1684–1691 *Nucleic Acids Research, 2000, Vol. 28, No. 8*

Degradation of ribosomal RNA precursors by the exosome

Christine Allmang, Philip Mitchell, Elisabeth Petfalski and David Tollervey*

Institute of Cell and Molecular Biology, Swann Building, King's Buildings, The University of Edinburgh, Mayfield Road, Edinburgh EH9 3JR, UK

Received February 2, 2000; Revised and Accepted March 2, 2000

ABSTRACT

The yeast exosome is a complex of 3′→5′ exonucleases involved in RNA processing and degradation. All 11 known components of the exosome are required during 3′ end processing of the 5.8S rRNA. Here we report that depletion of each of the individual components inhibits the early pre-rRNA cleavages at sites A_0, A_1, A_2 and A_3, reducing the levels of the 32S, 20S, $27SA_2$ and $27SA_3$ pre-rRNAs. The levels of the 27SB pre-rRNAs were also reduced. Consequently, both the 18S and 25S rRNAs were depleted. Since none of these processing steps involves 3′→5′ exonuclease activities, the requirement for the exosome is probably indirect. Correct assembly of *trans*-acting factors with the pre-ribosomes may be monitored by a quality control system that inhibits pre-rRNA processing. The exosome itself degrades aberrant pre-rRNAs that arise from such inhibition. Exosome mutants stabilize truncated versions of the 23S, 21S and A_2-C_2 RNAs, none of which are observed in wild-type cells. The putative helicase Dob1p, which functions as a cofactor for the exosome in pre-rRNA processing, also functions in these pre-rRNA degradation activities.

INTRODUCTION

Ribosome biogenesis in eukaryotes mainly occurs in a specialized nuclear compartment, the nucleolus. The synthesis of rRNAs is not achieved by simple transcription of the individual rRNA species but requires a complex series of post-transcriptional processing steps. The mature 5.8S, 18S and 25S rRNAs are transcribed by RNA polymerase I as a single precursor, the 35S pre-rRNA. In addition to the mature rRNA sequences, this contains two external transcribed spacers, the 5′-ETS and 3′-ETS, and two internal transcribed spacers, ITS1 and ITS2 (Fig. 1).

In *Saccharomyces cerevisiae*, a large number of *trans*-acting factors are required for the removal of these spacers (reviewed in 1,2). Some of these factors have been characterized as nucleases: either endonucleases (RNase MRP, Rnt1p), 5′→3′ exonucleases (Rat1p; Xrn1p) or 3′→5′ exonucleases (the exosome complex). However, the majority of the *trans*-acting factors do not appear to participate directly in rRNA processing. These include small nucleolar ribonucleoprotein (snoRNP) particles and putative RNA helicases, both of which may act to modify the structure of the pre-rRNA, as well as a large number of factors for which no clear function is known. These tend to be classed as putative ribosome assembly factors, but for only a few factors is there clear evidence for such a role (reviewed in 1,2). It is assumed that pre-rRNA processing is inhibited in the absence of correct assembly of the pre-ribosomal particles in order to prevent the synthesis of defective ribosomes. Supporting this model, depletion or mutation of several ribosomal proteins was shown to inhibit pre-rRNA processing (3,4). The requirement for correct assembly could be envisaged to be active or passive. In the latter case, the processing enzymes might only be able to recognize their substrates if correctly assembled/folded in the pre-ribosomal particle. However, it appears more likely that quality control involves an active system which detects the absence of processing components and inhibits processing. An active system of quality control is best exemplified by the 18S rRNA dimethylase Dim1p. This is required both for rRNA methylation and processing, but these functions can be separated by specific mutations (5). Also consistent with an active mechanism was the, initially surprising, observation that mutation of many factors required for 60S subunit accumulation had strong effects on early pre-rRNA processing at sites A_0, A_1 and A_2 on the pathway of 40S synthesis (reviewed in 1,2).

