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The 5′ end of the 18S rRNA can be positioned from within the mature rRNA

KISHOR SHARMA,[1] JAAP VENEMA[1,3] and DAVID TOLLERVEY[1,2]

[1]European Molecular Biology Laboratory, Gene Expression Programme, 69012 Heidelberg, Germany
[2]Institute of Cell and Molecular Biology, University of Edinburgh, King's Buildings, Edinburgh EH9 3JR, United Kingdom

ABSTRACT

In yeast, the 5′ end of the mature 18S rRNA is generated by endonucleolytic cleavage at site A_1, the position of which is specified by two distinct signals. An evolutionarily conserved sequence immediately upstream of the cleavage site has previously been shown to constitute one of these signals. We report here that a conserved stem-loop structure within the 5′ region of the 18S rRNA is recognized as a second positioning signal. Mutations predicted to either extend or destabilize the stem inhibited the normal positioning of site A_1 from within the 18S rRNA sequence, as did substitution of the loop nucleotides. In addition, these mutations destabilized the mature 18S rRNA, indicating that recognition of the stem-loop structure is also required for 18S rRNA stability. Several mutations tested reduced the efficiency of pre-rRNA cleavage at site A_1. There was, however, a poor correlation between the effects of the different mutations on the efficiency of cleavage and on the choice of cleavage site, indicating that these involve recognition of the stem-loop region by distinct factors. In contrast, the cleavages at sites A_1 and A_2 are coupled and the positioning signals appear to be similar, suggesting that both cleavages may be carried out by the same endonuclease.

Keywords: nucleolus; pre-rRNA; RNA processing; small nucleolar RNA; yeast

INTRODUCTION

The organization of the rRNA genes is essentially identical in all Eukaryotes. The small subunit rRNA gene (18S rRNA in yeast) and the two large subunit rRNA genes (5.8S and 25S rRNAs in yeast) are cotranscribed into a large precursor (the 35S pre-rRNA in yeast) by RNA polymerase I. Within this pre-rRNA the mature rRNA sequences are separated by two internal transcribed spacers (ITS1 and ITS2) and flanked by two external transcribed spacers (5′ ETS and 3′ ETS) (see Fig. 1). The mature eukaryotic rRNAs are synthesized by a complex maturation pathway that requires both endonucleolytic cleavage and exonuclease digestion, as well as covalent modification of many nucleotides in the rRNA sequences.

The signals in the pre-rRNA that are used to identify the positions of cleavage at site A_1 have been investigated (Venema et al., 1995). Surprisingly, substitution mutations across site A_1 did not affect the efficiency or accuracy of cleavage. However, the insertion or deletion of 2 nt immediately 3′ to site A_1 led to heterogeneous cleavage, generating two major forms of the 18S rRNA differing by 2 nt at the 5′ end. Substitution of an evolutionarily conserved sequence immediately 5′ to site A_1 (i.e., within the 5′ ETS) abolished the use of one of these sites. These observations led to the conclusion that site A_1 is positioned with respect to two elements; the 5′ flanking sequence is one element, whereas the other lies within the mature 18S rRNA (Venema et al., 1995).

A stem-loop structure lies at the 5′ end of the 18S rRNA; in yeast the base of the stem lies 3 nt 3′ to the position of A_1 cleavage (see Fig. 2). This forms part of the central pseudoknot, a long-range interaction that is one of the most conserved regions of the ribosome (Alksne et al., 1993). To investigate the role of this structure in A_1 cleavage we constructed and analyzed a number of mutations in the 5′ stem and loop within the 18S rRNA.

RESULTS

Effects of mutations in 18S rRNA on pre-rRNA processing

Five mutations were generated in the 18S stem-loop/pseudoknot structure (see Fig. 2 and Materials and

[3] Present address: Department of Biochemistry and Molecular Biology, Vrije Universiteit, de Boelelaan 1083, 1081 HV Amsterdam, The Netherlands.

Reprint requests to: David Tollervey, Institute of Cell and Molecular Biology, University of Edinburgh, King's Buildings, Edinburgh EH9 3JR, United Kingdom; e-mail: d.tollervey@ed.ac.uk.

