Ribosomal 5 S rRNA Maturation in *Saccharomyces cerevisiae*

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The maturation of the ribosomal 5 S RNA in *Saccharomyces cerevisiae* is examined based on the expression of mutant 5 S rRNA genes, in vivo, and a parallel analysis of RNA processing, in vitro. Both types of analysis indicate that 5 S rRNA processing is not dependent on the nucleotide sequence of either the external transcribed spacer or the mature 5 S rRNA. The results further indicate the RNA is processed by an exonuclease activity which is limited primarily or entirely by helix I, the secondary structure formed between the mature and interacting termini. The 5 S RNA binding protein (YL3) also appears not to influence directly the maturation process, but rather to play a role in protecting the rRNA from further degradation by “housekeeping” nucleases. Taken together, the results continue to support a “quality control” function which helps to ensure that during maturation only normal precursors are processed and assembled into active ribosomes.

In bacteria, the ribosomal 5 S RNA component is encoded in a single long transcript which is cleaved and matured as three individual RNA components (the 16, 23, and 5 S rRNAs) that constitute the RNA portion of active ribosomes. In eukaryotes, the 5 S RNA of cytoplasmic ribosomes is encoded by a separate gene and transcribed by an alternate RNA polymerase III (see Ref. 11). Despite this uncoupled synthesis and the small size of the 5 S RNA molecule (~120 nucleotides), all known eukaryotic 5 S RNAs still are transcribed as slightly longer precursors with the 3′-sequence extensions removed as the RNA is processed. Studies in toad (2, 3), fly (4, 5), yeast (6, 7), and even mammalian cells (8, 9) have documented this maturation process or provided some detail of the underlying mechanism. Some differences in precursor size and enzymatic requirements have been observed between higher and lower eukaryotes; in *Drosophila* and yeast the 7–15 extra nucleotides appeared to be removed by a single rapid endonucleolytic step (6, 7, 10). More recent detailed studies in *vitro* have begun to define the structural features in 5 S RNA transcripts of *Drosophila melanogaster* which are essential for RNA processing (see Ref. 11). These studies initially concluded (10) that a base paired C at position 118 followed by a stretch of pyrimidines was the processing signal which is recognized by the processing enzyme and/or a required accessory factor. Subsequent studies have shown that a number of more distal structural features in the mature 5 S RNA also appear to affect the maturation process (11–14).

A potential role for the extended sequence in the 5 S RNA precursor and its subsequent processing was suggested when studies on rRNA termination indicated that accurate termination of the 5 S rRNA gene transcripts is critical to transcript stability and subsequent integration into ribosomal structure (15). Deletions or substantial displacements of the termination signals resulted in highly unstable transcripts which were not incorporated into stable ribosomes. Such results suggested that transcript termination coupled with rapid processing was essential and served as a quality control mechanism to help eliminate altered RNA molecules.

To further elucidate the structural features which underlie eukaryotic 5 S rRNA maturation and perhaps its role as a quality control mechanism, we have examined 5 S rRNA maturation in *Saccharomyces cerevisiae* using targeted mutagenesis to evaluate the effect of specific changes, both in vivo and in vitro. Our results indicate that, while both the structure of the 5 S RNA and the presence of the cognate ribosomal 5 S RNA binding protein, YL3 (16), are essential for the efficient integration of the 5 S rRNA into the ribosomal structure, the actual processing of the 5 S RNA is largely or entirely dependent only on stem I, the helix between the interacting of the mature RNA molecule.

**MATERIALS AND METHODS**

**Construction and Expression of Mutant 5 S rRNA Genes—**Mutations were introduced into a yeast 5 S rRNA gene (17) containing a structural marker mutation (18) by using mutant oligonucleotide primers during PCR amplification. Single step amplification was used for mutations in the 3′-end transcribed spacer region, and a modified two-step PCR strategy (19) was used for internal mutations. Mutant oligonucleotides and normal primers were synthesized using a Cyclone Plus automated DNA synthesizer (Millipore Corp., Milford, MA). Mutant genes were cloned into pYP404, a high copy (30–40 copies per cell) yeast shuttle vector (20), and the mutant sequence was confirmed by DNA sequencing (21). To express the mutant 5 S rRNA genes, the recombinant plasmids were purified and used to transform a LEU2-deficient yeast strain (AH22) as described by Hinne et al. (22).

