Substrate recognition by ribonucleoprotein ribonuclease MRP

OLGA ESAKOVA, ANNA PEREDERINA, CHAO QUAN, IGOR BEREZIN, and ANDREY S. KRASILNIKOV
Department of Biochemistry and Molecular Biology, The Pennsylvania State University, University Park, Pennsylvania 16802, USA

ABSTRACT
The ribonucleoprotein complex ribonuclease (RNase) MRP is a site-specific endoribonuclease essential for the survival of the eukaryotic cell. RNase MRP closely resembles RNase P (a universal endoribonuclease responsible for the maturation of the 5' ends of tRNA) but recognizes distinct substrates including pre-rRNA and mRNA. Here we report the results of an in vitro selection of Saccharomyces cerevisiae RNase MRP substrates starting from a pool of random sequences. The results indicate that RNase MRP cleaves single-stranded RNA and is sensitive to sequences in the immediate vicinity of the cleavage site requiring a cytosine at the position +4 relative to the cleavage site. Structural implications of the differences in substrate recognition by RNases P and MRP are discussed.

Keywords: ribonuclease MRP; RNase MRP; Saccharomyces cerevisiae; in vitro selection; ribonucleoprotein

INTRODUCTION
Ribonuclease (RNase) MRP is a universal eukaryotic site-specific endoribonuclease (Chang and Clayton 1987a,b; Karwan et al. 1991) closely related to RNase P, an RNA-based enzyme responsible for the maturation of 5' ends of tRNA (Altman and Kirsebom 1999). RNase MRP is an essential enzyme (Schmitt and Clayton 1992) found in practically all eukaryotes analyzed (Piccinelli et al. 2005; Rosenblad et al. 2006). RNase MRP is involved in the maturation of rRNA (Schmitt and Clayton 1993; Chu et al. 1994; Lygerou et al. 1994, 1996) and the degradation of a specific mRNA involved in the regulation of the cell cycle (Cai et al. 2002; Gill et al. 2004). A very small fraction of RNase MRP is found in the mitochondria (Chang and Clayton 1987a; Kiss and Filipowicz 1992); however, mitochondrial RNase MRP has a distinct composition and substrate specificity (Lu et al. 2010) and is beyond the scope of this work. Mutations in the RNA component of human RNase MRP result in a variety of pleiotropic diseases (Ridanpaa et al. 2001; Martin and Li 2007 and references therein).

RNase MRP is a ribonucleoprotein with a composition closely resembling that of eukaryotic RNase P (for review, see Esakova and Krasilnikov 2010). In Saccharomyces cerevisiae, RNase MRP contains 10 essential proteins, eight of which are also found in RNase P (Chamberlain et al. 1998, Salinas et al. 2005). The RNA component of RNase P (Fig. 1A) is the enzyme's catalytic moiety (Guerrier-Takada et al. 1983; Pannucci et al. 1999; Thomas et al. 2000; Kikovska et al. 2007); the RNA component of RNase MRP (Fig. 1B) shows clear similarity to that of RNase P. In particular, the catalytic (C-) domain of RNase P (a generally phylogenetically conserved domain that contains the active site) appears to have the same overall architecture as the corresponding part of RNase MRP (Domain 1, Fig. 1A,B; Esakova et al. 2008 and references therein). Several nucleotides that are universally conserved in RNase P are also found in RNase MRP (Lopez et al. 2009). Moreover, the P3 subdomains (Perederina et al. 2010) of the two enzymes (Fig. 1A,B) can be interchanged (Lindahl et al. 2000). The similarity of the C-domain of RNase P to the corresponding part of RNase MRP strongly suggests that the two enzymes use a common mechanism of catalysis.

The specificity (S-) domain of RNase P (Fig. 1A) is responsible for pre-tRNA substrate recognition by this enzyme (for review, see Esakova and Krasilnikov 2010). This domain contains a structurally conserved region that is involved in substrate recognition (Krasilnikov et al. 2003, 2004; Reiter et al. 2010). In general, RNase P does not recognize specific sequences but appears to use recognizable sequence preferences for the recognition of pre-tRNA.
structural features in its substrates (for review, see Kirsebom 2007).

