Accurate Processing of a Eukaryotic Precursor Ribosomal RNA by Ribonuclease MRP in Vitro

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Very few of the enzymes required for eukaryotic precursor ribosomal RNA (pre-rRNA) processing have been identified. Ribonuclease (RNase) MRP was characterized as a nuclease that cleaves mitochondrial replication primers, but it is predominantly nucleolar. Previous genetic evidence revealed that this ribonucleoprotein is required, directly or indirectly, for cleavage of the yeast pre-rRNA in vivo at site A_3. Here, an in vitro processing system that accurately reproduces this cleavage is described. Biochemical purification and the use of extracts depleted of the MRP RNA demonstrate that endonucleolytic cleavage of the pre-rRNA is directly mediated by RNase MRP. This establishes a role for RNase MRP in the nucleolus.

Three of the four eukaryotic ribosomal RNAs are produced by processing a long precursor RNA (Fig. 1A). Genetic analysis in the yeast *Saccharomyces cerevisiae* provides a means to dissect this processing pathway and identify the factors and steps involved (*1*). Nevertheless, study of the biochemical mechanisms underlying pre-rRNA processing would be facilitated by the development of tractable in vitro systems. The ribonucleoprotein RNase MRP was identified as an endonuclease that cleaves mitochondrial replication primers in vitro (*2*). However, its predominantly nucleolar localization (*3*) and the reported existence of another enzyme able to cleave mitochondrial primers (*4*) have led to some controversy about RNase MRP's cellular function. Mutations in two components of yeast RNase MRP, the MRP RNA (*5*, *6*) or Pop1p protein (*7*), inhibit in vivo cleavage of the pre-rRNA at a site, designated A_3, located upstream of the 5.8*S* rRNA (*7*, *8*). It was, however, unclear whether RNase MRP participated directly in this cleavage event.

Pop1p is a component of both RNase P and RNase MRP (*7*). A tagged version of Pop1p fused to two immunoglobulin G (IgG)–binding regions of *Staphylococcus aureus* protein A (ProtA-Pop1p) is functional in vivo and efficiently coprecipitates the RNase P and MRP RNAs (*7*). We reasoned that the activities of both RNase P (*9*) and RNase MRP might be enriched from extracts containing ProtA-Pop1p by affinity selection with IgG agarose beads (*10*). We first tested whether yeast RNase P activity (*11*) could be detected by this strategy. A ProtA-Pop1p precipitate cleaved a radiolabeled pre-tRNA (Fig. 1B). This reaction was most likely mediated by RNase P for the following reasons. (i) Cleavage was dependent on the presence of ProtA-Pop1p (Fig. 1B). (ii) Cleavage was accurate (*12*, *13*). (iii) Micrococcal nuclease treatment of the precipitate inhibited cleavage (*13*). (iv) The sup3e-A1 mutant pre-tRNA, which is defective for cleavage by RNase P (*14*), was not processed in our assay (*13*). Thus, affinity selection of ProtA-Pop1p can be used to detect associated enzymatic activities.

We tested next whether the same precipitates could process the 35*S* pre-rRNA. Because of the large size of the pre-rRNA substrate (7 kb), the products of the reaction were analyzed by primer extension (*10*). A primer extension stop appeared after incubation of the 35*S* pre-rRNA substrate with a ProtA-Pop1p precipitate (Fig. 1C). This stop mapped to site A_3 (Fig. 1C) and was not detected when extracts from a strain expressing nontagged Pop1p were used (Fig. 1C), showing that ProtA-Pop1p or associated factors (or both) mediate this reaction (*15*). The processing activity contains an essential RNA