**Analysis of Mutant 5 S rRNA Expression—**For all analyses, transformed cells were grown with shaking under selective conditions at 30 °C. Whole cell RNA was prepared by sodium dodecyl sulfate-pheno1 extraction and the 5 S rRNAs were fractionated at room temperature by SDS-phenol, fractionated on nondenaturing polyacrylamide gels and, described previously (23). The labeled RNA was again extracted with orthophosphate and collected by filtration on glass microfiber filters as described previously (23). The labeled RNA was again extracted with SD-sodium, fractionated on nondenaturing polyacrylamide gels and, after autoradiography, the film images were captured to quantify the 5 S rRNA bands as described above. For RNA half-life determinations, briefly labeled cultures were diluted with phosphate-buffered medium and aliquots were filtered again for RNA extraction and quantification (23).

The cellular plasmid copy number was determined by genomic hy-

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1 The abbreviations used are: PCR, polymerase chain reaction; DTT, dithiothreitol; MES, morpholinoethanesulfonic acid; PMSF, phenylmethylsulfonyl fluoride.
bridization analyses, as described previously (23), using a nick-translated yeast LEU2 gene as a probe. After autoradiography, the images were scanned for quantification as described above; the copy number was calculated from the intensity of the plasmid-derived band and the alternatively migrating genomic LEU2 band.

Preparation of 5 S rRNA Precursors—To produce 5 S rRNA precursor maturation of ribosomal 5 S RNA in S. cerevisiae 15207

Yeast nuclei were isolated as described by Aris and Blobel (24) with minor modifications. S. cerevisiae, strain AH22, was grown at 30 °C in YEPD medium (1% (w/v) Bacto-yeast extract, 2% (w/v) Bacto-peptone, 2% (w/v) glucose) medium to an absorbance of 1 at 600 nm (4 × 10^7 cells/ml). Cells were harvested by centrifugation for 5 min at 4000 g, the pellets were resuspended in 30 ml of lysis buffer containing 10% (v/v) glycerol, 10 mM MgCl_2, 10 mM EDTA, and collected by centrifugation. To prepare nuclei, visible in the 40% layer and at the 30–40% and 40–50% interfaces, were collected with a Pasteur pipette, frozen in liquid nitrogen, and stored at −70 °C.

The nuclear extracts were prepared from frozen nuclei using a modification of the procedure described by Hennighausen and Luon (25). The nuclei were diluted 10-fold in cold buffer containing 1 mM MgCl_2, 20 mM potassium phosphate, pH 6.45, and PIC, collected by centrifugation for 10 min at 10,000 g, and then lysed with 20 strokes of a Dounce homogenizer with a loose-fitting pestle. The lysate was chilled on ice for 10 min, cleared by centrifugation for 15 min at 13,000 g, and 25 ml were layered on a Ficoll step gradient (5 ml layers of 30, 40, and 50% Ficoll 400 in 1 mM MgCl_2, 10 mM potassium phosphate, pH 6.45, and PIC) formed in a SW28 rotor ultracentrifuge tube (Beckman Instruments). The gradients were fractionated at 18,000 rpm (55,400 × g) and 2 °C for 60 min. Nuclei, visible in the 40% layer and at the 30–40% and 40–50% interfaces, were collected with a Pasteur pipette, frozen in liquid nitrogen, and stored at −70 °C for several weeks.