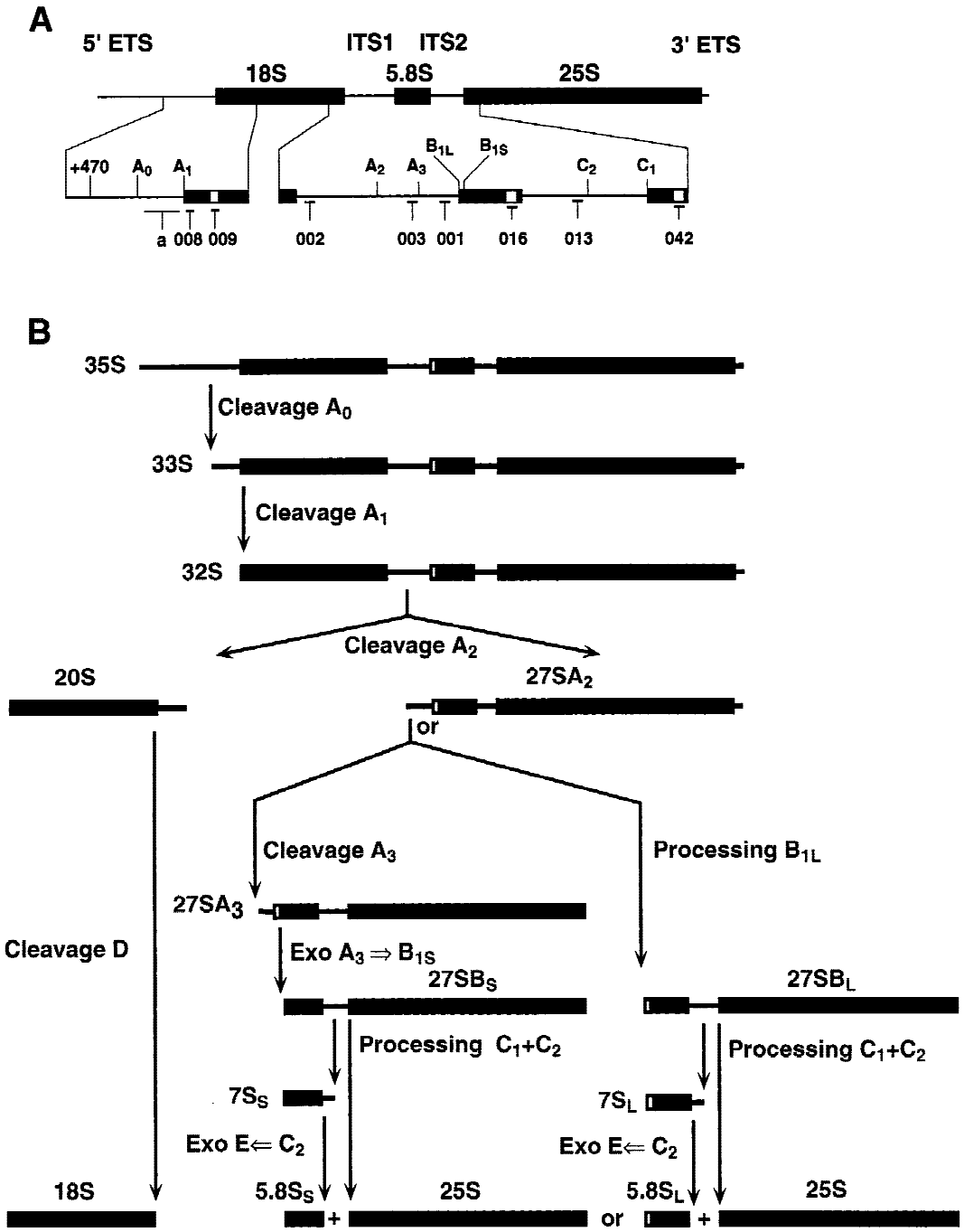


FIGURE 1. **A**: Structure of the pre-rRNA and location of oligonucleotide hybridization probes. Thick bars represent the mature rRNAs, thin bars indicate the transcribed spacer regions. The 18S, 5.8S, and 25S rRNAs are flanked by the 5′ and 3′ external transcribed spacers (5′ ETS and 3′ ETS) and separated by internal transcribed spacers 1 and 2 (ITS 1 and ITS2). Probe a is a riboprobe complementary to the A_0–A_1 fragment. Probes 009, 016, and 042 hybridize to the tags within the mature 18S, 5.8S, and 25S rRNAs, respectively. Probe 008 hybridizes to the mature 18S rRNA. Probes 002, 003, and 001 hybridize to ITS1 at positions 5′ to site A_2, between A_2 and A_3 and 3′ to site A_3, respectively. Probe 013 hybridizes to the 5′ region of ITS2. **B**: Major pre-rRNA processing pathway in *Saccharomyces cerevisiae*. The 35S precursor is cleaved at site A_0, giving rise to the 33S precursor. 33S is rapidly cleaved at site A_1, the 5′ end of the mature 18S rRNA, yielding the 32S precursor. 32S is cleaved at site A_2 in ITS1, yielding the 20S and $27SA_2$ pre-rRNA. This cleavage separates the pre-rRNAs destined for the small and large ribosomal subunit. The 20S precursor is endonucleolytically processed at site D, generating the mature 18S rRNA. The majority of the $27SA_2$ precursor is cleaved at site A_3 by RNase MRP, yielding the $27SA_3$ pre-rRNA, which is processed to the mature $5.8S_S$ and 25S rRNAs. An alternative, minor pathway generates the $5.8S_L$ rRNA.

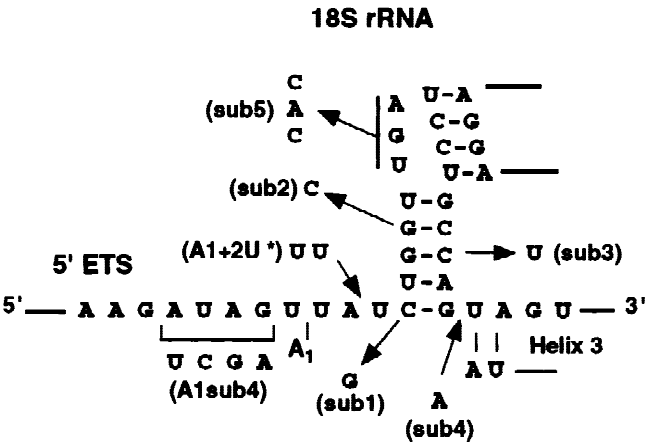


FIGURE 2. Predicted secondary structure of the 18S stem-loop/pseudoknot. Nucleotides that have been substituted (sub1, sub2, sub3, and sub5) or inserted (sub4) are indicated. The identical mutants, but containing two inserted nucleotides at the base of the stem (+UU), were also generated. These are designated in the text as sub1*–sub5*. The otherwise wild-type sequence with the 2-nt insertion is designated A1+2U. The A1sub4 mutation is also shown (Venema et al., 1995).

