabling efficient processing of all pre-tRNAs [Sun et al., 2006]. The example of RNase P in bacteria serves as a model system and provides fundamental information to understanding other examples of RNase P that contain more protein subunits.

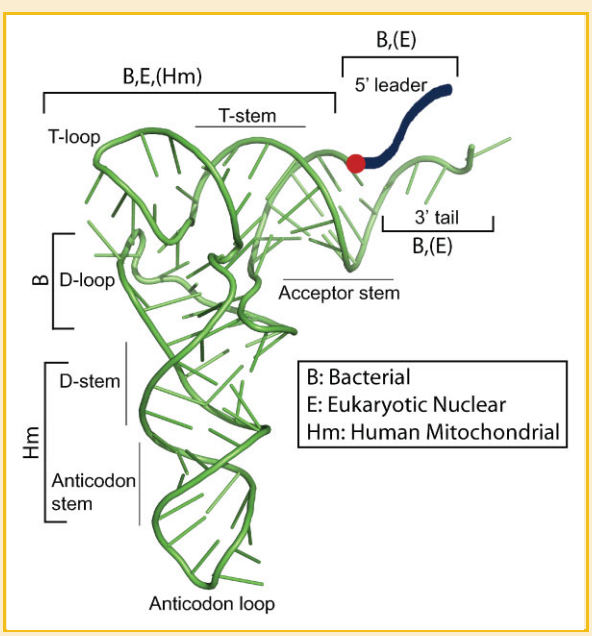

Fig. 2. Important structural regions for RNase P recognition have variations between types of RNase P. Crystal structure of yeast $tRNA^{Phe}$ (PDB code: 1EHZ) [Shi and Moore, 2000]. Cleavage site is indicated by a red dot. Parenthesis around type of RNase P indicate probable interactions but lack of supporting data. Figure created with MacPyMol; http://www.pymol.org.

SUBSTRATE RECOGNITION

RNase P's best-studied substrate, pre-tRNA, has varied primary sequence but common structural features that are important for cleavage. One of the techniques used to investigate how RNase P recognizes tRNA is to make deletions of regions in the substrate to determine the minimal structure needed for successful cleavage. Bacterial RNase P can cleave a minimal substrate that contains just the T-arm and acceptor-stems of the tRNA (Fig. 2). These stems stack to form a coaxial helix that is recognized and cleaved by RNase P in bacteria [Shi and Moore, 2000; Hansen et al., 2001].

In addition to minimal substrate data, biochemical data has indicated that in vitro at high salt, the RNA alone makes multiple contacts with the pre-tRNA substrate: near the cleavage site, D-loop, T-stem, T-loop, acceptor-stem, and CCA tail (Fig. 2) [Kirsebom, 2007]. The DNA-encoded CCA 3′-tail found in most bacterial pre-tRNAs has been shown to make specific contacts with the cognate RNA subunits in the P15 loop [Kirsebom, 2007]. Where this interaction is missing, for example in *Chlamydia*, the protein subunit appears to be able to compensate for the loss of the 3′ interaction with the RNA subunit [Kirsebom, 2007]. It appears that the importance of the P15 RNA subunit interaction with substrate seems to be overshadowed when protein is present.

Further characterization has shown that there are important contacts between the protein subunit and substrate [reviewed in Smith et al., 2007]. In pre-tRNAs, this interaction is clearly with the substrate leader sequences immediately upstream of the cleavage site. The protein was initially known to be important because the holoenzyme binds pre-tRNA substrate better than mature tRNA product, while the RNA subunit alone does not. Residues in the central cleft region of the protein structure have been shown to directly contact the pre-tRNA 5′ leader approximately 4–7 nucleotides 5′ to the cleavage site. This interaction serves to normalize recognition of varied substrates and compensates for

inefficient cleavage by the RNA subunit alone [Kirsebom and Vioque, 1995; Kirsebom, 2007; Smith et al., 2007]. It is worth reiterating that all of these effects on pre-tRNA cleavage are being produced by binding of a relatively small (~14 kDa) protein that is about one-tenth the mass of the RNA subunit and about half the mass of the pre-tRNA substrate.

