RNA (1996), 2:51–62. Cambridge University Press. Printed in the USA.

Recognition of cleavage site A_2 in the yeast pre-rRNA

CHRISTINE ALLMANG, YVES HENRY, HEATHER WOOD, JOHN PATRICK MORRISSEY, ELISABETH PETFALSKI, and DAVID TOLLERVEY
European Molecular Biology Laboratory, Gene Expression Programme, Postfach 10.2209, 69012 Heidelberg, Germany

ABSTRACT

Processing of the yeast pre-rRNA at site A_2 in internal transcribed spacer 1 (ITS1) has been shown to require several small nucleolar ribonucleoprotein particles (snoRNPs) as *trans*-acting factors. Here we report a detailed mutational analysis of the *cis*-acting signals required to specify the site of A_2 cleavage. Initial mutations established that the signals required for accurate cleavage of site A_2 lie in the 3′-flanking sequence; deletion or substitution of nucleotides in this region strongly inhibits processing, and residual cleavage is inaccurate at the nucleotide level. In contrast, the deletion of the 5′-flanking nucleotides has no detectable effect on processing. An evolutionarily conserved sequence, ACAC, is located at the site of cleavage. Substitution of the 3′ AC leads to heterogeneous cleavage, with activation of cleavage at an upstream ACAC sequence. In all mutants that retain an ACAC element, a site of cleavage is detected immediately 5′ to this sequence, showing that this element is recognized. An ACAC sequence is, however, not essential for accurate cleavage of site A_2. An additional signal is also present 3′ to A_2, in a region that has the potential to form a stem-loop structure that is evolutionarily conserved, but of low stability. As has been found for site A_1 (the 5′ end of the yeast 18S rRNA), the identification of the site of processing at A_2 relies on multiple recognition elements.

Keywords: ribosome; RNA processing; *Saccharomyces cerevisiae*; snoRNA; snoRNP

INTRODUCTION

The synthesis of eukaryotic ribosomes is a complex process that takes place in a specialized nuclear compartment, the nucleolus. This process includes transcription, processing and modification of the pre-ribosomal RNA (pre-rRNA), as well as assembly with ribosomal proteins to give the functional ribosomal subunits. In most eukaryotes, the nuclear ribosomal DNA (rDNA) genes have the same arrangement: the small subunit rRNA gene (18S rRNA in yeast) and two of the large subunit rRNA genes (5.8S and 25S rRNA in yeast) are cotranscribed by RNA polymerase I into a large precursor RNA (35S pre-rRNA in yeast). The pre-rRNA then undergoes sequential processing to generate the mature rRNAs (see Fig. 1A,B). This pre-rRNA processing has been studied in a number of eukaryotic systems, but is best understood in the budding yeast *Saccharomyces cerevisiae* (for a recent review see Venema & Tollervey, 1995).

Reprint requests to: David Tollervey, European Molecular Biology Laboratory, Gene Expression Programme, Postfach 10.2209, 69012 Heidelberg, Germany; e-mail: tollervey@embl-heidelberg.de.

The rRNA-coding regions are flanked by 5′ and 3′ external transcribed spacers (5′ and 3′ ETS) and separated by internal transcribed spacers 1 and 2 (ITS1 and ITS2) (see Fig. 1B). The yeast pre-rRNA is not simply cleaved at the ends of mature rRNA sequences, but undergoes cleavage and exonucleolytic digestion of the transcribed spacer regions (reviewed by Warner, 1989; Raué & Planta, 1991; Venema & Tollervey, 1995). The earliest well-characterized cleavage in the 35S pre-rRNA takes place within the 5′ ETS at site A_0, generating the 33S pre-rRNA (Hughes & Ares, 1991). This is followed by cleavage at site A_1, the 5′ end of 18S rRNA, generating the 32S pre-rRNA. Processing at site A_2 in ITS1 then splits the molecule into 5′ and 3′ fragments, the 20S and $27SA_2$ pre-rRNAs, which are destined to form the small and large ribosomal subunit rRNAs, respectively (Udem & Warner, 1972; Veldman et al., 1980, 1981). The 20S precursor is converted into the mature 18S rRNA by cleavage at its 3′ end (site D) in the cytoplasm (Udem & Warner, 1973; Veldman et al., 1980). The $27SA_2$ pre-rRNA is processed by two alternative pathways, giving rise to the two forms of 5.8S rRNA; the major, short form is designated $5.8S_S$, and the minor, long form, $5.8S_L$ (see Fig. 1B). Formation

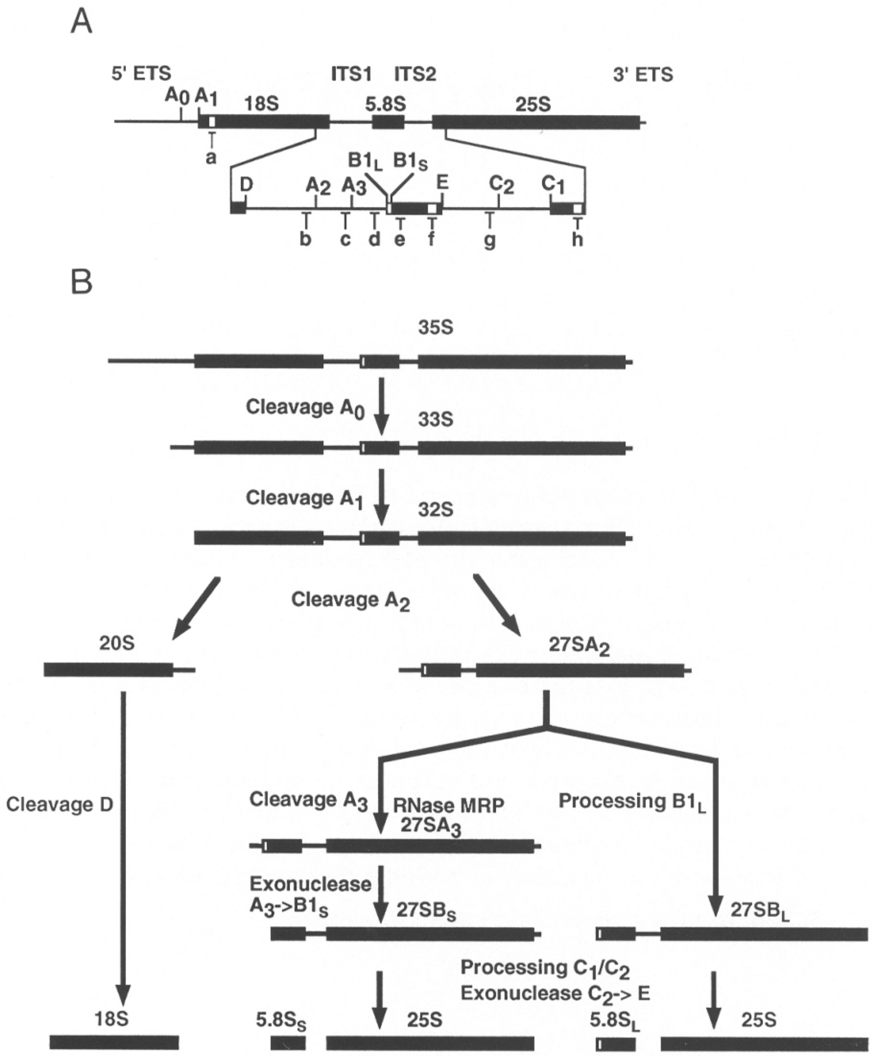

FIGURE 1. Structure and processing pathway of pre-rRNA in *Saccharomyces cerevisiae*. **A:** Structure of the 35S pre-rRNA and position of oligonucleotides. Bars represent the mature rRNA sequences and lines the transcribed spacers. Regions to which oligonucleotides a–h hybridize are indicated. Open boxes within the 18S, 5.8S, and 25S rRNA regions indicate the location of the tags. **B:** Processing pathway of pre-rRNA. The pathway is described in the Introduction (for more detailed descriptions see Lafontaine & Tollervey, 1995; Venema & Tollervey, 1995).

of $5.8S_S$, but not $5.8S_L$, requires cleavage in ITS1 at site A_3.

A large number of *trans*-acting factors have been shown to be required for yeast pre-rRNA processing. Many of these are small nucleolar RNAs (snoRNAs), which are associated with proteins to form ribonucleoprotein particles (snoRNPs). Four snoRNAs (U3, U14, snR10, snR30) and three snoRNP proteins (Nop1p, Sof1p, Gar1p) have been shown to play roles in the synthesis of 18S rRNA (Tollervey, 1987; Li et al., 1990; Hughes & Ares, 1991; Tollervey et al., 1991; Girard et al., 1992; Jansen et al., 1993; Morrissey & Tollervey, 1993; reviewed in Lafontaine & Tollervey, 1995). Genetic depletion of any of these components results in a loss of 18S rRNA synthesis, whereas synthesis of 25S and 5.8S rRNAs is unaffected; in each case, cleavage at both sites A_1 and A_2 is inhibited. A different snoRNP complex, RNase MRP, is responsible for cleavage at site A_3 (Shuai & Warner, 1991; Lindahl et al., 1992; Schmitt & Clayton, 1993; Chu et al., 1994; Lygerou et al., 1994).