RNase MRP does not have a structural element resembling the specificity domain of RNase P but possesses a distinct element, Domain 2, instead (Fig. 1B). The divergence between the specificity domain in RNase P and the corresponding part in RNase MRP is consistent with distinct specificities of the two closely related enzymes. RNase MRP Domains 2 can vary significantly when enzymes from different organisms are compared; the only phylogenetically conserved part of Domain 2 is a GARAR (where R is G or A) (Lopez et al. 2009) is outlined in gray. (C) A putative secondary structure of yeast pre-rRNA internal transcribed spacer 1 near the RNase MRP cleavage site A3. (Arrow) The A3 site. The diagrams are based on Esakova and Krasilnikov (2010).

**RESULTS**

**In vitro selection of RNase MRP substrates**

The RNase MRP holoenzyme was purified from *S. cerevisiae* using an affinity tag attached to the C terminus of the protein component Rmp1 (see Materials and Methods). The purified holoenzyme contained all known RNase MRP protein components; the RNA component of the purified complex was essentially pure (Supplemental Fig. 1). To select RNase MRP substrates, we used a procedure generally based on the approach previously described in Pan and Uhlenbeck (1992) and Pan (1995). Briefly, a library of deoxyribonucleotides was used to create a pool of circularized RNA molecules that contained a stretch of 68 random nucleotides. The starting number of RNA molecules (1.8 × 10^{13}) was significantly smaller than the number of possible sequences in the 68-nt-long random stretch (8.7 × 10^{40}); thus, practically each RNA molecule was expected to be unique. The pool of these circular RNA molecules was subjected to a cleavage with RNase MRP; the cleavage resulted in the linearization of RNA. The linearized molecules were separated from the remaining circular ones using electrophoresis in a denaturing polyacrylamide gel, and then extracted from the gel and self-ligated using T4 RNA ligase 1. The ligation resulted in a pool of circular RNA molecules similar to the starting pool but enriched in sequences cleavable by RNase MRP. Due to the specificity of T4 RNA ligase 1, only RNA molecules containing 5'-phosphate and 3'-hydroxyl termini (such as the ones produced after RNase P/MRP cleavage but not after typical nonspecific RNA degradation) were re-circularized. Recircularized RNA was used as a template for reverse transcription, followed by PCR to produce template DNA for the subsequent round of selection. After six rounds of selection, most of the RNA in the pool was cleavable by RNase MRP.

After the sixth round of selection, the pool of final DNAs was inserted into a plasmid vector, which was used to transform *Escherichia coli* cells. Plasmid DNA from individual colonies was isolated, sequenced, and used to produce RNAs by run-off transcription with T7 RNA polymerase. The resultant individual RNAs were labeled with ^{32}P and subjected to RNase MRP cleavage, followed by the analysis of the cleavage products on nucleotide-resolution polyacrylamide gels. In total, 144 cleavage sites were identified and analyzed (Supplemental Table 1).

**Analysis of identified RNase MRP substrates**

The analysis of the results of the substrate selection shows that all cleavage sites are located in single-stranded regions of RNA (as judged by the secondary structure modeling with RNAstructure) (Mathews et al. 2004).

More than 80% of all substrates have one of these five sequences in the positions +2 to +4: CUC, UUC, CGC, AUC, or CAC (Fig. 2A,B). When only the strongest 40% of all sites are analyzed (Supplemental Table 1), the distribution of the sequences in these positions changes considerably,
with CUC dominating in the strongest substrates (Fig. 2C). All identified substrates have a cytosine located in the position +4 from the cleavage site (Fig. 2A). The position −1 is typically occupied by a pyrimidine, while guanines were selected against at both +1 and −1 (Supplemental Table 1). Positions from −2 to −7 are enriched in uridines (Fig. 2A). An analysis of the sequences of individual substrates shows that the majority of them have U-rich stretches located in this area (Supplemental Table 1).

