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Structural insight into precursor ribosomal RNA processing by ribonuclease MRP

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Ribonuclease (RNase) MRP is a conserved eukaryotic ribonucleoprotein complex that plays essential roles in pre-ribosomal RNA (pre-rRNA) processing and cell cycle regulation. In contrast to RNase P, which selectively cleaves tRNA-like substrates, it has remained a mystery how RNase MRP recognizes its diverse substrates. To address this question, we determined cryo-EM structures of *Saccharomyces cerevisiae* RNase MRP alone and in complex with a fragment of pre-rRNA. These structures combined with biochemical studies reveal that co-evolution of both protein and RNA subunits has transformed RNase MRP into a distinct ribonuclease that processes single-stranded RNAs by recognizing a short, loosely-defined consensus sequence. This broad substrate specificity suggests that RNase MRP may have myriad yet unrecognized substrates that could play important roles in various cellular contexts.

Ribonuclease mitochondrial RNA processing (RNase MRP) is an essential eukaryotic ribonucleoprotein (RNP) that cleaves RNA substrates in a site-specific manner and plays pivotal roles in RNA metabolism (1–3). Its importance is manifested by the fact that a proteinaceous endoribonuclease that can cleave RNAs with sequence specificity has not been found in nature (4). RNase MRP is structurally and evolutionarily closely related to RNase P, another essential RNase that catalyzes the maturation of the 5'-end of precursor-transfer RNA (pre-tRNA) (3, 5–8). In contrast to RNase P, RNase MRP processes much more diversified substrates. *Saccharomyces cerevisiae* RNase MRP is predominantly localized in the nucleolus, where it is responsible for precursor ribosomal RNA (pre-rRNA) processing at a specific A3 site in the internal transcribed spacer region 1 (ITS1) to release the mature 5.8S rRNA (9–12). *S. cerevisiae* RNase MRP also asymmetrically and temporally exists in a single discrete cytoplasmic spot in daughter cells, where it regulates the cell cycle by cleaving the 5'-UTR of the cyclin B2 (CLB2) mRNA (13, 14). Similar roles of human RNase MRP in ribosome assembly and cyclin-dependent cell cycle regulation have also been established (15, 16). Several protein subunits of human RNase MRP have been considered immunological targets in autoimmune diseases (17–19), and mutations in the RNA subunit of human RNase MRP (RMRP) cause a spectrum of severely debilitating human diseases, including cartilage-hair hypoplasia (CHH) (20, 21), underscoring RNase MRP's essential role in human health.

RNase MRP holoenzyme contains a large catalytic RNA and multiple essential proteins (22, 23). Except for one or two species specific proteins, the catalytic RNA and most of the protein subunits of RNase MRP are conserved from yeast to humans (3). In *S. cerevisiae* RNase MRP, the majority of the protein components are shared with *S. cerevisiae* nuclear RNase P with the exception of Rmp1 and Snm1 (3, 22). Previous secondary structure analyses predicted that the RNA component of *S. cerevisiae* RNase MRP (Nme1) has a similar RNase P-type catalytic (C) domain (24–26). In particular, key nucleotides in the predicted active center highly resemble those in RNase P RNA, suggesting that both ribozymes likely share the same catalytic mechanism (3, 27). In sharp contrast to the C domain, the rest of the Nme1 RNA is completely different from the specificity (S) domain of RNase P RNA (3). In fact, it is even not clear whether this part of Nme1 is involved in the specific recognition of substrates. It has been speculated that this diversified RNA region and specific protein components of RNase MRP have been co-evolved during evolution, giving rise to altered substrate specificity (28, 29). Despite extensive biochemical and genetic studies, the lack of high-resolution structure of RNase MRP severely hinders our understanding of how this essential RNP could process such diversified substrates as pre-rRNA and CLB2 mRNA.

Here we present the cryo-electron microscopy (EM) structures of *S. cerevisiae* RNase MRP holoenzyme alone and in complex with an ITS1 fragment containing the A3

cleavage site. These structures reveal that, although the catalytic center of RNase MRP is nearly identical to that in RNase P, a striking local refolding of key protein subunits transforms RNase MRP into a distinct ribonuclease to process single-stranded RNAs by recognizing a short consensus sequence.

Characterization of *S. cerevisiae* RNase MRP holoenzyme

We purified the endogenous RNase MRP holoenzyme from *S. cerevisiae* using a two-step affinity purification scheme with a protein-A tag on subunit Pop4 and a triple-Flag tag on the RNase MRP-specific subunit Rmp1, the latter of which effectively prevented the contamination from RNase P (fig. S1A) (30, 31). The purified RNase MRP complex was subjected to mass spectrometry analysis, confirming the presence of all previously identified protein components in *S. cerevisiae* RNase MRP (fig. S1, A and B, table S1, and supplementary materials and methods).