Analyses of 3′ end maturation of the 5.8S rRNA led to the identification of the exosome, a complex of 3′→5′ exoribonucleases (6,7). The nuclear form of the exosome complex is composed of 11 components, all of which except Csl4p have either been shown to be 3′→5′ exoribonucleases *in vitro*, or are predicted to have this activity based on sequence homology (7–9; reviewed in 10). Six of the exosome components (Rrp41p, Rrp42p, Rrp43p, Rrp45p, Rrp46p and Mtr3p), are homologous to the *Escherichia coli* exonuclease RNase PH. Rrp44p/Dis3p is homologous to *E.coli* RNase R (a member of the RNase II family) and Rrp6p to *E.coli* RNase D. Rrp4p and Rrp40p are homologous to each other and contain a predicted S1 RNA-binding motif, as does Csl4p (8,11). Recombinant Rrp4p, Rrp41p, Rrp44p and Rrp6p were demonstrated to have 3′→5′ exonuclease activity *in vitro* (7,9). All components of the exosome are essential for viability (7,8), with the exception of Rrp6p the absence of which causes temperature-sensitive (ts) lethality (12). Nuclear and cytoplasmic forms of the complex exist, which can be distinguished by the presence of Rrp6p exclusively in the nuclear complex (8). The cytoplasmic

*To whom correspondence should be addressed. Tel: +44 131 650 7092; Fax: +44 131 650 7040; Email: d.tollervey@ed.ac.uk

The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors

Table 1. Yeast strains used in this work

Strain	Genotype	Reference
YDL401	*MATa his3Δ200 leu2Δ1 trp1 ura3-52 gal2 galΔ108*	38
P79	*MATa ade1-100 his4-519 leu2-3, 112 ura3-52 GAL10::protA-rrp4*	7
P147	as YDL401 but *GAL10::rrp40*	8
P118	as YDL401 but *GAL10::prot.A-RRP41*	38
P106	as YDL401 but *GAL10::rrp42*	7
P107	as YDL401 but *GAL10::rrp43*	7
P108	as YDL401 but *GAL10::rrp44*	7
YCA20	as YDL401 but *GAL10::rrp45*	8
YCA21	as YDL401 but *GAL10::rrp46*	8
YCA12	*MATa ade2-1 his3-Δ200 leu2-3, 112 trp1-1 ura3-1 can1-100 RRP6::Kl TRP1*	8
YCA31	as P118 but *RRP6::Kl TRP1*	14
YTK100	*MATa mtr3-1 ura3-52*	39
P170	as YDL401 but *GAL10::CSL4*	8
GAL::DOB1	*MATa ura3-1 ade2-1 his3-11,15 leu2-3 112 trp1-1 dob1::HIS3MX6* + [pAS24-DOB1]	16
JH84	*MATa leu2-3, 112 ura3-52 his3-Δ200 ade2-1 can1-100 UASgal::snr17A-URA3 snr17B::LEU2*	20

complex was shown to function in mRNA turnover (13) and mRNA deadenylation (P.Mitchell and D.Tollervey, unpublished data). In addition to its role in 3′ end synthesis of 5.8S rRNA, the nuclear exosome also functions in pre-snRNA and pre-snoRNA processing (14,15), as well as nuclear pre-mRNA turnover (C.Bousquet-Antonelli, C.Presutti and D.Tollervey, unpublished data) and degradation of the excised 5′-ETS region of the pre-rRNA (14,16). The exosome therefore functions in many aspects of RNA metabolism. The 3′ end processing of 5.8S rRNA, degradation of the 5′-ETS and normal pre-snoRNA processing each require the putative RNA helicase Dob1p/Mtr4p (16). Dob1p therefore appears to function as a cofactor of the exosome in many of its nuclear functions in pre-rRNA processing and degradation.

We report here that in addition to their specific roles in the 3′ maturation of the 5.8S rRNA, mutations in all components of the exosome inhibit other pre-rRNA processing steps, as was recently reported for Rrp43p (17).

MATERIALS AND METHODS

Strains

Growth and handling of *S.cerevisiae* were by standard techniques. GAL-regulated strains were pre-grown in RSG medium, containing 2% raffinose, 2% sucrose, 2% galactose, 0.67% yeast nitrogen base (DIFCO), and harvested at intervals following a shift to medium containing 2% glucose and 0.67% yeast nitrogen base. Temperature-sensitive strains were first grown in YPD at 23°C and harvested at intervals after a shift to the non-permissive temperature (37°C). Yeast strains used in this study are listed in Table 1.

