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Identification of *cis*-acting elements involved in 3′-end formation of *Saccharomyces cerevisiae* 18S rRNA

CATELIJNE A. VAN BEEKVELT, RIENK E. JEENINGA,[1] JAN VAN'T RIET, JAAP VENEMA,[2] and HENDRIK A. RAUÉ

Department of Biochemistry and Molecular Biology, IMBW, BioCentrum Amsterdam, Vrije Universiteit, de Boelelaan 1083, 1081 HV Amsterdam, The Netherlands

ABSTRACT

In yeast, the 3′ end of mature 18S rRNA is generated by endonucleolytic cleavage of the 20S precursor at site D. Available data indicate that the major *cis*-acting elements required for this processing step are located in relatively close proximity to the cleavage site. To identify these elements, we have studied the effect of mutations in the mature 18S and ITS1 sequences neighboring site D on pre-rRNA processing in vivo. Using clustered point mutations, we found that alterations in the sequence spanning site D from position –5 in 18S rRNA to +6 in ITS1 reduced the efficiency of processing at this site to different extents as demonstrated by the lower level of the mature 18S rRNA and the increase in 20S pre-rRNA in cells expressing only mutant rDNA units. More detailed analysis revealed an important role for the residue located 2 nt upstream from site D (position –2), whereas sequence changes at position –1, +1, and +2 relative to site D had no effect. The data further demonstrate that the proposed base pairing between the 3′ end of 18S rRNA and the 5′ end of ITS1 is not important for efficient and accurate processing at site D, nor for the formation of functional 40S ribosomal subunits. These results were confirmed by analyzing the accumulation of the D-A_2 fragment derived from the mutant 20S pre-rRNA in cells that lack the Xrn1p exonuclease responsible for its degradation. The latter results also showed that the accuracy of cleavage was affected by altering the spacer sequence directly downstream of site D but not by mutations in the 18S rRNA sequence preceding this site.

Keywords: processing; ribosome/RNA; yeast

INTRODUCTION

The synthesis of eukaryotic ribosomal subunits is a highly complex process that takes place mainly in the nucleolus (Hadjiolov, 1985; Warner, 1989; Raué & Planta, 1991; Mélèse & Xue, 1995). There the four rRNAs are processed from longer precursor transcripts, while at the same time, the ribosomal proteins are assembled onto the various precursor intermediates in a distinct order. The pre-40S and 60S subunits are then exported to the cytoplasm where, after some finishing touches, they take up their role in translation.

In eukaryotes, three of the four rRNAs (18S, 5.8S, and 25/28S rRNA) are encoded by a large number of tandemly repeated rDNA units (in yeast, 150–200 copies). These units are transcribed by RNA polymerase I into a single, large precursor RNA that contains external transcribed spacers, the 5′-ETS and the 3′-ETS, at either end as well as two internal transcribed spacers, ITS1 and ITS2, that separate the mature rRNA sequences (Fig. 1). The transcribed spacers are removed by a series of endonucleolytic cleavages and exonucleolytic degradation steps (Eichler & Craig, 1994; Venema & Tollervey, 1995, 1999; Raué & Planta, 1995) to produce the mature rRNA species.

So far, the pre-rRNA processing pathway has been studied most extensively in the yeast *Saccharomyces cerevisiae* (for reviews see Venema & Tollervey, 1995, 1999; Raué & Planta, 1995). In this organism, the first detectable pre-rRNA species containing the 18S, 5.8S, and 25S sequences is the 35S precursor, which has already lost most of the 3′-ETS. Processing of 35S pre-rRNA starts with endonucleolytic cleavages at sites A_0 (within the 5′-ETS), A_1 (the 5′ end of 18S rRNA), and A_2 (within ITS1) to yield the small subunit

Reprint requests to: H.A. Raué, Ph.D., Department of Biochemistry and Molecular Biology, Vrije Universiteit, de Boelelaan 1083, 1081 HV Amsterdam, The Netherlands; e-mail: raue@chem.vu.nl.

[1]Present address: Department of Human Retrovirology, Academic Medical Center, University of Amsterdam, Meibergdreef 15; 1105 AZ Amsterdam, The Netherlands.

[2]Present address: Solvay Pharmaceuticals, Department of Biotechnology, P.O. Box 900, 1380 DA Weesp, The Netherlands.