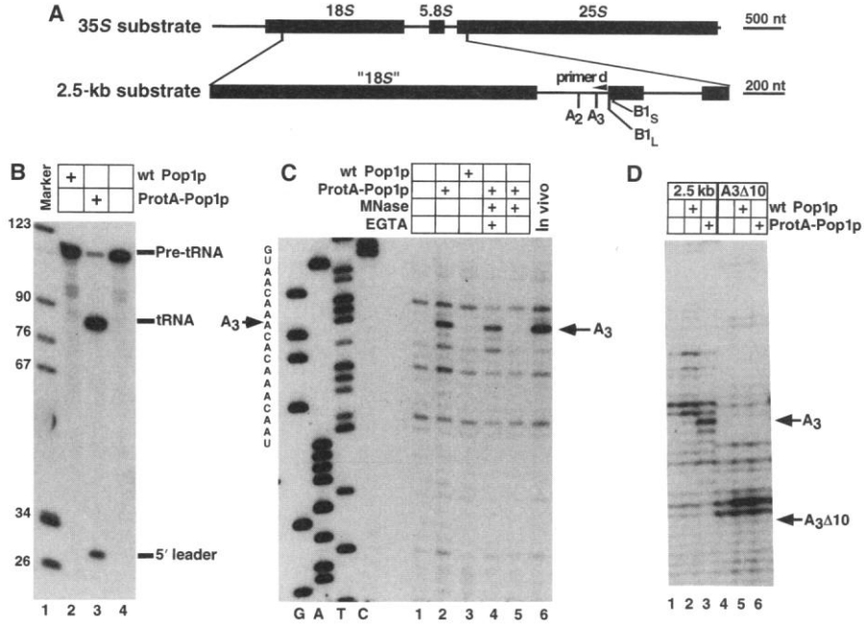

Fig. 1. In vitro processing of pre-tRNA and pre-rRNA by affinity-selected ProtA-Pop1p pellets. (**A**) Structure of the 35*S* pre-rRNA transcript and of the 2.5-kb substrate. Mature rRNAs are shown as boxes and spacers as lines. The A_2 and A_3 cleavage sites, the 5′ ends of the 5.8*S* rRNA ($B1_S$ and $B1_L$), and primer d are indicated on an enlarged drawing of the 2.5-kb transcript region. Scale bars are on the right. (**B**) Endonucleolytic cleavage of a ^{32}P-labeled SupS1 pre-tRNA transcript (*24*). Lane 1, molecular size marker with sizes indicated on the left in nucleotides; lane 2, control reaction with a precipitate from a wild-type extract (wt Pop1p); lane 3, transcript processed with a ProtA-Pop1p precipitate; lane 4, mock-treated substrate. The pre-tRNA, mature tRNA, and 5′ leader are indicated. (**C**) Processing of a nonlabeled 35*S* pre-rRNA at site A_3 analyzed by primer extension. Lane 1, mock-treated substrate depicting nonspecific primer extension stops (for example, because of secondary structure); lane 2, transcript processed with a ProtA-Pop1p precipitate; lane 3, control reaction with a precipitate from a wild-type extract; lane 4, EGTA addition prevents the micrococcal nuclease inactivation of the A_3 processing activity; lane 5, the A_3 processing activity of the ProtA-Pop1p precipitate is micrococcal nuclease (MNase)–sensitive; lane 6, primer extension on cellular RNA depicting the primer extension stop corresponding to the in vivo A_3 cleavage. Lanes G, A, T, and C are the cognate sequence ladder. (**D**) Mutant $A_3\Delta10$ is not processed in vitro. A 2.5-kb wild-type transcript (lanes 1 to 3) and a mutant derivative bearing a 10-nt deletion immediately 3′ to site A_3 (*8*) (lanes 4 to 6) were assayed by primer extension for in vitro cleavage at site A_3. Lanes 1 and 4, mock-treated substrates; lanes 2 and 5, substrate incubated with the IgG precipitate from a wild-type extract; lanes 3 and 6, substrate incubated with a ProtA-Pop1p precipitate. Positions corresponding to cleavage at A_3 of the wild-type and $A_3\Delta10$ substrates are indicated.

component because it was inactivated by micrococcal nuclease (Fig. 1C). A 10-nucleotide (nt) deletion immediately 3′ to site A_3 prevents processing at this site in vivo (*8*). A 2.5-kb substrate (Fig. 1A) carrying this mutation was not processed in vitro, whereas a control wild-type 2.5-kb transcript was processed (Fig. 1D), indicating that the in vitro reaction mimics processing in vivo. Our results show that a micrococcal nuclease–sensitive activity associated with Pop1p accurately cleaves the pre-rRNA at site A_3 in vitro.