Methods). The sub1 mutation is predicted to shorten the stem, moving the base of the stem 1 nt further from site A_1. The sub2 mutant is predicted to severely destabilize the stem structure. In the sub3 mutant, a G-C base pair was replaced by a noncanonical G-U base pair; the equivalent mutation confers a dominant cold-sensitive lethal phenotype in *Escherichia coli* (Dammel & Noller, 1993). The sub4 mutant is an insertion of 1 nt at the 3′ end of the stem that is predicted to lengthen the stem (by three A-U base pairs in the sub4 construct and by 2 base pairs in the sub4* construct). The sub5 mutation alters the three loop nucleotides that are not engaged in the pseudoknot interaction. In addition, each of the mutations was combined with the insertion of two U residues immediately 5′ to the stem, to form the sub1*–sub5* mutants. This insertion allows the positioning of site A_1 with respect to sequences 5′ to the site of cleavage to be resolved from positioning with respect to sequences within the 18S rRNA (Venema et al., 1995).

All the mutant constructs were cloned into plasmids that express the entire pre-rRNA under the control of the RNA polymerase II (pol II) *GAL7* promoter (Henry et al., 1994). These were expressed in strain NOY504 that is temperature sensitive for RNA polymerase I (pol I) (Nogi et al., 1991). When cells are shifted to 37 °C for 6 h in galactose-containing medium, chromosomal rDNA synthesis is reduced to a low level allowing the analysis of the processing of the mutant pre-rRNAs. Short oligonucleotide tags present in the mature 18S, 5.8S, and 25S rRNA sequences allow their synthesis to be monitored (Beltrame & Tollervey, 1992; Henry et al., 1994).

Growth of the pol I temperature-sensitive strains was assessed at 37 °C and 25 °C. Only the strain transformed with the wild-type rDNA plasmid was able to grow at 37 °C. None of the mutant pre-rRNAs (sub1–sub5) were able to support growth at the nonpermissive temperature, indicating that functional ribosomes were not synthesized from the mutant pre-rRNA (data not shown). At 23 °C, growth of the strains was not inhibited on galactose medium, indicating that the sub3 mutation is not dominant negative in yeast. Under these conditions, the pre-rRNA expressed from the plasmid contributes approximately 30% of the ribosomes (data not shown). In *E. coli* the equivalent C_{23}-U mutation is dominant negative, although at higher expression levels (50–60% of wild-type; Dammel & Noller, 1993).

Northern analysis was used to determine whether the mutant pre-rRNAs are processed to mature 18S and 25S rRNAs (Fig. 3, lanes 2–6). These RNAs were detected using the oligonucleotide tags present within the mature sequence (probes 009 and 042, Fig. 1). Synthesis of the 25S rRNA is not expected to be affected by mutations within the 18S rRNA coding region, and this was observed (Fig. 3D). The levels of 18S rRNA in the sub1 and sub3 mutants were comparable to the wild-type control (Fig. 3G, lanes 2 and 4), showing that the inability of these constructs to support growth is not due to the absence of the rRNA. The level of 18S rRNA was reduced in the sub2 mutant (Fig. 3G, lane 3) and strongly reduced in the sub4 and sub5 mutants (Fig. 3G, lanes 5 and 6). The negative control strain, which contains a plasmid lacking the rDNA, does not show any background signal (Fig. 3, lane 8).

Analysis of the processing of the sub1 and sub2 pre-rRNAs by Northern hybridization (Fig. 3, lanes 2 and 3) revealed that the levels of the major precursors on the pathway of 18S rRNA synthesis, the 35S, 32S, $27SA_2$, and 20S pre-rRNAs, are little affected by the mutations. In sub5 the 35S pre-rRNA was mildly accumulated, but other intermediates were unaffected (Fig. 3, lane 6). In the sub3 pre-rRNA a mild reduction in the levels of the $27SA_2$ and 20S pre-rRNAs was observed (Fig. 3C, lane 4), indicative of some inhibition in processing at site A_2, but synthesis of the 18S rRNA was unaffected. Some reduction in the pre-rRNA levels was also seen in the RNA extracted from the strain expressing sub4, but a similar reduction was seen in the level of mature 25S rRNA (Fig. 3, lane 5), indicating that this is due to differences in loading. The negative control shows only very low background hybridization (Fig. 4, lane 8). We conclude that the underaccumulation of the 18S rRNA synthesized from the sub2, sub4, and sub5 pre-rRNAs is not because of a failure of pre-rRNA processing, and is therefore likely to be due to destabilization of the mature rRNA.

As previously reported (Venema et al., 1995) the insertion of 2U residues immediately 5′ to the 18S stem-loop structure (see Fig. 2A, A1+2U) did not affect the processing of an otherwise wild-type pre-rRNA (Fig. 3, lane 1). However, the combination of the 2-nt insertion