To test if the features that are typical to the identified substrates (CUC/UUC/CGC/AUC/CAC in the positions +2 to +4, no guanines in the positions +1 and −1, and a U-rich stretch located 5′ to the cleavage site) are sufficient for RNase MRP cleavage, we tested a series of oligonucleotides with sequences that were random, except for their middle parts where the sequence features of interest were located (e.g., 5′-N19-UUUU-H*H-CUC-N13-3′, where N is a random nucleotide [G, A, U, C] and H is a random mix of A, U, and C; the random nucleotides were used to eliminate a possible influence of the upstream and downstream sequences or secondary structures on the results). Cleavage assays demonstrate that these oligonucleotides are cleaved by RNase MRP at the position indicated by the asterisk, consistent with the results of the substrate selection (Fig. 3).

The most salient feature of the identified substrates is the conserved cytosine in the position +4 (Fig. 2A). To test if the presence of a cytosine in the position +4 is required for RNase MRP cleavage, we replaced this cytosine with other nucleobases (G, A, and U). The results of the cleavage assays show that the presence of a cytosine in the position +4 is required for RNase MRP cleavage (Fig. 4).

An analysis of identified substrates indicates that guanines in positions +1 and −1 (i.e., immediately flanking the cleavage site) are selected against (Fig. 2A; Supplemental Table 1). To test if the presence of guanines in the immediate vicinity of the cleavage site affects cleavage, we compared RNase MRP cleavage of a substrate that does not have guanines in positions +1 and −1 with that of substrates that have guanines in positions +1 or −1, or both. The results show that while the presence of a guanine next to the cleavage site does not eliminate RNase MRP cleavage, the efficiency of cleavage is considerably reduced (Fig. 5). The presence of guanines at both the +1 and the −1 positions effectively eliminates cleavage (Fig. 5).

Most of the identified substrates have a U-rich stretch (typically three to five uridines) localized several (typically two to five) nucleotides 5′ to the cleavage site; the localizations, sizes, and compositions of these stretches vary (Supplemental Table 1). Removal of the U-rich stretch

**FIGURE 2.** Sequences of in vitro selected RNase MRP substrates. (A) Consensus sequence logo. (Arrow) RNase MRP cleavage site. (B) Frequencies of the occurrence of different sequences at positions +2 to +4 in all RNase MRP substrates. (C) Frequencies of the occurrence of different sequences at positions +2 to +4 in 40% of the strongest RNase MRP substrates.

**FIGURE 3.** RNase MRP cleavage of partially randomized sequences. Random sequences were used to eliminate the potential influence of the upstream and downstream sequences or secondary structures on the results. (A) 5′-N19-UUUU-H*H-AUC-N13-3′. (B) 5′-N19-UUUU-H*H-CUC-N13-3′. (C) 5′-N19-UUUU-H*H-CGC-N13-3′. In all panels, N is a random nucleotide (G, A, U, C); H is a random mix of A, U, and C; asterisk (*) designates the cleavage site (indicated by an arrow in the figure). (Lane 1) Digest with RNase T1 (a marker); (lane 2) alkaline hydrolysis (a marker); (lane 3) untreated RNA substrate; (lanes 4, 5) substrates digested with RNase MRP. Substrates were 5′-end-labeled with 32P and separated on a 6% denaturing polyacrylamide gel.
does not eliminate RNase MRP cleavage but does result in a reduction in the efficiency of cleavage (data not shown).

All identified RNase MRP cleavage sites are located in regions of RNA that are expected to be single-stranded. To test if RNase MRP can cleave double-stranded RNA, cleavable RNase MRP substrates were extended using a complementary sequence so the resultant molecules contained cleavage site sequences as part of a double-stranded RNA hairpin. RNase MRP was not able to cleave double-stranded RNA (data not shown).