Preparation of 5 S rRNA Precursors—To produce 5 S rRNA precursor complexes, the 5 S RNA precursor was dephosphorylated using calf intestinal alkaline phosphatase and labeled at the 5′ end using T4 polynucleotide kinase and [γ-32P]ATP as described previously (29). In most experiments, the precursor was labeled at the 5′ end to ensure that full length products were obtained. For 5′-end-labeled RNA, 3′,5′-[32P]P bisphosphate and T4 RNA ligase were used instead (28). The labeled RNA was purified on an 8% denaturing polyacrylamide gel, detected by autoradiography, and recovered by homogenization as described above. For the RNA processing assays, the labeled RNA (32 pmol of precursor) was incubated at 30 °C with 10 ml of nuclear extract in a final volume of 20 ml containing 37.5 mM NaCl, 3 mM MgCl_2, 100 mM KCl, 3 mM DTT, 10% glycerol, 0.25 mM PMSF, 50 mM EDTA, and 30 mM HEPESE-KOH, pH 7.9. Reactions were stopped by the addition of 0.4 ml of stop buffer (0.3 M sodium acetate, 0.1% SDS, 10 mM EDTA, and 40 mg/ml of yeast tRNA as a carrier). The RNA was then digested with pancreatic enzyme and 32P-labeled bands were visualized by autoradiography.

RESULTS

The mutagenic strategies used in these studies were based on PCR amplification (19), and mutant genes were expressed in vivo using a high copy, autonomously replicating yeast shuttle vector (20). As illustrated in Fig. 1 (A99), under normal conditions, 80–90% of the cellular 5 S RNA was mutant, easily detected using a well characterized and neutral (18, 23) single base substitution, Y5A99. In the course of this study, additional mutations were introduced in three regions of the 5 S RNA gene: the nontranscribed spacer downstream of the termination site, the 3′ nonconserved spacer, which is transcribed but removed during RNA processing, and the actual 5 S RNA sequence. The mutations examined in Fig. 1 illustrate features in the 3′-nonconserved spacer that are essential for chain termination, but which do not affect RNA maturation. As originally suggested by the gene sequence (31) and subsequently confirmed by transcription in vitro (6), or brief labeling in vivo (7), the extended cluster of adenylate residues in the coding sequence, beginning with A + S and encoding a poly(U) tract in the 3′ end of the precursor, signals termination. This systematic analysis (Fig. 1) shows that five adenylate acid residues (lane c) are essential for efficient termination in vivo, but some termination still occurs when only four residues are present (lane g). Furthermore, it is also clear that the position of the adenylate cluster is not critical, and a shorter extension encoded by adenylate residues still functions in efficiently terminated and matured 5 S RNA (lane d). Only traces of mutant RNA were evident with three or fewer adenylate acid residues (lanes a and b).

Because previous in vitro analyses have indicated that the extended sequence itself plays a role in RNA maturation (10), base substitutions also were introduced into the extension pre-
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Expression and stability of internal mutations in the yeast 5 S rRNA

| Region | Mutation | Steady-state | Briefly labeled | Internal promoter |
|--------|----------|-------------|----------------|-------------------|
| Stem 2' | Y5C20 | 21 | 77 |
| Y5C22U23 | 0 | 84 |
| Y5U29 | 82 | 83 |
| Y5A39 | 83 | 72 |
| Y5G39 | 65 | 84 |
| Y5G56U57 | 9 | 49 |
| Y5G61 | 10 | 28 |
| Y5G54 | 15 | 15 |
| Stem 3' | Y5G69 | 9 | 78 |
| Y5A70 | 11 | 40 |
| Y5C56 | 18 | 17 |
| Y5G59 | 33 | 86 |
| Y5G97 | 83 | 77 |
| Y5G98 | 72 | 71 |
| Y5A99 | 78 | 80 |
| Y5C101–103 | 6 | 84 |

* Based on the universal 3-stem or "wishbone" model for the 5 S RNA (see Lee et al. (23)).

\[\text{Percent radioactive mutant RNA after 2 min of labeling and acrylamide gel fractionation.} \]

\[\text{Percent radioactive mutant RNA after min of labeling and acrylamide gel fractionation.} \]

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mature rRNA molecules, in earlier studies (23) and as noted in Table I, the initial presence of mature rRNA and the instability were confirmed by brief labeling and half-life determinations. In most cases the reduced levels of mutant RNA were related to RNA instability and a brief labeling revealed much higher initial levels. In five examples (Y5U56U57, Y5C61, Y5C64, Y5A70, and Y5A86); however, the reduced levels corresponded closely with changes in the eukaryotic 5 S rRNA gene internal promoter sequence (23). Taken together these data indicate that, in S. cerevisiae, none of the internal sequences or structures that were examined was critical to RNA maturation but changes to them could substantially affect the stability of the mature RNA or its initial transcription.