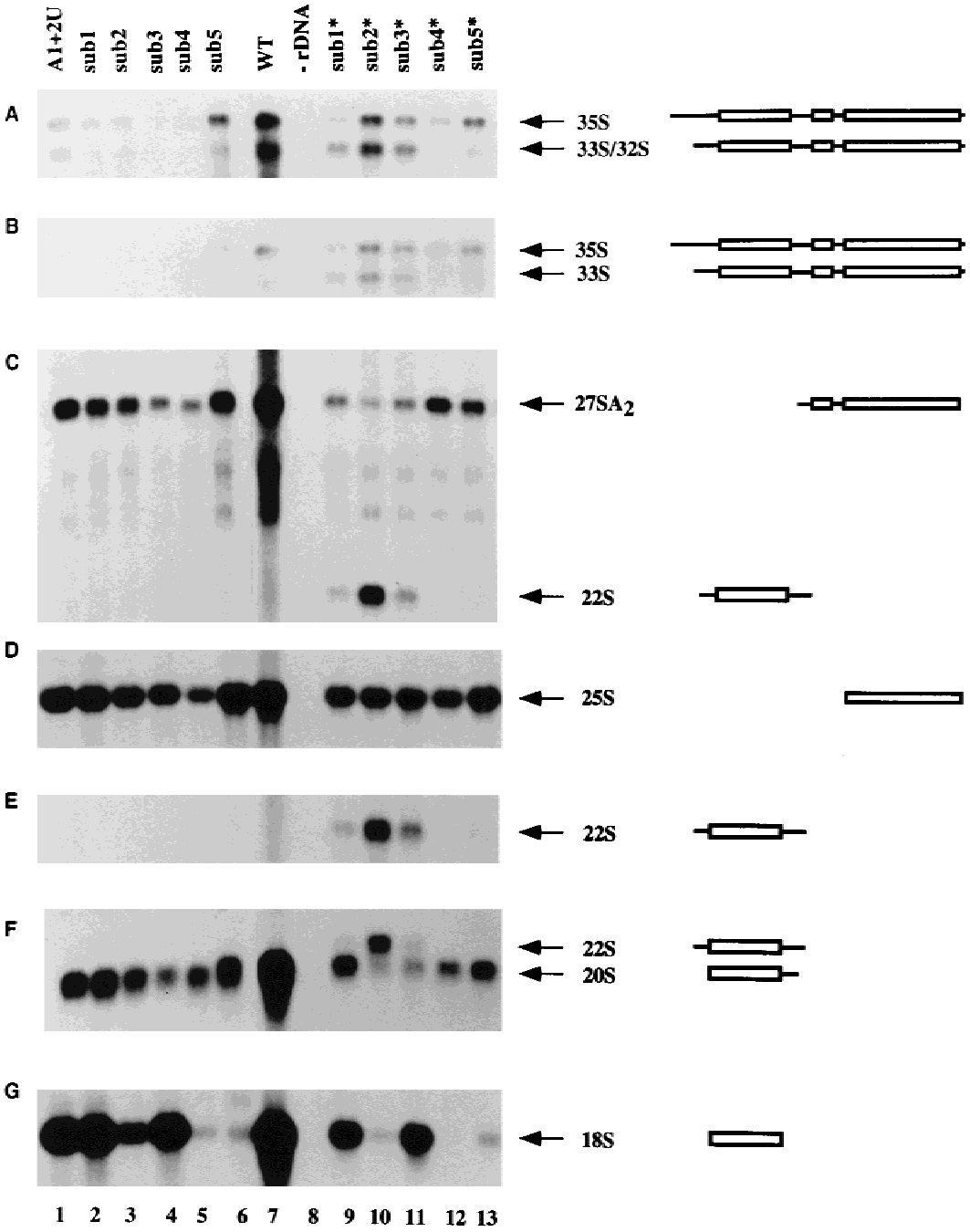


FIGURE 3. Effects of 18S rRNA mutations on pre-rRNA processing. RNA was extracted from strains carrying a plasmid with the wild-type rDNA (lane 7), lacking the rDNA sequence (lane 8), or with the rDNA containing the mutations indicated, and analyzed by Northern hybridization. A schematic representation of the precursors is also shown. **A**,**C**: Probe 003, hybridizing in ITS1 between A_2 and A_3. **B**,**E**: Probe a, hybridizing in the 5′ ETS between A_0 and A_1. **D**: Probe 042, complementary to the 25S rRNA tag. **F**: Probe 002, hybridizing in ITS1 5′ to A_2. **G**: Probe 009, complementary to the 18S rRNA tag. The locations of the sub1–sub5 mutations are indicated in Figure 2. The sub1*–sub5* mutations additionally have a 2U insertion at the 5′ end of the 18S rRNA.

with the sub2 and, to a lesser extent, the sub1 and sub3 mutations (in sub1*, sub2*, and sub3*) led to the accumulation of the 33S pre-rRNA (Fig. 3B) and the appearance of the aberrant 22S RNA (Fig. 3E). In contrast, processing of the sub4* and sub5* pre-rRNAs was unaffected (Fig. 4, lanes 12 and 13). The 33S pre-rRNA is the product of cleavage at site A_0 in the 5′ ETS. This is a normal processing intermediate but is not readily detected in the wild-type pre-rRNA by Northern hybridization because it is very rapidly cleaved at sites A_1 and A_2. The identity of this precursor was verified by a probe that hybridizes between A_0 and A_1

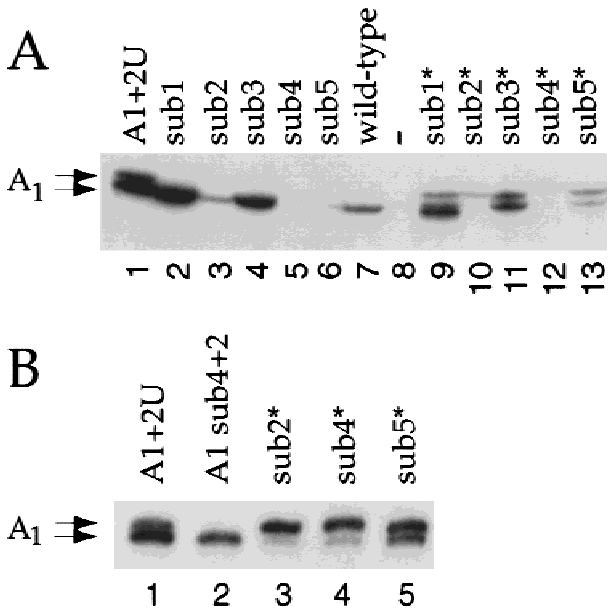


FIGURE 4. Effects of 18S rRNA mutations on the position of A_1 cleavage. RNA was extracted from strains carrying a plasmid with the wild-type rDNA (lane 7), lacking the rDNA sequence (lane 8), or with the rDNA containing the mutations indicated, and analyzed by primer extension with oligo 009, complementary to the 18S rRNA tag. **A**: Primer extension on equal amounts of RNA from each strain. **B**: Fivefold more RNA from strains expressing sub2*, sub4*, and sub5* has been loaded to allow the alteration in the use of the two A_1 sites to be visualized.