The purified RNase MRP holoenzyme was fully active to process a 123-nucleotide (nt) ITS1 fragment of yeast pre-rRNA containing the A3 site with no detectable activity to pre-tRNA (Fig. 1A and fig. S1C) (10, 32). Sequence and phylogenetic analysis of ITS1 regions from multiple species in Saccharomycetaceae revealed a highly conserved 12-nt fragment containing the A3 cleavage site with a GC-rich stem and a long stretch of AU-rich sequence at its 5' and 3' termini, respectively (Fig. 1B and fig. S2). Notably, neither the stem nor the AU-rich sequence is required for the substrate recognition by RNase MRP (Fig. 1A and fig. S3). Further experiments showed that a 21-nt fragment with the A3 site at the center can be efficiently processed by RNase MRP (Fig. 1A and fig. S3), suggesting that, unlike RNase P, which recognizes the three-dimensional structural feature of pre-tRNA, RNase MRP processes short, single-stranded substrates with a distinct sequence specificity (Fig. 1A and fig. S3). This is consistent with a previous *in vitro* selection of *S. cerevisiae* RNase MRP substrates (32). Hereafter, we will refer to this 21-nt fragment as ITS1_{A3} (Fig. 1A). For simplicity, we define the nucleotide 3' to the cleavage site as the "center" (position 1) of ITS1_{A3} (Fig. 1A).

Structures of RNase MRP and its complex with the ITS1_{A3} substrate

To gain insights into the architecture of RNase MRP and the molecular basis of substrate recognition by RNase MRP, we determined the cryo-EM structures of *S. cerevisiae* RNase MRP holoenzyme alone and its complex with the ITS1_{A3} substrate at resolutions of 2.5 Å and 2.9 Å, respectively (Fig. 1, C and D, fig. S4, and table S2). The cryo-EM density maps were of sufficient quality, enabling us to build the atomic model of RNase MRP (Fig. 1, C and D and fig. S5, A to C).

Nucleotides A(-2) to A5 in the ITS1_{A3} substrate were clearly discernable in the density map (fig. S5D). In addition, the EM map also revealed a stacking of the bases of A6 and A7 with A5, allowing us to generate a model of ITS1_{A3} containing nine continuous nucleotides A(-2) to A7 (Fig. 1D and fig. S5D). The structure of apo RNase MRP can be superimposed onto that of the RNase MRP-ITS1_{A3} complex with a root mean square deviation (RMSD) value of ~0.5 Å (fig. S6A), suggesting that RNase MRP adopts a rigid conformation before substrate binding. Hereafter we will focus on the RNase MRP-ITS1_{A3} complex structure, unless stated otherwise.

In the RNase MRP holoenzyme, the Nme1 RNA forms a compact structure occupying the central region of the complex (Fig. 1C). Eleven protein subunits sequentially contact one another and interlink together to form a hook-shaped architecture, with Pop1, Rmp1, and part of Pop4 as the head, and Pop6-Pop7, Pop5-(Rpp1)₂-Pop8, and Pop4-Snm1-Pop3 subcomplexes being the arm (Fig. 1C). This protein hook tightly wraps around and stabilizes Nme1, involving intricate interactions between Nme1 and the protein head module at one side and an extended, flat interface between Nme1 and the arm module at the other (Fig. 1C). Together, these RNA-protein interactions shape the holoenzyme into a configuration with a deep, narrow groove at the catalytic center, in which snugly lies the single-stranded ITS1_{A3} substrate (Fig. 1D). The Pop1-Pop4-Rmp1 ternary motif in the head module mediates specific interactions with the substrate (Fig. 1D). The EM density map of this ternary motif is well-ordered in the complex, but it is of poor quality in the apo structure, suggestive of a local induced fit mechanism for substrate recognition by RNase MRP (fig. S6B). It is noteworthy that the ITS1_{A3}-binding surface on the Pop1-Pop4-Rmp1 motif is highly conserved in Saccharomycetaceae, underscoring the important function of this ternary motif in substrate recognition (fig. S7, A to C).

Structure of the Nme1 RNA

The Nme1 RNA adopts an extended single-layered configuration with a compact core from which extend out four stems P3', P10, P11 and P15 to different directions (Fig. 2, A and B). The RNA core is characterized by three parallelly packed helices, which are composed of coaxially stacked stems P19-P2-P3, P1-P4 and P9-P8, respectively (Fig. 2, A and B). Except for the tip of P19, helices P19-P2-P3 and P1-P4 highly resemble their counterparts in the RNase P structure (fig. S8, A and B) (30, 33). In particular, three conserved regions CR-I, CR-IV and CR-V in the core of Nme1 fold into a pseudoknot motif, which is identical to the structure observed in the catalytic center of RNase P (fig. S8, A and B) (30, 34–37).

