Terminal Structure Mediates 5 S rRNA Stability and Integration during Ribosome Biogenesis*

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Formation of the eukaryotic ribosomal 5 S RNA-protein complex has been shown to be critical to ribosome biogenesis and has been speculated to contribute to a quality control mechanism that helps ensure that only normal precursors are processed and assembled into active ribosomes. To study the structural basis of these observations, the RNA-protein interface in the 5 S RNA-protein complex of the yeast (Saccharomyces cerevisiae) ribosome was examined based on a systematic introduction of targeted base substitutions in the RNA sequence. Most base substitutions had little or no effect on the efficiency of complex formation, but large effects were observed when changes disrupted helix I, the secondary structure formed between the interacting termini. Again, only modest effects were evident when the extended 3′ end of the mature RNA molecule was altered, but essentially no complex was formed when the 5′ end of the mature 5 S RNA sequence was artificially extended by one nucleotide. In vitro analyses demonstrated that this extension also dramatically altered the maturation of 5 S RNA precursor molecules as well as the stability of the mature 5 S RNA. Taken together, the results indicate that in the course of RNA maturation, the 5 S RNA-binding protein binds precisely over or “caps” the termini in a critical manner that protects the RNA from further degradation.

While first recognized as a discrete ribosomal component almost four decades ago, the function of the 5 S RNA remains unclear and often disputed (see Ref. 1). Nevertheless, numerous studies on 5 S RNAs from diverse origins have demonstrated intriguing evolutionary changes in the localization and expression of genes encoding this RNA, while molecular features have been shown to be highly conserved both with respect to the primary and secondary structures (see Refs. 2 and 3) and even aspects of the tertiary structure (4, 5). Included in these features is a strong interaction between the RNA termini, which results in a 9–10 base pair helix that is usually staggered at the 3′ end (see Fig. 1). Previous studies on ribosomes, both in bacteria and eukaryotic organisms, also have shown that the 5 S RNA molecules can be dissociated from ribosomes as an RNA-protein complex (e.g. Refs. 6, 7). Three ribosomal proteins have been isolated from complex prepared using ribosomes of Escherichia coli (3); in eukaryotes a single ribosomal protein (YL3 or Rp15p in yeast) of approximately equal total molecular weight has been observed to constitute the protein fraction (8). Mutations in either the yeast 5 S RNA (1) or the YL3 protein (9) result in strikingly unstable ribosomal subunits, consistent with a critical role in ribosome assembly (10).

Because the 5 S RNA-protein complex is relatively small and easily purified, it has been an attractive model for the study of RNA-protein interactions in ribosomes and even ribonucleoprotein complexes, in general. Extensive studies on complexes from E. coli have characterized the binding sites for all three ribosomal proteins (see Ref. 11) and indicated interactions with three of the four helices (I, II, and IV) in the bacterial 5 S RNA. Based on x-ray crystallography, recent analyses now show that the complex, together with a part of the 23 S rRNA, constitutes the central protuberance of the large ribosomal subunit, and at least in bacteria, the protein components mediate the integration of the 5 S RNA into the ribosome, permitting specific tertiary interactions with the 23 S rRNA (12–14). While crystal analyses are not available for eukaryotic ribosomes, past studies on the structure of the yeast 5 S RNA-protein complex, based on protection from ribonuclease digestion (8) or modification exclusion (15), have suggested that the helix formed by the termini of the 5 S RNA molecule (helix I) represents the primary protein binding site with further influence by helix II and IV. Naturally arising differences in the nucleotide sequence that do not alter these helices appear not to affect the formation of this complex (16), an observation that suggests a recognition of secondary or tertiary structure (17) rather than the actual nucleotide sequence.