Recently developed techniques have allowed the analysis of the roles of *cis*-acting sequences in the pre-rRNA and their interaction with *trans*-acting factors. A number of *cis*-acting elements required for 18S synthesis have been identified in the 5′ ETS, including the binding site for the U3 snoRNA (Beltrame & Tollervey, 1992, 1995; reviewed in Venema & Tollervey, 1995). Detailed analysis of the sequences surrounding site A_1, the 5′ end of the 18S rRNA, identified two distinct recognition signals that define the position of the endonucleolytic cleavage at this site (Venema et al., 1995). One mechanism involves recognition of a conserved sequence upstream of A_1 in the 5′ ETS, whereas the second mechanism positions the A_1 cleavage at a fixed distance from structures within 18S rRNA.

Within ITS1, structural elements required in *cis* for processing are evolutionarily conserved because the ITS1 sequence can be functionally replaced in the *S. cerevisiae* pre-rRNA by the corresponding regions from the yeast species *Torulaspora delbrueckii*, *Kluyveromyces lactis*, and *Hansenula wingei* (Van Nues et al., 1994). Each of these sequences can be drawn in secondary structures resembling that proposed by Yeh et al. (1990) for *S. cerevisiae* ITS1. Despite the low overall conservation of primary sequence, phylogenetic comparison revealed the presence of conserved sequences in proximity to both the A_2 and A_3 cleavage sites (Van Nues et al., 1994). In particular, a conserved 4-nt sequence, ACAC, located immediately 3′ to site A_2, could constitute a *cis*-acting element (see Fig. 2). Deletion of this sequence and other sequences surrounding A_2 reduces the formation of the 20S and 27SA_2 pre-rRNAs (Lindahl et al., 1994; Van Nues et al., 1994).

Previous analyses have established that site A_2 can be accurately cleaved in pre-rRNAs carrying large deletions of either the 5′ or 3′ regions of ITS1 (Henry et al., 1994), demonstrating that recognition does not depend on higher-order structure or long-range interactions. As an initial step toward understanding the mechanism by which site A_2 is recognized and cleaved, we have undertaken a detailed analysis of the effects of mutations at site A_2 on the accuracy and efficiency of cleavage.

RESULTS

Experimental system

To test the effects of mutations at A_2 on ribosome synthesis, we have used a system allowing the conditional expression of mutant and wild-type pre-rRNA (Henry et al., 1994). Mutant pre-rRNAs were expressed from the pGAL::rDNA plasmid containing the entire pre-rRNA-coding region placed between an inducible RNA polymerase II *GAL7* promoter and terminator sequences (Nogi et al., 1991). Neutral tags are present in the 18S, 5.8S, and 25S rRNA coding sequences to allow the identification of the mature rRNAs synthesized from the plasmid-borne rDNA. These plasmids were

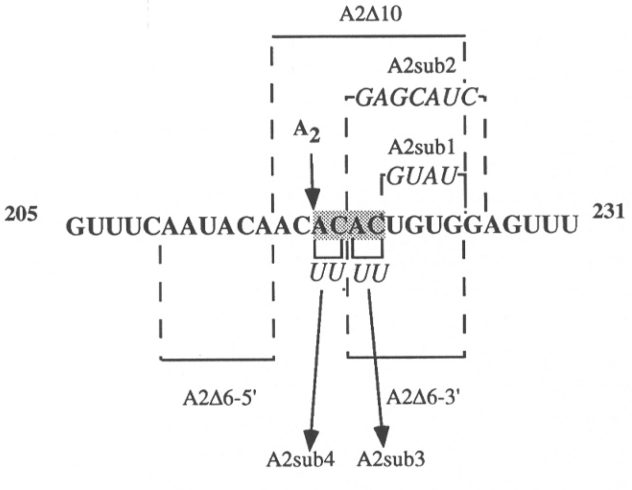

FIGURE 2. Location of the mutations introduced at sites A_2. Deletion and substitution mutations around A_2 are indicated on the primary sequence of ITS1. Nucleotides are numbered according to their positions within ITS1. The evolutionarily conserved nt ${}_{218}$ACAC${}_{221}$ are boxed.

expressed in an *rpa12* strain, NOY504 (Nogi et al., 1993), which is temperature sensitive for the transcription of the chromosomal rDNA by RNA polymerase I. After 6 h growth at 37 °C, the nonpermissive temperature, >90% of the transcription of the endogenous rDNA is lost and cells become dependent on the expression of the plasmid rDNA (Henry et al., 1994). Using this system, mutations in the pre-rRNA were tested for their effects on pre-rRNA processing by primer extension and northern hybridization.

Analysis of effects of deletions around A_2

Previous analyses have shown that deletion of nt 43–188 in the 5′ region of ITS1 (mutant ITS1Δ5′) does not affect processing at A_2, whereas deletion of nt 236–330 in the 3′ region of ITS1 (mutant ITS1Δ3′) somewhat reduces the efficiency of cleavage at A_2 but does not affect the accuracy (Henry et al., 1994). It was, therefore, anticipated that the signals necessary for specifying the site A_2 would be located close to the site of cleavage. In order to understand the importance of nucleotides surrounding the A_2 processing site and identify the *cis*-acting sequences necessary for accurate A_2 cleavage, an initial series of deletions was constructed. It was unclear whether the primary or the secondary structure was recognized, or whether the sequence across the site of cleavage or the flanking nucleotides was required. As an initial experiment, the deletion of 10 nt across the cleavage site (A2Δ10) and deletions of the 5′ and 3′ flanking nucleotides (A2Δ6-5′ and A2Δ6-3′), were tested. The positions of these deletions are shown in Figure 2.

The mutations were functionally analyzed by expression in the pGAL::rDNA construct. Surprisingly, all of the deletion mutants support growth of an *rpa12* strain on galactose plates at the nonpermissive temperature, showing that they do not prevent the synthesis of functional ribosomes. Strains complemented by the A2Δ6-3′ and A2Δ10 mutants have, however, doubling times of 11.5 h, compared to 6.5 h for the strain complemented by the wild-type pGAL::rDNA (data not shown).

The effects of these deletions on the accuracy and efficiency of A_2 cleavage were analyzed by primer extension using oligonucleotide d (Fig. 3A; summarized in Fig. 4). The level of primer extension products terminating at site A_2 reflects the abundance of the 27SA_2 pre-rRNA. Low levels of 27SA_2 pre-rRNA, due to residual transcription of the chromosomal rDNA, were detected in the −rDNA control. These levels do not interfere with the interpretation of the effects of the mutations. The A2Δ6-5′ mutation did not detectably affect A_2 cleavage (Fig. 3A, lane 5) as compared to the strain expressing the wild-type ITS1 sequence (Fig. 3A, lane 2). In contrast, the A2Δ6-3′ and A2Δ10 mutations strongly inhibited overall cleavage at A_2 (Fig. 3A, lanes 3 and 4). In the A2Δ6-3′ mutant, residual cleavage was also het-

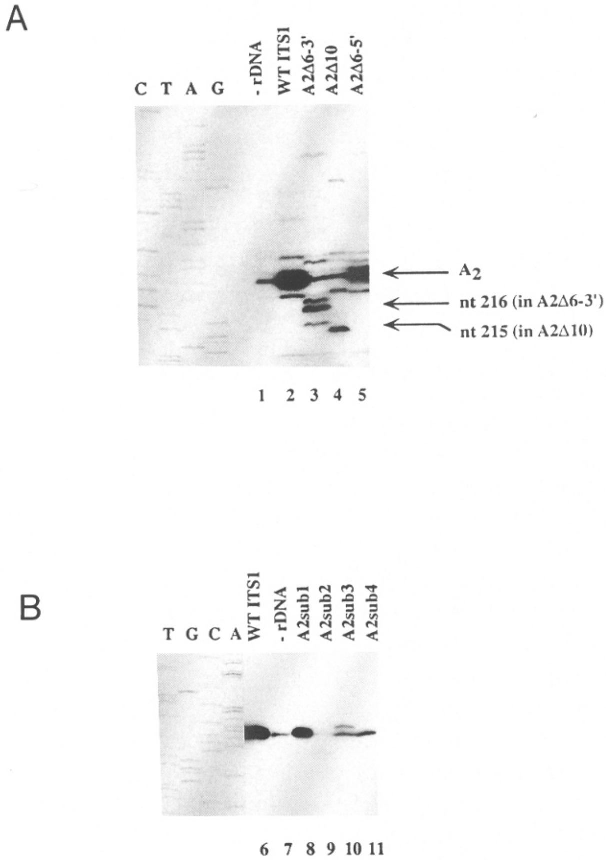

FIGURE 3. Primer extension analysis of A_2 mutations. RNA was extracted from NOY504 transformants expressing mutant pre-rRNAs following growth for 6 h at 37 °C. **A,B:** Primer extension was performed using oligonucleotide d, which hybridizes in the 3′ region of ITS1. A dideoxynucleotide sequence generated with the same oligonucleotide was run in parallel. Lanes 1 and 7, RNA extracted from a strain carrying a plasmid without rDNA sequences; lanes 2 and 6, RNA extracted from a strain carrying a plasmid with the wild-type rDNA sequence; lane 3, A2Δ6-3′ mutation; lane 4, A2Δ10 mutation; lane 5, A2Δ6-5′ mutation; lane 8, A2sub1 mutation; lane 9, A2sub2 mutation; lane 10, A2sub3 mutation; lane 11, A2sub4 mutation. The position of the wild-type A_2 cleavage site is indicated by an arrow, as is the major site of residual cleavage in mutants A2Δ6-3′ and A2Δ10.