RNA extraction, northern hybridization and primer extension

RNA was extracted as described previously (18). For high molecular weight RNA analysis, 8 µg of total RNA was separated on a 1.2% agarose gel containing formaldehyde and transferred for northern hybridization as described previously (18). Primer extension was performed as described previously (19) on 4 µg of total RNA using primer 033.

For pre-rRNA and rRNA analysis the following oligonucleotides were used:

001, 5′-CCAGTTACGAAAATTCTTG;
002, 5′-GCTCTTTGCTCTTGCC;
003, 5′-TGTTACCTCTGGGCCC;
004, 5′-CGGTTTTAATTGTCCTA;
005, 5′-ATGAAAACTCCACAGTG;
006, 5′-AGATTAGCCGCAGTTGG;
007, 5′-CTCCGCTTATTGATATGC;
008, 5′-CATGGCTTAATCTTTGAGAC;
013, 5′-GGCCAGCAATTTCAAGTTA;
017, 5′-GCGTTGTTCATCGATGC;
020, 5′-TGAGAAGGAAATGACGCT;
026, 5′-CCAGATAACTATCTTAAAAG;
033, 5′-CGCTGCTCACCAATGG;
041, 5′-CTACTCGGTCAGGCTC.

RESULTS

Exosome mutants affect early pre-rRNA processing steps

Processing of the pre-rRNA was analyzed in strains carrying mutations in the 11 known components of the exosome. *GAL*-regulated constructs were used to deplete Rrp4p, Rrp40p, Rrp41p, Rrp42p, Rrp43p, Rrp44p, Rrp45p, Rrp46p and Csl4p, while ts-lethal *mtr3-1* and *rrp6*-Δ mutations were used to assess the roles of Mtr3p and Rrp6p. The effects of each of the exosome mutants was analyzed by northern hybridization and compared to the isogenic wild-type strain (WT). The wild-type pre-rRNA processing pathway is shown in Figure 1; processing pathways seen in the exosome mutants are shown in Figure 2. Examples (*GAL::rrp40*, *GAL::cls4*, *rrp6*-Δ,