The broadening of substrate recognition by the protein subunit has also had the effect of expanding the use of the ribozyme's catalytic domain beyond specifically pre-tRNA (Table II). One set of non-tRNA substrates in bacteria appears to form structures that are similar to tRNA. These RNAs include the following: tmRNA precursors from *Escherichia coli* (10Sa) and *Cyanelle*, TYMV viral RNA, ColE1 RNA, and long-nuclear retained RNA (lncRNA) [Giegé et al., 1993; Komine et al., 1994; Jung and Lee, 1995; Gimple and Schön, 2001; Wilusz et al., 2008]. RNase P cleavage of these substrates further illustrates that the shape of the substrate is what is most important for recognition and cleavage and not the primary sequence.

The 4.5S RNA substrate represents a different sort of substrate cleaved by bacterial RNase P. The structure of 4.5S RNA is thought to be a long hairpin that is distinct from tRNA [Peck-Miller and Altman, 1991]. RNase P RNA can cleave pre-4.5S RNA weakly without protein in vitro, but the protein subunit lowers the K_m immensely [Peck-Miller and Altman, 1991]. Another substrate, bacteriophage ϕ80-induced RNA, is also cleaved by RNase P. It is thought to form a structure very similar to 4.5S RNA [Bothwell et al., 1976]. This versatility of substrate recognition by RNase P was further investigated by in vitro selection of RNA that can be cleaved with or without the protein subunit. When the RNA enzyme was present without protein, most cleaved substrates resembled tRNA in structure, but when the protein was added to the RNA subunit, non-tRNA substrates were more readily cleaved [Liu and Altman, 1994]. This is consistent with the observed effect of protein on substrate recognition, expanding the active site to accommodate many different substrates.

The trend of increased substrate recognition was extended further when it was found that the holoenzyme, but not the RNA alone, could cleave single stranded RNA as small as 5 nt [Hansen et al., 2001]. The products of these cleavages were chemically consistent with a normal RNase P mechanism. Cleavage was relatively fast with single turnover rates of 0.1–0.7 min^{-1} depending on identity. In addition, transient structures in riboswitches were shown to be cleaved by RNase P, further illustrating its general RNA cleavage ability [Altman et al., 2005].

General RNA cleavage ability is expanded further with examples of RNase P cleaving RNAs that are intergenic, mRNAs, or antisense transcripts of coding regions. For example, bacterial RNase P has been shown to work in concert with RNase E to process polycistronic mRNA [Alifano et al., 1994]. This RNA is very unstable in precursor form, however, after cleavage by RNase P its half-life increases almost 10-fold [Alifano et al., 1994]. A larger role for various intergenic regions in polycistronic mRNA in *E. coli* was also indicated by microarray analysis [Li and Altman, 2003]. In addition, antisense RNA precursor C4 from bacteriophages P1 and P7 is cleaved by RNase P, which results in inhibition of antirepressor (Ant) synthesis [Hartmann et al., 1995]. RNase P cleaved C4 RNA is required for this inhibition to occur. Combined, these results clearly illustrate that adding protein can increase the capacity for substrate recognition and suggests that the more complex eukaryotic RNase P could have significantly more substrates than the bacterial version.

ARCHAEAL RNase P: INCREASED PROTEIN COMPLEXITY

STRUCTURE AND HOMOLOGOUS PROTEINS

Archaeal RNase P serves as an evolutionary intermediate that contains an RNA subunit and 4–5 protein subunits (Fig. 1 and Table I). Two main branches of archaeal RNase P are delineated by RNA subunit structure as ancestral, type A, and type M, which is mainly from *Methanococci* [Harris et al., 2001]. The main difference is that most type A RNase P RNAs have been shown to have activity without protein subunits while none of the type M RNase P RNAs have activity without protein. The in vitro catalytic activity of the archaeal RNA is more dependent on salt then the bacterial RNA, suggesting more dependence on protein subunits for folding or substrate binding. The protein subunits in archaeal RNase P are related to eukaryotic proteins and most were identified via sequence homology to yeast protein subunits (Fig. 1). For *Methanothermobacter thermoautotrophicus* (Mth) these proteins are, with names of the corresponding yeast proteins in parentheses, Mth11p (Pop4), Mth687p (Pop5), Mth688p (Rpp1), and Mth1618p (Rpr2) (Table I). Yeast two-hybrid analysis has indicated protein–protein interactions for these RNase P subunits (Fig. 1) [Hall and Brown, 2004]. In addition, one of the protein subunits, Mth687p (Pop5), appears to adopt a fold similar to that of the bacterial protein subunit (Fig. 1) [Wilson et al., 2006]. This indicates that Mth687p (Pop5) might carry out some of the same functions as the bacterial RNase P protein, namely, protein contacts with pre-tRNA substrate. The structures of the other protein subunits in archaeal RNase P have been determined also [Evans et al., 2006]. Mth11p (Pop4) was shown to adopt an oligonucleotide fold, which is present in many other RNA-binding proteins indicating probable RNA-binding roles. Mth688p (Rpp1) folds into an αβ barrel similar to the metallo-dependent hydrolase superfamily of proteins, while Mth1618p (Rpr2) folds into two α-helices with interactions at hydrophobic amino acids at the N-terminus along with a central domain comprised of an unstructured loop and a C-terminal zinc ribbon [Hall and Brown, 2002; Evans et al., 2006]. These structures are useful since the high degree of sequence homology between archaea and eukarya proteins is expected to extend to homology of tertiary, and possibly quaternary structures.