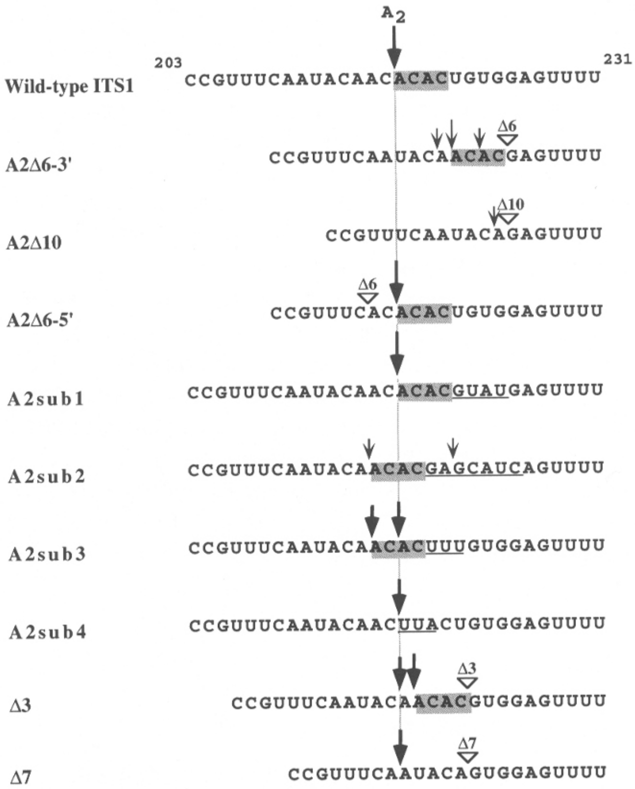

FIGURE 4. Summary of the effects of A_2 mutations on cleavage at site A_2. Primer extension results for the A_2 mutations are summarized and compared to the results of Lindahl et al. (1994) for mutants Δ3 and Δ7. Sequences are aligned in respect to the 3′ sequences. Cleavage at site A_2 is represented by an arrow; the approximate efficiency of the cleavage, as reflected by the intensity of the primer extension stop (see Fig. 3), is represented by the size of the arrow. Large arrow, strong cleavage. Small arrow, weak cleavage. Sites of deletions are represented by a triangle and nucleotide substitutions are underlined. ACAC nucleotides are boxed. Position of the wild-type cleavage is marked by a vertical bar. For mutants Δ3 and Δ7, the intensities of the primer extension stops are taken from the results of Lindahl et al. (1994).

erogeneous; the major primer extension stop was located at A_{216}, with minor stops at A_{215} and at A_{218} (the wild-type position with respect to the 5′ flanking sequence) (Fig. 3A, lane 3; Fig. 4). In the A2Δ10 mutant, residual cleavage was at position A_{215} (Fig. 3A, lane 4; Fig. 4). It is notable that the deletion of 6 nt 3′ to A_2 inhibited cleavage to the same extent as the larger deletion across A_2.

Primer extension through sites A_0 and A_1 revealed that processing at these sites was not altered by deletions at A_2 (data not shown). In particular, no accumulation of the 33S pre-rRNA, indicated by the level of the A_0 primer extension stop, was observed. The 33S pre-rRNA is processed by cleavage at site A_1 and this result therefore indicates that the kinetics of processing at site A_1 were not affected by the A_2 mutations.

The effects of the deletions at A_2 on pre-rRNA processing were also examined by Northern hybridization. The steady-state levels of the mature rRNAs were determined using oligonucleotides complementary to the tags present in the 25S (Fig. 5A-IV) and 18S rRNA (Fig. 5A-VII). In the A2Δ6-5′ mutant, the 18S and 25S rRNAs levels were comparable to that of the wild-type control. Some reduction in 18S and 25S rRNA levels were, however, observed for A2Δ6-3′ and A2Δ10 (Fig. 5A-IV and 5A-VII, lanes 5 and 4). Transformants carrying a plasmid without the rDNA unit did not show any background signal (Fig. 5A-IV and 5A-VII, lane 2), indicating that only tagged, plasmid-derived 18S and 25S rRNA were detected. The A2Δ6-5′ mutation did not detectably affect pre-rRNA processing, and normal levels of all the major intermediates were detected (Fig. 5A). The A2Δ6-3′ and A2Δ10 mutations resulted in some accumulation of 32S (Fig. 5A-I) and a strong decrease in the level of $27SA_2$ (Fig. 5A-II and 5A-III) as