Previous sequence analyses predicted a long stem ymP5

in the Nme1 RNA, but failed to assign it as the P9 stem in that the predicted ymP5 contains a much longer duplex than P9 normally has in RNase P (3, 25, 26). Therefore, it was unanticipated that stems P8 and P9 in the S domain of Nme1 could coaxially stack together to form a continuous helix in the RNA core with a duplex region that resembles helix P9-P8 in the RNase P structure (fig. S8, B and C). In fact, the P9 stem in Nme1 contains a very large loop, part of which (U132-A136) is disordered and the rest folds back to its own minor groove and mediates non-Watson-Crick interactions with three consecutive base pairs A121-U153, G122-C152 and A123-A151 (fig. S8, C and D), allowing P9 to contact P1 in the same fashion as in RNase P (fig. S8, B and C). Similarly, on the other end of the P9-P8 helix, the pentaloop of stem P8 (₁₀₉GAAAA₁₁₃) that corresponds to previously identified consensus sequence GARAR (R: purine) in RNase MRP RNAs interacts with the minor groove of stem P4, closely resembling the P8-P4 interface in RNase P RNA (fig. S8, B and C) (30). Together, we conclude that the compact RNA core with three parallelly packed helices is conserved in both RNase MRP and RNase P RNAs (Fig. 2A and fig. S8, A and B) (30). In addition to stem P9, the structure of Nme1 also reveals a three-base-pair stem P5 immediately adjacent to P4, which has not been predicted by previous analyses but is very likely conserved in all RNase MRPs (Fig. 2, A and B) (3, 25-27).

Except for the P9-P8 helix, the rest of the S domain in Nme1 (stems P10 and P11, previously named as ymP6 and ymP7) is markedly different from the S domain in RNase P RNA (fig. S8E), and only makes very limited contacts with protein subunits in RNase MRP (Fig. 1C). Both P10 and P11 can be considerably reduced in size without significant effects on viability or pre-rRNA processing *in vivo*, indicating that these stems are not critical for RNase MRP function (38). This is in sharp contrast to the P10/11-P12 branch of the RNase P RNA in which conserved regions CR-II and CR-III fold into a T-loop structure and play an important role in pre-tRNA substrate binding (fig. S8, A and B) (30, 39, 40). Collectively, we conclude that the S domain of Nme1 is distinct from its counterpart in RNase P RNA both structurally and functionally.

Protein subunits and their interactions with Nme1

The overall architecture of the protein hook in RNase MRP resembles that of RNase P (fig. S9A). In particular, the Pop6-Pop7 heterodimer, the Pop5-(Rpp1)₂-Pop8 heterotetramer, and the Pop4-Snm1-Pop3 heterotrimer are identical to their counterparts in RNase P (fig. S9, A to D) (30, 33, 41). These three protein subcomplexes together form an elongated arm and cover the surface of one side of the Nme1 RNA (Fig. 1C and fig. S9A). The protein head module contains Pop1, Rmp1 and the N-terminal motif of Pop4

(Pop4_{NTM}) (Fig. 3A). Pop1, the largest protein subunit in both RNase MRP and RNase P, is composed of three motifs: an N-terminal motif (NTM), an internal motif (INM), and a large C-terminal globular domain (CTD) (Fig. 3A). Pop1_{CTD} is separated from the rest of the head module, covering the RNA core in the same fashion as in RNase P from the other side of the RNA opposite to the arm module (Fig. 3A and fig. S9E).

Notwithstanding these similarities, marked structural differences do exist in both the RNA and protein subunits in the vicinity of the substrate-binding site. In RNase P, Pop1_{NTM} adopts a helical structure enriched with positively charged residues to stabilize the open junction between CR-IV, stems P4, P7 and P15 of the Rpr1 RNA, serving as an anchor for pre-tRNA substrate recognition (Fig. 3B and fig. S10, A and B) (30). Pop4_{NTM}, on the contrary, folds into a small compact motif and binds the Rpr1 RNA from the opposite side of Pop1_{NTM} (Fig. 3B and fig. S10, A and C) (30). Strikingly, in RNase MRP, both Pop1_{NTM} and Pop4_{NTM} completely refold themselves and adopt extended conformations, wrapping around one end of the helix bundle of Rmp1 (Fig. 3B and fig. S11, A to D). This intricate ternary subcomplex sits atop stem P5 and mediates intimate interactions with the pseudoknot of Nme1, forming part of the substrate-binding groove and playing an important role in specific recognition of single-stranded substrates (Fig. 3B). It should be noted that, although Pop1_{NTM} adopts completely different conformations in RNase MRP and RNase P, a short stretch of arginine residues (R94, R97, R98 and R99) occupies exactly the same location and mediates similar stacking and electrostatic interactions with the RNA pseudoknot, underscoring the crucial function of these residues in stabilizing the catalytic center in both RNPs (fig. S12, A and B) (30). In addition to Pop1_{NTM}, Pop1_{INM} in RNase MRP also reorganizes itself with a distinct fold, sitting in a deep cleft formed by the junction among stems P5 and P8 to P11 (Fig. 3B and fig. S11B). In comparison, Pop1_{INM} in RNase P forms a different topology and associates with the P7-P8'-P8-P9-P10/11 junction from the opposite direction. (Fig. 3B and fig. S10B) (30).

