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REPORT

Ngl2p is a Ccr4p-like RNA nuclease essential for the final step in 3′-end processing of 5.8S rRNA in *Saccharomyces cerevisiae*

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ABSTRACT

***Saccharomyces cerevisiae* contains three nonessential genes (*NGL1*, *NGL2*, and *NGL3*) that encode proteins containing a domain with similarity to a Mg^{2+}-dependent endonuclease motif present in the mRNA deadenylase Ccr4p. We have investigated a possible role of these proteins in rRNA processing, because for many of the pre-rRNA processing steps, the identity of the responsible nuclease remains elusive. Analysis of RNA isolated from cells in which the *NGL2* gene has been inactivated (*ngl2*Δ) demonstrates that correct 3′-end formation of 5.8S rRNA at site E is strictly dependent on Ngl2p. No role in pre-rRNA processing could be assigned to Ngl1p and Ngl3p. The 3′-extended 5.8S rRNA formed in the *ngl2*Δ mutant is slightly shorter than the 6S precursor previously shown to accumulate upon combined deletion of the 3′ → 5′ exonuclease-encoding *REX1* and *REX2* genes or upon depletion of the exosomal subunits Rrp40p or Rrp45p. Thus, our data add a further component to the set of nucleases required for correct 3′-end formation of yeast 5.8S rRNA.**

Keywords: **Ccr4p; Ngl2p; processing; ribosomal RNA; RNA nuclease**

INTRODUCTION

In eukaryotic cells, three of the four mature rRNAs are formed from a polycistronic primary transcript by a complex, ordered series of endo- and exonucleolytic cleavages. These cleavages remove the external (ETS) and internal (ITS) transcribed spacers from the transcript, leaving the mature 18S, 5.8S, and 25/28S rRNA molecules with precisely defined 5′ and 3′ ends. The eukaryotic processing pathway has been most extensively characterized in the yeast *Saccharomyces cerevisiae* (Kressler et al., 1999; Venema & Tollervey, 1999; Lafontaine & Tollervey, 2001; see Fig. 1). In these cells, the first detectable intermediate is the 35S pre-rRNA, which has already lost most of the 3′ ETS through cotranscriptional cleavage by Rnt1p (Abou-Elela et al., 1996; Kufel et al., 1999), the yeast homolog of bacterial RNase III. The 35S precursor is cleaved endonucleolytically at sites A0, A1, and A2, resulting in a 3′-extended 20S precursor to 18S rRNA and a 5′-extended $27SA_2$ precursor to 5.8S/25S rRNA that also still contains the complete ITS2, as well as a short remnant of the 3′ ETS. The mature 3′ end of 18S rRNA is formed by endonucleolytic cleavage of 20S pre-rRNA at site D, which takes place in the cytoplasm (Stevens et al., 1991; Moy & Silver, 1999; Vanrobays et al., 2001). The major pathway for converting the $27SA_2$ precursor into 5.8S and 25S rRNA starts with cleavage at site A3 by the endonuclease RNaseMRP (Schmitt & Clayton, 1993; Chu et al., 1994; Lygerou et al., 1996), followed by exonucleolytic digestion of the $27SA_3$ precursor by exonucleases Xrn1p and Rat1p to site $B1_S$, the 5′ end of mature $5.8S_S$ rRNA (Henry et al., 1994; Petfalski et al., 1998). A minor pathway, starting with either the $27SA_2$ or $27SA_3$ intermediate, leads to $5.8S_L$ rRNA, whose 5′ end, located 6 nt upstream from $B1_S$, is probably the result of an endonucleolytic event. The resulting two 27SB precursors are processed identically. Cleavage at C2 within ITS2 results in formation of 7S and 25.5S pre-rRNA (Geerlings et al., 2000). Maturation of the 7S precursor to 5.8S rRNA is a multistep process involving the exosome complex as well as the 3′ → 5′ exonucleases Rex1p and Rex2p (Mitchell et al., 1996; Allmang et al., 1999; Van Hoof et al., 2000). Rex1p is also re-