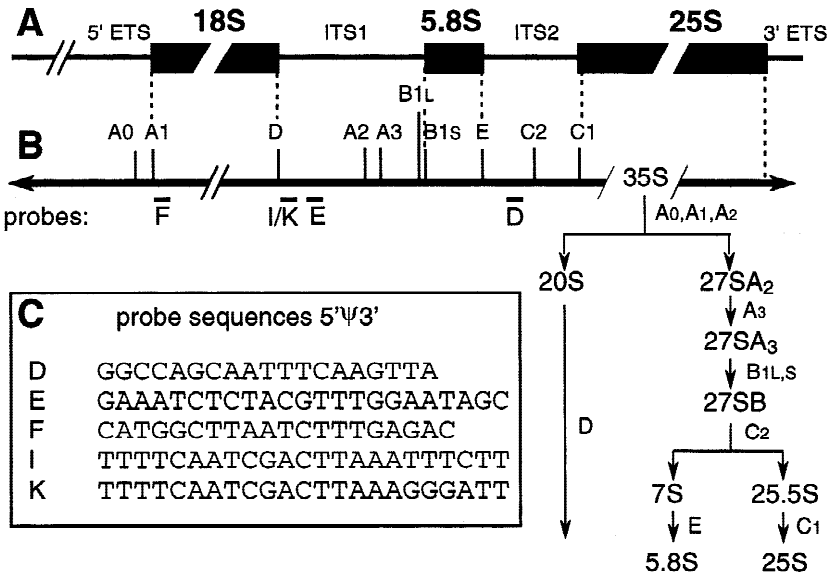

FIGURE 1. Biogenesis of yeast rRNA. **A:** The yeast rRNA operon. Mature and spacer sequences are represented by the bars and lines, respectively. **B:** The yeast pre-rRNA processing scheme. The positions of the processing sites as well as those of the probes used in northern hybridization are indicated and the major pre-rRNA intermediates are shown (Venema & Tollervey, 1999; Geerlings et al., 2000). **C:** Sequence of the various probes used in northern hybridization.

20S pre-rRNA and the large subunit $27SA_2$ pre-rRNA (cf. Fig. 1B). Whereas further processing of the latter into 5.8S and 25S rRNA occurs in the nucleolus, the 20S pre-rRNA is exported as part of a 43S preribosomal subunit to the cytoplasm, where cleavage at site D removes the remaining portion of ITS1 to produce mature 18S rRNA (Udem & Warner, 1973; Trapman & Planta, 1976; Stevens et al., 1991; Moy & Silver, 1999).

Accurate and efficient processing of yeast pre-rRNA involves a large number of *trans*-acting factors as well as specific primary and secondary structural features (*cis*-acting elements) within the pre-rRNA (reviewed in Van Nues et al., 1995a; Kressler et al., 1999; Venema & Tollervey, 1999). In vivo mutational analysis has shown that removal of most of the 3′-ETS by Rnt1p, the yeast homolog of RNase III, requires a hairpin located within this spacer (Kufel et al., 1999). Production of the 20S and $27SA_2$ species by cleavage of the 35S pre-rRNA at sites A_0, A_1, and A_2 depends upon a single-stranded region of 10 nt, located in the 5′-ETS (Beltrame & Tollervey, 1992, 1995; Beltrame et al., 1994), as well as sequences at the 5′ end of 18S rRNA (Hughes, 1996; Sharma & Tollervey, 1999), both of which are recognized by the U3 snoRNA. Cleavage at A_1 is guided by two additional *cis*-acting signals, a conserved sequence upstream from A_1 in the 5′-ETS and the 5′-terminal stem-loop/pseudoknot structure in 18S rRNA (Venema et al., 1995b). The 5′ region of ITS1 contains several further *cis*-acting elements involved in cleavage at site A_2 (Lindahl et al., 1994; Van Nues et al., 1994; Allmang et al., 1996a, 1996b; Liang & Fournier, 1997), whereas *cis*-acting elements required for processing of the $27SA_2$ precursor at sites A_3, $B1_L$, and $B1_S$ are located within the 3′ region of this spacer (Henry et al., 1994; Lindahl et al., 1994; Van Nues et al., 1994; Allmang et al., 1996a, 1996b). Base pairing between the 3′ terminus of ITS2 and the 5′ terminus of 5.8S rRNA (Van Nues et al., 1994) and structural features within some of the expansion segments of the mature 25S rRNA sequence also appear to play a role in the latter three processing steps (Jeeninga et al., 1997). Finally, removal of ITS2 depends upon both primary and secondary structural elements distributed throughout this spacer (Van der Sande et al., 1992; Van Nues et al., 1995a), including base pairing between the 3′ end of 5.8S and the 5′ end of 25S rRNA (Peculis & Greer, 1998), as well as the integrity of the region in domain III of 25S rRNA that contains the binding site for r-protein L25 (Van Beekvelt et al., 2000). It should be realized that this inventory of *cis*-acting elements is likely to be far from complete. Moreover, in most cases the manner in which these elements function is still unknown.