The RNase MRP and RNase P RNAs are the major RNA species found in a ProtA-Pop1p precipitate when assayed by end-labeling (*13*); therefore, one of the corresponding endonucleases is most likely to mediate the in vitro pre-rRNA processing reaction. RNase P and MRP were separated by biochemical purification (*16*) (Fig. 2A). In the most purified MRP fraction (M2), no RNase P RNA could be detected, whereas the purest RNase P fraction (P2) contained only trace amounts of MRP RNA (Fig. 2B). The peak RNase MRP and P fractions from each column were affinity-selected on IgG agarose beads and assayed for cleavage of a pre-tRNA substrate and the 35S pre-rRNA substrate (Fig. 2, C and D). Accurate endonucleolytic cleavage of the pre-tRNA was detected in the RNase P–containing fractions but not in the fractions highly enriched for RNase MRP (Fig. 2C). Conversely, processing of the 35S pre-rRNA was specifically detected in the RNase MRP fractions (Fig. 2D).

We used an in vivo depletion strategy (*17*) to demonstrate that RNase MRP, rather than a copurifying ribozyme, is responsible for the processing reaction. Extracts were prepared from cells that conditionally transcribe the MRP RNA (*6*) and also express ProtA-Pop1p. In vitro cleavage of the 35S pre-rRNA at site A_3 was lost in extracts prepared after in vivo depletion of MRP RNA (Fig. 3A). MRP RNA depletion did not affect RNase P activity (Fig. 3B). Extracts prepared from wild-type cells grown under the same conditions were active for processing at site A_3 (Fig. 3A) and RNase P activity (Fig. 3B). Therefore, depletion of the MRP RNA specifically affected processing at site A_3. We conclude that RNase MRP directly and accurately processes the pre-rRNA at site A_3 in vitro.

To better define the substrate requirements for pre-rRNA processing, we tested a 141-nt transcript overlapping site A_3 (Fig. 4A). This short substrate was processed accurately at site A_3 by a precipitate of fraction M2 (and M1) containing highly purified RNase MRP (Fig. 4B). Incubation of internally labeled 141-nt substrate with a purified RNase MRP precipitate produced fragments of 81 and 60 nt (Fig. 4C), corresponding to the 5′ and 3′ cleavage products, respectively. Cleavage at site A_3 by RNase MRP is therefore endonucleolytic, and the information re-