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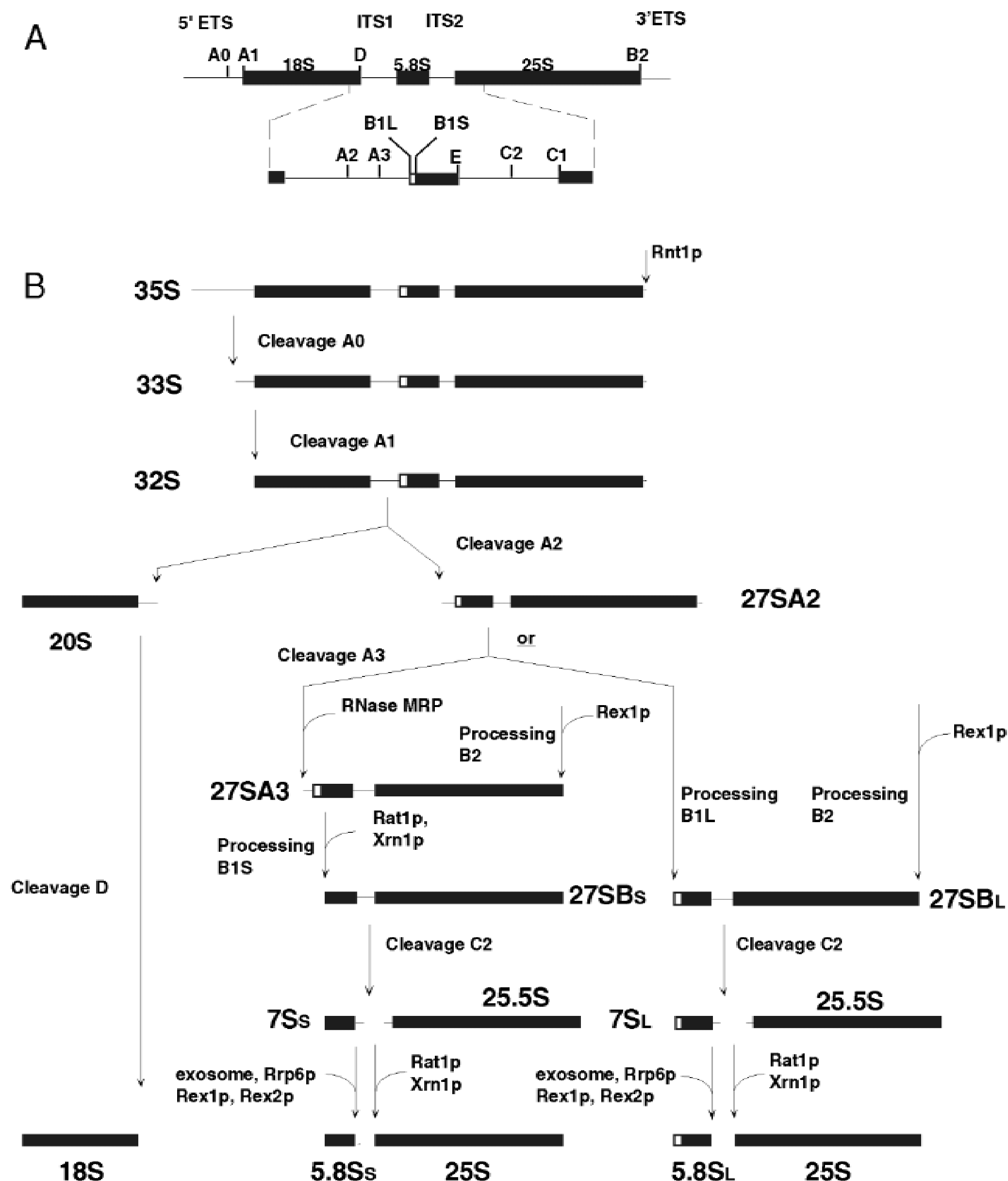

FIGURE 1. Pre-rRNA processing in *Saccharomyces cerevisiae*. **A**: Structure of the pre-rRNA operon depicting the location of the processing sites. **B**: Schematic representation of the processing pathway. Known endo- and exonucleases involved in the various processing steps are indicated.

sponsible for the final stage of 3′-end maturation of 25S rRNA, as well as formation of the mature 3′ end of 5S rRNA (Van Hoof et al., 2000). The 5′ end of mature 25S rRNA again results from exonucleolytic digestion by Xrn1p and Rat1p that removes the remaining portion of ITS2 from the 25.5S pre-rRNA (Geerlings et al., 2000).