Site D, the 3′ end of yeast 18S rRNA, is the only processing site for which no distinct *cis*-acting elements have been identified yet. As mentioned above, cleavage at site D is a cytoplasmic event and most likely involves a single endonucleolytic cleavage of the 20S pre-rRNA that separates the mature 18S rRNA sequence from the D-A_2 fragment of ITS1. Structural analysis data indicate that site D is located at the apical end of a hairpin formed by base pairing between the 3′-end of 18S rRNA and the 5′ end of ITS1 (Yeh et al., 1990; see Fig. 2)

In mammalian cells, the *cis*-acting elements involved in formation of the 3′ end of 18S rRNA, which in contrast to yeast occurs in the nucleus (Bowman et al., 1981), were found to be limited to a region extending from 60 nt upstream to 103 nt downstream from the cleavage site. Mutational analysis using a minigene system identified the ultimate helix 45 of 18S rRNA and its 3′-terminal sequence CAUUA as being important for

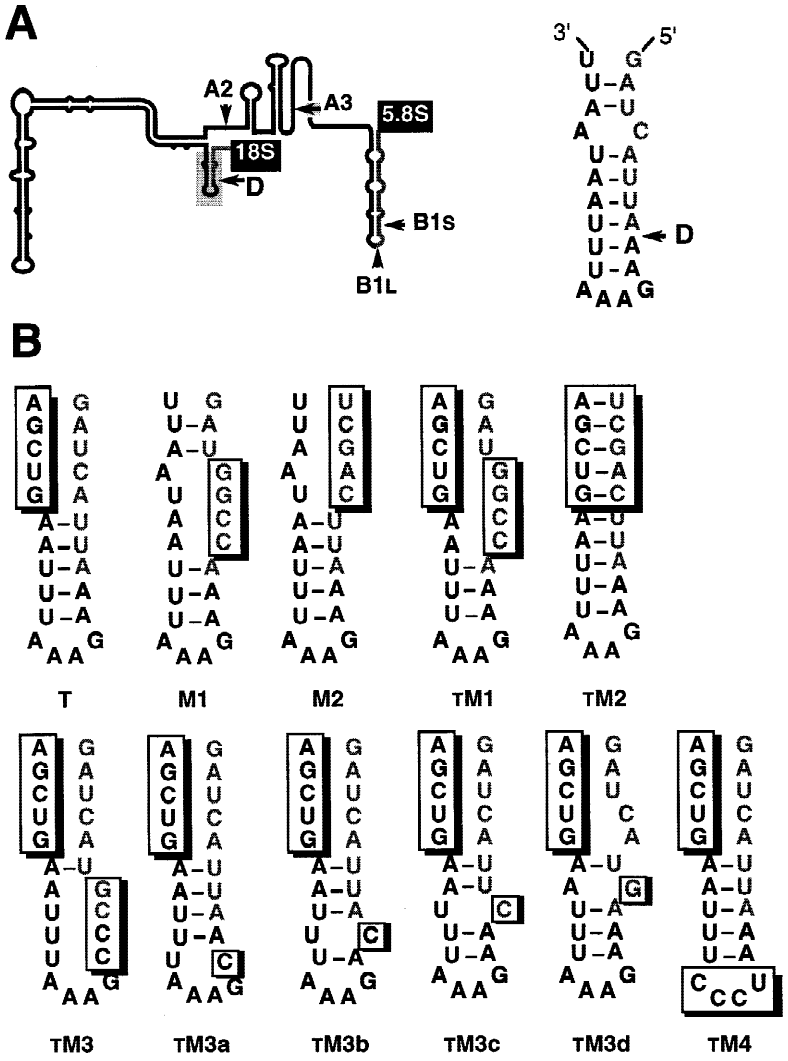

FIGURE 2. Mutations used in this study. **A:** Schematic representation of the secondary structure of yeast ITS1 and the adjacent mature 18S rRNA and 5.8S regions according to Yeh et al. (1990). The wild-type sequence of the shaded region spanning site D is shown. Nucleotides of the mature sequence are printed in gray, those of ITS1 in black. **B:** Sequence of the region spanning site D in the different mutant pre-rRNAs. Spacer nucleotides are shown in black, residues of the mature 18S rRNA sequence in gray. Nucleotides differing from the wild-type sequence are boxed.

efficient and accurate 3′-end processing (Cavaillé et al., 1996). The available data for yeast indicate that as far as ITS1 is concerned, no *cis*-acting elements required for cleavage at site D are present beyond the 5′-terminal 31 nt of the spacer (Van Nues et al., 1994). So far no *cis*-acting elements within the yeast mature 18S rRNA sequence involved in this processing step have been identified.