(probe a in Fig. 1; Fig. 3B). The 22S precursor was also detected by probe a (Fig. 3E) and by a probe hybridizing between sites A_2 and A_3 (oligo 003, Fig. 3C), but not by oligo 001 that hybridizes 3′ to site A_3 or by oligo 013 in ITS2 (data not shown). The 22S RNA therefore extends from A_0 to A_3 and is the product of cleavage of the 33S RNA at site A_3 in the absence of cleavage at sites A_1 and A_2. The level of the 20S pre-rRNA was strongly reduced in sub2* and reduced to a lesser extent in sub3* (Fig. 3F, lanes 10 and 11). The level of the $27SA_2$ pre-rRNA was also reduced in sub1*–sub3* (Fig. 3C, lanes 9–11). Some delay in cleavage at A_0 was indicated by the accumulation of the 35S pre-rRNA in these strains.

The levels of the 27SB pre-rRNAs were not strongly affected by the sub1–sub5 mutations and primer extension did not reveal any clear differences in cleavage at site A_3 or processing at the alternative B_{1L} and B_{1S} sites (data not shown). No accumulation of the 7S pre-rRNA species was observed and the ratio of $5.8S_L$:$5.8S_S$ was unaffected as judged by Northern hybridization with probe 016 (data not shown). Processing at sites on the pathway of 5.8S/25S rRNA synthesis was therefore unaffected by the mutations in the 18S rRNA.

We conclude that the combination of the 2U insertion with the sub1–sub3 mutations, each of which are predicted to destabilize the stem structure (Fig. 2), inhibits processing at sites A_1 and A_2.

The sub2* mutation also showed decreased 18S rRNA accumulation compared to the sub2 mutation (Fig. 3, lanes 3 and 10). This may be due to the combination of inhibition of 18S synthesis and its destabilization.

Mutants that affect the 18S stem structure inhibit the spacing mechanism for A_1 positioning

The position and efficiency of A_1 cleavage was assessed by primer extension using primer 009, complementary to the 18S tag. In the sub1–sub5 pre-rRNAs the position of A_1 cleavage is unaltered and the signal strength is in good agreement with the level of 18S rRNA detected by Northern hybridization (Fig. 4A, lanes 2–6).

Insertion of 2 nt between the stem and site A_1, in the A1+2U pre-rRNA (Fig. 4A, lane 1) and sub1*–sub5* constructs (Fig. 4A, lanes 9–13), led to heterogeneity at the 5′ end of 18S rRNA. The upper band, which is displaced with respect to the 18S rRNA sequence by the insertion, corresponds to the site selected by recognition of the 5′ flanking sequence at site A_1. Formation of the upper band was selectively inhibited by substitution of 4 nt 5′ to A_1 (mutation A1 sub4+2), which abolishes utilization of the 5′ cleavage site (Fig. 4B, lane 2) (Venema et al., 1995). The lower band corresponds to a cleavage that remains at the same distance from the primer despite the insertion and is therefore assumed to be positioned with respect to some signal that lies 3′ to the site of the inserted nucleotides.

In the sub1* and sub3* pre-rRNAs (Fig. 4A, lanes 9 and 11) the ratio between the two A_1 sites was similar to the otherwise wild-type, A1+2U control RNA (Fig. 4A, lane 1) with some preference for the 3′ processing site. In contrast, both the sub2* and sub4* mutations greatly reduced utilization of the 3′ cleavage site and the sub5* mutation more weakly inhibited its utilization (Fig. 4A, lanes 10, 12, and 13). Since site A_1 is the 5′ end of the 18S rRNA, the overall signal was reduced in line with the reduced level of the 18S rRNA. In Figure 4B we present a primer extension analysis in which fivefold more RNA was used for sub2*, sub4*, and sub5* to compensate for this, allowing the data to be more clearly visualized. In all of the mutants, the positions of cleavage correspond to the two positions cleaved in A1+2U pre-rRNA, with the exception of sub4*. Unexpectedly, the sub4* mutation reproducibly led to an increase in the separation between the two primer extension stops from 2 to 3 nt. Because of the 1-nt insertion, the position of the 5′ processing site was displaced by 1 nt. However, the site of the residual 3′ processing was not displaced (Fig. 4B, lane 4).