In all the eukaryotes that have been examined, the 5 S RNA molecule is transcribed as a slightly longer precursor molecule with a short sequence extension at the 3′ end (12 nucleotides in Saccharomyces cerevisiae. Although this feature seems unnecessary and wasteful, the extra sequence is removed by nuclelease cleavage during RNA maturation and integration into the ribosomal structure. Studies in the toad (18), fly (19), yeast (20), and mammalian cells (21) have documented this maturation process and provided some detail regarding the underlying mechanism. For example, in Drosophila the extra nucleotides appear to be removed by a single rapid endonucleolytic step (22, 23), while in yeast it is primarily or entirely removed by an exonuclease (24). Studies of the 5 S RNA maturation process in yeast also indicate that, although the process is essentially independent of the nucleotide composition, it is surprisingly dependent on the length and higher order structure of the extended sequence. Transcripts that are not normally terminated or terminated much later are highly unstable and not incorporated into ribosomes (25). Furthermore, this is equally true when helical structure is introduced into this extended sequence (24). Taken together, these observations have led to the suggestion that the precursor sequence and its removal during RNA maturation serve, at least in part, as a quality

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control mechanism that ensures that mutated transcripts are not integrated into active ribosomes.

Since the 5 S rRNA-binding protein appeared to interact primarily with the helix formed by the termini of the 5 S rRNA molecule, in this study specific changes were introduced into this region to further examine the influence on protein binding and the integration of the molecule into stable ribosomes. The results indicate an unusual and critical dependence on the 5′ end structure, a feature which suggests that a protein "cap" at the termini mediates the quality control function.

**EXPERIMENTAL PROCEDURES**

**Construction and Expression of Mutant 5 S rRNA Genes—**Mutations were introduced into a yeast 5 S rRNA gene (26) containing a structural marker mutation (27) by using mutant oligonucleotide primers during PCR amplification; a modified two-step PCR strategy (28) was used to introduce mutations into coding sequence. Mutant oligonucleotides and normal primers were synthesized using a Cyclone Plus automated DNA synthesizer (Millipore Corp., Milford, MA). Mutant genes were cloned into pYF404, a high-copy (32) in a 50-

To express the mutant 5 S rRNA genes, the recombinant plasmids were used to transform a LEU2-deficient yeast strain (AH22) as described by Hinnen et al. (31).

**Preparation of Mutant 5 S rRNAs and Precursors—**To prepare mutant 5 S rRNAs with internal nucleotide changes, the transformed cells were grown with shaking under selective conditions at 30 °C. Whole cell RNA was prepared by SDS-phenol extraction, and the 5 S rRNAs were fractionated at room temperature by electrophoresis on 12% non-denaturing polyacrylamide gels (27).

To synthesize precursor molecules of 5 S rRNAs with changes at the 5′ or 3′ end, normal and mutant oligonucleotide primers were used to prepare T promoter/5 S rRNA-protein complex (5 in a 50-

**Preparation of 5 S rRNA-Protein Complex—**To express the mutant 5 S rRNA genes, the recombinant plasmids were transformed into pYF404, a high-copy (30

**Stability of the 5 S rRNA-Protein Complex**—Several mutants with insertions or deletions in the 3′ end structure, a feature which suggests that a protein

**In Vitro RNA Processing—**For in vitro RNA processing, the 5 S rRNA precursor was labeled at the 5′ end using T4 polynucleotide kinase and [γ-32P]ATP (34) or 3′ end with cytidine[5′-32P]ribophosphate and T4 RNA ligase (35). The labeled RNA was purified on an 8% polyacrylamide gel, and detected by autoradiography, and recovered by ethanol precipitation as described above. For the in vivo processing reactions, labeled RNA (10,000 cpm) was incubated at 30 °C with 10 μl of nuclear extract in a final volume of 20 ml containing 37.5 mM NaCl, 3 mM MgCl2, 100 mM KCl, 3 mM DTT, 10% glycerol, 0.5 mM EDTA, 0.1 mM DTT, 0.5 mM EDTA, 0.1 mM DTT, 0.5 mM MgCl2, 0.1 mM MgCl2, 0.1 mM EDTA, 0.1 mM DTT, 0.5 mM MgCl2, 0.1 mM magnesium phosphate, pH 6.6, and 100 μM (35) and collected by centrifugation for 10 min at 6000