Another distinct structural feature of RNase MRP occurs at the interface between stem P9 of Nme1 and the Pop4-Snm1-Pop3 heterotrimer. In both RNase P and RNase MRP, a long helix of Pop4 simultaneously associates with the termini of stems P1 and P9, enhancing the long-range RNA-RNA interactions (Fig. 3C). In RNase MRP, Pop4^{Tyr78} in the helix mediates a stacking interaction with nucleotide U125 of Nme1, stabilizing the loop of stem P9 in a folded conformation (Fig. 3C). This in turn allows the Pop4 helix to maintain its direction so that it can be connected to Pop4_{NTM} in the head module for substrate recognition (Fig. 3C). By contrast, in RNase P the local structure of Rpr2 and the

bulge in stem P9 change the direction of Pop4 helix with a sharp turn of $\sim 90^\circ$, which otherwise would have a severe collision with the T-loop of the RNA if the helix adopts the same continuous conformation as in RNase MRP (Fig. 3D). Furthermore, the folded loop in stem P9 in RNase MRP occupies the location of the T ψ C loop of pre-tRNA in the RNase P complex, indicating that pre-tRNA could not fit into the substrate-binding site in RNase MRP (Fig. 3E). Taken together, we conclude that, although RNase MRP shares the same overall structural organization as RNase P, several key structural differences in both protein and RNA subunits define RNase MRP as a distinct ribozyme to process non-pre-tRNA substrates.

The RNase MRP-ITS1_{A3} interface

Although ITS1_{A3} is a single-stranded substrate, it adopts a helical-like conformation, resembling the 5' strand of the pre-tRNA acceptor stem in the RNase P complex (fig. S13, A and B). ITS1_{A3} is accommodated deeply in the substrate-binding site, sitting on the minor groove of stem P4 in the Nme1 pseudoknot with its backbone partially or completely buried in a solvent-excluded contact area of $\sim 1,200 \text{ \AA}^2$ (Fig. 4A). The overall conformation of the ITS1_{A3} substrate is mainly governed by two sets of the π - π stacking interactions among bases; A(-2) stacks with A(-1), A1 and C2, and A3 with A5, A6 and A7 (Fig. 4, A and B). The A(-2)-A(-1)-A1-C2 stack is further extended into the Nme1 RNA by the base of U266 in CR-IV of Nme1 (Fig. 4, A to C). Notably, this intermolecular continuous base stack highly resembles the stacking between the 5'-leader of pre-tRNA and nucleotide A314 in CR-IV of the Rpr1 RNA in the RNase P-pre-tRNA complex structure (Fig. 4C and fig. S13, C and D) (30). In addition to the stacking, the sugar-ring and the phosphate backbone of A(-1) in ITS1_{A3} mediate two electrostatic interactions with the main chain of Pop5 N-terminal residue Val2 (Fig. 4C and fig. S13C), which are also conserved in the RNase P-pre-tRNA interactions (fig. S13D) (30). Together, these observations indicate that both RNase MRP and RNase P utilize the same molecular mechanism to non-specifically bind nucleotides of the substrates 5' to the cleavage site (fig. S13, A to D) (30).

The most prominent features of the ITS1_{A3} substrate are the two cytosine, C2 and C4, that define the sequence-specific recognition of ITS1_{A3} by RNase MRP. The side chain of Pop4^{Arg28} points to the ITS1_{A3} substrate and coordinates two electrostatic interactions with the Watson-Crick donor and acceptor groups of C2 in ITS1_{A3} (Fig. 4, B and D and fig. S13E). When the RNase MRP-ITS1_{A3} structure is overlaid onto that of the RNase P-pre-tRNA complex, the short helix in Pop4^{NTM} occupies the equivalent position of pre-tRNA G71-C72 in the RNase P-pre-tRNA complex and the C2-Pop4^{Arg28} interaction strikingly mimics the C2-G71 base pair-

ing in pre-tRNA, stabilizing the single-stranded ITS1_{A3} in the helical conformation (fig. S13, E and F) (30). This Pop4^{Arg28}-mediated 'pseudo-Watson-Crick' interaction together with a hydrogen bond between the sugar-ring hydroxyl group of C2 and the side chain of Rmp1^{Gln97} prefers a cytosine at position 2 in the ITS1_{A3} substrate (Fig. 4, B and D). In addition to the interaction with C2, the guanidino group of Pop4^{Arg28} also makes a stacking interaction with the base of A3, serving as a stabilizing connector between C2 and A3 (Fig. 4, B and D).