To obtain more detailed information on the primary and secondary structural features required for processing at site D in yeast, we mutagenized the ITS1 and 18S rRNA sequences flanking this site. The effect of these mutations on processing was analyzed in two different ways. On the one hand, we assessed the levels of mature 18S rRNA and its 20S precursor in yeast cells that express only the mutant rDNA (the "in vivo Pol II system"; Venema et al., 1995a). On the other hand, we determined the level of the (tagged) ITS1 fragment derived from the mutant 20S pre-rRNA in cells that stably accumulate this fragment because they lack the Xrn1p exonuclease (Stevens et al., 1991; Moy & Silver, 1999).

RESULTS AND DISCUSSION

In a first series of experiments, we introduced three different sets of clustered point mutations, T, M1, and M2 (Fig. 2), which alter the upstream and downstream flanking sequences of site D, into the *GAL*-driven rDNA unit of plasmid pJV12, which carries a neutral tag in the 18S as well as the 25S rRNA sequence (Musters et al., 1989; Beltrame et al., 1994). We then performed an initial characterization of these mutations by analyzing the growth of YJV100 cells transformed with the mutant rDNA units on glucose-based medium, which allows them to express only the mutant rDNA (Venema et al., 1995a). The results showed that the M1 mutation reduced the growth rate about threefold compared to control cells transformed with wild-type rDNA units, whereas the M2 mutation was lethal (data not shown).

The T mutation, however, did not have any detectable effect on growth. This indicates that this mutation, even though it destroys the same set of base pairs as the M2 mutation (Fig. 2B), is neutral with respect to ribosome biogenesis and can be used as a tag to mark the ITS1 of the mutant rDNA unit. This conclusion was confirmed by northern analysis of total RNA isolated from the YJV100 transformants 16 h after the shift to glucose-based medium. Using probe F complementary to the tag in 18S rRNA and probe E complementary to a wild-type sequence in the 5′ portion of ITS1 (Fig. 1B,C) we detected normal levels of both mature 18S rRNA and its 20S precursor in glucose-grown T mutant cells (Fig. 3A,B, cf. lane 3 to lane 2). The effect of the various mutations on processing at site D was therefore analyzed using rDNA units carrying the additional tag in the ITS1 sequence.

As shown in Figure 3, the M1 mutation, which alters nt −2 through −5 of the mature 18S rRNA sequence relative to site D, has a negative effect on the efficiency of processing: after being shifted to glucose-based medium, TM1 transformants contain a significantly reduced level of mature 18S rRNA, whereas the level of the 20S precursor is clearly increased (lane 4). The processing phenotype and growth rate of these transformants is identical to that of cells expressing M1 mutant rDNA units that lack the ITS1 tag (data not shown). The M2 mutation, which changes nt −4 through −8 of 18S rRNA, and thus overlaps mutation M1 by 2 nt (Fig. 2B), causes a completely different and rather peculiar phenotype. TM2 transformants grown on glucose lack detectable amounts of both 18S rRNA and the 20S precursor species (Fig. 3A,B, lane 5). Again, YJV100 cells expressing M2 mutant rDNA units that lack the ITS1 tag show an identical phenotype (data not shown). It should be noted that the T and M2 mutations were chosen in such a way that complementarity between the two sequences is maintained (Fig. 2B). Restoration of the base-pairing potential in the upper portion of the proposed hairpin containing site D, therefore, does not reverse the effect of the M2 mutation. The neutral character of the T mutation, on the other hand, shows that abolishing base pairing, in itself, does not affect production of mature 18S rRNA. Therefore, we conclude that secondary structure in the upper region of the helix containing site D is not important for fully efficient processing at this site. Neither is it required for production of functional 40S ribosomal subunits.