The effect of the protein on archaeal RNase P has been studied with the aid of bacterial precedence and eukaryotic homology. Fundamental roles of protein in the simple bacterial RNase P system have been preserved in the archaeal system, partially supported by the effect of adding protein to the archaeal RNase P RNA in reconstitution experiments. These experiments showed the addition of one to four of the protein subunits lowered the in vitro salt requirement for cleavage significantly while K_{cat} increased 25-fold and K_m decreased 170-fold [Tsai et al., 2006]. It is not clear to what extent these changes are due to structural stabilization of the RNA

subunit versus direct substrate interactions as both are probably occurring.

EUKARYOTIC RNase P: DIVERSE FUNCTIONS FROM RELATED ORIGINS

Like many other biosynthetic processes in eukaryotes, eukaryotic RNase P has been partitioned and specifically localized into subcellular locations to allow for additional functions and more complex regulation. This partitioning has been accompanied by a split into multiple and distinct enzymes, composed of varying levels of protein and RNA, and in some cases no RNA at all. The comparably simple bacterial and archaeal RNase P holoenzymes have been replaced by nuclear RNase P, RNase MRP, mitochondrial RNase P, and chloroplast RNase P. The archaeal trend of increased protein content compared to bacteria is further extended in these complexes, presumably to keep pace with a massive increase in the complexity of the RNA biosynthetic pathways in these systems.

RNase MRP

In eukaryotes another enzyme is added into the RNase P milieu, RNase MRP, which is closely related to nuclear RNase P but has entirely different substrates. RNase MRP has only been found in eukaryotes thus far and shares many of the protein subunits with RNase P. Except for the RNase P-specific protein Rpr2, RNase MRP in yeast has all the RNase P proteins and two additional RNase MRP-specific proteins, Snm1 and Rmp1 (Table I). RNase MRP also has its own RNA subunit, encoded by the *NME1* gene in yeast, which is clearly evolutionarily related to RNase P RNA. In humans, RNase MRP also has a unique RNA subunit (7-2 RNA) which likely combines with seven of the 10 human RNase P proteins: Rpp20, 25, 29, 30, 38, hPop5, and hPop1 (Table I) [Walker and Engelke, 2006]. Many of these proteins show homology with yeast proteins and the RNA subunit is also similar (Table I). The overlap in protein identity with RNase P points towards similar evolutionary origins for the complexes.

RNase MRP was originally shown to cleave mitochondrial RNA primers for DNA replication in vitro, leading to the enzyme's name (RNase Mitochondrial RNA Processing). Interestingly, bacterial RNase P has also been shown to have this capability (Table II) [Potuschak et al., 1993]. This RNase MRP cleavage result was controversial, as most RNase MRP was shown to localize to the nucleolus. RNase MRP has since been shown to process pre-rRNA by being required for cleavage at the A3 site within the ITS1 spacer, generating mature 5.8S rRNA in vivo. This substrate has also been shown to be cleaved by yeast RNase P at similar sites in vitro, further indicating the fundamental relatedness of these two complexes [Chamberlain et al., 1996]. It is worth noting, however, that the specificity of RNase P's cleavage of pre-rRNA is questionable as there were multiple sites cut by RNase P. Recent localization of RNase MRP has shown that a minor fraction of the enzyme is also present in cytoplasmic P-bodies in yeast, where it is proposed to be involved in processing CLB2 mRNA and possibly other mRNAs [Gill et al., 2006, 2004]. This localization is relatively transient and dependent on the cell cycle. Thus, the main population of RNase MRP seems to be in the nucleolus but significant micro-populations can appear where its involvement in other RNA processing pathways is needed.