In contrast to C2, C4 flips out from the helical position and points into a pocket formed by the protein head module and the Nme1 RNA (Fig. 4, B and E, and fig. S13G). The base of C4 is sandwiched by the side chains of Rmp1^{Arg24} and Pop1^{His103} through stacking interactions in the center of the pocket (Fig. 4, B and E). The structure shows that the cavity of the pocket is not optimal to accommodate bulky purine nucleotides (Fig. 4E). Notably, the edge of the base of C4 is surrounded with three hydrogen-bonding interactions with Nme1 U226, Rmp1^{Arg24} and Pop4^{Thr38}, providing a strong preference for cytosine at this position (Fig. 4E). In addition to these sequence specific interactions, the backbone phosphate group of C4 accepts one hydrogen bond from Rmp1^{Gln28}, further stabilizing the base of C4 in the pocket (Fig. 4E). Collectively, the RNase MRP-ITS1_{A3} complex structure demonstrates that RNase MRP recognizes a short single-stranded region of ITS1_{A3} and two nucleotides C2 and C4 make sequence specific interactions with RNase MRP.

Consensus substrate sequence

To further define the consensus substrate sequence for RNase MRP, we mutated each nucleotide from C(-3) to A5 in ITS1_{A3} and evaluated their effects using the in vitro activity assay. Consistent with the structure, substitution of C4 with any other nucleotides all led to reduced activity, confirming that position 4 relative to the cleavage site prefers a cytosine (Fig. 4F) (32). In contrast to C4, mutational analysis of C2 resulted in a hierarchical ranking of C>U>A>G, from the most to the least preferred nucleotide for position 2 in ITS1_{A3} (Fig. 4F). Notably, this nucleotide ranking is in perfect agreement with the RNase MRP-ITS1_{A3} interface at C2. First, despite the shape of uracil resembling cytosine, its imino group does not provide compatible hydrogen bond acceptors to the guanidino group of Pop4^{Arg28} (Fig. 4D). Second, due to the small size of the pocket, a pyrimidine is preferred at position 2 (Fig. 4D). Third, if a purine could be fit into position 2 by some local structural adjustment, adenine is more favorable than guanine due to the same consideration of hydrogen bond compatibility with the side chain of Pop4^{Arg28} (Fig. 4D).

In accordance with the observation that nucleotides other than C2 and C4 in ITS1_{A3} make no sequence specific interactions with RNase MRP, single nucleotide replace-

ment at positions -3, -2, -1, or 5 in ITS1_{A3} had marginal effect on the processing activity of RNase MRP (Fig. 4F). Surprisingly, substitution of A1 or A3 with a uracil or cytosine led to a detectable reduction in activity (Fig. 4F). Close examination of complex structure suggested that the stacking interaction between A3 and the guanidino group of Pop4^{Arg28} will likely be weakened by a pyrimidine replacement of A3 (Fig. 4F). Similarly, a pyrimidine at position 1 would also attenuate the stacking interaction with C2 (Fig. 4F). Therefore, the mutational data are consistent with the structural information, suggestive of a preference for purines for positions 1 and 3 in the substrate. In aggregate, combination of our structural and biochemical data defines a short consensus sequence for RNase MRP substrate as 5'-*RCRC-3' (*: cleavage site). Notably, an A-to-C substitution at position -1 would generate two additional optimal cleavage sites between positions -5 and -4 and between -3 and -2 (Fig. 1A). Indeed, the activity assay of this mutant ITS1_{A3} substrate generated three equal-amount products with the expected sizes (Fig. 4F), providing an independent validation of the consensus sequence for RNase MRP substrates.

It should be noted that there was a minor band below the consensus product in the in vitro assay, indicating a cleavage event between A(-5) and A(-4) in ITS1_{A3} using 5'-*ACAA-3' as the substrate recognition sequence (Fig. 4F). This is in agreement with the mutational data that the majority of the single-nucleotide substitutions in ITS1_{A3} were not deleterious and can still be cleaved by RNase MRP (Fig. 4F). In addition to ITS1_{A3}, several substrates of yeast RNase MRP have been identified, and among them the cleavage regions in the 5'-UTRs of CLB2 mRNA and chitinase CTS1 mRNA have been biochemically characterized (13, 42). In light of the RNase MRP-ITS1_{A3} structure, we performed multiple sequence alignment of these cleavage regions. The resulting cleavage sequences of this alignment largely abides by the 5'-*RCRC-3' rule derived from the structural and mutational analysis of the RNase MRP-ITS1_{A3} interaction yet with some exceptions (fig. S14), suggesting that, despite the preferred 5'-*RCRC-3' substrate sequence, RNase MRP can still process a broader spectrum of single-stranded RNA substrates.