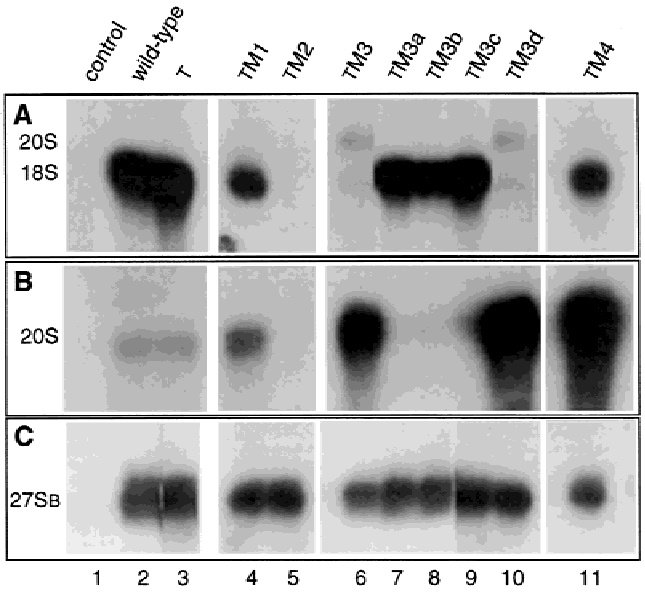

FIGURE 3. Effect of the various mutations on the levels of mature and precursor rRNA. YJV100 cells transformed with pJV12 plasmids carrying the different mutant rDNA units were shifted from galactose- to glucose-based medium to block expression of the endogenous wild-type rDNA units (Venema et al., 1995a). Sixteen hours after the shift total RNA was isolated, separated by electrophoresis on a 1.2% agarose gel, and analyzed by northern hybridization. Lane 1: RNA from cells transformed with an "empty" plasmid. Lane 2: RNA from cells transformed with wild-type rDNA plasmid containing only the tags in 18S and 25S rRNA. Lanes 3–11 RNA from cells transformed with the various mutant rDNA plasmids indicated at the top. **A:** Level of mature 18S rRNA. The blot was hybridized with probe F complementary to the tag in the mature 18S rRNA sequence. **B:** Level of 20S pre-rRNA. The blot was hybridized with probe E complementary to a sequence in the 5′-region of ITS1. **C:** Level of 27SB pre-rRNA. The blot was hybridized with probe D complementary to a sequence within ITS2. See Figure 1 for the location and sequence of the probes.

Next we analyzed two additional clustered point mutations. Mutation M3 spans site D, altering nt −2 through +2, whereas mutation M4 changes the 4 nt of the loop of the hairpin containing site D (Fig. 2B). The first of these two mutations severely inhibits processing. After having been shifted to glucose, YJV100 cells transformed with the TM3 mutant rDNA units contain a very low amount of mature 18S rRNA whereas the 20S precursor accumulates to extremely high levels (Fig. 3A,B, lane 6). The M4 mutation also has a considerable, though less severe, negative effect on processing. The level of 18S rRNA in TM4 mutant cells upon growth on glucose is comparable to that observed for the TM1 mutant (Fig. 3A, cf. lane 11 to lane 4). However, the level of the 20S precursor in TM4 cells is again very high, indicating a substantial kinetic delay in processing at site D (Fig. 3B, lane 11).

Surprisingly, even though YJV100 cells transformed with TM4 and TM1 mutant rDNA units have very similar levels of mature 18S rRNA, the former mutant grows on glucose-based medium at about twice the rate of its TM1 counterpart (data not shown). This may indicate a functional defect in 18S rRNA carrying the M1 mutation. It should be noted that this mutation changes the CAUU sequence that is highly conserved in eukaryotic 18S rRNA.

Mutation M3 was dissected by altering each of its four residues individually (Fig. 2B, mutants TM3a through TM3d). As shown in Figure 3A,B, an A → C sequence change at position +2 or +1 in ITS1 (mutants TM3a, lane 7 and TM3b, lane 8) or position −1 in 18S rRNA

(mutant TM3c, lane 9) has no effect on processing as judged from the normal steady-state levels of 18S rRNA and the 20S precursor in the respective transformants grown on glucose-based medium. Changing the U residue at position −2 in 18S rRNA into G (mutant TM3d), however, causes the same phenotype as the M3 mutation, that is, 18S rRNA becomes virtually undetectable and 20S pre-rRNA accumulates very strongly, indicating an (almost) complete block of processing at site D (Fig. 3A,B, lane 10). This processing phenotype must be the result of the U → G sequence change rather than destruction of the base pairing because the U → C sequence change at position −2 that is part of the M1 mutation has a much less severe effect on processing. Furthermore, mutations M3a, M3b, and M3c all disturb the proposed base pairing around site D without any deleterious effect on cleavage, demonstrating that efficient processing and formation of functional 18S rRNA do not depend upon secondary structure in this region.