In the present study, a correct maturation in these examples was established further in vitro, using a nuclear cell free extract prepared from S. cerevisiae. As illustrated in Fig. 3d, the extract was effective with both normal and efficiently expressed mutant 5 S rRNA precursors, resulting in stable and fully processed mature 5 S rRNA. As also illustrated by the examples shown in Fig. 3, b–e, this was equally true for precursors with mutations in the 3' end-spacer sequence. Nucleotide substitutions in the spacer (b and c), a shorter spacer (d), or a longer spacer (e), all resulted in a normal 5 S rRNA molecule. The only exceptions were precursors which contained altered secondary structure in the spacer region. As illustrated in Fig. 3f, in this case a new mature terminal was evident at the beginning of the altered secondary structure and the resulting molecule was highly unstable. Unlike the other examples, the amount of labeled precursor plus full-length 5 S RNA dropped rapidly and internally cleaved fragments were evident.

Similar results also were obtained when the 5 S rRNA sequence mutations were examined. As illustrated by the examples shown in Fig. 4, in all cases the mature 5 S rRNA was evident but, depending on the specific mutation, the stability of the product varied greatly. For example, a change which partially disrupted the terminal helix (e) always resulted in a slightly shorter and very unstable product, but a change which only slightly altered the helix (f) resulted in a more stable product. Similarly, more internal changes (b–d) resulted in correctly processed 5 S rRNA for which the stability, in vitro, generally reflected that which was observed in vivo.

Taken together, the present results, both in vivo and in vitro, strongly suggest that an exonuclease activity simply trims the precursor, at least in most instances. This conclusion is supported by the absence of dependence on sequence specificity in any of the experiments which are described, as well as the critical nature of altered secondary structure (stem I). The helix formed between the termini appears to be the key controlling element in the maturation process, an observation which suggests that the structure itself, at least in part, limits the action of the exonuclease. To examine this possibility further, additional changes were introduced to either the precursor or the assay conditions. In the first instance, the 3' end was phosphorylated by the addition of pCp using RNA ligase (28) to inhibit exonuclease cleavage. As shown in Fig. 5 (middle), this action fully inhibited normal processing, in vitro. The precursor molecules did disappear slowly because of nonspecific internal cleavage into much shorter fragments which migrated from the gel. In the second experiment (right), a large amount of unlabelled 5 S rRNA precursor was added to the assay mixture. This resulted in a much slower processing reaction in which the intermediated steps were much more evident. Finally, in a third experiment, oligonucleotides, complementary to either the 5' or 3' end of the mature 5 S rRNA were hybridized with precursor, prior to in vitro assay. As shown in Fig. 6, the oligonucleotide which was complementary to the 3' end (left) did not disrupt the maturation process, but with 5' end specific oligonucleotide, processing was very abnormal with shortened
degradation products clearly evident (right).

A variety of studies on the structure of the 5 S rRNA has indicated that the helical stem which forms between the termini is essential to an interaction with the ribosomal 5 S RNA binding protein (29, 32), an interaction which has been shown important for ribosome assembly (33–35), and even 5 S RNA storage (36). Our previous studies of the yeast 5 S RNA-protein complex have indicated that three helical regions are important for this interaction, the primary site being the helix formed by the termini (32, 37). Since 5 S RNP is likely to be present in any nuclear extract and since 5 S RNA can readily exchange into such a complex (29, 32), the effect of the the 5 S RNA ribosomal binding protein also was examined with respect to RNA processing. In the experiments shown in Fig. 7, the processing of 5 S rRNA precursor was compared in the presence of a large pool of 5 S rRNA binding protein (right) or of mature 5 S rRNA (middle). In all cases the results were entirely comparable with an efficient and complete processing of the 5 S rRNA precursor. The presence of excess 5 S RNA, which would be expected to remove 5 S RNA binding protein from the extract did not prevent RNA maturation and the presence of excess protein also had no effect.