Active site

The cryo-EM density map unveils three putative metal ions (M1, M2 and M3) in the vicinity of the scissile phosphate, which would stabilize the ITS1_{A3} substrate at the catalytic center of RNase MRP (fig. S15, A and B). The EM density map is of sufficient quality, allowing us to define the locations of the putative M1 and M3 ions (fig. S15B). M1 corresponds to one of the putative catalytic magnesium ions (Mg²⁺) in the RNase P-pre-tRNA structure (Fig. 5, A and B) (30), and is coordinated by inner-sphere contacts with the

scissile phosphate of ITS1_{A3} and non-bridging phosphoryl oxygens of Nme1 A86 and G87 (Fig. 5A). Although the local density map for M2 is not discrete, we could model a putative Mg²⁺ ion in the density (fig. S15B), so that it is stabilized by A86, A305 and A306 of Nme1 as well as the O3' leaving group of A(-1) in the ITS1_{A3} substrate (Fig. 5A), occupying the equivalent position of the other catalytic ion in the RNase P-pre-tRNA structure (Fig. 5, A and B) (30). In contrast to catalytic M1 and M2 ions, the third putative metal ion M3 may play a structural role by mediating contacts with the backbone of Nme1 G87 and the scissile phosphate of ITS1_{A3}, helping stabilize the local substrate geometry at the active site (Fig. 5A).

The configuration of this RNA-based catalytic center of RNase MRP is identical to that in the RNase P-pre-tRNA complex, except for the conformation of the conserved uridine in stem P4 of the RNA subunits (Fig. 5, A and B) (30). Upon pre-tRNA binding in RNase P, U93 in Rpr1 rotates a large angle to point into the P4 stem and interacts with the putative M1 Mg²⁺ ion, transforming the ribozyme into a fully active state (Fig. 5B) (30). In contrast, the corresponding uridine U88 of Nme1 in the RNase MRP-ITS1_{A3} complex structure still maintains a flipped-out conformation (Fig. 5A). We propose that the snapshot captured in the RNase MRP-ITS1_{A3} complex structure represents a state of initial substrate binding to RNase MRP, in which U88 is still in an inactive conformation (Fig. 5A). Given that less than half of the nucleotides in ITS1_{A3} can be observed in the EM density map, it is possible that the rest of ITS1_{A3}, although disordered in the structure, may help induce the conformational change of U88 to fully activate the ribozyme. How RNase MRP is fully activated by ITS1_{A3} warrants future investigations. Thus, although the biochemistry and cryo-EM structure of the RNase MRP-ITS1_{A3} complex reveal clear substrate recognition differences compared to RNase P-pre-tRNA interactions, both essential RNase P and MRP ribonucleoprotein complexes appear to share a common two metal-ion S_N2-type catalytic mechanism (Fig. 5C) (30, 37).

Discussion

In sharp contrast to RNase P that has a penchant for the tRNA-like fold, it has remained a mystery how RNase MRP recognizes its substrates. So far, only a few RNase MRP substrates have been identified, severely impeding our understanding of RNase MRP function. The short consensus sequence derived from our structural and biochemical studies implies that RNase MRP may have myriad substrates that have not been identified but could play important roles in various cellular contexts. Future work is required to identify and characterize these novel RNase MRP substrates.

Mutations in the RNA component of human RNase MRP causing the highly pleiotropic disease CHH harkens to

RNase MRP's essential and irreplaceable role in humans (20, 21). Comparative analysis of the RNA components of yeast and human RNase MRP shows that, except for peripheral stems P10, P11 and P15, most of the structural elements in yeast Nme1 RNA are conserved in human RMRP (Fig. 2B and figs. S16 and S17A) (21). Three-dimensional mapping of disease-derived mutations onto the corresponding positions in Nme1 structure unveils that, although these incapacitating mutations are found in a wide range of locations in RMRP, most of them are spatially clustered in the helical core near the catalytic center of the RNA (fig. S17B). In particular, A70 > G is the most prevalent mutation that has been found in over 90% of the CHH patients (21). This adenine nucleotide is conserved in all RNase P and MRP RNAs and forms a key Hoogsteen base-pair with a uracil or cytosine (C250 in human RMRP) in the center of the catalytic pseudoknot (fig. S17C). Structure-based analysis suggests that, although these positions are not directly involved in substrate binding and processing, their mutations very likely induce conformational changes of the RNA, leading to defective substrate binding and processing. Further structural and functional studies of the human RNase MRP holoenzyme will be required to fully understand the effects of these disease mutations.