To establish whether the various mutations affect the processing pathway leading to 5.8S/25S rRNA, we analyzed the level of 27SB pre-rRNA in the different YJV100 transformants 16 h after the shift to glucose-based medium using probe D complementary to ITS2 (Fig. 1). In all cases, the intensity of the hybridization signal obtained was very similar to that observed for control cells transformed with wild-type, doubly tagged rDNA units (Fig. 3C, cf. lanes 3–11 to lane 2), indicating that the mutations do not have a significant effect on the processing cleavages involved in production of the large subunit rRNAs. This is in agreement with previous data showing that mutations in 18S rRNA or the 5′-region of ITS1 did not affect the processing steps leading to 25S/5.8S rRNA (Van Nues et al., 1995b; Venema et al., 1995b; Sharma & Tollervey, 1999).

The data presented above make it clear that efficient processing at site D depends upon sequence information in both the mature and spacer sequences neighboring the cleavage site. The nucleotides immediately flanking site D (positions +2, +1, and −1), however, appear not to be involved. The residue at position −2, on the other hand, is of particular importance. The processing phenotypes caused by the M1 and M3d mutations, respectively, suggest that the presence of a pyrimidine at position −2 is essential for cleavage to occur. U may be the preferred residue, but this will have to be confirmed by further analysis because we cannot exclude that the negative effect on processing seen for mutation M1 is due to one or more of the sequence changes at positions −3, −4, and −5. That the processing machinery involved in 3′-end formation of 18S rRNA does not tolerate a purine at position −2 also finds support in the data of Cavaillé et al. (1996). These authors reported that a mutation that changes the same conserved (−5)CAUU(−2) sequence at the 3′ end of mouse 18S rRNA into UCGA, and thus introduces a U → A substitution at position −2, completely blocks processing of the minigene precursor transcript.

Processing at site D can also be monitored by analyzing the accumulation of the D-A_2 fragment in cells lacking Xrn1p 5′ → 3′ exonuclease, the enzyme primarily responsible for its degradation (Stevens et al., 1991; Moy & Silver, 1999). We, therefore, introduced the pJV12 plasmids carrying the ITS1-tagged, mutant rDNA units into yeast strain D183, in which the *XRN1* gene is inactivated (kindly provided by Dr. D. Tollervey). Total RNA was isolated from the different, exponentially growing transformants, separated on an 8% polyacrylamide gel and analyzed by northern hybridization, using probe I complementary to the ITS1 sequence containing mutation T (Fig. 1B,C). The results in Figure 4 demonstrate that this probe specifically recognizes the D-A_2 fragment carrying the tag. Whereas RNA prepared from cells expressing wild-type, ITS1-tagged rDNA units produces a clear hybridization signal (Fig. 4, lane 3), RNA isolated from cells transformed with either an empty plasmid (Fig. 4, lane 1) or a plasmid carrying an untagged, wild-type rDNA unit (Fig. 4, lane 2) does not hybridize.

The remaining data shown in Figure 4 confirm the conclusions drawn above concerning the effect of the various mutations on 3′-end formation of 18S rRNA. The D-A_2 fragment is readily detectable in D183 cells expressing the TM1 (Fig. 4, lane 4), TM3a, TM3b, and TM3c (Fig. 4, lanes 7–9) mutant rDNA units, whereas no signal corresponding to this fragment can be seen for RNA derived from the TM2, TM3, and TM3d transformants (Fig. 4, lanes 5, 6, and 10, respectively). In our initial analysis, probe I gave at best a very faint signal with RNA isolated from D183 cells transformed with the TM4 mutant rDNA units (data not shown). However, when we used probe K, which spans the same region of ITS1 as probe I but has a 3′-terminal se-

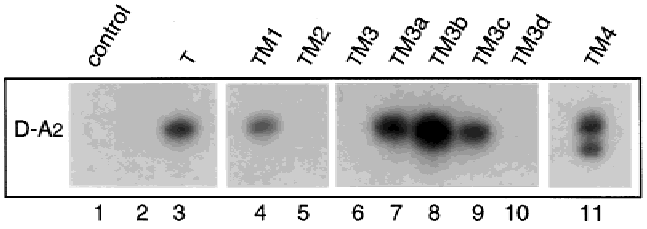

FIGURE 4. Effect of the various mutations on accumulation of the tagged D-A_2 fragment in cells lacking the Xrn1p exonuclease. Total RNA was extracted from exponentially growing D183 (*xrn1*$^-$) transformants, separated on an 8% polyacrylamide gel and analyzed by northern hybridization using probe I complementary to the ITS1 sequence carrying the T mutation (lanes 1–10), or probe K complementary to the ITS1 sequence carrying both the T and the M4 mutation (lane 11). See Figure 1 for the location and sequence of the probes. Lane 1: RNA from cells transformed with an "empty" plasmid. Lane 2: RNA from cells transformed with wild-type rDNA plasmid containing only the tags in 18S and 25S rRNA. Lanes 3–11 RNA from cells transformed with the various mutant rDNA plasmids as indicated at the top.