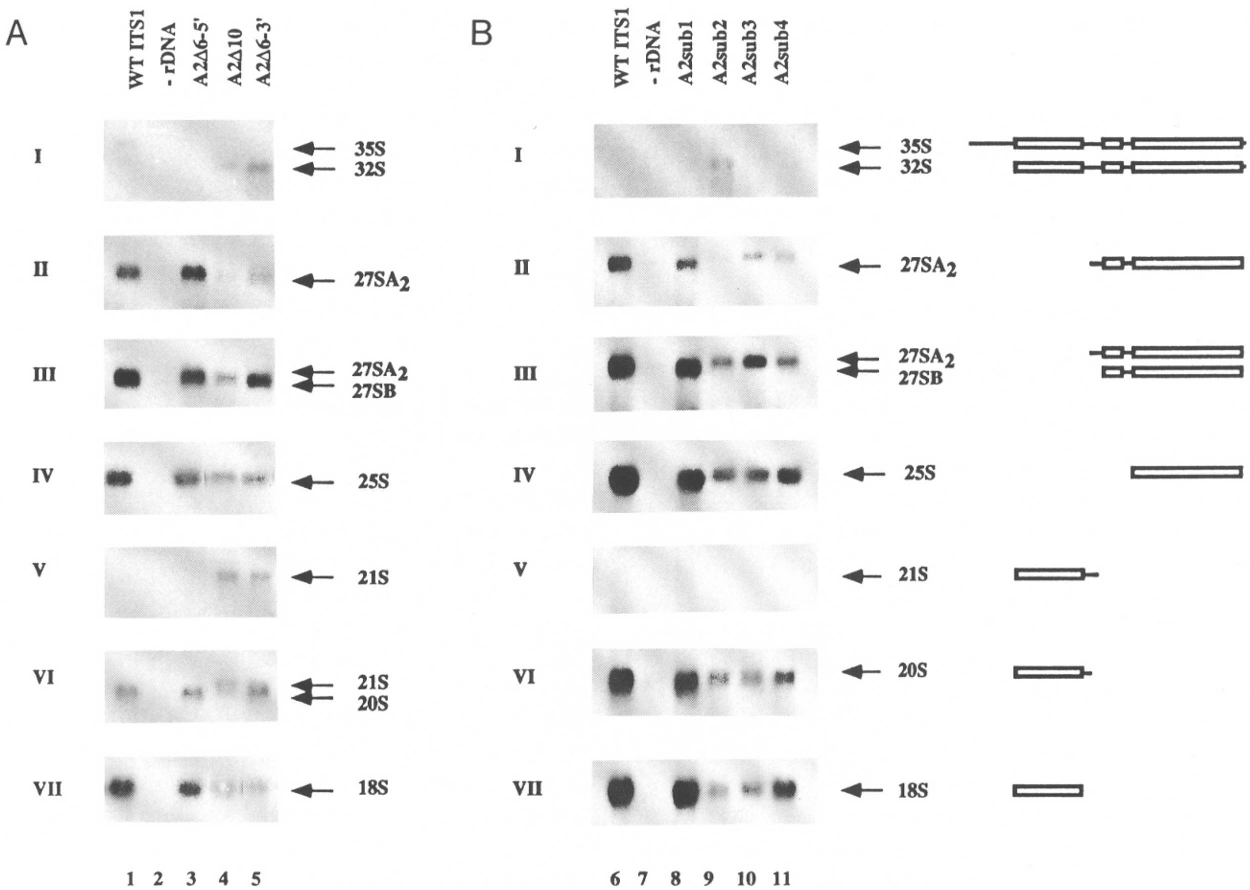

FIGURE 5. Northern analysis of high molecular weight RNA from strains expressing A_2 mutations. **A,B:** RNA was extracted from NOY504 transformants expressing mutant pre-rRNAs following growth for 6 h at 37 °C. The RNA was separated on a 1.2% agarose gel containing formaldehyde and transferred to Hybond N^+ membranes (Amersham) for northern hybridization. Lanes 1 and 6, strain carrying a plasmid with the wild-type rDNA sequence; lanes 2 and 7, strain carrying a plasmid without rDNA sequences; lane 3, A2Δ6-5′ mutation; lane 4, A2Δ10 mutation; lane 5, A2Δ6-3′ mutation; lane 8, A2sub1 mutation; lane 9, A2sub2 mutation; lane 10, A2sub3 mutation; lane 11, A2sub4 mutation. Hybridization with: I, oligonucleotide c, hybridizing in ITS1 between sites A_2 and A_3; II, oligonucleotide d, hybridizing in the 3′ region of ITS1; III, oligonucleotide g, hybridizing in ITS2; IV, oligonucleotide h, hybridizing to the tag in 25S rRNA; V, oligonucleotide c, hybridizing in ITS1 between sites A_2 and A_3; VI, oligonucleotide b, hybridizing in ITS1 5′ to A_2; VII, oligonucleotide a, hybridizing to the tag in 18S rRNA. For simplicity, we only show selected regions of each northern; together these show all the pre-rRNA and rRNA species that we have detected. The position of the various pre-rRNA and rRNA species is indicated on the right and the RNAs are schematically represented; boxes represent the mature rRNA sequences and thin lines the transcribed spacers.

compared to the wild-type control, indicating a delay in A_2 processing. Consistent with this, a 3′ extended form of the 20S pre-rRNA was detected in these mutants (Fig. 5A-V). This aberrant intermediate, the 21S pre-rRNA, extends to site A_3, because it can be detected by oligonucleotide c (hybridizing between sites A_2 and A_3) (Fig. 5A-V) and oligonucleotide b (hybridizing 5′ to A_2 in ITS1) (Fig. 5A-VI), but not by oligonucleotide d (located 3′ to the A_3 cleavage site) (data not shown). A concomitant decrease in 20S pre-rRNA synthesis in the A2Δ6-3′ and A2Δ10 mutations is detected with oligonucleotide b (Fig. 5A-VI). This shows that A_3 cleavage is occurring prior to cleavage at A_2, clearly indicating that A_2 processing is inhibited in the A2Δ6-3′ and A2Δ10 mutants. The level of 27SB pre-rRNA is clearly reduced only for mutant A2Δ10 (Fig. 5A-III). Consistent with the results of primer extension, no accumulation of the 35S or 33S pre-rRNAs was seen in the A_2 mutants (Fig. 5A-I), indicating that processing at sites A_0 and A_1 is unaffected. The 33S pre-rRNA is not readily detected by northern hybridization in wild-type cells, but strongly accumulates if cleavage at A_1 is inhibited while processing continues at A_0 (Lafontaine et al., 1995; Venema et al., 1995).

Altogether these results indicate that sequences located immediately 5′ to A_2 are not required for A_2 cleavage because a 6-nt deletion in this region (A2Δ6-5′) has no effect on A_2 cleavage or pre-rRNA processing. Sequences located 3′ to A_2 are, however, required both for the efficiency and accuracy of A_2 cleavage. The importance of the 3′ flanking nucleotides was therefore investigated in more detail.

Detailed analysis of *cis*-acting sequences required for A_2 cleavage

Four overlapping substitution mutants were analyzed (see Fig. 2); the 6 nt in the 3′ flanking region shown to be important by the deletion mutations were mutated in the A2sub1 and A2sub2 mutants (substituting 4 and 7 nt, respectively). The role of the evolutionarily conserved $_{218}$ACAC$_{221}$ sequence was tested in the A2sub3 and A2sub4 mutants. When tested in the pGAL::rDNA construct, all the substitution mutations supported growth at nonpermissive temperature in an *rpa12* strain, showing that they allow the synthesis of functional ribosomes.

A_2 cleavage was analyzed by primer extension using primer d (Fig. 3B; summarized in Fig. 4). The A2sub1 and A2sub4 mutations did not clearly affect the accuracy of A_2 cleavage (Fig. 3B, lanes 8 and 11). In contrast, the 7-nt substitution, A2sub2 (Fig. 3B, lane 9), completely abolished cleavage at the wild-type position (A_{218}); the intensity of the primer extension stop at this position was reduced to that of the −rDNA control (Fig. 3B, lane 7). On longer exposures, two minor stops were detected, at positions 216 and 222 (Fig. 3B, lane 9; Fig. 4). The A2sub2 mutation mainly differs from the 4-nt substitution, A2sub1, by the substitution of two additional nucleotides located at position +3 and +4 from A_2. Substitution of these two nucleotides in the A2sub3 mutant also resulted in heterogeneous cleavage. Two primer extension stops were detected, one at the position of the wild-type A_2 cleavage and one located 2 nt 5′ to A_2, at A_{216} (Fig. 3B, lane 10; Fig. 4).

Northern hybridization with probes against the tags in the 25S (Fig. 5B-IV) and 18S (Fig. 5B-VII) rRNAs showed that the A2sub2, A2sub3, and A2sub4 mutations each reduced the levels of both 25S and 18S rRNA. Stronger effects on 18S rRNA synthesis were observed for A2sub2 and A2sub3 (Fig. 5B-VII, lanes 9 and 10). The pre-rRNA processing pathway was more strongly affected in the A2sub2 mutant than in the other mutants (Fig. 5B). The A2sub2 mutant showed accumulation of the 32S pre-rRNA (Fig. 5B-I, lane 9) and a strong decrease in the level of the 27SA$_2$ pre-rRNA, indicating a delay in A_2 processing; the levels of the 27SB and 20S pre-rRNAs were also reduced (Fig. 5B-II, 5B-III, and 5B-VI). In the other substitution mutants, the level of 27SA$_2$ was decreased to a lesser extent. A slight decrease in 20S pre-rRNA was also observed for A2sub3 and A2sub4 (Fig. 5B-VI, lanes 10 and 11). The decrease in the levels of the 20S pre-rRNA in the A2sub2, A2sub3, and A2sub4 mutants correlates well with the decrease in the levels of 18S rRNA.

We have previously observed that the 5′ end of the snoRNA snR30 exhibits sequence complementarity to the 3′ flanking sequences shown here to be important for A_2 cleavage (Henry et al., 1994). Mutant forms of snR30, which carried compensatory mutations restoring perfect complementarity to the A2sub1 and A2sub2 mutations, were constructed and expressed. When expressed as the sole form of snR30, these mutations did not interfere with growth or A_2 cleavage of the wild-type pre-rRNA, nor did their expression restore processing of the A2sub1 or A2sub2 mutant pre-rRNAs at sites A_2 or A_3 (see also the accompanying paper, Allmang et al., 1996) (data not shown). It therefore appears unlikely that base pairing with this region of snR30 is the major mechanism for the recognition of site A_2.

Conformational studies of the A_2 substitution mutants

Structural analyses using chemical probing were performed to determine whether any of the A_2 substitution mutations (A2sub1 to A2sub4) lead to the sequestration of the site in regions of secondary structure or lead to changes in the structure of more distant regions of ITS1. In view of the effects of the A_2 substitution mutants on the cleavage of site A_3 (see Allmang et al., 1996), it was particularly important to determine whether the structure of this site was altered. In vitro RNA transcripts, generated from wild-type and mutant plasmids (see the Materials and methods), were used for this study and compared to a full-length 35S transcript. The four bases were each tested at one of their Watson–Crick positions: A(N-1) and C(N-3) with DMS; G(N-1) and U(N-3) with CMCT. The probes and their use have been described (Ehresmann et al., 1987). To estimate the degree of stability of helices, chemical probing was performed in the presence (native conditions) and in the absence (semi-denaturing conditions) of magnesium ions. Incubation controls were run in parallel in order to detect pauses or artifactual stops of reverse transcriptase due to the structure of the template or proton-catalyzed hydrolysis of the RNA chain, frequent at pyrimidine–adenine phosphodiester bonds (Dock-Bregeon & Moras, 1987). Such phenomenon result in a loss of information for the positions affected (shown as nd, not determined, in Fig. 7). Examples of primer extension analysis are shown in Figure 6. The deduced reactivity patterns are given in Figure 7A and B.