The structure of yeast RNase MRP explains why RNase MRP is observed in eukaryotes but not in bacteria and archaea (2, 6, 43). It has long been appreciated that the RNase P and MRP family of ribonucleases share a common ancestor from the "RNA world", presumably a ribozyme that can catalyze the 5'-end maturation of pre-tRNA (44). The overall architecture of this ribozyme has been faithfully succeeded by bacterial and archaeal RNase Ps, in particular the two RNA-based anchors (A-anchor and the T-loop anchor) that can specifically recognize the three-dimensional structure of pre-tRNA through the double-anchor mechanism (Fig. 5D) (30, 37, 45). It is noteworthy that this high specificity endowed by the RNA anchors, on the other hand, also constrains the ability of bacterial and archaeal RNase Ps to process non-pre-tRNA-like substrates. Although bacteria and archaea had acquired additional protein subunits during evolution (Rpp for bacteria, and (Pop5-Rpp30)₂ and Rpp29-Rpp21-Rpp38 for archaea), these proteins did not replace the essential function of RNA-based anchors for pre-tRNA recognition (Fig. 5D) (45–47). The evolution of protein subunits Pop1 and Pop4 in eukaryotic RNase P and MRP released this limitation; Pop1 is a new addition in eukaryotes whereas Pop4 has evolved from its archaeal ancestor Rpp29 with a new N-terminal motif (48, 49). In eukaryotic nuclear RNase P, whereas the T-loop anchor has been retained, the A-anchor was replaced by the NTM of Pop1, which folds into a compact module and locks the cleavage site of pre-tRNA in the catalytic center (Fig. 5D) (30). In

contrast, RNase MRP had abandoned both RNA-based anchors during evolution, and the NTMs of Pop1 and Pop4 adopt extended conformations that are completely different from those in RNase P (Fig. 5D). Consequently, these refolded motifs together with an RNase MRP specific subunit Rmp1 specifically recognize single-stranded substrates with a short consensus sequence (Fig. 4, A to F, and Fig. 5D). Therefore, the replacement of the rigid RNA-based apparatus by malleable protein subunits Pop1 and Pop4 in eukaryotic RNase P and MRP not only allows eukaryotes to maintain the activity to accurately process pre-tRNA and pre-tRNA-like substrates, but also endows them with the capability of processing much more diversified single-stranded substrates using the same two-metal-ion catalysis mechanism (Fig. 5, C and D).

RNase MRP is one of the two remaining RNA-based RNP catalysts that are found only in eukaryotic cells. The other is spliceosome, which assembles with nuclear pre-mRNAs and splices out the major class of introns (50). But, why is RNase MRP specifically needed, given that there are many proteinaceous endoribonucleases in eukaryotic cells? The known RNA endonucleases either cleave their targets through recognition of specific structures or have very limited or no sequence specificity (51–55). On the other end, some molecular machineries have sequence-specific RNA cleavage activities but require a guide RNA for target recognition (54). A proteinaceous endoribonuclease that can cleave only RNA in a sequence-specific manner has not yet been found. Therefore, the evolution of RNase MRP RNP likely reflects the need to process single-stranded RNAs with sequence specificity in eukaryotes, which contain many more RNA transcripts than bacteria and archaea. The identification and characterization of these RNase MRP substrates will help unveil the in vivo function of this abundant essential RNP.

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SUPPLEMENTARY MATERIALS

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Materials and Methods

Figs. S1 to S17

Tables S1 and S2

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Figure 1

Fig. 1. Overall structures of the *S. cerevisiae* RNase MRP holoenzyme alone and in complex with the ITS1_{A3} substrate. (A) Schematic diagram of the *S. cerevisiae* 35S pre-rRNA transcript. Mature rRNAs and spacers are shown as black boxes and lines, respectively, along with predicted secondary structure of the 5'-stem in ITS1. The numbering scheme of the A3 cleavage site is shown under the sequence of ITS1_{A3}, and the consensus recognition sequence is boxed with C2 and C4 colored in red. (B) Multiple sequence alignment of the ITS1 region from representative species (*Saccharomyces cerevisiae*; *Saccharomyces kudriavzevii*; *Saccharomyces arboricola*; *Saccharomyces eubayanus*; *Lachancea thermotolerans*; *Eremothecium cymbalariae*; *Naumovozyma castellii*; *Tetrapispora blattae*; *Kazachstania naganishii*; *Kluyveromyces lactis*; *Zygosaccharomyces rouxii*; *Torulaspora delbrueckii*) in Saccharomycetaceae. Nucleotides important for substrate recognition are highlighted by blue triangles. (C) Front and back views of the overall structure of the *S. cerevisiae* RNase MRP complex. The protein and RNA subunits of RNase MRP are shown in cartoon representation and colored according to the scheme shown on the right. The boundary between the head and arm modules in the protein hook is denoted by a red dashed line. (D) Overall structure of the *S. cerevisiae* RNase MRP complex with the single-stranded ITS1_{A3} substrate. The RNase MRP complex and the ITS1_{A3} substrate are shown in cartoon and colored as in (C). A close-up view of the substrate-binding pocket is shown in surface representation on the right. Pop1_{NTM} and Pop4_{NTM}: N-terminal motifs of Pop1 and Pop4.