**Cleavage of known RNase MRP substrates**

RNase MRP is essential for the survival of the eukaryotic cell (Schmitt and Clayton 1992). Thus far, two natural RNase MRP substrates outside the mitochondria have been identified. First, RNase MRP was shown to cleave the 5' UTR of the CLB2 (Cyclin B2) mRNA (Gill et al. 2004). (RNase MRP, generally a nucleolar enzyme, was shown to transiently accumulate in a discrete cytoplasmic spot where it co-localizes with the CLB2 mRNA [Gill et al. 2006]). In vitro cleavage assays performed on the 5' UTR of CLB2 mRNA confirm previously reported (Gill et al. 2004) RNase MRP cleavage at sites that are consistent with the identified consensus (Supplemental Fig. 2).

The other known RNase MRP substrate is the internal transcribed spacer 1 (ITS1), which is cleaved by RNase MRP at the A3 site (Fig. 1C; Lygerou et al. 1996). As expected on the basis of the results of the analysis of RNase MRP specificity, mutations of the cytosine located 4 nt 3' to the A3 site eliminate RNase MRP cleavage (Fig. 6A,B). A hairpin that may potentially fold in the immediate vicinity of the A3 site (Fig. 1C) is not essential for RNase MRP cleavage (Fig. 7).

**DISCUSSION**

The results of the substrate selection combined with cleavage assays indicate that cleavage by RNase MRP occurs in single-stranded regions of RNA and requires the presence of...
a cytosine at the position +4 from the cleavage site (Fig. 2A).
Positions +2, +3 are typically occupied by CU, UU, CG, AU,
or CA (Fig. 2B,C), and a presence of a U-rich region 5’
9
of the cleavage site improves the efficiency of RNase MRP
cleavage, while the presence of a guanine at positions +1 or
/C0
1 substantially reduces it.

The specificity demonstrated by RNase MRP in vitro
appears to be surprisingly broad. Several explanations can
be suggested to reconcile the discrepancy between the
abundance of sequences cleavable by RNase MRP in vitro
and the apparent rarity of cleavage sites in vivo.

It should be noted that a large fraction of the potential
RNase MRP cleavage sites on pre-rRNA is inaccessible to
RNase MRP cleavage because of the formation of secondary
structures (both in the transcribed spacers and, especially,
in the regions corresponding to the mature rRNAs). The
binding of ribosomal proteins or factors governing the
ribosomal synthesis is likely to further reduce the number of
pre-rRNA sites accessible to RNase MRP cleavage in vivo. In general, the relative rarity of “naked” single-stranded RNA in the cell should substantially reduce the number of sites available for RNase MRP cleavage, albeit not necessarily to the extent required to explain the apparent rarity of actually observed sites in vivo.

If RNase MRP has broad substrate specificity in vivo, the
cell must employ additional mechanisms to direct RNase
MRP cleavage. One of the likely mechanisms might involve
the localization of RNase MRP, which could be actively
governed by its interactions with other elements of cellular
machinery to co-localize the enzyme with its proper sub-
strates such as pre-rRNA. Such co-localization of RNase
MRP with proper substrates, when combined with the
restrictions imposed by the secondary structure of RNA
and the presence of bound proteins, could dramatically
reduce the number of in vivo cleavage sites even with the
inherently broad specificity of the enzyme itself. The
existence of additional “specificity factors” that can interact
with RNase MRP in vivo and serve to direct the enzyme to
its proper cleavage sites is also a possibility. Indeed, effects
of the depletion of Rrp5, an essential nucleolar pro-
tein involved in the maturation of rRNA (Venema and
Tollervey 1996), are similar to those of the inactivation of

FIGURE 6. Cytosine in the position +4 \([\text{C}(+4)]\) is required for RNase
MRP cleavage at the A3 site. (A) Cleavage of a 56-nt-long substrate
containing the A3 site. (Arrow) The location of the cleavage site. (B)
Cleavage of the same substrate as in A, but with C(+4) replaced with
a random mix of G, A, and U. (Lanes 1,5) Alkaline hydrolysis
(markers); (lanes 2,6) untreated RNA substrates; (lanes 3,4,7,8) RNase
MRP digests. All substrates were 5’-end-labeled with \(^{32}\text{P}\) and
separated on a 6% denaturing polyacrylamide gel.