The secondary structure of the complete ITS1 region of *S. cerevisiae* was previously determined by Yeh et al. (1990), using the same technique, who presented two possible structures for the region of the A_2 cleavage site (nt 210–250). One of these, containing a hairpin 3′ to A_2 (nt 223–243), was favored by Van Nues et al. (1994) on the basis of phylogenetic conservation. The results obtained for the structure of ITS1 in the 35S in vitro transcript were in good agreement with the model of Van Nues et al. (1994) (Fig. 7A), however, nt 223–243 do not appear to fold into a stable and defined structure. The chemical probing pattern can, nevertheless, be reconciled with the formation of a hairpin in

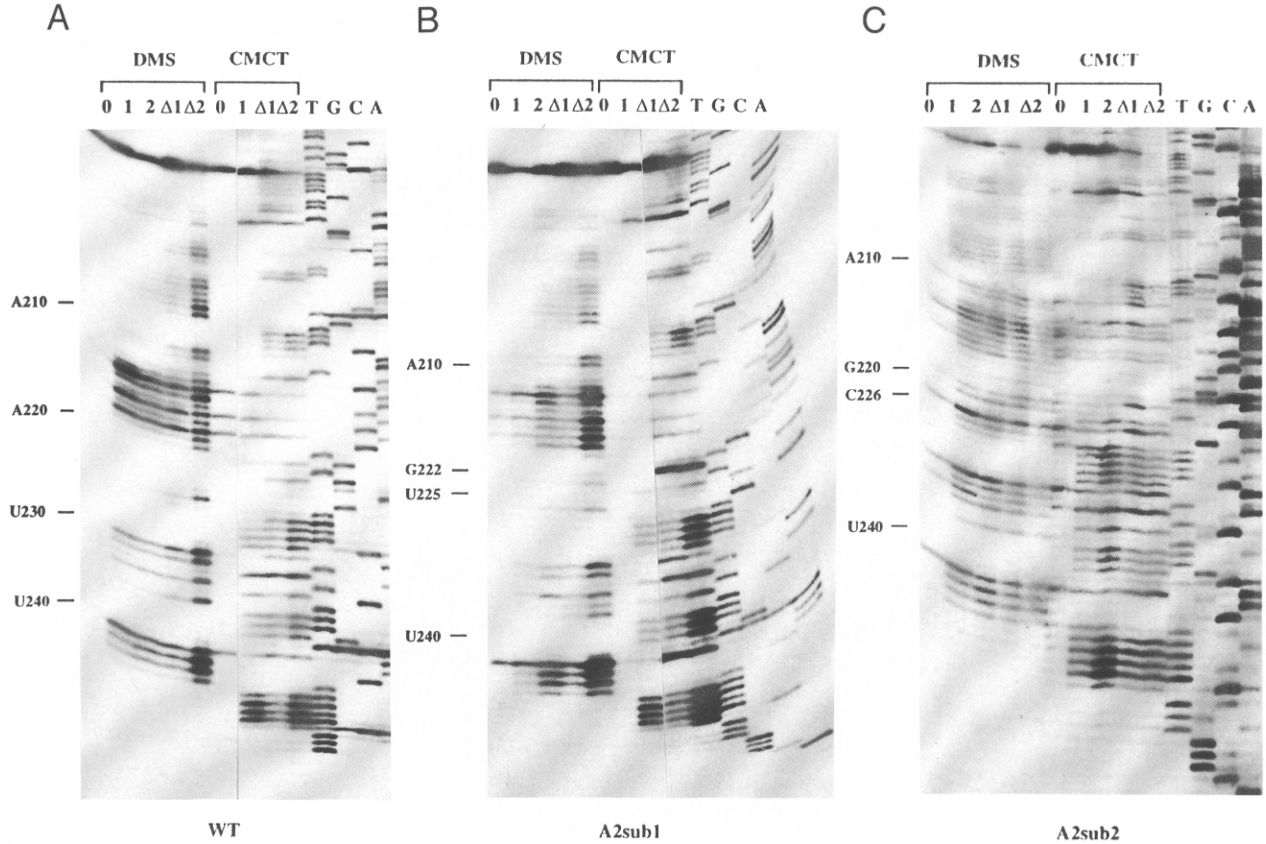

FIGURE 6. Polyacrylamide gel fractionation of cDNA products obtained after primer extension showing the reactivity of the WT, A2sub1, and A2sub2 ITS1 regions after treatment with DMS and CMCT. Chemical reactions were carried out at 37 °C in the appropriate buffer (see the Materials and methods). Native conditions: 0, incubation control; 1, DMS 1 min, CMCT 2 min; 2, DMS 2 min, CMCT 5 min; Δ1, DMS 2 min, CMCT 2 min; Δ2, DMS 4 min, CMCT 5 min. A dideoxynucleotide sequence generated with the same oligonucleotide was run in parallel. Positions of some nucleotides are indicated.

this region that is particularly unstable and requires magnesium for correct folding. The stem showed an unusual pattern of accessibility. Nucleotides 223–228, predicted to lie on one strand, were protected from chemical modifications, with the exception of A_{227}, which was reactive under native conditions. In contrast, nucleotides on the other strand (238–243) were more accessible to modification, they were weakly reactive under native conditions and reactive under semi-denaturing conditions. Similar patterns of chemical reactivity have been reported previously (Philippe et al., 1990; Gilmer et al., 1993). The composition of the stem, consisting largely of G-U base pairs, allows several potential foldings, but none of these account unambiguously for the observed pattern of reactivity. In the loop (229–237), nt 234–236 were clearly reactive under native conditions, whereas most of the nucleotides displayed a weak reactivity that was increased in the absence of magnesium. This indicates their probable involvement in interactions such as internal structure within the loop or long-range tertiary interactions. This structure may be stabilized in vivo by the binding of *trans*-acting factors. Furthermore, irregular and flexible structures are often required for RNA–protein interactions or RNA function (Nagai, 1992; Mattaj, 1993; Pyle & Green, 1995).

The wild-type ITS1 transcript (WT RNA) extending from the tag in 18S rRNA to the tag located in 25S rRNA (Fig. 1A) showed a pattern of chemical modification very similar to that of the 35S RNA transcript. Differences in reactivity were only observed for loop nt U_{229}–U_{230}, which show a slight decrease in reactivity under native conditions compared to the 35S RNA. The WT RNA can thus be used as a reference. It is noticeable that, for the 35S RNA as well as for the mutant RNAs, several reverse transcriptase stops, attributed to the previously observed phenomenon of pyrimidine–adenine phosphodiester bond hydrolysis (Dock-Bregeon & Moras, 1987), mask the reactivity of some nucleotides in particular in the CA-rich region of the A_2 cleavage site. As a general observation, the changes in reactivity induced by the mutations were exclusively localized in the hairpin immediately 3′ to A_2. We therefore present the reactivity data of the mutants only for nt 213–250 (Fig. 7B). Mutant A2sub1 displayed a very similar reactivity pattern to the WT RNA, with the exception of nt A_{224} and U_{225}, that became slightly reactive under