In the past decade, a multitude of nonribosomal, *trans*-acting factors has been identified that are crucial for correct and efficient pre-rRNA processing. Surprisingly, however, this collection includes only a few of the enzymes directly involved in the actual nucleolytic events. In particular, the identity of almost all of the processing endonucleases remains to be established (cf. Fig. 1).

Recently, bioinformatics has been used to identify proteins containing a domain similar to the enzymatic core of Mg^{2+}-dependent endonucleases (Dlakic, 2000; Dupressoir et al., 2001; Chen et al., 2002). This class of nucleases was founded by bovine DNase I and apurinic/apyrimidinic (AP) DNA repair endonuclease, as well as the *Escherichia coli* exonuclease III and the human AP endonuclease APE1. The proteins share a set of five highly conserved motifs, which, as shown by site-directed mutagenesis and crystallographic studies, are involved in catalysis and metal-ion binding.

In *Saccharomyces cerevisiae*, this set of nuclease signature motifs occurs at the C-terminus of a group of proteins that include the DNA repair AP endonuclease

encoded by *APN2* and Ccr4p. The latter protein was shown to be involved in cytoplasmic mRNA deadenylation (Tucker et al., 2001), a function that has been confirmed by in vitro experiments, showing that Ccr4p is a $3' \rightarrow 5'$ poly(A) exonuclease (Chen et al., 2002). Three further genes that encode proteins of unknown function showing this signature motif have been called *NGL1* (YOL042), *NGL2* (YMR285), and *NGL3* (YML118; Dupressoir et al., 2001; Chen et al., 2002). All three *NGL* genes are nonessential (Winzeler et al., 1999) and individual deletion has little or no effect on the cellular growth rate in YPD. Because the *NGL* genes encode potential endonucleases, we decided to determine their possible involvement in pre-rRNA processing. Our results show that Ngl2p is essential for the final stage in 3′-end maturation of 5.8S rRNA. We did not detect any defects in pre-rRNA processing in *ngl1*Δ or *ngl3*Δ mutant cells.

RESULTS AND DISCUSSION

To assess the possible involvement of the *NGL* genes in pre-rRNA processing, we isolated total RNA from yeast mutants in which each of the three genes had been individually inactivated and compared it to RNA isolated from the parental strain (all obtained from EUROSCARF). Agarose and polyacrylamide gel electrophoretic analysis did not reveal any peculiarities with regard to 25S and 18S rRNA in these strains (data not shown). However, the *ngl2* deletion strain (*ngl2*Δ) did show a clear abnormality in its 5.8SS rRNA (Fig. 2A). Although *ngl2*Δ cells still produce a short and a long form of 5.8S rRNA in the normal ratio of about 10:1, both species migrate slightly slower than their wild-type counterparts (Fig. 2A, lane 4), indicating an increase in size. The extent of this increase is 5–6 nt as concluded from the fact that the $5.8S_S$ from the *ngl2*Δ cells migrates at about the same position as the $5.8S_L$ from wild-type cells. None of the *ngl* deletion strains shows an altered size of 5S rRNA (Fig. 2A).

We investigated the nature of the 5.8S rRNA extension in the *ngl2*Δ cells by reverse transcription analysis, using an oligonucleotide complementary to the 5′ region of 5.8S rRNA. Figure 2B shows that RNA obtained from the parental or the *ngl2*Δ strain shows identical primer extension stops corresponding to the $B1_S$ and $B1_L$ positions separated by 6 nt. Therefore, the extension of the 5.8S rRNA from the *ngl2*Δ mutant must be located at its 3′ rather than its 5′ end. This was confirmed by northern hybridization using a probe that spans site E, the 3′ end of wild-type 5.8S rRNA (cf. Fig. 1). This probe does not hybridize to mature 5.8S rRNA, but does detect 3′-extended precursors of this rRNA, like the normal 7S pre-rRNA and the 5.8S + 30 and 6S species that are intermediates in 3′-end processing of 5.8S rRNA accumulating in exosome mutants (Mitchell et al., 1996; Allmang et al., 1999). Whereas in the RNA samples from wild-type, *ngl1*Δ, and *ngl3*Δ cells, only faint signals corresponding to the latter intermediates can be detected, RNA isolated from