To confirm that the loss of the 3′ A_1 site is due to the inhibition of cleavage rather than selective destabilization of the 18S rRNA, the excised A_0–A_1 fragment was analyzed by Northern hybridization (Fig. 5). As previ-

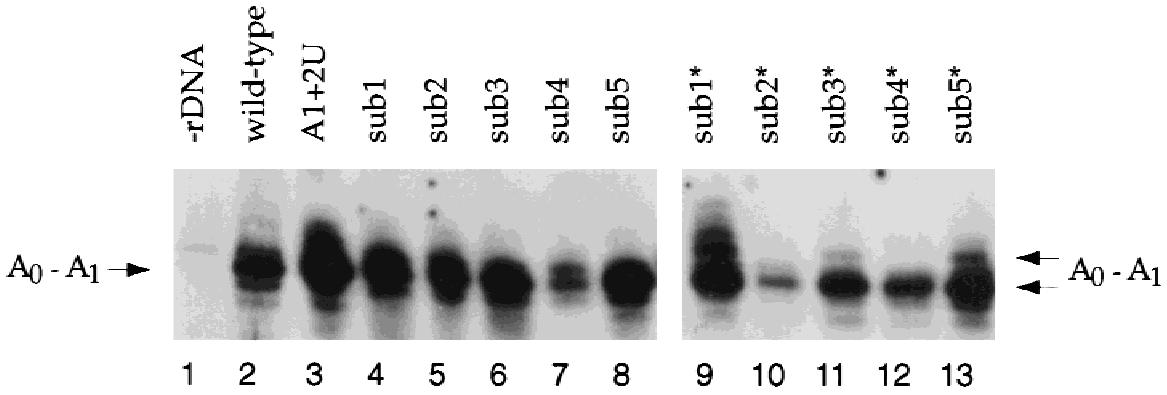


FIGURE 5. Effects of 18S rRNA mutations on the accumulation of the excised A_0–A_1 fragment. RNA was extracted from strains carrying a plasmid with the wild-type rDNA (lane 2), lacking the rDNA sequence (lane 1), or with the rDNA containing the mutations indicated. RNA was separated using a gel system that allows the resolution of single nucleotide differences and analyzed by Northern hybridization using a riboprobe complementary to the A_0–A_1 region.

ously reported (Venema et al., 1995), there is a 1-nt heterogeneity between the rDNA in the host genome and that present on the GAL::rDNA plasmid. This gives rise to a fragment 1 nt longer than the major band in the wild-type rDNA lane (Fig. 5, lane 2), which is also present in the −rDNA control (Fig. 5, lane 1) and all other lanes. In the A1+2U sample, an additional band appears that is 2 nt longer than the major band in the wild-type lane (Fig. 5, lane 3), corresponding to the heterogeneity in cleavage at site A_1. The upper (i.e. longer) band represents cleavage at the 3′ site. Comparison of the sub2* and sub4* samples (Fig. 5, lanes 10 and 12) with the A1+2U control (Fig. 5, lane 3) or sub1* (Fig. 5, lane 9) shows a great reduction in the longer A_0–A_1 fragment, confirming that pre-rRNA cleavage at the 3′ site is inhibited by the 18S mutations. The longer form of the A_0–A_1 fragment was also reduced in the sub5* lane (Fig. 5, lane 13), consistent with the altered ratio seen in the mature rRNA. The reason for the apparent reduction in the longer A_0–A_1 fragment in the sub3* strain is unclear.

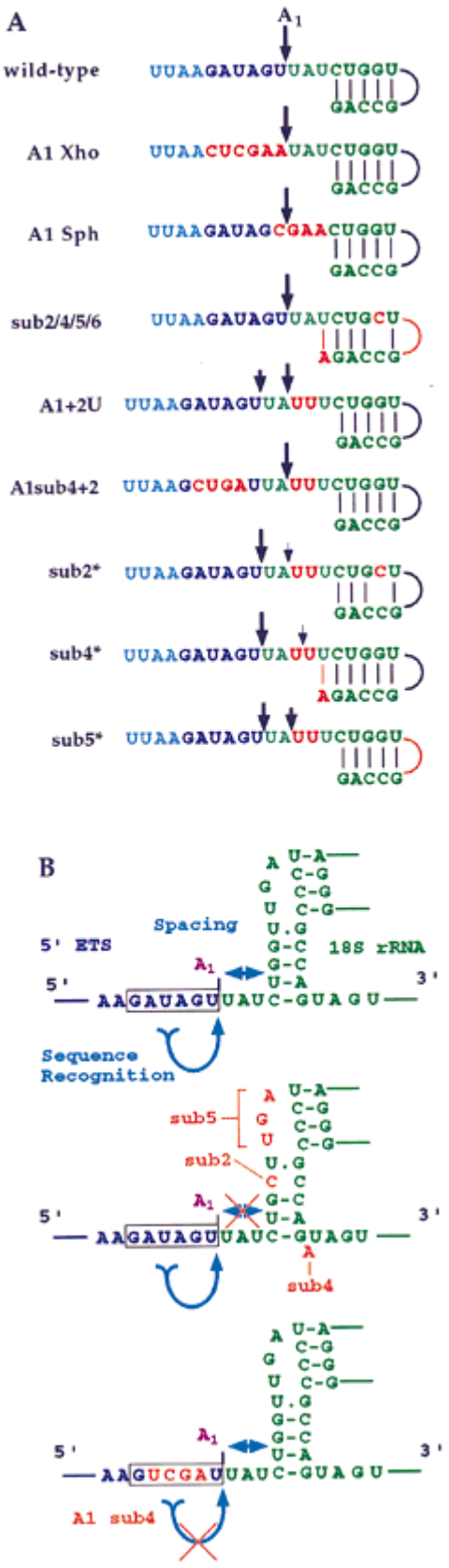
A summary of the effects of the 18S mutations on the sites of A_1 cleavage is shown in Figure 6. We conclude that the stem-loop structure within the 18S rRNA is indeed recognized to position the site A_1 cleavage. This recognition is normally redundant with the recognition of the 5′ flanking sequence; in the wild-type pre-rRNA both elements are used to select the same site of cleavage.