MITOCHONDRIAL RNase P

Mitochondrial RNase P activities are relatively diverse. Two of the best-studied examples are from yeast and human. In *Saccharomyces cerevisiae* the holoenzyme is composed of an essential RNA subunit, Rpm1 (490 nt but varied by strain), that is encoded in the mitochondrial genome, and a nuclear encoded protein, Rpm2 (105 kDa) [Walker and Engelke, 2006]. Rpm1 has lost structural complexity compared to nuclear RNase P RNA Rpr1 but does still share a few conserved regions [Seif et al., 2003]. Given the relatively large size of the Rpm2 protein, the loss in complexity of the RNA could be compensated for by the protein subunit. In contrast to Rpm1, the protein subunit in *S. cerevisiae* shows even less sequence similarity to other RNase P proteins. The protein can localize to the nucleus, as well as the mitochondrion, and act as a transcriptional activator of mitochondrial mRNAs used for mitochondrial chaperones and import [Stribinskis and Ramos, 2007]. In addition it appears that it plays a role in coordination of transcription and mRNA decay and storage in cytoplasmic P-bodies [Stribinskis and Ramos, 2007].

Despite the widespread use of RNA subunits in yeast mitochondrial RNase P, it is increasingly accepted that organelles in other organisms may have developed alternative RNase P activities through convergent evolution to solve the same problem without RNA. There were initial suggestions of this in work with plant chloroplasts and human mitochondria, but the nature of these enzymes was initially controversial. Recent evidence shows that at least human mitochondrial RNase P does not contain an RNA subunit, as only three protein subunits were required to reconstitute pre-tRNA cleavage activity [Holzmann et al., 2008]. These proteins are as follows: a tRNA methyltransferase (MRPP1), a short-chain dehydrogenase/reductase-family member (MRPP2), and a previously unidentified metallonuclease (MRPP3) [Holzmann and Rossmanith, 2009]. This collection of protein components, none of which are homologous to known RNase P proteins, combines to provide specific pre-tRNA recognition and cleavage products that are indistinguishable from other examples of RNase P.

Not surprisingly, changes in substrate recognition are seen with human mitochondrial RNase P. Due to the lack of RNA in the complex, key determinants for recognition appear to be drastically different from "traditional" RNase P. Mutations in the D-domain and anticodon stem were shown to specifically affect processing by mitochondrial RNase P but not nuclear RNase P (Fig. 2) [Rossmanith and Karwan, 1998]. There are presumably contacts near the active site as cleavage is the same as canonical RNase P processing, though this has not yet been investigated in detail.

CHLOROPLAST RNase P

The nature of chloroplast RNase P appears to mirror mitochondrial RNase P in its varied RNA content. *Cyanelle* of primitive alga

Cyanophora paradoxa have been shown to have both RNA and protein subunits that are required for activity [Cordier and Schön, 1999]. This is in contrast to spinach chloroplasts were there has been no RNA subunit identified [Thomas et al., 2000]. Like human mitochondrial RNase P the protein only reaction appears to be relatively efficient and as it can bind pre-tRNA with a K_d of 16 nM [Thomas et al., 2000]. It is interesting that the fundamental reaction of pre-tRNA cleavage can be "passed" between an RNA active site supported by protein to a protein only active site while maintaining efficient cleavage.

NUCLEAR RNase P

One example of nuclear RNase P is in *S. cerevisiae* where the complex is composed of an RNA subunit with nine essential proteins. The RNA subunit has conserved features of the bacterial RNA, however, certain regions are added or deleted in the structure (Fig. 1) [Frank et al., 2000; Evans et al., 2006]. The RNA subunit and all of the protein subunits are required for RNase P activity, which is essential for life in yeast. Also, RNase P is present in relatively low numbers in yeast cells (200–400 copies/cell). This low copy number combined with the large complex makes in vitro assembly difficult and so large-scale biochemical purifications have been carried out to investigate this complex in yeast.