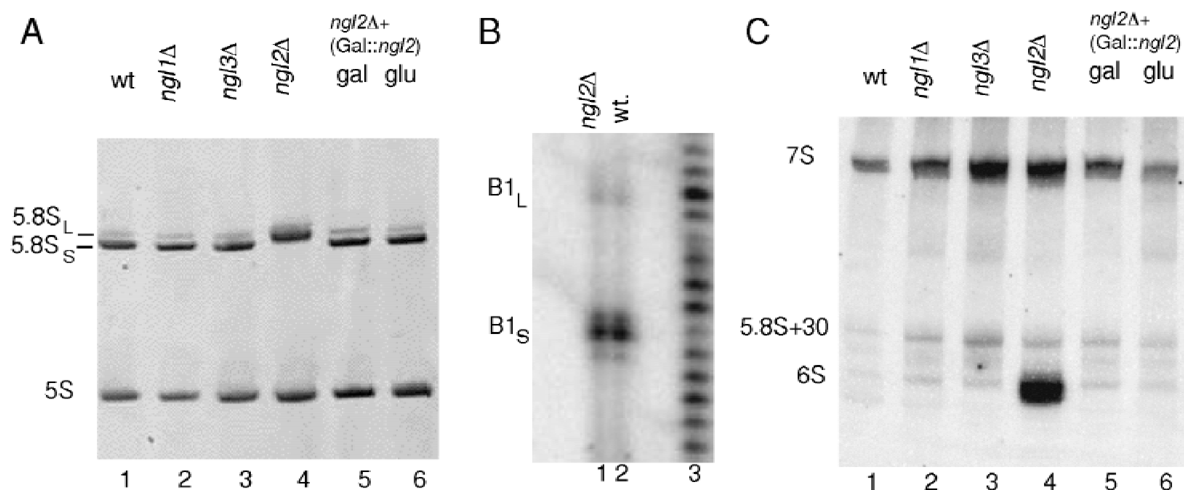

FIGURE 2. Inactivation of *NGL2* causes accumulation of a 3′-extended form of 5.8S rRNA. **A**: Total RNA was isolated from the parental strain BY4741 (wild-type) as well a derivatives in which the *NGL1*, *NGL2*, or *NGL3* had been inactivated by replacement with the kanamycin gene (all strains were obtained from EUROSCARF). RNA was separated on an 8% polyacrylamide gel and stained with EtBr. Lane 1: wild-type BY4741; lane 2: *ngl1*Δ; lane 3: *ngl2*Δ; lane 4: *ngl3*Δ. Lanes 5 and 6 show RNA isolated from *ngl2*Δ cells carrying a plasmid-encoded, wild-type *NGL2* gene under control of the *GAL* promoter after growth on galactose (lane 5) or glucose (lane 6). **B**: The 5′ ends of 5.8S rRNA in wild-type and *ngl2*Δ cells are identical. RNA from BY4741 (wild-type) and *ngl2*Δ cells was subjected to primer extension analysis using a probe complementary to the 5′ region of 5.8S rRNA. The stops corresponding to the mature ends of $5.8S_L$ and $5.8S_S$ rRNA are indicated. A primer extension ladder is used as size marker. Lane 1: wild-type; lane 2: *ngl2*Δ. **C:** The gel shown in **A** was blotted and hybridized with a probe spanning the 3′ end of 5.8S rRNA. The positions of the different intermediates in the conversion of 7S pre-rRNA to 5.8S rRNA previously identified are indicated.