FIGURE 7. A putative hairpin located 5’ to the A3 site in the internal
transcribed spacer 1 of pre-rRNA (Fig. 1C) is not essential for RNase
MRP cleavage. (Lanes 1–6) RNase MRP cleavage of an ITS1 fragment
encompassing nucleotides –81 to 66 from the A3 site. (Lanes 7–12)
RNase MRP cleavage of a short ITS1 fragment encompassing
nucleotides –47 to 7 from the A3 site with the putative hairpin
(nucleotides –39 to –7) removed. (Lanes 1,7) Alkaline hydrolysis
(markers); (lanes 2,8) RNase T1 digests (markers); (lanes 3,9) un-
treated RNA substrates; (lanes 4,5,10,11) RNase MRP digests; (lanes
6,12) RNase A digests (markers). The mobilities of the short products
of RNase MRP digestion differ slightly from those of the products of
alkaline hydrolysis due to the presence of additional terminal
phosphates in the latter; RNase A digest markers (lanes 6,12) were
 treated with T4 polynucleotide kinase to eliminate this effect (Brown
and Bevilacqua 2005). All substrates were 5’-end-labeled with \(^{32}\text{P}\) and
separated on a 6% denaturing polyacrylamide gel.
RNase MRP (Venema and Tollervey 1996; Lindahl et al. 2009).

While the purified enzyme contained all known essential RNase MRP components (Supplemental Fig. 1), the potential presence of a fraction of the molecules that is missing one or more components could, in principle, result in a broadened specificity. The presence of such a fraction could be the result of a partial component loss during RNase MRP purification (which does not seem to be likely since RNase MRP proved to be very stable even in elevated salt or in the presence of moderate concentrations of denaturants [O Esakova and AS Krasilnikov, unpubl.]), or, more likely, could result from the existence of several subpopulations of RNase MRP in the cell. The latter seems to be a plausible explanation considering that human RNase MRP holoenzymes were suggested to exist in at least two forms that differed in their protein compositions (Welting et al. 2006). The different forms may play a biological role: A change in the composition of RNase MRP could, among other things, result in different specificities in different cellular compartments and/or during different phases of the cell cycle. (RNase MRP was shown to participate in the regulation of the cell cycle [Cai et al. 2002; Gill et al. 2004, 2006].) The potential presence of more than one form of RNase MRP could in principle result in the broadening of the specificity of the purified enzyme; however, the existence of different forms of RNase MRP in yeast and the effects of the potential changes in the composition on RNase MRP specificity need further studies.

The identification of potential “specificity factors” (whether they are novel factors transiently associated with RNase MRP in vivo or bona fide RNase MRP components) would be interesting, especially in light of recent data indicating that the involvement of RNase MRP in the processing of pre-tRNA may go beyond the known cleavage at the A3 site (Lindahl et al. 2009; Schneider et al. 2010).

In addition, it cannot be excluded that RNase MRP interacts with more than one region of its substrate in vivo. It should be noted that pre-tRNA recognition by the related RNase P holoenzyme involves several parts of the substrate, each part contributing to recognition (for review, see Kirsebom 2007; Esakova and Krasilnikov 2010). Given a sufficient sequence or structural complexity of the putative additional parts of the substrate, they could potentially be missed in our in vitro selection.