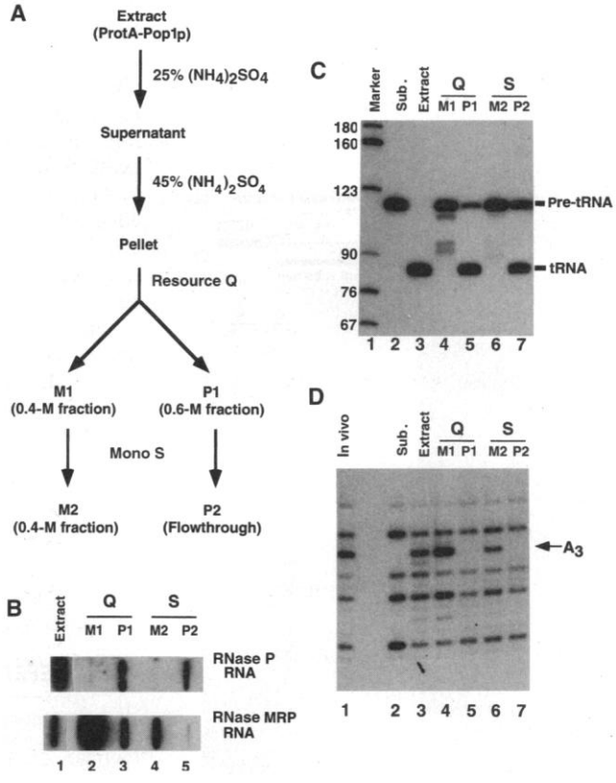

Fig. 2. Purified RNase MRP processes pre-rRNA at the A_3 site. (**A**) Fractionation scheme. The fractionation conditions and fraction names are indicated. Activities present in the M1, P1, M2, and P2 fractions were tested after further purification by affinity selection on IgG agarose. (**B**) Distribution of RNase P and MRP RNAs in the fractions. Lane 1, RNAs extracted from total extract; lanes 2 and 4, RNAs from the peak RNase MRP fractions after Resource Q and Mono S chromatography, respectively; lanes 3 and 5, RNAs from the peak RNase P fractions after Resource Q and Mono S chromatography, respectively. Slot-blots of the duplicate filters were hybridized either with an RNase P (top panel) or MRP RNA probe (lower panel). Lane 1 was exposed for a shorter period than were the other lanes, but identical exposure lengths are shown for the P and MRP hybridizations, allowing direct comparison of the ratio of RNase P and MRP RNAs. Traces of RNase MRP RNA could be detected in fraction P2 after longer exposure, whereas only a background signal could be detected in the slot containing the M2 fraction probed for RNase P RNA. (**C**) Assay of the pre-tRNA processing activity by precipitates of the fractions. Precipitates from total extract (lane 3), the Resource Q M1 and P1 fractions (lanes 4 and 5), and the Mono S M2 and P2 fractions (lanes 6 and 7) were assayed for pre-tRNA processing. A mock-treated substrate is presented in lane 2 and a molecular size marker in lane 1 with the size of the corresponding bands (in nucleotides) indicated on the left. Some nonspecific degradation is apparent in lanes 4 and 6, but no specific cleavage is detected. (**D**) Assay of the pre-rRNA processing activity in precipitates of the fractions. In lanes 3 to 7, pre-rRNA processing was assayed with the same fractions as for the pre-tRNA processing in (C). Lane 2 shows a mock-treated RNA, and lane 1, RNA extracted from wild-type cells. Sub., substrate.

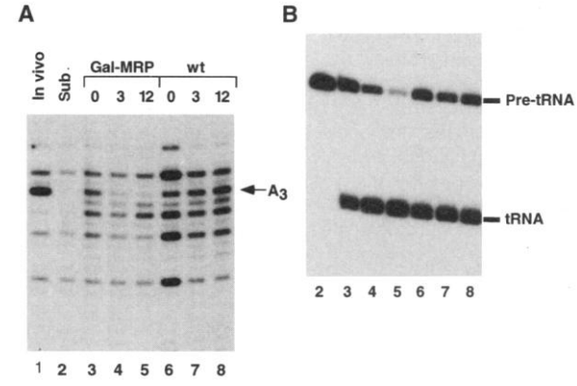

Fig. 3. Precipitates of extracts made from cells depleted in vivo of RNase MRP RNA do not process pre-rRNA at site A_3. (**A**) In vitro cleavage of the pre-rRNA substrate (Sub.) by ProtA-Pop1 precipitates. A plasmid carrying the ProtA-Pop1 construct was introduced into strain MES124, which contains a galactose-regulated *NME1* gene (Gal-MRP, lanes 3 to 5), and into the otherwise isogenic wild-type strain MES123 (lanes 6 to 8) (*6*). Extracts were prepared from cells grown on galactose (lanes 3 and 6) and from cells grown on glucose for 3 hours (lanes 4 and 7) and 12 hours (lanes 5 and 8). Under these conditions the MRP RNA amount reaches a minimum after 8 hours (*6*). Lane 1, RNA extracted from wild-type cells; lane 2, mock-treated RNA. (**B**) Pre-tRNA processing by precipitates of the same extracts. Lanes are as for (A), except that lane 1 is omitted.