*ngl*Δ*2* cells produces a strong band migrating slightly faster than the 6S pre-rRNA (Fig. 2C, lane 4), which still contains about 8 nt of ITS2 (Mitchell et al., 1996). We, therefore, conclude that Ngl2p acts late in 3′-end maturation of 5.8S rRNA, being essential for the removal of the final 5–6 nt of ITS2. Consistent with this conclusion, we do not see significant alterations in the levels of the 7S or 5.8S + 30 precursors in the *ngl2*Δ cells.

To ascertain that the effect on 5.8S rRNA maturation in the *ngl2*Δ strain was indeed due to the absence of Ngl2p, we cloned the *NGL2* gene behind the *GAL* promoter in pTL26 (Lafontaine & Tollervey, 1996). The resulting plasmid was then introduced into the *ngl2*Δ mutant and the transformants were grown on either galactose or glucose. RNA analysis shows that in galactose-grown cells, the normal, wild-type size of 5.8S rRNA is restored (Fig. 2A, lane 5). Moreover, the signal corresponding to the 3′-extended form of 5.8S rRNA disappears (Fig. 2C, lane 5). This is convincing evidence that Ngl2p is required for proper maturation of the 3′ end of 5.8S rRNA. Surprisingly, analysis of the rRNA from the glucose-grown *ngl2*Δ transformants also shows restoration of the wild-type phenotype for 5.8S rRNA (Fig. 2A, C, lane 6). Because the cells were grown on glucose from the start, we conclude that expression of the *GAL*-driven plasmid-encoded *NGL2* gene, even when repressed, is still sufficient for normal processing of the 5.8S + 5 rRNA. To our knowledge, this is the first example of a *trans*-acting processing factor that is required in only such low amounts.

Formation of the 3′ end of yeast 5.8S rRNA is a multistep process involving a number of different 3′ → 5′ exonucleases acting in sequence (Briggs et al., 1998; Allmang et al., 1999). The 7S precursor formed by the initial cleavage at C2 is first converted into the 5.8S + 30 species, which requires all of the essential components of the exosome. The 5.8S + 30 intermediate is then shortened to the 6S species, still containing ~8 nt of ITS2 by Rrp6p, a component specific to the nuclear exosome (Burkard & Butler, 2000). The 6S precursor appears to be a substrate for the 3′ → 5′ exonucleases Rex1p or Rex2p (Van Hoof et al., 2000), which are not part of the exosome. Our results show that trimming of the 6S precursor stops about 5 nt before the actual 3′ end, and that still another nuclease, Ngl2p, is required to complete the maturation process.

Inactivation of the *NGL2* gene causes a much tighter phenotype than deletion of the *RRP6* and *REX1* + *REX2* genes or depletion of any of the exosome components. Whereas in the latter cases the cells still contain almost wild-type levels of mature 5.8S rRNA (Allmang et al., 1999; Van Hoof et al., 2000), no mature 5.8S rRNA can be detected in the *ngl2*Δ null mutant (Fig. 2). In contrast to the preceding steps, therefore, removal of the final 5–6 nt of ITS2 is absolutely dependent on Ngl2p.

Formation of the mature 3′ end of 25S rRNA also is a multistep process starting with cleavage of the primary Pol I transcript by Rnt1p to give a discrete 3′ end at position +210 downstream from the mature 25S sequence (Kufel et al., 1999). This precursor is then shortened in at least two steps, one of which requires the Rex1p exonuclease (Kempers-Veenstra et al., 1986; Van Hoof et al., 2000), leaving 10–15 nt of the 3′ ETS. These similarities prompted us to investigate the possible involvement of the *NGL* genes in removal of this last remnant of the 3′ ETS, which occurs concomitantly with 5′-end maturation of the 5.8S rRNA (Kufel et al., 1999). Therefore, we analyzed RNA isolated from each of the three *ngl*Δ strains by northern hybridization using a probe overlapping site B2 (cf. Fig. 1). As shown in Figure 3, this probe does not hybridize to mature 25S rRNA (lane 2) but does detect the 3′-extended 25S as well as the 35S and 27SA$_2$ species accumulating in *rnt1*Δ cells (lane 1; Abou-Elela et al., 1996; Kufel et al., 1999). No signal can be detected in any of the three *nlg* deletion strains, however (Fig. 3, lanes 3–5). Thus, either the Ngl proteins are not involved in the final step in 3′-end formation of 25S rRNA or they have redundant functions in this process.