The RNA component of RNase P in *S. cerevisiae* is Rpr1 and is 369 nt long in mature form. It is transcribed by RNA polymerase III as a 487 nt precursor that is processed at some point during assembly with the protein subunits (Fig. 1) [Srisawat et al., 2002]. The Protein subunits interact with both the RNA subunit and each other to form the RNase P complex. In yeast these subunits are Pop1, Pop3, Pop4, Pop5, Pop6, Pop7, Pop8, Rpp1, and Rpr2 (Fig. 1 and Table I). It appears that Pop1 and Pop4 make contacts with the RNA subunit, with Pop1 interacting with the eukaryote-specific P3 loop [Houser-Scott et al., 2002]. In addition, bacterially expressed Pop6/Pop7 were shown to form a heterodimer and bind specifically to the P3 loop of the RNA subunit [Perederina et al., 2007]. Thus, the P3 loop in the RNA subunit appears to accommodate many protein contacts. The other proteins have not been shown to bind directly to the RNA subunit in yeast but have been shown to bind to other protein subunits in the complex (Fig. 1) [Houser-Scott et al., 2002]. There are, however, two proteins that appear to be added after an active precursor complex has been formed: Pop3 and Rpr2 [Srisawat et al., 2002]. The roles of Pop3 and Rpr2 can be inferred not to be essential for pre-tRNA binding and cleavage in vitro, but the fact that they are present in the majority of RNase P in the cell and that Rpr2 is a unique protein subunit of RNase P, points towards important roles in the complex [Srisawat et al., 2002].

Nuclear RNase P from humans has also been extensively studied. Human RNase P has a single RNA subunit, H1, and at least 10 proteins, 7 of which are homologous to yeast RNase P proteins (Table I) [Jarrous, 2002]. In human RNase P it has been shown that Rpp29 and Rpp21 can bind the tRNA substrate in vitro [Jarrous, 2002]. One difficulty with these types of experiments is that most RNase P proteins have large patches of basic amino acids (KKD/E) that have a high potential for binding single stranded RNA [Xiao et al., 2002]. Further, seven of the yeast proteins have calculated pI values higher than 9 except for Pop8 and Pop5, which have pIs of 4.6 and 7.8. These motifs could combine to serve as specific RNA-binding sites for either substrates or the RNA subunit when correctly assembled in vivo but when overexpressed in vitro these binding sites could be relatively non-specific.

Like its evolutionarily related cousin RNase MRP, RNase P is found primarily in the nucleolus in yeast [Walker and Engelke, 2006]. In humans the localization is less constant, with proteins and/or the RNA subunit found in the nucleoplasm, cytoplasm, cajal bodies, and the perinucleolar compartment [Jarrous, 2002]. Multiple localizations of RNase P would be consistent with the behavior of the highly related RNase MRP, which in turn is consistent with the discovery of multiple types of substrates (Table II) [Gill et al., 2006].

Substrate recognition. Despite the high-protein content in nuclear RNase P it appears that the mechanism of pre-tRNA cleavage remains the same and is housed in the RNA subunit. Early phosphothioate substitution experiments with substrates showed that yeast RNase P has the same type of Mg^{2+} dependence and chemical products that bacterial RNase P does [Pfeiffer et al., 2000]. Also it was recently shown that the human RNase P RNA can cleave tRNA without protein, albeit with extremely low activity and at high salt [Kikovska et al., 2007]. It is interesting to note that with all the increased complexity of eukaryotic RNase P, compared to bacterial RNase P, the overall mechanism of pre-tRNA cleavage appears to be roughly the same with an initial burst of tRNA formation followed by a rate-limiting step which is most likely product release [Hsieh et al., 2008]. In addition, the same study suggested there appears to be a kinetically important conformational change during catalysis akin to the bacterial RNase P.

In eukaryotes the nature of pre-tRNA transcripts is somewhat different from those in bacteria. Pre-tRNAs are synthesized by RNA polymerase III and are terminated by a 3′-polyuridine (U_{4-6}) sequence soon after the end of the aminoacyl stem. This tail sequence is usually present when RNase P cleaves the 5′ leader, and usually has the capacity to form a short Watson–Crick stem with the 5′ leader sequence. However, if this 5′ leader-3′ trailer pairing forms a continuous extension of the acceptor-stem, RNase P is unable to cleave, suggesting that the 5′ leader and 3′ trailer might need to be separated for cleavage to occur [Lee et al., 1997]. Another change in recognition by nuclear RNase P is that the bacterial-type P15 loop has been lost, possibly in response to the lack of encoded CCA in the 3′ trailers of nuclear transcripts [Evans et al., 2006]. It is not yet clear what portion of the RNase P holoenzyme interacts with the pre-tRNA leader and trailer, since removal of these sequences has relatively minor effects on substrate binding [Ziehler et al., 2000].

In addition to the substrate differences minimal substrate requirements are altered in eukaryotic RNase P. The same major contacts that are important in bacterial RNase P are required with pre-tRNAs in eukaryotes, namely, the T-stem plus acceptor-stem coaxial structure, but there is an extra requirement of a bulge between the two stems for eukaryotic RNase P (Fig. 2) [Yuan and Altman, 1995]. This bulge can be as small as one nucleotide but more flexibility appears to improve cleavage.