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quired for substrate recognition is contained in a 141-nt fragment of the pre-rRNA.

A low level of aberrant processing of the 141-nt substrate 1 nt 3′ to site A_3 was observed with our purest RNase P preparation (*18*) (Fig. 4, B and C, lane 4 in each). The 141-nt substrate may be recognized by RNase P because of a structural resemblance to pre-tRNA or because of its binding to Pop1p. Only low levels of aberrant cleavage could be detected with the longer substrates (Figs. 1 to 3), possibly because folding of these longer RNAs interferes with RNase P binding or catalysis or both. This cleavage was not detectable in vivo (*7, 8, 13*). Another in vitro substrate for RNase MRP, the mitochondrial replication primer, is also cleaved by RNase P (*19*). These observations are consistent with a recent model proposing that RNase MRP and its role in eukaryotic pre-rRNA processing evolved from RNase P (*20*).

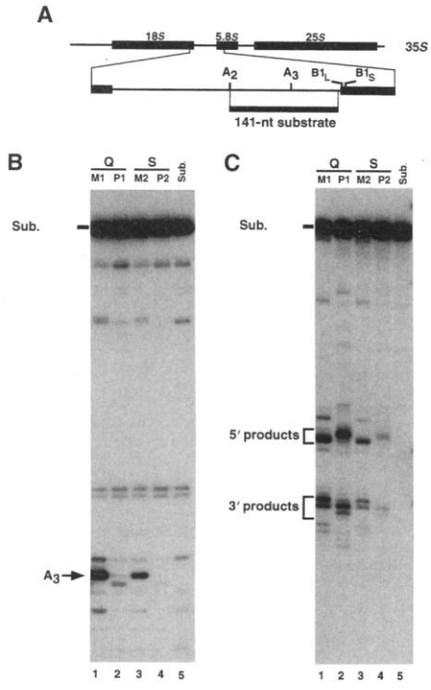

Fig. 4. A 141-nt pre-rRNA substrate is recognized and cleaved endonucleolytically by RNase MRP. (**A**) Location of the 141-nt substrate. The 35*S* pre-rRNA is shown on top, and the region surrounding site A_3 is shown enlarged below. The 141-nt substrate extends from 3 nt downstream of the A_2 site to 9 nt upstream of the $B1_L$ site (*8*). (**B**) In vitro processing of an unlabeled 141-nt substrate (Sub.). The products of the reaction with IgG precipitates of the peak RNase MRP (lanes 1 and 3) and P (lanes 2 and 4) fractions were detected by primer extension. The bands corresponding to the substrate RNA and the A_3 cleaved product are indicated. Lane 5 is a mock-treated substrate. Note that about 250 fmol of the substrate were used, approximately 50-fold more than the labeled substrate used for the experiment depicted in (C). (**C**) Endonucleolytic cleavage of the 141-nt substrate by RNase MRP. Internally labeled 141-nt substrate was incubated with IgG precipitates of the peak RNase MRP (lanes 1 and 3) and P (lanes 2 and 4) fractions, and the products were detected after gel electrophoresis. The positions of migration of the substrate and the 5′ and 3′ cleavage products are indicated. The 3′ product always appears as a doublet, because of a 1-nt heterogeneity at the 3′ end of the substrate, generated during in vitro transcription. The 3′ and 5′ fragments were identified by processing end-labeled substrates (*13*).

Few reactions that reproduce steps of the eukaryotic pre-rRNA processing in vitro have been described (*21*). We have shown that steps of yeast pre-rRNA processing can be accurately reproduced in vitro by the genetic identification of the components implicated and the use of tagged proteins to specifically enrich for the desired activity. A similar strategy could be applied to the study of other complex cellular processes. Our results demonstrate that RNase MRP accurately cleaves pre-rRNA at site A_3 in vitro. From this and previous in vivo studies of RNase MRP mutants, we conclude that RNase MRP is directly implicated in rRNA processing, consistent with its nucleolar localization.