Ngl2p, as well as Ngl1p and Ngl3p, belongs to the Mg^{2+}-dependent *ExoIII/HAP1* nuclease family that also contains yeast Ccr4p (Dupressoir et al., 2001). The latter protein was shown to be a 3′ → 5′ exonuclease (Chen et al., 2002), but the family also encompasses several proteins with endonuclease activity. Biochemical experiments, therefore, are required to determine whether Ngl2p is an endo- or an exonuclease. Also, the

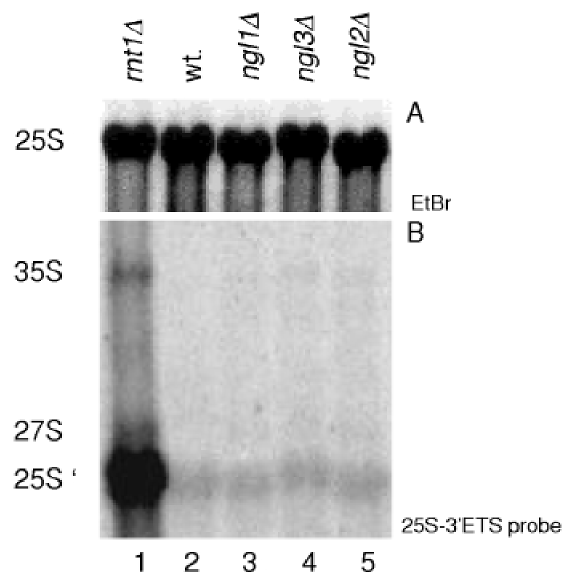

FIGURE 3. Deletion of individual *NGL* genes does not impair 3′-end maturation of 25S rRNA. RNA isolated from the wild-type parental strain (lane 2) and the various *ngl*Δ derivatives (lanes 3–5) was separated on a 1.2% agarose gel, blotted, and hybridized with a probe spanning the mature 3′ end of 25S rRNA. RNA isolated from an *rnt1*Δ strain was used as a control (lane 1). **A**: EtBr staining of 25S rRNA. **B**: Northern hybridization. The positions of the different known processing intermediates are indicated.

subcellular localization of the protein remains to be established. We have noted the presence of a strong consensus nuclear localization signal spanning residues 41–53 of Ngl2p (HKKKGKKGKKSKPI), suggesting that the protein resides in the nucle(ol)us. Nevertheless, cytoplasmic localization of Ngl2p can not be excluded, because it is known that 60S subunits containing 3′-extended forms of 5.8S rRNA can be exported from the nucleus (Briggs et al., 1998).

According to the model of Yeh and Lee (1990) ITS2 folds into a set of helical segments encompassing almost the whole of the spacer (Fig. 4A). The 3′ end of the 5.8S + 30 species, the first distinct intermediate in the maturation of the 7S precursor, is located at the end of the first of these helices (helix II), which includes the terminal 2 nt of 5.8S rRNA. Although this might explain why at this stage processing is taken over by another exonuclease, the exosome apparently is quite capable of progressing through helix IV, which is at least as, if not more, stable than helix II. It seems more likely, therefore, that the handover (Allmang et al., 1999) is necessitated by the decreasing distance to helix I