quence complementary to mutation M4 (Fig. 1C), we obtained two clear, closely spaced hybridization signals (Fig. 4, lane 11).

Figure 4 also provides the important additional information that, with exception of M4, none of the mutations studied affects the accuracy of processing at site D, as probe I detects a single band located at the same position as in the wild-type control (Fig. 4, cf. lanes 4, 7, 8, and 9 to lane 3). In the case of the M4 mutation, however, about half of the ITS1 fragment cleaved from mutant 20S pre-rRNA has the correct size, while the other half is slightly smaller, indicating that cleavage has occurred shortly downstream from the actual site D (Fig. 4, lane 11). Thus, mutation M4 identifies a *cis*-acting element within ITS1 that is important not only for fully efficient but also for fully accurate cleavage. Although the precise location of the aberrant cleavage was not determined, the observation that probe I recognizes the tagged D-A_2 fragment carrying the M4 mutation very poorly, whereas probe K does so efficiently, indicates that it must be located within less than 5 nt downstream from the correct site.

The conclusion that a *cis*-acting element important for 3′-end formation of 18S rRNA is present within the region of ITS1 bordered by nt +3 and +6 seems to be in conflict with the observation of Liang and Fournier (1997), who observed normal amounts of yeast small subunit rRNA in cells that express an rDNA fragment encoding the 5′-ETS and mature 18S sequences followed by only 3 nt of ITS1 and flanked by *GAL* promoter and terminator sequences. We can envisage several possible explanations for this apparent contradiction. First, assuming that normal processing has indeed occurred, either the G at position +3 may be the critical determinant or the *cis*-acting element may have been restored in the fusion construct used by these authors. However, the northern hybridization data shown do not exclude the possibility that the precursor transcript in question has been processed aberrantly. The presence of an incorrect 3′ end might be an alternative explanation for the observation (Liang & Fournier, 1997) that the 18S rRNA is nonfunctional.

Nucleotides +3 through +6 of ITS1 are unlikely to contain the sole signal directing the endonuclease to the correct site, because about half of the mutant 20S precursor is still processed correctly (Fig. 4). This is reminiscent of the situation described for processing at site A_1, where two distinct, independent signals ensure correct 5′-end formation of 18S rRNA (Venema et al., 1995b). The nature of the additional signal(s) ensuring accurate cleavage at site D remains obscure, however. Cavaillé et al. (1996) have reported data indicating that CAUUA^{i}A sequence from position −5 to +1 in mouse pre-rRNA may be involved in defining the 3′ end of 18S rRNA. This conclusion was based on the observation that insertions upstream from this sequence move the cleavage site downstream by the corresponding number of nucleotides and that altering the two A residues on either side of site D causes aberrant cleavages within ITS1 in addition to the correct one. Our data do not support such a role for the corresponding CAUUAA sequence in yeast 20S rRNA because none of the mutations affecting this sequence (M1, M3, M3a, M3b, and M3c) causes heterogeneity in processing (Fig. 4).

The phenotype of mutation M2 is rather unusual in that it prevents the stable formation of 18S rRNA without concomitant accumulation of the 20S precursor (Fig. 3). The fact that we were unable to detect a D-A_2 fragment derived from the mutant pre-rRNA in D183 transformants (Fig. 4) indicates that the absence of 18S rRNA is not due to its rapid turnover. Furthermore, we observed no accumulation of precursor species preceding 20S pre-rRNA in the processing pathway (data not shown), which suggests that processing up to this precursor is unaffected. The only reasonable explanation for the absence of both 18S rRNA and the 20S precursor in the TM2 transformants, therefore, is the rapid turnover of the latter. This explanation finds additional support in the dramatic difference in the level of 20S pre-rRNA between the TM1 and TM4 transformants, despite their very similar steady-state levels of mature 18S rRNA (Fig. 3). The lower level of the precursor in the former mutant, which overlaps the TM2 mutation by 2 nt might also reflect a higher turnover rate. Possibly, altering the sequence in this region covered by residues −3 through −8 disturbs the assembly of the small subunit in a manner that destabilizes the 43S ribosomal precursor particle.