Domain 1 of RNase MRP and the catalytic domain of the closely related RNase P have multiple common features (Fig. 1A,B) and are likely to have similar structural organizations (Esakova et al. 2008 and references therein). Substrate recognition by RNase MRP, however, appears to be markedly different from pre-tRNA recognition by RNase P. Eukaryotic RNase P does not recognize specific sequences in its pre-tRNA substrates, but rather uses conserved structural features of the substrates for recognition (Esakova and Krasilnikov 2010 and references therein). The key conserved structural features used by RNase P for its recognition of pre-tRNA (T- and D-loops) (Torres-Larios et al. 2005, 2006; Reiter et al. 2010) are located at a considerable distance from the cleavage site. As a result, the RNase P domain responsible for substrate recognition (the specificity domain) (Fig. 1A; for review, see Esakova and Krasilnikov 2010) is positioned away from the catalytic domain (Torres-Larios et al. 2005, 2006; Reiter et al. 2010).

In contrast, RNase MRP proved to be sensitive to sequence features of its substrates that are located in the immediate vicinity of the cleavage site. This suggests that elements of RNase MRP responsible for substrate recognition (most likely, parts of Domain 2) are positioned in the immediate vicinity of the catalytic site, folding back into the catalytic domain and forming an overall more compact (perhaps less flat) structure than RNase P. The only phylogenetically conserved element in Domain 2 of RNase MRP, the GARAR sequence (Fig. 1B), is positioned close enough to the putative catalytic center to fulfill the role of the element folding back to the catalytic domain and interacting with the substrate. This hypothesis is currently under investigation.

MATERIALS AND METHODS

Yeast strains

The S. cerevisiae strain OE1004 (MATα RMP1::TAPHIS8::TRP1 sep 1::URA3 pep4::LEU2 nucl1::LEU2 ade2-1 trpl1-1 his3-11,15 can1-100 ura3-1 leu2-3,112) used for RNase MRP purification was constructed using strain LSY389-34A (MATα sep 1::URA3 pep4::LEU2 nucl1::LEU2 ade2-1 trpl1-1 his3-11,15 can1-100 ura3-1 leu2-3,112; a generous gift from Mark Schmitt) (Salinas et al. 2005) as the starting point. The affinity tag fused to the C-terminus of the Rmp1 gene (Salinas et al. 2005) was generated using PCR-based genomic tagging. To generate the tag, the plasmid pBS1479, which contains the TAP fusion cassette (Rigaut et al. 1999), was amplified using primers Rmp1-HisLg-A (5’-AAA GAAAAAGAAAGAAACAAATCGACATTGATGGCATATTCCGA CATCACCATCATTCACTCATGATTGATGATATTCCAACT ACTG-3’) and Rmp1-HisLg-B (5’-TACITGGGCCAGACAAGGTCA TTAATGATATTGATGATATTCCAGTATATAGGG-3’).

The resultant tag TAPHIS8 was similar to the standard TAP tag (Rigaut et al. 1999), but with eight histidine residues replacing the calmodulin-binding fragment. The genomic sequence of the Rmp1 gene with the fused tag was confirmed by sequencing. The insertion of the tag did not affect 5.8S rRNA processing in vivo (Fig. 8).

RNase MRP purification

RNase MRP holoenzyme was purified from S. cerevisiae strain OE1004 (above) using a tandem affinity tag attached to the C-terminus of RNase MRP protein component Rmp1. Rmp1 (Salinas et al. 2005) is not found in RNase P, which eliminated the possibility of the contamination of purified RNase MRP with this closely related ribonucleoprotein complex. The tandem affinity tag was based on the commonly used TAP tag (Rigaut et al. 1999). The resultant tag TAPHIS8 was similar to the standard TAP tag (Rigaut et al. 1999), but with eight histidine residues replacing the calmodulin-binding fragment. The genomic sequence of the Rmp1 gene with the fused tag was confirmed by sequencing. The insertion of the tag did not affect 5.8S rRNA processing in vivo (Fig. 8).
with 0.5 mL of Ni-NTA Agarose (QIAGEN) for 5 h at 4°C. The sample was incubated treated to the final volume of 2 mL using an Amicon-Ultra 15 (100 kDa MWCO) concentrator (Millipore). The sample was incubated for 12 h at 4°C with light agitation. The resin was washed six times with 10 mL of buffer containing 20 mM Tris-HCl (pH 7.9), 150 mM KCl, 1 mM Mg-Acetate, 10% glycerol, 1 mM PMSF, and 0.1% (v/v) Tween 20, and RNase MRP purification.