Fig. 2. Structure of the Nme1 RNA. (A) Two orthogonal views of the overall structure of the Nme1 RNA. CR-I, CR-IV and CR-V are colored in slate, orange and cyan, respectively. The helical RNA core is highlighted by a red box. (B) Secondary structure diagram of the Nme1 RNA. The canonical Watson-Crick and non-canonical base-pairing interactions are shown as solid lines and dots, respectively. The long-range base-pairing and tertiary interactions are denoted as black and blue dotted lines, respectively. Nucleotides that are universally conserved in all known MRP RNAs are highlighted with shaded circles.

Fig. 4. Substrate recognition by RNase MRP. (A) The ITS1_{A3} substrate adopts a deformed helical conformation and is accommodated deeply in the substrate-binding groove. The ITS1_{A3}-binding groove is shown in ribbon (left) and surface (right) representation and colored as in Fig. 1C. The ITS1_{A3} substrate is shown in cartoon and colored in cyan. Base stacking interaction, recognitions of C2 and C4 are highlighted in green, blue and red boxes, respectively; enlarged in (C) to (E). (B) Schematic diagram of the RNase MRP-ITS1_{A3} interaction. Nucleotides in ITS1_{A3} and Nme1 are shown in cyan and gray, respectively. Residues in Pop1, Pop4, Rmp1 and Pop5 are colored as in Fig. 1C. Hydrogen bonds and electrostatic interactions are denoted as black dashed lines. (C) Close-up view of the stacking interactions among the nucleotide bases in ITS1_{A3} and between ITS1_{A3} and U266 in CR-IV of Nme1. (D) Detailed interactions of ITS1_{A3} C2 with Pop4^{Arg28} and Rmp1^{Gln97}. (E) ITS1_{A3} C4 flips into a pocket formed by Pop1^{NTM}, Pop4^{NTM}, Rmp1 and the Nme1 RNA. (F) In vitro cleavage activity assay of wild-type and single-nucleotide substituted ITS1_{A3} substrates. A 12-nucleotide oligo was used as a marker for the correctly processed product by RNase MRP. A minor cleavage between A(-5) and A(-4) is denoted with a red star. Three equal-amount cleavage products due to the A-to-C substitution at position -1 are denoted with red triangles.

Fig. 5 (preceding page). Implications for catalysis and evolution of RNase MRP. (A) Left: Overview of the catalytic center in yeast RNase MRP. The pseudoknot of Nme1 and the ITS1_{A3} substrate are colored in orange and cyan, respectively. Right inset: A close-up view of the catalytic center. The coordination of modeled ions M1 and M2 is highlighted in magenta dashed lines and that of M3 in yellow dashed lines. The conserved nucleotide U88 of Nme1 flips out and points away from the catalytic center in the complex structure. (B) A close-up view of the catalytic center in yeast RNase P (PDB: 6AH3) (30). Coordination of modeled ions M1 and M2 are essentially identical to those in RNase MRP except for nucleotide U93. In presence of the pre-tRNA substrate, the corresponding conserved nucleotide U93 points into the catalytic center to coordinate the M1 metal ion. (C) Proposed reaction mechanism for the processing of ITS1_{A3} by yeast RNase MRP. The reactive oxygens are colored in red, the ITS1_{A3} scissile phosphate is depicted in a pre-attack state, and the interactions between catalytically important nucleotides and reactive oxygens mediated by modeled ions M1 and M2 are shown as magenta dashed lines. M1 coordinates an attacking hydroxyl nucleophile during the cleavage reaction. (D) A schematic cladogram depicting the possible evolution pathway of RNase P and RNase MRP is shown at upper-left (LUCA: last universal common ancestor). Proteins that mainly function to stabilize the catalytic RNA subunit and adopt similar architectures in both RNase P and RNase MRP are shown in grey surface. Single-stranded regions 5' to the cleavage site in pre-tRNA and ITS1_{A3} are colored in red and the rest of the substrates in cyan.