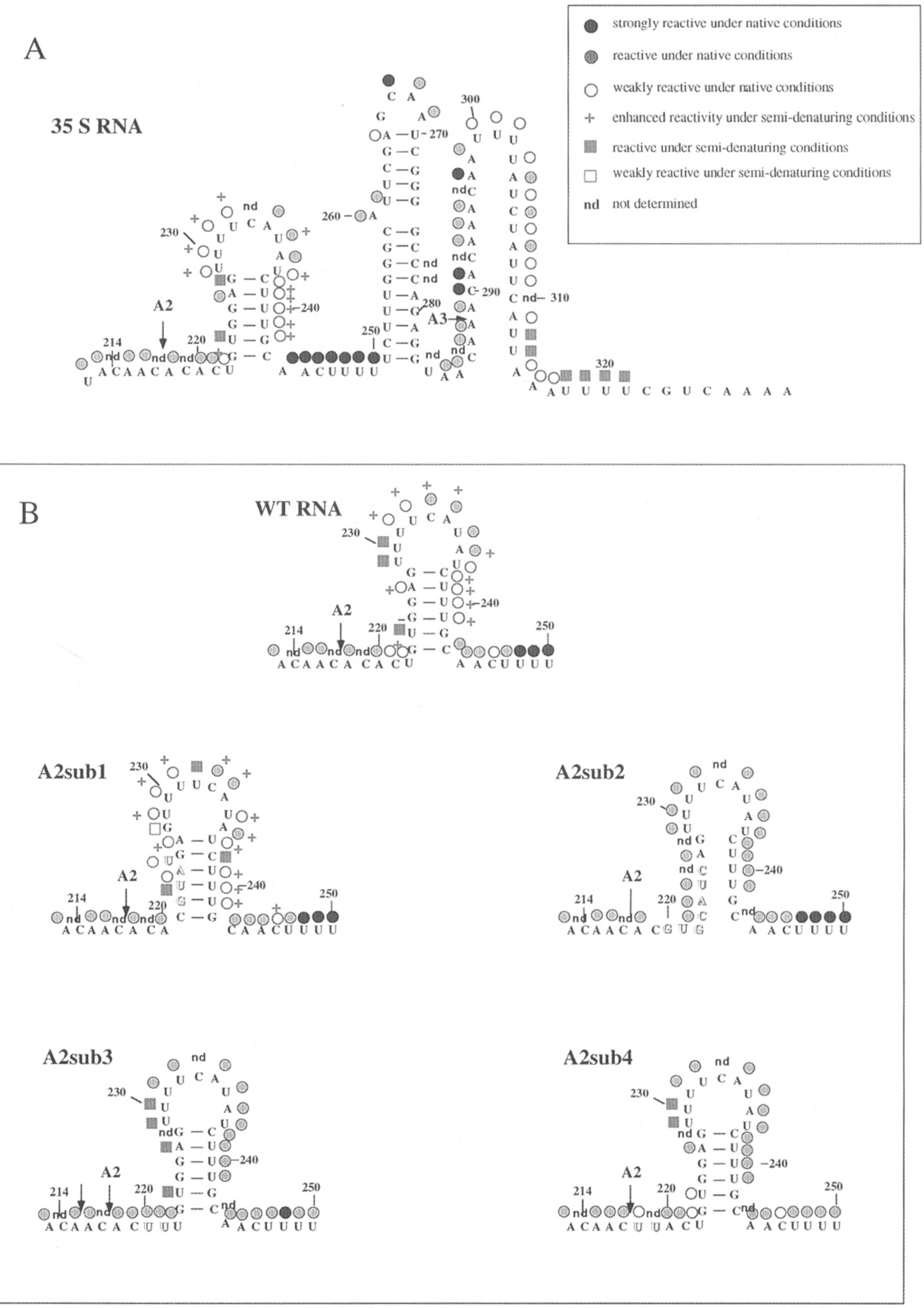

FIGURE 7. Summary of reactivity toward the chemical probes DMS and CMCT of the wild-type and A_2 substitution mutants in the vicinity of the A_2 cleavage site. Results are displayed on the secondary structure model (Van Nues et al., 1994). The degree of reactivity of the Watson–Crick positions toward the chemical probes is indicated by the symbols tabulated at the upper right of the figure. Nucleotides nonreactive under both native and semi-denaturing conditions have no symbol. **A:** Results obtained for nt 212–329 of the 35S RNA transcript. **B:** Results obtained for the *Bam*H I-*Xho* I RNA transcripts of the wild-type and A_2 substitution mutants for nt 213–250.

native conditions, indicating that one of these nucleotides is probably bulged out of the helix. The 4-nt substitution in strand 223–228 apparently does not prevent base pairing with strand 238–243, but may slightly change the base pairing scheme. Because nt C_{239} showed a slight decrease in reactivity, base pairing can be proposed between nt C_{221}–G_{242}; G_{222}–U_{241}; U_{223}–U_{240}; A_{224}–U_{239}; G_{226}–C_{238}; and A_{227}–U_{237} at the base of the stem. In the loop, a decrease in the reactivity of U_{232}, which became reactive only under semi-denaturing conditions, was observed. Overall, similar folding can be proposed for A2sub1 and the WT RNA. For mutant A2sub2, however, the pattern of chemical reactivity was completely altered because nt 213–250 were fully reactive under native conditions, with the exception of nt C_{219}–G_{222}. This indicates that the whole region is single stranded and no hairpin or other clear structure can be formed. The mutations in A2sub3 and A2sub4 are located in a region predicted to be single-stranded, and both displayed the same pattern of chemical reactivity. This pattern was not significantly different from the WT RNA except for nt 238–242, which became more reactive under native conditions, indicating a decrease of stability, whereas nucleotides on the opposite strand (223–228) remained unreactive.

This study revealed that none of the substitutions prevent the formation of the hairpin structure predicted to be located 3′ to A_2 except A2sub2, in which this region becomes single stranded. None of the substitution mutations alter the structure of more distant regions of ITS1. In particular, no alteration of the structure around site A_3 was detected.

DISCUSSION

The aim of this research was to identify and characterize the *cis*-acting elements required for cleavage of the yeast pre-rRNA at site A_2 in ITS1. Two types of *cis*-acting signal are predicted to be present. (1) The signals required for the activation of cleavage at site A_2 are expected to be located both close to site A_2 and at distant locations. They include, for example, the U3-binding site in the 5′ ETS and site A_1, the 5′ end of the mature 18S; mutations at either of these sites (which are located approximately 2 kb from site A_2) have been shown to interfere with A_2 cleavage (Beltrame et al., 1994; Venema et al., 1995). (2) The signals that specify the nucleotides to be cleaved are expected to be located close to the site of cleavage and are the principal subject of the investigation reported here.

Mutations in the sequences around site A_2 were introduced into the pre-rRNA expressed from a plasmid under the control of an RNA polymerase II *GAL7* promoter. In a strain that is temperature sensitive for RNA polymerase I, growth at the nonpermissive temperature is dependent on the synthesis of mature rRNAs from the mutant pre-rRNAs. Deletions of the 5′ and 3′ flanking sequences (A2Δ6-5′ and A2Δ6-3′, respectively) and across A_2 (A2Δ10) were analyzed. The A2Δ6-5′ deletion had no effect on processing at site A_2, whereas the A2Δ6-3′ and A2Δ10 mutants greatly reduced the efficiency of A_2 cleavage and residual cleavage was inaccurate at the nucleotide level (see Fig. 4). This can be correlated with alterations of the pre-rRNA processing pathway of these mutants. Both A2Δ10 and A2Δ6-3′ showed an accumulation of 32S pre-rRNA, a reduction of $27SA_2$ pre-rRNA synthesis, and the formation of a 3′ extended form of 20S pre-rRNA up to the A_3 cleavage site, the 21S RNA. Because the overlapping A2Δ10 and A2Δ6-3′ mutants have similar effects on the efficiency of A_2 cleavage, it can be assumed that the 6 nt (220–225) located 3′ to A_2 are important and involved in accurate A_2 cleavage. Additionally, these results indicate that sequences located 5′ to A_2 are not required for the efficiency or accuracy of this processing reaction.

The importance of sequences in the 3′ flanking region (nt 220–225) was further tested by analyzing four overlapping substitution mutants. These analyses indicate that the evolutionarily conserved $_{218}$ACAC$_{221}$ sequence represents one important recognition element. In fact, this sequence is part of a 6-nt repeat $_{216}$AC.ACAC$_{221}$, where the period represents the site of cleavage. However, whereas the $_{218}$ACAC$_{221}$ sequence 3′ to the site of cleavage is conserved in other yeast ITS1 sequences (Van Nues et al., 1994), the 5′ $_{216}$AC$_{217}$ sequence is not conserved. Substitution of the 3′ $_{220}$AC$_{221}$ nt in the A2sub3, A2sub2, and A2Δ6-3′ mutants resulted in cleavage 5′ to the residual $_{216}$ACAC$_{219}$ sequence. These data would be consistent with the recognition of the ACAC element as defining the 3′ side of the cleavage site. This element is not, however, essential for cleavage, because the A2sub4 mutant was accurately processed in the absence of an ACAC sequence. Nor is it sufficient for efficient cleavage, as shown by the low level of cleavage in the A2sub2 and A2Δ6-3′ mutants, which retain, and apparently recognize, an ACAC sequence. Moreover, in the A2sub3 mutant, substantial cleavage occurred at the position of the wild-type A_2, despite the absence of a 3′ ACAC sequence. Together these data make it clear that other elements are able to correctly position A_2 cleavage independently of the presence of an ACAC element.

Substitution of nt 220–226 in mutant A2sub2 or deletion of nt 220–225 in mutant A2Δ6-3′ resulted in heterogeneous A_2 cleavage; A2sub2 completely abolished A_2 cleavage at the normal position (see Fig. 4). Overall cleavage was also strongly reduced in these mutants, leading to an accumulation of 32S pre-rRNA with concomitant decrease in 20S, $27SA_2$, and 27SB pre-rRNA levels. It is, therefore, likely that the second signal requires recognition of nucleotides including 220–225.

The wild-type cleavage lies between C-A residues, but this is not a requirement for processing. Mutant A2sub4 is cleaved between C-U residues and the upstream cleavage site in A2sub3 is between A-A residues.

Our interpretation of these data is that the A_2 cleavage site can be positioned by recognition of either an ACAC element located immediately 3′ to the site of cleavage or recognition of sequences located further 3′. In the wild-type pre-rRNA, both signals contribute to position cleavage at the same site; in mutants where a discrepancy exists between the position of A_2 specified by these two elements, heterogeneous cleavage results. This model also explains the results recently reported by Lindahl et al. (1994) (see Fig. 4). They observed that a 3-nt deletion of nt 220–222 ($\Delta 3$) leads to heterogeneous cleavage; the major cleavage occurred 5′ to the residual ${}_{216}ACAC_{219}$ sequence, whereas minor cleavage occurred at the wild-type position with respect to the 3′ flanking sequence. In the $\Delta 7$ mutant, which lacks an ACAC element but retains the sequences further 3′, cleavage occurred only at the wild-type position with respect to the 3′ flanking sequence.