DISCUSSION

Two signals specify the site of A_1 cleavage

The data presented here strongly support the recognition of the 5′ stem-loop region within the 18S rRNA in positioning the site of A_1 cleavage, which generates the 5′ end of the mature 18S rRNA (see Fig. 6). Previous data indicated that the sequence immediately 5′ to the site of cleavage is also recognized to position this site (Venema et al., 1995). The contributions made by these two elements can be resolved by the insertion of 2 nt immediately 3′ to site A_1 that results in the use of two cleavage sites, 2 nt apart. In an otherwise wild-type pre-rRNA, both sites are used, showing that both signals contribute to the normal positioning of site A_1. The combination of the 2-nt insertion with the substitution of 4 nt immediately 5′ to site A_1 inhibited the use of the 5′ A_1 site, indicating that this is positioned with respect to the sequence 5′ to the site of cleavage (Venema et al., 1995). In contrast, three different mutations in the 18S stem-loop region inhibited cleavage at the 3′ site, showing this site to be positioned with respect to this structure. The sub2 mutation is predicted to destabilize the stem, the sub4 mutation is predicted to extend the stem, and the sub5 mutation alters the loop sequence. While these data do not establish whether the structure or sequence of this region is being recognized, it is notable that there appears to be a correlation between predicted effects on the overall structure of the stem and loop and the inhibition of its use in positioning site A_1. Moreover, the sub4 mutation is located 13 nt away from sub2 in the primary sequence. We postulate that the normal positioning of site A_1 involves recognition of the structure of the 18S stem and loop.

The 5′ flanking sequence at site A_1 is conserved among Ascomycete fungi, including *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, and a related sequence is conserved among plants, but no similar conservation was observed in vertebrates (Venema et al., 1995). This suggests that recognition of the stem structure may be the major positioning mechanism in humans. In yeast, increasing the size of the insertion in the 5′ region of the 18S rRNA (i.e., increasing the distance between the sites of cleavage that would have been specified by the two positioning elements) resulted in only a single cleavage site that was correctly positioned with respect to the 18S stem, indi-



cating that this is the dominant mechanism (Venema et al., 1995).

The 5′ stem-loop structure of 18S is highly conserved in evolution as, indeed, are the ribosomal proteins that associate with this region (Alksne et al., 1993). The sub1 and sub3 mutations alone allowed 18S rRNA synthesis at, or close to, wild-type levels, but the mutant rRNAs were unable to support growth, indicating that the structure of this region is crucial to ribosome function. The sub3 mutation changes a G-C to G-U base pair; the equivalent mutation in the *E. coli* 16S rRNA shows a dominant cold-sensitive phenotype (Dammel & Noller, 1993) but no negative effect of expression of sub3, or other mutant RNAs, was observed in a strain also expressing the wild-type pre-rRNA at 23 °C. The sub2, sub4, and sub5 mutations reduce accumulation of the 18S rRNA. Cleavage at A_1 is not obviously affected by the mutations, as shown by the levels of the 20S pre-rRNA and excised A_0–A_1 fragment, indicating that the mature rRNA is destabilized by the mutations. We speculate that this represents the activity of a quality control system; in the absence of correct ribosomal protein assembly on the 5′ region of the 18S rRNA, the mature rRNA may be degraded by the Rat1p and/or Xrn1p 5′ → 3′ exonucleases, which are also responsible for degradation of the excised A_0–A_1 fragment (Petfalski et al., 1998). In *E. coli* a point mutation that destabilized the pseudoknot interaction resulted in ready loss of ribosomal proteins and destabilization the 30S ribosomal subunits (Poot et al., 1996).

Some correlation was observed between the effects of the mutations on 18S rRNA stability and their effects on positioning of site A_1. One possibility is that the correct assembly of the rRNA and ribosomal proteins is required both to allow specification of the site of cleavage and to prevent degradation of the rRNA. Three ribosomal proteins have been identified that bind to this region of the 18S rRNA, Rps4p (*SUP44*), Rps13p (*SUP46*), and Rps28p; the homologs of *E. coli* S5, S4, and S12, respectively (All-Robyn et al., 1990; Vincent & Liebman, 1992; Alksne & Warner, 1993; Alksne et al., 1993). Strains carrying translational suppressor alleles of each of these proteins (generously provided by S. Liebman) were tested for expression of the A1+2U pre-rRNA. However, no clear effects on the formation of the two 18S rRNA 5′ ends were observed (data not shown). The suppressor mutations presumably result

FIGURE 6. Positioning of the 5′ end of 18S rRNA. **A:** Cartoon showing the locations of the primer extension stops detected in the various pre-rRNAs. For constructs in which heterogeneous processing was observed, the relative efficiency is approximately indicated by the size of the arrows. The sub2, sub4, and sub5 mutations are drawn superimposed. The data for the A1 Xho and A1 Sph mutations are taken from Venema et al., 1995. **B:** Summary of the effects of mutations in the 18S rRNA stem-loop structure and 5′ flanking sequence on the positioning of the site of A_1 cleavage.

in subtle changes in the structure of the proteins, and it may be that more drastic alterations would have clearer effects.

Recognition of A_1, and indeed all pre-rRNA processing sites, is likely to have two components—the signals that identify the site as being a pre-rRNA cleavage site and the signals that specify the nucleotide to be used as the site of cleavage. These signals were apparently partially separated by the mutations. The relative effects of the different mutations on processing efficiency were quite different from their effects on the specificity of the site of A_1 cleavage. None of the single sub1–sub5 mutations markedly affected pre-rRNA processing. However, sub1, sub3, and, particularly, sub2 each showed a synergistic inhibition of processing when combined with the insertion of 2U in the 3′ flanking sequence at A_1, between the stem structure and the site of cleavage (sub1*–sub3*). The 2U insertion alone did not affect the efficiency of processing. In each case the 33S pre-rRNA was accumulated together with a 22S RNA that extends from site A_0 to site A_3, showing that cleavage at sites A_1 and A_2 was specifically inhibited. Each of these mutations is predicted to destabilize the stem, in contrast to sub4 and sub5. The sub2 mutation, which is predicted to be the most destabilizing, had the greatest effect on processing when combined with the alteration of the 3′ flanking sequence at A_1. Loss of cleavage at site A_2 is very likely to be a direct consequence of the inhibition of A_1 cleavage—no mutation *in cis* or *in trans* has been identified that allows A_2 cleavage in the absence of prior cleavage at A_1.