While RNA processing remained a constant in most experiments, as already noted, large differences in product stability were observed. Because the 5 S RNA binding protein would be expected to affect the susceptibility of 5 S RNA to random nuclelease digestion, the affinity of the mutant RNAs for this ribosomal protein also was examined. As previously shown for mature labeled 5 S RNA, labeled 5 S rRNA precursor also can readily exchange into purified 5 S ribonucleoprotein complex (Fig. 8 (RNP)). More important, as illustrated in Fig. 8, many of the precursors for unstable 5 S RNA mutants were observed to exchange less effectively. For example, a mutation in the spacer sequence (G122), which results in normal levels of 5 S rRNA in vivo, was observed to efficiently interact with protein to form RNP (lane f), but changes in the terminal helix resulted in interactions which were compromised to various degrees (lanes a–e). This reduced interaction often correlated with the

FIG. 4. Effect of 5 S rRNA mutations on the processing of 5 S RNA precursors in vitro. The 5 S rRNA precursors containing normal (a) or altered internal sequences; Y5U56–57 (b), Y5G69 (c), Y5C101–103 (d), Y5G116 (e), or Y5C118 (f) were prepared in vitro by T7 RNA polymerase transcription. The RNA was labeled at the 5’ end and incubated for 0, 30, or 90 min with nuclear extract, SDS/phenol extracted and fractionated by gel electrophoresis. The positions of the precursor and mature 5 S RNA are indicated by arrowheads.

FIG. 5. Inhibition of 5 S rRNA precursor processing in vitro. The 5 S rRNA precursor was prepared and labeled as described in Fig. 3 before being incubated for 0, 30, or 90 min with nuclear extract, SDS/phenol extracted and fractionated by gel electrophoresis (left). A second aliquot (middle) was extended at the 3’ end with pCp using RNA ligase before incubation with nuclear extract, and a third aliquot (right) was incubated with nuclear extract together with unlabeled precursor RNA. The positions of the original precursor and the normal mature 5 S RNA are indicated by arrowheads.

FIG. 6. Effect of complementary oligonucleotides on the processing of 5 S rRNA precursors in vitro. The 5 S rRNA precursor was prepared and labeled as described in Fig. 3, and aliquots were hybridized with oligonucleotides complementary to the 3’ (left) or 5’ (right) end of the mature 5 S RNA sequence before being incubated for 0, 30, or 90 min with nuclear extract, SDS/phenol extracted and fractionated by gel electrophoresis. The positions of the original precursor and the normal mature 5 S RNA are indicated by arrowheads.
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interfere with processing and mature RNA production by prematurely limiting the progress of the exonucleolytic cleavage.

The importance of the YL3 protein to product stability and ribosomal integration is clear, but its significance in 5 S RNA processing appears limited at best. Even in the presence of a large excess of free 5 S RNA, RNA processing proceeds normally (Fig. 7) and to completion without aberrant products. Taken together with previous studies on the YL3 protein (35), these results suggest that the protein is likely to be much more relevant to ribosome assembly and/or function than RNA processing.

The results of the present study lead to a strikingly different conclusion about 5 S RNA processing than previously reached in *Drosophila*. Based on *in vitro* analyses, Levering and co-workers concluded (see Ref. 11) that 5 S RNA processing in the fly is mediated primarily by an endonuclease activity and that a variety of changes in the 5 S RNA can dramatically affect the maturation process. Most point mutations in loop B, helix III, and loop C severely inhibited processing changes which appear to have little or no effect in *S. cerevisiae* (see Table I). At present, it appears that the two organisms utilize very different primary mechanisms to mature the 5 S RNA, a feature which raises further questions about the need for a 5 S RNA precursor and its processing.