MATERIALS AND METHODS

Strains and transformation procedure

Growth and handling of *S. cerevisiae* and *Escherichia coli* were performed by standard techniques. The *E. coli* strains Sure™ Strain (Stratagene, La Jolla, California, USA), JM109 and BMH 71-18 *mutS* were used as bacterial hosts. *S. cerevisiae* strain YJV100 [Matα, *rpa135::LEU2, ade2-1, his3-11, leu2-3,112, trp 1-1, ura3-1, can1-100, RDN1::pGRIM(TRP1d, GAL7-rDNA)*; Venema et al., 1995a] is defective in RNA polymerase I. This strain is rescued by multiple integrated copies of a wild-type rDNA unit under control of an inducible *GAL7* promoter. Strain D183 (Matα, rar5-1, ade2-1, his3-11, 15, leu2-3, trp1-1, ura3-52), which lacks the Xrn1p 5′ → 3′ exonuclease, was kindly provided by Dr. David Tollervey (University of Edinburgh, UK). Transformation of yeast cells with the various plasmids described below was carried out by the freeze–thaw method (Klebe et al., 1983). Transformants were selected at 30 °C on minimal plates containing YNB and 2% (w/v) galactose and 2% (w/v) minimal agar (Difco Laboratories, Michigan, USA).

Plasmids

Plasmid pJV12 (2μ, *URA3*) is derived from Yeplac195 (Gietz & Sugino, 1988), which contains an *S. cerevisiae* rDNA unit

under control of the *PGK1* promoter (Venema et al., 1995a). The rDNA unit is wild-type in sequence except for the presence of small, functionally neutral oligonucleotide tags in the 18S rRNA (Beltrame et al., 1994) and 25S rRNA (Musters et al., 1989). pGEM3-18S•SacSph and pUK19-18S•SacSph were obtained by cloning a 1-kb *Sac*I-*Sph*I fragment from pJV12, encompassing the 3′ portion of the 18S rRNA (from nt −559 relative to site D), the complete ITS1 as well as 5.8S rRNA, into pGEM3 (Promega, Madison, Wisconsin, USA) or pUK19, respectively.

Site-directed mutagenesis

Mutations M1, M2, and M3 (Fig. 2B) were introduced by a two-step PCR procedure using pGEM3-18S•SacSph as a template. In the first round, a mutagenic primer and a primer complementary to the T7 promoter were used. The resulting PCR fragment was purified by gel electrophoresis using QIAEX II (Qiagen, Germany) and used as a primer in the second round of PCR, together with a primer complementary to the Sp6 promoter sequence. The resulting PCR fragment was cut with *Sac*I-*Sph*I and the restriction fragment was used to replace the corresponding wild-type fragment of pJV12. The mutant *Sac*I-*Sph*I fragment of the resulting pJV12 derivative was completely sequenced to ascertain that only the desired mutation was present. Mutations TM1 and TM2 were obtained in a similar way except that pGEM3-18S•SacSph carrying the M3 mutation was used as the template. Mutations TM3, TM3a, TM3b, TM3Dc, TM3d, and TM4 were introduced by a two-step PCR procedure using pUK19-18S•SacSph as the template. In the first round a mutagenic primer and a primer overlapping the *Stu*I site in 18S rRNA (nt −114/−138 relative to site D) were used. The resulting PCR fragment was used in the second round PCR together with a primer overlapping the *Afl*II site in ITS1 (nt +95/+118 relative to site D). The resulting PCR fragment was digested with *Stu*I and *Afl*II and used to replace the corresponding wild-type fragment of pUK-18S•SacSph. Selected mutants were checked by sequencing. Subsequently the mutant *Stu*I-*Afl*II fragment was used to replace its wild-type counterpart in pJV12.

RNA analysis

Total RNA was isolated as described previously (Venema & Tollervey, 1996; Venema et al., 1998) from YJV100 transformants 16 h after the shift from galactose- to glucose-based medium. For northern analysis, 5 μg of RNA were separated electrophoretically on a 1.2% (w/v) agarose gel, blotted, and hybridized to different oligonucleotide probes, each of which recognizes (a) specific pre-rRNA(s) (Fig. 1B,C). For detection of the D-A_2 fragment, the RNA was separated on an 8% polyacrylamide gel.

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