**Immunoblot Analyses—**For immunoblotting, whole cell protein extracts (38) or purified YLS35 S rRNA complex protein was fractionated on a 12% SDS-polyacrylamide resolving gel with a 5% stacking gel and
transferred to nitrocellulose. The membrane was rinsed in TBST (20 mM Tris-HCl, 137 mM sodium chloride, 0.3% Tween 20, pH 7.6), stained (39) with India ink (1:250 dilution in TBST), destained, and blocked for 1 h with 5% dried milk powder in TBST (blocking buffer). The filter was then incubated with antibody (anti-Lhp1p diluted 1:1000 in blocking buffer) for 1 h, washed three times with TBST, and incubated for 1 h with a horseradish peroxidase-linked secondary antibody (Amersham Biosciences) diluted 1:2500 in blocking buffer. After four washes the membrane was incubated in ECL (Amersham Biosciences) detection reagents as described by the manufacturer and exposed on X-OMAT AR film (Kodak).

**RESULTS**

**Influence of the 5 S RNA Sequence on the Formation of Ribonucleoprotein Complex**—Past studies on the eukaryotic 5 S RNA-protein complex from yeast ribosomes indicated a single protein constituent, which based on peptide mapping and partial sequence analyses (8, 40) was identified as the YL3 protein. Subsequent studies showed that this complex could be efficiently labeled through RNA exchange by incubating unlabelled complex in the presence of $^{32}$P-labeled 5 S RNA (see Fig. 2, Ctl). This efficient exchange also has provided for a simple assay of essential structural features in the 5 S RNA and previously was combined with nuclease digestion (4, 8) or modification exclusion (15) to determine general features in the protein interface. As indicated in Fig. 1, both approaches identified the universally conserved terminal helix (I) as a primary binding site but also suggested interactions or at least protein contact with helix II and IV. To more directly access the contributions of individual nucleotides, in the present study mutations were introduced systematically in a yeast 5 S rRNA gene containing a neutral structural marker (Y5A99) mutation (27), either by the methods of Kunkel (41) or by a modified two-step PCR procedure (28). Normal or mutant RNAs were purified from cells transformed with mutant 5 S RNA genes or prepared in vitro using T7 RNA polymerase (24). RNAs labeled at either the 5' or 3' end with polynucleotide kinase after pretreatment with alkaline phosphatase (34) or the 3' end using RNA ligase (35) were equally efficient during RNA exchange (15, 24); the one-step 3' end label was used in most experiments. As shown in Fig. 2, Ctl, when compared with natural 5 S RNA (Ctl), the in vitro prepared 5 S RNA also was equally efficient in RNA exchanges. As summarized in Fig. 1, the efficiency of RNA exchange varied dramatically depending on the specific mutant RNA. Most changes had little or no effect on the exchange efficiency with levels of exchange $-90-105\%$ of that observed with normal yeast 5 S rRNA. Some changes, however, (e.g. Y5A33 and Y5U90i5) did have modest but reproducible effects.

**Fig. 1.** Formation of 5 S rRNA-protein complexes by RNA exchange. Normal or mutant 5 S RNAs were prepared, labeled, and incubated with purified 5 S RNA-protein complex as described under “Materials and Methods.” Protein-bound and free RNA fractions were separated on an 8% nondenaturing polyacrylamide gel, visualized by autoradiography and quantified by scintillation counting. The nucleotide changes in each RNA are indicated in bold letters together with the amount of ribonucleoprotein complex expressed as a percentage of that which was observed with normal 5 S rRNA. Values represent averages for three to six replicate experiments. The helical regions also are identified (I–V) together with the RNA-protein interface as initially determined by ribonuclease protection studies (dark shading) and extended (light shading) using modification exclusion (4, 15, 16).