Structural studies in solution using chemical probing techniques show that, among the four substitution mutants, only mutant A2sub2 clearly prevented the formation of a small unstable hairpin located immediately 3′ to the ACAC sequences. This was also the only substitution mutant in which no cleavage occurred at the wild-type position with respect to the 3′ flanking nucleotides. Overall cleavage efficiency was also strongly reduced. In contrast, substitution of nt 222–225 within this sequence in A2sub1 had no effect on A_2 cleavage and did not prevent formation of this hairpin. Mutation A2Δ6-3′ is also strongly predicted to prevent both formation of this stem and accurate processing. On the basis of phylogenetic comparisons, this region was proposed to be structurally conserved (Van Nues et al., 1994) and a small hairpin closed by a U-rich loop can be drawn for several organisms related to *S. cerevisiae*, including *K. lactis*, *T. delbrueckii*, and *H. wingei*. Because stem-loop structures are potentially formed in all the mutants cleaved at A_2 (A2sub1, A2sub3, A2sub4) and absent in the noncleaved or weakly cleaved mutants (A2sub2, A2Δ6-3′, A2Δ10), this might constitute a 3′ recognition element required for efficient A_2 cleavage. The 3′ recognition element could also, more simply, consist of the conserved primary sequence. An alternative possible explanation for the inhibition of A_2 cleavage in the mutants was that some of the mutations lead to the sequestration of the site in regions of secondary structure. The structural analyses make it unlikely that this is the case.

There are interesting similarities between pre-rRNA processing at sites A_1 (Venema et al., 1995) and A_2 (this work). In both cases, two major recognition signals are thought to act to position a site of endonucleolytic cleavage. In the wild-type pre-rRNA, the two signals position cleavage at the same nucleotide. However, at both A_1 and A_2, mutations that create a discrepancy between the sites of cleavage specified by the two recognition signals result in heterogeneous cleavage, indicating that the two signals are active concurrently on the wild-type pre-rRNA. At both sites, one of the signals lies 3′, probably in a stem-loop structure, whereas the other signal is an evolutionarily conserved sequence at the site of cleavage; although at A_1, the conserved sequence is 5′ to the cleavage site, at A_2, it is 3′. Moreover, at both A_1 and A_2, processing is surprisingly insensitive to alterations in the nucleotides at the site of cleavage.

All known mutations in the RNA or protein components of the U3, U14, snR10, or snR30 snoRNP particles simultaneously inhibit cleavage at sites A_1 and A_2 (Tollervey, 1987; Li et al., 1990; Hughes & Ares, 1991; Tollervey et al., 1991; Girard et al., 1992; Jansen et al., 1993; Morrissey & Tollervey, 1993). This has led to the suggestion that these sites are cleaved within a multi-snoRNP complex (Maxwell & Fournier, 1995; reviewed by Morrissey & Tollervey, 1995). Recognition of both A_1 and A_2 may involve multiple interactions between snoRNP components and sequences surrounding the cleavage sites.

MATERIALS AND METHODS

Strains and media

Growth and handling of *S. cerevisiae* used standard techniques. The strain used was NOY504: α, *rpa12::LEU2, leu2-3, 112, ura3-1, trp1-1, his3-11, can1-100* (Nogi et al., 1993) (generously provided by M. Nomura).

Construction of *cis*-acting mutants

The wild-type plasmid was pGAL::rDNA (Henry et al., 1994). This plasmid, derived from pNOY102 (generously provided by M. Nomura) contains the entire yeast rDNA unit fused to the *GAL7* promoter (Nogi et al., 1993) in YEp24 (2 μm-URA3). In addition, small oligonucleotide tags have been inserted in the 18S, 5.8S, and 25S rRNA genes. The plasmid used as a negative control (−rDNA) was YEplac 195 (2 μm-URA3) (Gietz & Sugino, 1988). All ITS1 mutants were produced by two-step PCR. In the first round, the mutagenic primer was used in combination with a primer complementary to sequences at the 3′ end of the mature 18S rDNA. The gel-purified product was combined with a downstream primer hybridizing in the 5.8S rDNA sequence and amplified by PCR to yield the final product. These latter PCR fragments, containing the various ITS1 mutations, were introduced into the pBSrDNAB-XFtag plasmid (Henry et al., 1994). This plasmid is a derivative of pBluescript II-KS containing a *Bam*H I–*Xho* I fragment of the rDNA. The ends of the latter fragment correspond to restriction sites found in the tags present in the 18S rDNA (*Bam*H I) and 25S rDNA (*Xho* I). Subcloning of the mutant PCR fragments was done using the following digests: a *Bsm* I + *Stu* I digest for A2sub1, A2sub2, A2sub3, A2sub4, and A2Δ20, a *Sna*B I + *Stu* I digest for A2Δ20/A3Δ10. *Sfi*I–*Xho* I fragments from the resulting pBSrDNAB-XFTag derivatives were then inserted into the pGAL::rDNA plasmid. Positions of the mutations are indicated in Figure 2. In the A2sub1 mutant, wild-type sequence $_{222}$UGUG$_{225}$ was substituted by $_{222}$GUAU$_{225}$, whereas in mutant A2sub2, nt $_{220}$ACUGUGG$_{226}$ were substituted by $_{220}$GAGCAUC$_{226}$. For the A2sub3 and A2sub4, nt $_{220}$AC$_{221}$ and $_{218}$AC$_{219}$, respectively, were substituted by UU.

RNA extraction

Prior to RNA extraction for northern analysis or primer extension, NOY504 strains were transformed with pGAL::rDNA or the ITS1 mutant plasmids and grown at 23 °C in minimal galactose medium until they reached mid-log phase. Cells were diluted to OD_{600} 0.09 and shifted for 6 h to 37 °C (Henry et al., 1994). Total RNA was extracted as previously described (Tollervey & Mattaj, 1987).

Northern hybridization

For each sample, 4 μg of total RNA was separated on 1.2% agarose-formaldehyde gels and transferred to Hybond N$^+$ membranes for northern hybridization as described (Tollervey & Mattaj, 1987). Northern hybridization was performed as previously described using the following oligonucleotides. a, 5′-CGAGGATCCAGGCTTT-3′; b, 5′-GCTCTTTGCTCTT GCC-3′; c, 5′-TGTTACCTCTGGGCCC-3′; d, 5′-CCAGTTAC GAAAATTCTTG-3′; g, 5′-GGCCAGCAATTTCAAGT-3′; h, 5′-ACTCGAGAGCTTCAGTAC-3′. Oligonucleotides a and h hybridize to the tags in 18S and 25S rRNAs, respectively.

Primer extension

Primer extension was performed as described previously (Beltrame & Tollervey, 1992) on 4 μg of total RNA using the following primers: d, 5′-CCAGTTACGAAAATTCTTG-3′; g, 5′-GGCCAGCAATTTCAAGT-3′; 18S+564, 5′-ACCAGACT TGCCCTCC-3′. To identify the position of primer extension stops, DNA sequencing reactions performed with the same oligonucleotides were run in parallel.

Structural analysis of the ITS1 RNA mutants by chemical probing

For structural studies, RNAs were transcribed from the *Bam*H I-*Xho* I fragment of the mutant rDNAs subcloned into the pBSrDNAB-XFtag plasmid. After *Xho* I digestion, the linearized plasmids were transcribed by bacteriophage T7 RNA polymerase. For chemical probing, 1 μg of the transcript, in the presence of 1 μg of *Escherichia coli* total tRNA, was treated with the chemical probes dimethylsulfate (DMS, Aldrich) and 1-cyclohexyl 3-(2 morpholinoethyl)carbodiimide metho-p-toluene (CMCT, Merck) following the method described by Ehresmann et al. (1987) in the same conditions as Allmang et al. (1994). For DMS modification under native conditions, the reaction was carried out for 1 and 2 min at 37°C in 20 μL of buffer N1 (50 mM sodium cacodylate, pH 7.0, 20 mM magnesium acetate, 300 mM KCl) in the presence of 2 μL of DMS freshly diluted 1/40 (v/v) in ethanol. DMS probing under semi-denaturing conditions was conducted in Buffer D1 (50 mM sodium cacodylate, pH 7.0, 1 mM EDTA) for 2 and 4 min. CMCT modifications under native conditions were performed at 37 °C in 20 μL of buffer N2 (50 mM sodium borate, pH 8.0, 20 mM magnesium acetate, 300 mM KCl) in the presence of 5 μL of CMCT (20 mg/mL in H_2O) for 2 and 5 min, the reactions in semi-denaturing conditions were done in buffer D2 (50 mM sodium borate, pH 8.0, 1 mM EDTA) for 2 and 5 min. For each reaction, a control was treated in parallel, omitting the chemical reagent. All the reactions were stopped and the RNA was precipitated by addition of 10 μL 7.5 M ammonium acetate and 90 μL of ethanol for 30 min at −80 °C. After centrifugation, RNA pellets were washed twice with 70% ethanol (v/v). The pellets were then dissolved in 8 μL H_2O. Sites for nucleotide modification were detected by primer extension as described above, using oligonucleotide d as a primer.

ACKNOWLEDGMENTS

We thank Dr. M. Nomura (University of California, Irvine) for the *rpa12* strain NOY504. We thank various colleagues at EMBL for critical reading of the manuscript. C.A. was the recipient of a grant from the EU, Y.H. and J.P.M. were the recipients of grants from EMBL, and H.W. was the recipient of a grant from EMBO.

Received October 20, 1995; returned for revision November 14, 1995; revised manuscript received December 5, 1995

REFERENCES

Allmang C, Henry Y, Morrissey JP, Wood H, Petfalski E, Tollervey D. 1996. Processing of the yeast pre-rRNA at sites A_2 and A_3 is linked. *RNA* 2:000–000.