While RNA processing, in general, has been widely documented for several decades, the role of these often complex events has not always been clear. As already noted, it is puzzling for example, why a short molecule such as the 5 S RNA should be transcribed as a slightly longer precursor, a feature which appears to be universally conserved in eukaryotes and even with alternate processing mechanisms. We believe that, taken with the earlier observation that termination is essential for efficient 5 S RNA production (15), the present study continues to be consistent with an editing model, namely, that the processing is a quality control mechanism which ensures a functional RNA. It is clear that defects in ribosomes can easily be lethal to cells as a single defective ribosome can dramatically alter the efficient functioning of the entire polyribosome. In this circumstance, the action of the trimming exonuclease together with other nuclease activities, rapidly destroy the nascent chain if the molecule does not efficiently interact with protein and integrate into ribosomal structure. Such interactions already have been shown to involve many structural features in the 5 S RNA (29, 32, 37, 38). Previous studies also have shown that when the 5 S RNA is not integrated into the ribosome, either because of changes in the RNA structure (38) or the cognate 5 S RNA binding protein (35), the large subunit remains highly unstable and degrades rapidly. In many laboratories, studies based on targeted mutations, often reveal striking ribosomal instabilities in response to surprisingly small changes. It is, therefore, attractive to postulate that in many of these instances, a mechanism as described in this study, serves to remove the defective RNAs before they severely disrupt protein synthesis. Basically, we propose that, once the nascent chain in vivo requires the presence of at least five adenylic acid residues in the coding DNA strand. Other features in nascent 3'-external transcribed spacer are not significant to the maturation process, the requirement only being a free 3' hydroxyl end to initiate the exonuclease cleavage. Changes in the position of the termination signal or marked changes in the composition of the extended sequence had no significant effect. The formation of artificial secondary structure by targeted changes (Fig. 3) did stability of RNA, both *in vivo* and *in vitro* suggesting that, while this interaction was not critical to RNA processing, it was important in the stabilization of processed RNA and perhaps its integration into ribosome structure (33, 35).

**DISCUSSION**

In the present study, 5 S RNA processing in yeast was further examined based on the expression of mutant genes, *in vitro* and a parallel analysis of RNA processing *in vitro*. The analyses reveal a simple trimming process by an exonuclease activity which is limited by the secondary structure of the 5 S RNA molecule. At the same time, the interaction of the 5 S RNA molecule with its cognate ribosomal binding protein appears to stabilize the secondary structure, and limits further degradation of the 5 S RNA by other nuclease activities. The efficient termination of the nascent chain in vivo requires the presence of at least five adenylic acid residues in the coding DNA strand. Other features in nascent 3'-external transcribed spacer are not significant to the maturation process, the requirement only being a free 3' hydroxyl end to initiate the exonuclease cleavage. Changes in the position of the termination signal or marked changes in the composition of the extended sequence had no significant effect. The formation of artificial secondary structure by targeted changes (Fig. 3) did

![Fig. 7. Effect of 5 S rRNA or the 5 S RNA-protein complex on the processing of 5 S RNA precursors *in vitro*. The 5 S rRNA precursor was prepared and labeled as described in Fig. 3, and aliquots were incubated for 0, 120, or 240 min with nuclear extract (left) and an excess of unlabeled 5 S rRNA (middle), or an equivalent amount of purified 5 S RNA-protein complex (right) before being SDS/phenol-extracted and fractionated by gel electrophoresis. The positions of the original precursor and the normal mature 5 S RNA are indicated by arrowheads.

![Fig. 8. Formation of 5 S RNA precursor-protein complexes by RNA exchange. Mutant 5 S RNA precursors Y5G116 (a), Y5A118 (b), Y5G119 (c), Y5G120 (d), Y5G121 (e), or Y5G122 (f) were prepared and labeled as described in Fig. 3, and aliquots were incubated with purified 5 S RNA-protein complex as described under "Materials and Methods" before fractionation on 8% nondenaturing polyacrylamide gels. The positions of the free precursor and 5 S RNA-protein complex are indicated by arrowheads; normal 5 S RNA (RNA) and 5 S RNA-protein-protein complex (RNP) are included as markers.

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