**Fig. 2.** Effect of a 5'-end nucleotide extension on the formation of the 5 S rRNA-protein complex by RNA exchange. Purified cellular 5 S rRNA (A), in vitro transcribed 5 S RNA with an extra guanylic acid residue at the 5'-end (B), and in vitro transcribed normal 5 S rRNA (Ctl) was labeled and incubated with purified 5 S RNA protein-complex as described under “Experimental Procedures.” Protein-bound (RNP) and free (RNA) fractions were separated on an 8% polyacrylamide gel and visualized by autoradiography.

**Stability of the 5 S rRNA-Protein Complex**

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with 75–85% levels of exchange, while still others (e.g., Y5G116 and Y5C118) resulted in very little RNA exchange (<10%). Consistent with the past studies based on nuclease digestion (4, 11) or modification exclusion (12), the mutational analyses indicated that the strongest effects were all linked to structural disruptions in helix I, providing clear and direct evidence that helix I constitutes the primary protein binding site.

Influence of the Terminal Structure on the Formation of Ribonucleoprotein Complex—To further explore essential features in helix I, additional changes were introduced in this region. Since 5 S RNA molecules normally have a sequence extension at the 3′ end, in the course of the additional analyses the 3′ end also was extended by one nucleotide. Surprisingly, this change had the most striking effect. As shown in Fig. 2, with one additional guanylic acid residue at the 5′ end (A), only a slight trace of complex was evident. This was in strong contrast with normal, in vivo (Ctl) or in vitro (B) transcribed 5 S RNA. The special nature of this change was further underlined when compared with other changes at the termini. As shown in Fig. 3, when the 3′ end extension was removed (lane a) or when the residue was changed (lane b) only moderate effects were evident. In contrast, when these changes were introduced in a 5 S RNA molecule containing the nucleotide extension at 5′ end (lanes c and d), again only trace amounts of ribonucleoprotein were evident, with complex formation being almost totally inhibited.

Internal changes in helix I would be expected to disrupt the secondary structure of this helix, an effect that is likely the cause of the observed strong reduction in complex formation. Indeed past structural analyses (1, 42) have confirmed such changes in the secondary structure. The changes at the termini, however, would not be expected to disrupt the helix. In view of the dramatic effects, with a 5′ end extension, the RNA sequences were further confirmed by sequence determination, and the possibility of structural rearrangements were eliminated by computer-aided modeling and actual secondary structure determination. The only interaction that could be envisaged would be a base pair extension in helix I but this G-U base pair at the end of an open helix clearly is unstable. Nevertheless, to fully exclude a structural rearrangement, the terminal-modified 5 S RNA molecules also were subjected to partial cleavage with T1 ribonuclease (Fig. 4). Since in vitro synthesized RNA contains the same terminal heterogeneity, doublet bands were evident with the two synthetic molecules (A and B) but the primary cleavage sites remained identical to those observed in the natural 5 S RNA molecule (Ctl). Such results again indicated that the effect on complex formation was not the result of a structural rearrangement but was due to a direct hindrance of protein binding.

Processing and Stability of the 5′ End-extended 5 S rRNA—Previous studies (24) on the maturation of the yeast 5 S rRNA have indicated that the 5 S RNA-binding protein (YL3) does not directly influence the processing of 5 S rRNA precursors, but it...
does play a significant role in protecting this rRNA from further degradation by “housekeeping” nucleases. To examine the influence of the 5′ end extension on RNA maturation and stability, both 5 S rRNA precursor and mature RNAs were incubated with a cell extract, previously demonstrated to effectively process 5 S rRNA precursors in vitro (24). As shown in Fig. 5, the normal precursor (A) was fully processed to stable mature 5 S RNA in ~15 min, but a 5′-extended molecule (B) revealed differences in both the maturation profile and product stability. Basically, two populations of product were evident, a more stable normal population and a rapidly degraded second population. Even the normal population, however, appeared less stable than product without the additional nucleotide at the 5′ end. About 50% of this population was degraded after 105 min of incubation. Abbreviated gel fractionations that would include short fragments of the 5′ end did not reveal any specific processing at the 5′ terminal (results not shown).