Allmang C, Mougel M, Westhof E, Ehresmann B, Ehresmann C. 1994. Role of conserved nucleotides in building the 16S rRNA binding site of *E. coli* ribosomal protein S8. *Nucleic Acids Res* 22:3708–3714.

Beltrame M, Henry Y, Tollervey D. 1994. Mutational analysis of an essential binding site for the U3 snoRNA in the 5′ external transcribed spacer of yeast pre-rRNA. *Nucleic Acids Res* 22:5139–5147.

Beltrame M, Tollervey D. 1992. Identification and functional analysis of two U3 binding sites on yeast pre-ribosomal RNA. *EMBO J* 11:1531–1542.

Beltrame M, Tollervey D. 1995. Base pairing between U3 and the pre-ribosomal RNA is required for 18S rRNA synthesis. *EMBO J* 14:4350–4356.

Chu S, Archer RH, Zengel JM, Lindahl L. 1994. The RNA of RNase MRP is required for normal processing of ribosomal RNA. *Proc Natl Acad Sci USA* 91:659–663.

Dock-Bregeon AC, Moras D. 1987. Conformational changes and dynamics of tRNAs: Evidence from hydrolysis patterns. *Cold Spring Harbor Symp Quant Biol* 52:113–121.

Ehresmann C, Baudin F, Mougel M, Romby P, Ebel JP, Ehresmann B. 1987. Probing the structure of RNA in solution. *Nucleic Acids Res* 15:9109–9128.

Gietz RD, Sugino A. 1988. New yeast-*Escherichia coli* shuttle vectors constructed with in vitro mutagenized yeast genes lacking six-base pair restriction sites. *Gene* 74:527–534.

Gilmer D, Allmang C, Ehresmann C, Guilley H, Richards K, Jonard G, Ehresmann B. 1993. The secondary structure of the 5′-noncoding region of beet necrotic yellow vein virus RNA3: Evidence for a role in viral RNA replication. *Nucleic Acids Res* 21:1389–1395.

Girard JP, Lehtonen H, Caizergues-Ferrer M, Amalric F, Tollervey D, Lapeyre B. 1992. GAR1 is an essential small nucleolar RNP protein required for pre-rRNA processing in yeast. *EMBO J 11*:673–682.

Henry Y, Wood H, Morrissey JP, Petfalski E, Kearsey S, Tollervey D. 1994. The 5′ end of yeast 5.8S rRNA is generated by exonucleases from an upstream cleavage site. *EMBO J 13*:2452–2463.

Hughes JMX, Ares MJ. 1991. Depletion of U3 small nucleolar RNA inhibits cleavage in the 5′ external transcribed spacer of yeast preribosomal RNA and impairs formation of 18S ribosomal RNA. *EMBO J 10*:4231–4239.

Jansen R, Tollervey D, Hurt EC. 1993. A U3 snoRNP protein with homology to splicing factor PRP4 and Gβ domains is required for ribosomal RNA processing. *EMBO J 12*:2549–2558.

Lafontaine D, Tollervey D. 1995. *Trans*-acting factors in yeast pre-rRNA and pre-snoRNA processing. *Biochem Cell Biol Special Issue. "Frontiers in Translation."* Forthcoming.

Lafontaine D, Vandenhaute J, Tollervey D. 1995. The yeast 18S rRNA dimethylase Dim1p is required for pre-rRNA processing. *Genes & Dev 9*:2470–2481.

Li HV, Zagorski J, Fournier MJ. 1990. Depletion of U14 small nuclear RNA (snR128) disrupts production of 18S rRNA in *Saccharomyces cerevisiae*. *Mol Cell Biol 10*:1145–1152.

Lindahl L, Archer R, Zengel JM. 1994. Alternate pathways for processing in internal transcribed spacer 1 in pre-rRNA of *Saccharomyces cerevisiae*. *Nucleic Acids Res 22*:5399–5407.

Lindahl L, Archer RH, Zengel JM. 1992. A new rRNA processing mutant of *Saccharomyces cerevisiae*. *Nucleic Acids Res 20*:295–301.

Lygerou Z, Mitchell P, Petfalski E, Séraphin B, Tollervey D. 1994. The *POP1* gene encodes a protein component common to the RNase MRP and RNase P ribonucleoproteins. *Genes & Dev 8*:1423–1433.

Mattaj IW. 1993. RNA recognition: A family matter. *Cell 73*:837–840.

Maxwell ES, Fournier MJ. 1995. The small nucleolar RNAs. *Annu Rev Biochem 35*:897–934.

Morrissey JP, Tollervey D. 1993. Yeast snR30 is a small nucleolar RNA required for 18S rRNA synthesis. *Mol Cell Biol 13*:2469–2477.

Morrissey JP, Tollervey D. 1995. Birth of the snoRNPs—The evolution of RNase MRP and the eukaryotic pre-rRNA processing system. *Trends Biochem Sci 20*:78–82.

Nagai K. 1992. RNA–protein interactions. *Curr Opin Struct Biol 2*:131–137.

Nogi Y, Yano R, Dodd J, Carles C, Nomura M. 1993. Gene *RRN4* in *Saccharomyces cerevisiae* encodes the A12.2 subunit of RNA polymerase I and is essential only at high temperatures. *Mol Cell Biol 13*:114–122.

Nogi Y, Yano R, Nomura M. 1991. Synthesis of large rRNAs by RNA polymerase II in mutants defective in RNA polymerase I. *Proc Natl Acad Sci USA 88*:3962–3966.

Philippe C, Portier C, Mougel M, Grunberg-Manago M, Ebel JP, Ehresmann B, Ehresmann C. 1990. Target site of *Escherichia coli* ribosomal protein S15 on its messenger RNA. *J Mol Biol 211*: 415–426.

Pyle AM, Green JB. 1995. RNA folding. *Curr Opin Struct Biol 5*: 303–310.

Raué HA, Planta RJ. 1991. Ribosome biogenesis in yeast. *Prog Nucleic Acid Res Mol Biol 41*:89–129.

Schmitt ME, Clayton DA. 1993. Nuclear RNase MRP is required for correct processing of pre-5.8S rRNA in *Saccharomyces cerevisiae*. *Mol Cell Biol 13*:7935–7941.

Shuai K, Warner JW. 1991. A temperature sensitive mutant of *S. cerevisiae* defective in pre-rRNA processing. *Nucleic Acids Res 19*: 5059–5064.

Tollervey D. 1987. A yeast small nuclear RNA is required for normal processing of pre-ribosomal RNA. *EMBO J 6*:4169–4175.

Tollervey D, Lehtonen H, Carmo-Fonseca M, Hurt EC. 1991. The small nucleolar RNP protein NOP1 (fibrillarin) is required for pre-rRNA processing in yeast. *EMBO J 10*:573–583.

Tollervey D, Mattaj IW. 1987. Fungal small nuclear ribonucleoproteins share properties with plant and vertebrate U-snRNPs. *EMBO J 6*:469–476.

Udem SA, Warner JR. 1972. Ribosomal RNA synthesis in *Saccharomyces cerevisiae*. *J Mol Biol 65*:227–242.

Udem SA, Warner JR. 1973. The cytoplasmic maturation of a ribosomal precursor ribonucleic acid in yeast. *J Biol Chem 248*:1412–1416.

Van Nues RW, Rientjes JMJ, Van der Sande CAFM, Zerp SF, Sluiter C, Venema J, Planta RJ, Raué HA. 1994. Separate structural elements within internal transcribed spacer 1 of *Saccharomyces cerevisiae* precursor ribosomal RNA direct the formation of 17S and 26S rRNA. *Nucleic Acids Res 22*:912–929.

Veldman GM, Brand RC, Klootwijk J, Planta RJ. 1980. Some characteristics of processing sites in ribosomal precursor RNA of yeast. *Nucleic Acids Res 8*:2907–2920.

Veldman GM, Klootwijk J, van Heerihuizen H, Planta RJ. 1981. The nucleotide sequence of the intergenic region between the 5.8S and 26S rRNA genes of the yeast ribosomal RNA operon. Possible implications for the interaction between 5.8S and 26S rRNA and the processing of the primary transcript. *Nucleic Acids Res 19*:4847–4862.

Venema J, Henry Y, Tollervey D. 1995. Two distinct recognition signals define the site of endonucleolytic cleavage at the 5′ end of yeast 18S rRNA. *EMBO J 14*:4883–4892.

Venema J, Tollervey D. 1995. Processing of pre-ribosomal RNA in *Saccharomyces cerevisiae*. *Yeast*. Forthcoming.

Warner JR. 1989. Synthesis of ribosomes in *Saccharomyces cerevisae*. *Microbiol Rev 53*:256–271.

Yeh LCC, Thweatt R, Lee JC. 1990. Internal transcribed spacer 1 of the yeast precursor ribosomal RNA. Higher order structure and common structural motifs. *Biochemistry 29*:5911–5918.