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10. Yeast extracts (*22*) were prepared from the ProtA-Pop1p–expressing strain BSY414 and the otherwise isogenic wild-type strain BSY17 (*7, 23*). ProtA-Pop1p was selected from 0.6 μl of extract by incubation for 2.5 hours at 4°C with 6 μl of a 50% suspension of IgG agarose beads in 120 μl of IPP150 buffer [150 mM NaCl, 10 mM tris-Cl (pH 8.0), 0.1% NP-40, and 0.1% NaN_3] (*7*). IgG pellets were incubated with 5 fmol of labeled pre-$tRNA^{SupS1}$ (*24*) for 30 min at 37°C in 20 mM tris-Cl (pH 8), 10 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 50 mM KCl, bovine serum albumin (50 μg/ml), and RNasin (60 U/ml). Pre-rRNA substrates were produced by in vitro transcription of the appropriate plasmids. Five femtomoles of pre-rRNA substrate were processed as described above for tRNA, except for Fig. 4B where 250 fmol of unlabeled 141-nt substrate were used. The products of the processing reactions with unlabeled pre-rRNA substrates were extracted and analyzed by primer extension (*23*) with radiolabeled oligonucleotide d (*8*) (Fig. 1A). Micrococcal nuclease was incubated for 20 min at 37°C with IgG precipitates after the addition of $CaCl_2$ to a final concentration of 4 mM. Before addition of the substrate, EGTA was added to a final concentration of 32 mM. For the control, EGTA was added to the IgG precipitates before addition of the micrococcal nuclease. Reaction products were fractionated by gel electrophoresis (*25*).
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15. Note that the use of ProtA-Pop1p precipitates was essential for the detection of in vitro processing. No activity was detected in complete extracts (*13*), probably because of the presence of inhibitors or the occurrence of competing reactions.
16. Extract from strain BSY414 was mixed with one-eighth volume 2M potassium phosphate (pH 8), and solid $(NH_4)_2SO_4$ was added to 25% saturation. After centrifugation, solid $(NH_4)_2SO_4$ was added to the supernatant to 45% saturation. The precipitate, in buffer Q6-50 [20 mM histidine (pH 6), 50 mM NaCl, 0.2 mM EDTA, 10% glycerol, 0.5 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), and 2 mM benzamidine] was loaded on a Resource Q column (Pharmacia). Elution was performed with a linear gradient of 300 to 800 mM NaCl. The RNase MRP (M1) and the RNase P (P1) fractions were loaded separately on Mono S in buffer S7-50 [50 mM $NaPO_4$ (pH 7), 50 mM NaCl, 0.2 mM EDTA, 10% glycerol, 0.5 mM DTT, 0.5 mM PMSF, and 2 mM benzamidine]. RNase P remained in the flowthrough (P2), whereas RNase MRP eluted in a 0.4-M NaCl step (M2). During the fractionation, we followed the RNase P and MRP RNAs by slot blot hybridizations and ProtA-Pop1p by protein immunoblotting (*25*). The RNase P and MRP peak fractions were affinity-selected with IgG agarose. Less than 0.01% of the starting protein was present in fractions M2 and P2. Because of the high amount of IgGs present on the beads, it is not possible to directly evaluate the final level of purification after the affinity selection. The RNase MRP present in fraction P1 is a minor amount of the total RNase MRP present in extracts, which is poorly functional possibly because it is missing some protein component. This is in agreement with its aberrant chromatographic behavior compared with the bulk of RNase MRP.
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18. The 141-nt transcript is processed by precipitates from whole-cell extracts containing ProtA-Pop1p both at site A_3 and 1 nt 3′ to site A_3. Processing at both sites is micrococcal nuclease–sensitive (*13*).
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