Differences in stability were further observed when mature RNAs were reincubated with the same extract. As shown in Fig. 6, when RNAs with differences at the termini were compared in vitro, the results correlated strongly with observations in vivo, and the efficiency of ribonucleoprotein complex formation. After 60 min of incubation, normal RNA (Ctl) was essentially undergraded. In contrast, RNA with a change at the 3′ end (A) was partially degraded, and RNA with a one nucleotide extension at the 5′ end (B) was almost completely degraded. As reported in a previous study for other mutations, the yield or stability of the 5 S RNA again correlated closely with its ability to form a complex with the 5 S rRNA-binding protein (YL3).

In eukaryotes virtually all nascent polymerase III transcripts initially appear to associate with the La protein or Lhp1p in S. cerevisiae (see Ref. 43). Apparently, the 5 S rRNA interacts with the ribosomal 5 S rRNA-binding protein after a transient association with the La protein (10, 45), which then directs the 5 S rRNA to the nucleolus (10, 46). Since the YL3 protein used in this study was purified as a 5 S rRNA protein complex, there was some possibility that La protein might be present. To eliminate this possibility an immunoblot analysis was undertaken using anti-Lhp1p protein (47). A shown in Fig. 7, no La protein was observed even when the gel was heavily loaded and the resulting membrane was somewhat overexposed. In a whole cell extract the La protein is clearly present, and visualized by autoradiography.

**DISCUSSION**

Past studies on the maturation of the ribosomal 5 S RNA in S. cerevisiae (25) have indicated that proper termination coupled with efficient processing is critical to the integration of 5 S RNA transcripts into stable ribosomes. Such studies also suggested that RNA processing, at least in part, acts as a “quality control” mechanism that helps to ensure that only normal rRNA precursors are effectively processed and assembled into active ribosomes. Subsequent studies (24) have further suggested that the 5 S RNA-binding protein (YL3) may not influence the maturation process directly but rather that it plays an important role in protecting the nascent 5 S RNA molecules from further degradation by housekeeping nucleases. The mutational analyses described in this report provide direct evi-
stability and perhaps even ribosome integration. As a result, this precise fit is critical to 5 S rRNA processing. In the course of RNA processing, the YL3 protein (8, 48) binds tightly over or in the 5 S rRNA termini to protect the RNA molecule from further degradation. A number of the changes indicate that structure associated with the 5 S rRNA termini is important to the RNA-protein interaction, but a single nucleotide addition at the 5' end is sufficient to sterically hinder the protein interaction to a degree that almost prevents the interaction entirely. We suggest that in the course of rRNA maturation the YL3 protein (8, 48) binds tightly over or in the 5 S rRNA termini and protects the RNA molecule from further degradation. As a result, this precise fit is critical to 5 S rRNA stability and perhaps even ribosome integration.

The observations in this study, while fully consistent with the previous reports, also provide new detail about the nature of the eukaryotic 5 S rRNA-protein complex and the way in which the 5 S RNA-binding protein probably acts to stabilize and protect the RNA molecule from further degradation. A number of the changes indicate that structure associated with the 5 S RNA molecules that inefficiently bind the cognate ribosomal protein probably acts to stabilize and protect the RNA molecule from further degradation. A question that remains is why the effect of an extension at the 5' end was much less critical than at the 5' end. Although less strongly bound, a natural slightly shorter processing variant also has been reported to form a complex with the YL3 protein (44), and our past study on the yeast 5 S RNA-protein complex has shown that the longer precursor RNAs form stable complexes with the YL3 protein (24) as well. The same study further indicated that in S. cerevisiae, the 5 S RNA is processed by an exonuclease activity that is limited primarily or entirely by the helix I structure. As a result it seems attractive to speculate that the YL3 protein, fixed critically relative to the 5' end, also fully caps the 3' end when the extended terminal has been removed, and as suggested above for the La protein, perhaps may even act to displace the exonuclease enzyme in the course of RNA processing. Whatever the case, the critical 5' end extension remains an intriguing structural feature of this essential ribosomal RNA-protein complex.

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