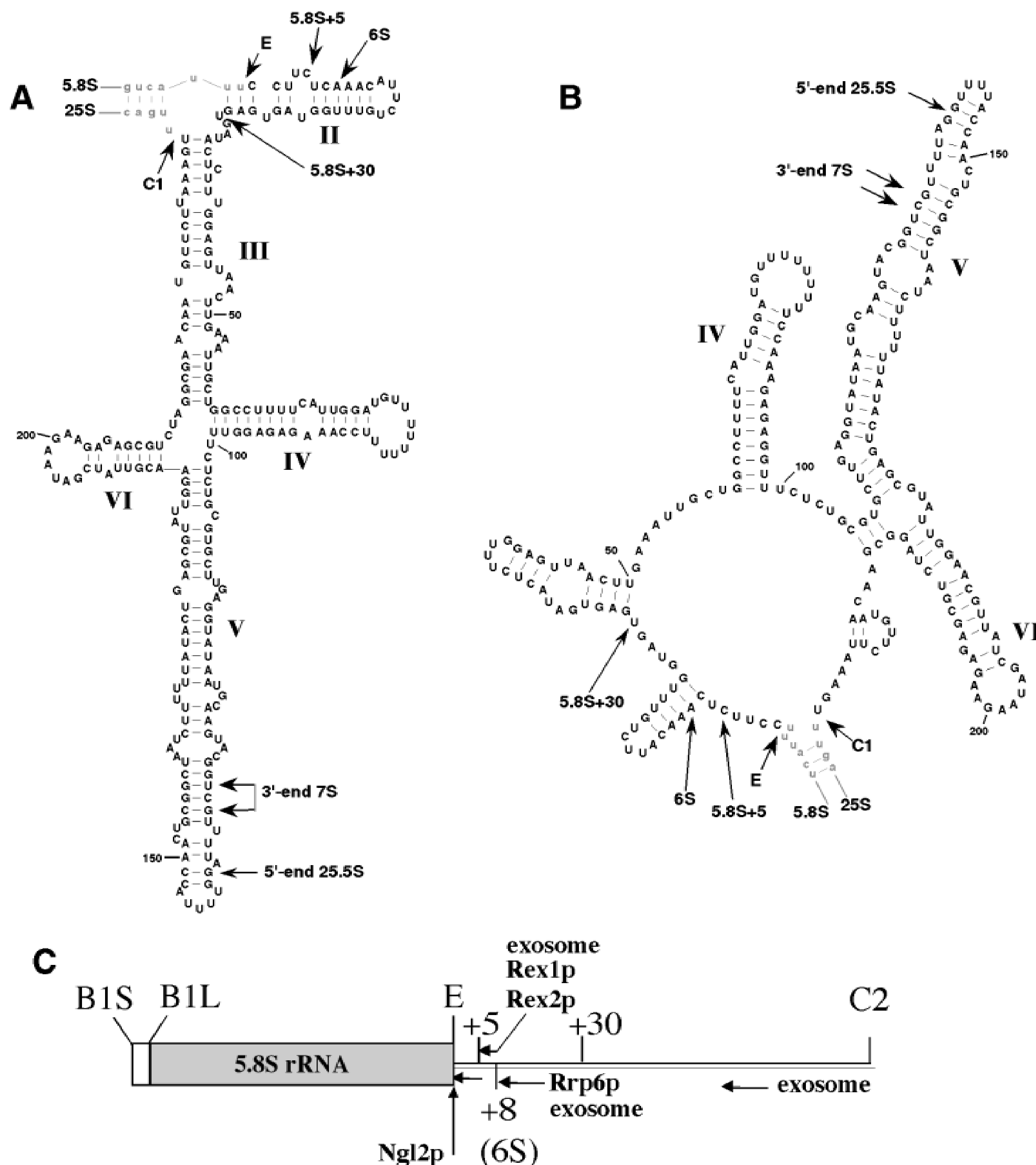

FIGURE 4. Structure of ITS2 and schematic representation of 3′ end processing of 5.8S rRNA. **A:** Secondary structure model of Yeh and Lee (1990). **B**: Model of Joseph et al. (1999). The positions of the 3′ ends of the 7S pre-rRNA, its intermediates in processing to 5.8S rRNA and the 5′ end of 25.5S rRNA are indicated. **C:** The processing pathway used in formation of the mature 3′ end of 5.8S rRNA in *S. cerevisiae*.

formed by the base-paired 3′ and 5′ ends of 5.8S and 25S rRNAs, respectively, and ribosomal proteins associated with this structure. Helix I has indeed been assigned a crucial role in the formation of the large-subunit rRNAs, but its disturbance already blocks the first step in ITS2 processing, that is, cleavage at C2 (Peculis & Greer, 1998; Côté & Peculis, 2001). Thus, it is unclear to what extent this helix is important in 3′-end formation of 5.8S rRNA. The absence of any sequence-specific recognition elements in helix I suggests that it does not act itself as a binding site for r-protein(s). However, this does not exclude the proximity effect as an explanation for the nuclease handover observed in 7S → 5.8S processing.