Accompanying the loss of some of the bacterial pre-tRNA contacts, nuclear RNase P has acquired new eukaryotic specific

single stranded RNA contacts. Eukaryotic RNase P binds more strongly to single stranded RNA then bacterial RNase P, inhibiting pre-tRNA cleavage only in yeast RNase P [Ziehler et al., 2000]. Proteins seem probable sites for these interactions, as most of the nine protein subunits are very basic. This binding showed a strong sequence dependence with RNA homopolymers (poly-G > U >> A >>> C) (Table II) [Ziehler et al., 2000]. In contrast to single stranded RNAs, a highly structured RNA, 5S rRNA, showed little or no competition with tRNA [Ziehler et al., 2000]. It seems likely that the tight binding resulting in inhibition is a collaboration between more than one individual RNA-binding site, since short homopolymers (U_7 and U_{11}) have no effect, and the 3′ oligoU trailer on pre-tRNAs does not strongly affect the K_M of the yeast nuclear RNase P [Ziehler et al., 2000].

In eukaryotes there have also been non-tRNA substrates discovered and a much larger number of possible substrates suggested, though this has not yet been explored extensively (Table II). One example is a non-coding, antisense RNA, HRA1, which is cleaved by RNase P in *S. cerevisiae* [Yang and Altman, 2007]. Recently, RNase P in yeast has been shown to be involved in one of the pathways for the maturation of box C/D intron encoded snoRNAs [Coughlin et al., 2008]. Although highly selective cleavage could not be reproduced in vitro using deproteinated intron substrates, the pre-snoRNP RNAs co-immunoprecipitated with RNase P and in vivo analysis of RNase P conditional mutants confirmed accumulation of precursor snoRNAs in RNase P-deficient strains.

A broad range of additional RNA has been identified as potential RNase P substrates in addition to the ones outlined in Table II. These RNAs were identified as copurifying with RNase P and whose abundance or size is affected by defects in either the RNase P RNA (Rpr1), the RNase P-specific protein subunit (Rpr2), and the largest protein subunit (Pop1). These studies found that several groups of RNA were affected by RNase Pmutation and associated physically with RNase P [Coughlin et al., 2008]. The RNA included mRNAs that encode protein subunits of the ribosome, mRNAs from subunits of RNA polymerases I, II, III, translation initiation factor mRNA, box C/D snoRNP protein mRNA, and transcripts from six intergenic regions. The methodology employed for the original binding studies did not differentiate between ''sense'' and ''antisense'' strands in each region, leaving open the possibility that RNase P might be interacting with either strand, or even possible sense/antisense hybrids. Thus, additional studies are needed to further parse the potential substrate dataset based on strand specificity.

Other potential yeast RNase P substrates were identified in a separate strand-specific study by depletion of Rpp1, a protein that is a subunit of both RNase P and RNase MRP [Samanta et al., 2006]. This data had relatively little overlap with the Coughlin et al. study, possibly indicating RNase MRP substrates. However, one interesting set of potential substrates identified were several novel non-coding RNAs that were either adjacent or antisense to protein coding genes [Samanta et al., 2006]. This dataset combined with the Coughlin et al. study suggests a large potential pool of RNase P and RNase MRP substrates, but extensive investigation will be required to confirm physiological significance of the various candidates.

CONCLUSION

The evolutionary pressure to retain the RNA subunit of RNase P appears to be very strong. Regardless of what additional substrates RNase P might have been co-opted to cleave, the need for pre-tRNA cleavage is fundamental. This was recently shown with *Nanoarchaeum equitans* in which the lack of pre-tRNA 5′ leader sequences in primary transcripts from this very compact and relatively simplified genome seems to have resulted in the loss of RNase P activity [Randau et al., 2008]. Contrasted with this leaderless tRNA genome, most organisms have retained the catalytic RNA core of the enzyme, while adding protein content to allow it to selectively recognize the increasing number of possible RNA substrates in more complex organisms and still maintaining pre-tRNA cleavage. It appears that the RNA processing ability of the RNA subunit has needed ''shoring up'' by more and more proteins to cope with further cellular complexity (Fig. 1). Although this discussion has focused on the likelihood that the extra proteins have increased potential for substrate recognition, protein complexity might also be required for correct cellular localization, RNA subunit stabilization, and cooperation with other RNA processing components. The end result is that all of these factors have provided increased functionality to the RNA core.

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