The purification procedure was generally based on one previously described (Gill et al. 2004), with modifications aimed to maintain the presence of magnesium at all purification steps and reflecting the change in the composition of the purification tag.

Sixteen liters of yeast was grown at 30°C with vigorous aeration on YPD media to the late logarithmic phase. The culture was cooled on ice; the cells (~150 g) were harvested by centrifugation at 4000g (4°C), washed with water, and resuspended in a buffer containing 50 mM Tris-HCl (pH 7.6), 10 mM MgCl$_2$, 10 mM DTT, 0.1% (v/v) Tween 20, and 100 nM DNA template, and 300 mM spermidine, 50 Ci n$^2$0 for run-off transcription with T7 RNA polymerase (0.1 μg/mL) in 40 mM Tris-HCl (pH 8.1), 6 mM MgCl$_2$, 5mM DTT, 1 mM spermidine, 50 μg/mL BSA, 1 mM ATP, 1 mM UTP, 1 mM CTP, 1 mM GTP, 5 mM GMP, 100 mM DNA template, and 300 nM of oligonucleotide 5'-TAATACGACTCACTATAGGGAGTCGGCTCTAG-3' (the introduced T7 RNA polymerase promoter sequence is underscored) and 5'-GTATTGAATCGTGAAAG-3'.

The resultant double-stranded DNA was extracted with phenol and used for run-off transcription with T7 RNA polymerase (0.1 μg/mL) in 40 mM Tris-HCl (pH 8.1), 6 mM MgCl$_2$, 5mM DTT, 1 mM spermidine, 50 μg/mL BSA, 1 mM ATP, 1 mM UTP, 1 mM CTP, 1 mM GTP, 5 mM GMP, 100 mM DNA template, and 300 mM of oligonucleotide 5'-TAATACGACTCACTATAGGGAGTCGGCTCTAG-3' (the introduced T7 RNA polymerase promoter sequence is underscored) and 5'-GTATTGAATCGTGAAAG-3'.

The resultant double-stranded DNA was extracted with phenol and used for run-off transcription with T7 RNA polymerase (0.1 μg/mL) in 40 mM Tris-HCl (pH 8.1), 6 mM MgCl$_2$, 5mM DTT, 1 mM spermidine, 50 μg/mL BSA, 1 mM ATP, 1 mM UTP, 1 mM CTP, 1 mM GTP, 5 mM GMP, 100 mM DNA template, and 300 mM of oligonucleotide 5'-TAATACGACTCACTATAGGGAGTCGGCTCTAG-3' (the introduced T7 RNA polymerase promoter sequence is underscored) and 5'-GTATTGAATCGTGAAAG-3'.

The linearized RNA (enriched in sequences cleavable by RNase MRP) was extracted from the gel, resuspended in the buffer for run-off transcription with T7 RNA polymerase (0.1 μg/mL) in 40 mM Tris-HCl (pH 8.1), 6 mM MgCl$_2$, 5mM DTT, 1 mM spermidine, 50 μg/mL BSA, 1 mM ATP, 1 mM UTP, 1 mM CTP, 1 mM GTP, 5 mM GMP, 100 mM DNA template, and 300 mM of oligonucleotide 5'-TAATACGACTCACTATAGGGAGTCGGCTCTAG-3' (the introduced T7 RNA polymerase promoter sequence is underscored) and 5'-GTATTGAATCGTGAAAG-3'.
RNAse MRP substrates

83°C, and cooled on ice. The RNA was self-ligated using T4 RNA ligase 1 in the presence of 15% (v/v) DMSO as described above. Following ligation, the RNA was extracted with phenol.