The fact that removal of the final 30 nt of ITS2 from pre-5.8S rRNA requires two additional handovers, one from Rrp6p to Rex1p/Rex2p and a further one to Ngl2p, might be explained in the same way, as the structure of helix II does not provide any obvious clues as to the necessity for such a complicated series of events. This is even more true for the alternative ITS2 model, proposed by Joseph et al. (1999; see Fig. 4B). An intriguing question remains as to why these final maturation steps are necessary at all. At least the immature 5.8S + 5 species seems to function perfectly well, as the *ngl2Δ* strain does not show a noticeable growth defect. On the other hand, the 5.8S + 30 species, although it can be incorporated into functional ribosomes, does disturb 60S subunit assembly, as evident from the underproduction of these subunits in cells that are defective in further processing of this intermediate (Briggs et al., 1998). Thus, even the 5.8S + 5 species might have subtle effects on ribosome function that, although undetected in a simple growth experiment, might still be disadvantageous.

In summary, the present data suggest the following pathway for the formation of mature 5.8S rRNA from the 7S precursor (Fig. 4C): conversion to the 5.8S + 30 species by the exosome followed by shortening to the 6S (5.8S + 8) precursor by Rrp6p. 3′-end formation of 5.8S rRNA is continued by the exonucleases Rex1p/Rex2p, resulting in the 5.8S + 5 species. The last few nucleotides are then removed by Ngl2p. Only this final step appears to be completely enzyme specific. For all preceding steps, there is a preferred enzyme, but its function can be taken over by (an)other enzyme(s), albeit not with the same efficiency.

MATERIALS AND METHODS

Strains

The parental strain BY4741 (MAT**a**; *his3D1*; *leu2D0*; *met15D0*; *ura3D0*) and its *ngl* deletion derivatives *ngl1Δ* (YOL042w::kanMX4), *ngl2Δ* (YMR285c::kanMX4), and *ngl3Δ* (YML118w::kanMX4) were obtained from EUROSCARF (http://www.uni-frankfurt.de/fb15/mikro/euroscaf/index.html). Strain *rnt1Δ* (MAT**a**, *his3*, *leu2*, *trp1*, *ura3*, *lys2Δ*, *rnt1::HIS3*) was obtained from Sherif Abou Elela, University of Sherbrooke, Canada. Strains were grown at 30 °C in YPD or in minimal medium containing either glucose or galactose. The *rnt1Δ* strain was grown at 23 °C.

Construction of a *GAL*-driven *NGL2* gene

The open reading frame of *NGL2* was amplified by PCR using as the 5′ primer GGATCCATCGATATATGACACAAGACAAAGAAGTC and as the 3′ primer TATTAAGGGCCCGCAAGATATTTATGCACGACA. The underlined *Cla*I and *Apa*I restriction sites were used to clone the PCR fragment into pTL26 (Lafontaine & Tollervey, 1996). The resulting plasmid was introduced into the *ngl2Δ* strain using LiAc.

Northern blot and primer extension analysis

Yeast strains were grown at 30 °C and total RNA was isolated from 10 OD_{660} units of cells. For northern analysis, 5 μg of RNA were separated on either a 1.2% (w/v) agarose or an 8% polyacrylamide/TBE/urea gel. The gels were blotted and hybridized according to standard protocols with different [^{32}P]ATP-labeled probes. The presence of 3′-extended forms of 5.8S rRNA was determined by hybridization at 37 °C of a probe spanning the 3′ end of 5.8S rRNA having the sequence 5′-GAATGTTTGAGAAGGAAATGACGCTC-3′. The presence of 3′-extended 25S rRNA species was analyzed by hybridization at 32 °C with a probe having the sequence 5′-GAAAGAAATAAAAAACAAATCAGACAAC-3′ that spans the mature 3′ end of 25S rRNA.. Primer extension was performed by standard procedures using 10 μg of RNA and a primer complementary to the 5′ region of 5.8S rRNA (5′-CTGCGTTCTTGATCGATGCG-3′).

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