There are interesting parallels between the signals in the pre-rRNA that specify the positions of sites A_1 and A_2. In both cases there appear to be two signals, a sequence at the site of cleavage that is directly recognized and a signal further 3′ that can be used to position the site of cleavage by some spacing mechanism (Venema et al., 1995; Allmang et al., 1996). In the case of A_2, the spacing mechanism appears to recognize a weak stem-loop structure that lies 5 nt 3′ to the site of cleavage (Allmang et al., 1996). We speculate that these similarities are a consequence of cleavage at both sites by the same endonuclease.

MATERIALS AND METHOD

Strains and media

Standard *S. cerevisiae* techniques were employed. The yeast strain NOY 504: *MATa*; *rpa12::LEU2*; *leu2-3, 112 ura3-1*; *trp1-1*; *his3-11*; *ade2-101*; *CAN1-100* (Nogi et al., 1993; generously provided by M. Nomura, University of California, Irvine) was used for all the experiments. Yeast strains were grown in minimal medium containing 2% galactose, 0.67% yeast nitrogen base plus nutrients and supplemented with the required amino acids (Sherman et al., 1986).

Plasmids and constructs used in this study

A plasmid containing the entire yeast rDNA repeat fused to an inducible *GAL7* promoter (pGAL::rDNA) was used as a wild-type control (Sherman et al., 1986; Nogi et al., 1991; Henry et al., 1994). Synthesis of ribosomes derived from this plasmid was monitored by hybridization to small oligonucleotide tags present within the 18S, 5.8S, and 25S rRNA sequences. A YEplac 195 plasmid (2μ, URA3) that does not contain an rDNA unit was used as a negative control (Gietz & Sugino, 1988). Mutations were generated via a two-step PCR approach. Two oligonucleotide primers, a 3′ mutagenic primer and a 5′ primer complementary to a sequence in the 5′ ETS were used. Using the tagged rDNA plasmid as template, a 200-nt fragment was amplified. This was then gel purified, digested with *Nde*I + *Hin*dIII and subcloned into vector pTH66, which contains the sequences of the 5′ ETS and 18S rRNA up to the *Bam*HI site in the tag. The 200 nt were sequenced to confirm the mutation and to eliminate any additional errors induced during amplification. Correct clones, and the wild-type pGAL::rDNA, were digested with *Bam*HI and the fragments exchanged (Venema et al., 1995).

Analysis of pre-rRNA processing

The plasmids containing the mutations and the positive and negative controls were transformed into the yeast strain NOY504 using the Li-acetate method as described (Gietz et al., 1992). Cells were grown at 23 °C to mid-log phase in minimal medium containing galactose, diluted to an OD_{600} of 0.1 and shifted to 37 °C for 6 h (Henry et al., 1994). Cells were harvested by centrifugation at 4 °C, washed with ice-cold water, centrifuged again, and stored at −80 °C. Frozen cell pellets were resuspended in 0.5 mL of 4 M guanidine thiocyanate (GTC) and RNA was extracted as previously described (Sharma et al., 1996). Total RNA was separated on 1.2% Agarose, 6% formaldehyde gels using 4 μg of total RNA per lane as previously described (Tollervey, 1987). The gel was then transferred to a Hybond N^+ membrane (Amersham) with 10× SSPE (1× is 0.18 M NaCl, 10 mM NaH_2PO4, and 1 mM EDTA) as transfer buffer. For the high resolution Northern shown as Figure 5, RNA was separated on a 40-cm × 20-cm × 1-mm gel containing 6% polyacrylamide, 7 M urea, and 1× TBE. The gel was run at 25 W to maintain an elevated temperature and transferred by electroblotting. Hybridizations were performed in 6× SSPE, 0.5% SDS and 5× Denhardt's solution (Maniatis et al., 1989). To detect the A_0–A_1 fragment and distinguish the 33S from the 32S pre-rRNA, the filter was hybridized with a riboprobe generated as previously described (Venema et al., 1995). This was performed in 40% formamide, 5× SSPE, 5× Denhardt's, 1% SDS, and 200 μg/mL Herring sperm DNA.

Oligonucleotides used as hybridization probes had the following sequences:

001 (27SA-2): CCAGTTACGAAAATTCTTG
002 (20S-2): GCTCTTTGCTCTTGCC
003 (27SA-3): TGTTACCTCTGGGCCC
008 (18S+34): CATGGCTTAATCTTTGAGAC
009 (18S-αTAG): CGAGGATCCAGGCTTT
013 (rna2.1): GGCCAGCAATTTCAAGTTA
016 (5.8S-Ftag): DGDDUDCUGGCGDdGdC
042 (25S Tag 1): ACTCGAGAGCTTCAGTACC.

Oligo 016 is largely composed of 2′-methyl RNA; D is diaminopurine.

Primer extensions

Primer-extension analysis was performed as previously described (Beltrame & Tollervey, 1992) using 4 μg of RNA. A sequencing ladder was run in parallel using the same oligonucleotide primer that had been 5′ phosphorylated with unlabeled ATP.

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