Extracted RNA was used to generate a library of double-stranded DNA similar to the initial one, but enriched with sequences cleavable by RNAse MRP using reverse transcription followed by PCR amplification. RNA was resuspended in the 15 μL of 5 mM Tris-HCl (pH 8.0), 10 mM KCl, and 0.1 mM EDTA, and annealed to a primer (5′-GTATTGAAATCGTAGG-3′; 1 μM) by heating for 5 min to 83°C followed by incubation for 15 min on ice. A reverse transcription reaction was performed in 20 μL of 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 5 mM MgCl₂, 1.5 mM DTT, 1 mM each of dNTPs, and 40 units of SuperScript II reverse transcriptase (Invitrogen) in the presence of the SUPERase-In RNAse inhibitor (Ambion) for 15 min at 47°C. PCR amplification was performed with the same primers that were used in the generation of the initial double-stranded DNA template (above). After 27 cycles of amplification, the PCR products were extracted with phenol and used in the next round of substrate selection.

Substrate selection was run as described above for six rounds; most of the RNA substrates generated in the sixth round were cleavable by RNAse MRP. Double-stranded DNAs generated at the end of the last selection round were cloned into the Sma I site of the pUC19 plasmid; the resulting plasmids were used to transform E. coli cells. One hundred and twenty individual colonies were randomly picked and used to sequence plasmid DNA.

Analysis of RNAse MRP cleavage sites

Plasmid DNA was amplified by PCR using the same primers as described above; RNA was synthesized using standard run-off transcription with T7 RNA polymerase (Milligan and Uhlenbeck 1989) followed by purification on 15% denaturing (8 M urea) polyacrylamide gel. The resulting RNA was dephosphorylated with calf alkaline phosphatase and 5′-end 32P-labeled with T4 polynucleotide kinase. Labeled RNA was gel-purified again and used in RNAse MRP cleavage assays performed in 20 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 50 mM NaCl, 1 mM DTT, and 50 μg/mL BSA at 37°C in the presence of SUPERase-In RNAse inhibitor (Ambion).

To analyze RNAse MRP cleavage of the CLB2 mRNA substrate, the substrate was synthesized using standard run-off transcription with SP6 RNA polymerase. Plasmid pJA108 (a generous gift from Mark Schmitt) (Gill et al. 2004) linearized with EcoRI was used as the template for in vitro transcription. RNAse MRP cleavage assays were performed as described above. Cleavage products were analyzed by primer extension using primers RT270-1 (5′-ACAAATG ATAAAAATTTCTCC-3′) and RT270-2 (5′-CAAAAAGGGAAACAG ATGCTC-3′) following the protocol that was previously described (Esakova et al. 2008); the same primers annealed to plasmid pJA108 were used to obtain sequencing ladders.

SUPPLEMENTAL MATERIAL

Supplemental material can be found at http://www.rnajournal.org.

ACKNOWLEDGMENTS

We thank Phil Bevilacqua, Joe Reese, and Mark Schmitt for valuable comments and suggestions. We thank the staff of the Proteomics Core Facility at the Huck Institute of Life Sciences (PSU) for their help with mass spectrometry analysis. This work was supported by NIH Grant GM085149 to A.S.K.

Received July 29, 2010; accepted November 15, 2010.

REFERENCES


Esakova et al.


Substrate recognition by ribonucleoprotein ribonuclease MRP

Olga Esakova, Anna Perederina, Chao Quan, et al.

RNA 2011 17: 356-364 originally published online December 20, 2010
Access the most recent version at doi:10.1261/rna.2393711

Supplemental Material
http://rnajournal.cshlp.org/content/suppl/2010/12/07/rna.2393711.DC1

References
This article cites 44 articles, 23 of which can be accessed free at:
http://rnajournal.cshlp.org/content/17/2/356.full.html#ref-list-1

License

Email Alerting Service
Receive free email alerts when new articles cite this article - sign up in the box at the top right corner of the article or click here.

To subscribe to RNA go to:
http://rnajournal.cshlp.org/subscriptions

Copyright © 2011 RNA Society