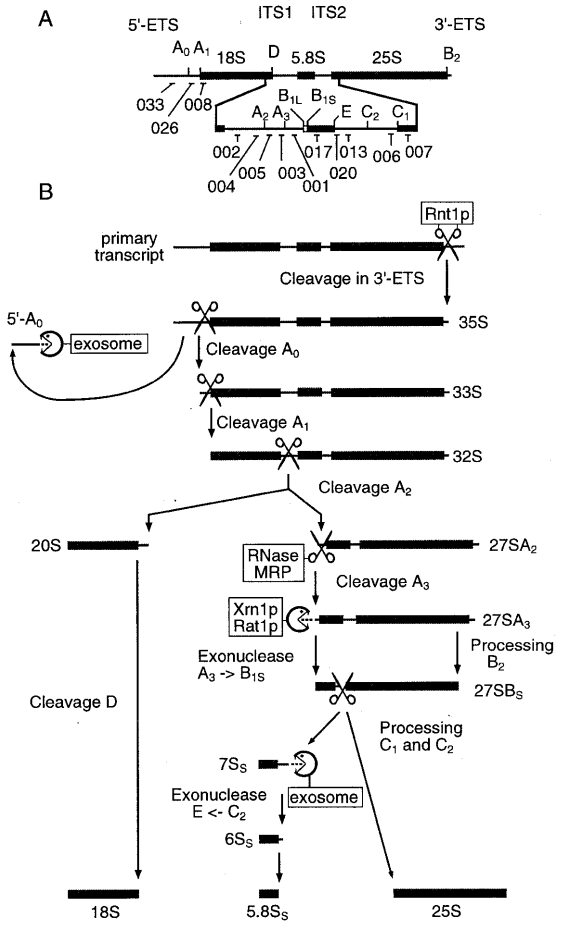

Figure 1. Structure and processing of the pre-rRNA in *S.cerevisiae*. (**A**) Structure of the 35S pre-rRNA with the location of oligonucleotide probes used for hybridization and primer extension. (**B**) Major pre-rRNA processing pathway. The primary transcript is processed by a series of sequential cleavages into the mature 18S, 5.8S and 25S rRNA. Initial cleavage in the 3′-ETS by Rnt1p yields the 35S pre-rRNA. The snoRNP-dependent cleavage at site A_0 in the 5′-ETS then generates 33S pre-rRNA, which is rapidly cleaved at site A_1, producing the 32S pre-rRNA. Cleavage at site A_2 in ITS1 then splits the 32S pre-rRNA into the 20S and $27SA_2$ pre-rRNAs, destined to form the RNAs of the small and large ribosomal subunit, respectively. The 5′ part of the molecule, 20S pre-rRNA, is exported to the cytoplasm and endonucleolytically cleaved at site D to generate mature 18S rRNA. The $27SA_2$ pre-rRNA is processed by two alternative pathways, giving rise to two forms of 5.8S rRNA, the major short form $5.8S_S$ and a minor long form $5.8S_L$. For simplicity, only the major pathway to $5.8S_S$ is shown. In this pathway, $27SA_2$ is cleaved by RNase MRP at site A_3 to generate $27SA_3$, which is processed by the 5′→3′ exonucleases Rat1p and Xrn1p to site B_{1S}, the 5′ end of the $27SB_S$ pre-rRNA and mature $5.8S_S$ rRNA. In the alternative pathway, processing occurs at site B_{1L}, the 5′ end of $27SB_L$ and $5.8S_L$ rRNA. The subsequent processing of both 27SB species is identical. Processing at sites C_1 and C_2 releases the mature 25S rRNA and the 7S pre-rRNA. The 7S pre-rRNA is 3′ processed by the exosome complex, generating the 6S pre-rRNA, which is then trimmed to the mature 5.8S. The exosome also degrades the excised spacer region from the 5′ end of the primary transcript to site A_0.

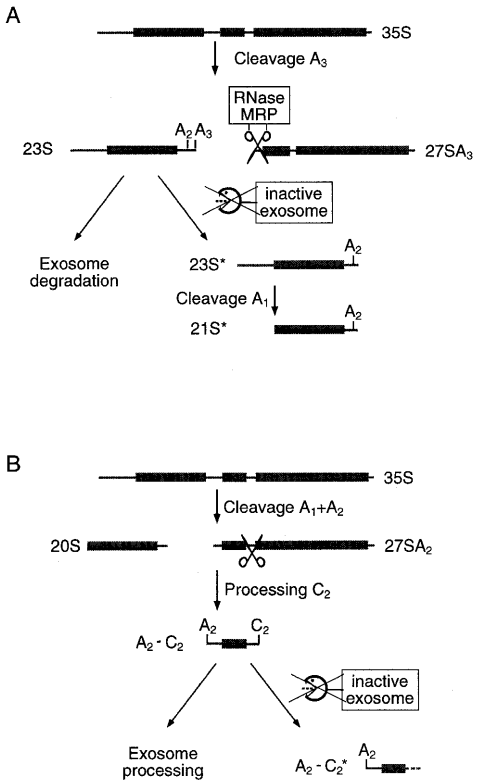

Figure 2. Pre-rRNA processing and degradation in exosome mutants. The inactivation of any of the exosome components results in the inhibition of the early pre-rRNA cleavages. The major intermediates observed in exosome mutants result from (**A**) inhibition of cleavage at sites A_0–A_2 or (**B**) inhibition of cleavage at site A_3 in ITS1. (A) The 23S RNA extends from the 5′ end of the primary transcript to site A_3 and is detected in strains mutant for several snoRNAs and many other processing components. The exosome mutants are unusual in accumulating the 23S* RNA (a slightly shortened form of 23S) and the 21S*, the product of cleavage of this RNA at site A_1. (B) The A_2–C_2 RNA extends from site A_2 in ITS1 to site C_2 in ITS2. Mutations in RNase MRP components also inhibit A_3 cleavage and lead to the synthesis of forms of the 5.8S rRNA that are 5′ extended to site A_2 but 3′ processed by the exosome to site D (the mature 3′ end of the 5.8S rRNA). The exosome mutants are unusual in accumulating the A_2–C_2* species that extend to heterogeneous sites in ITS2, between C_2 and the 3′ end of the 5.8S rRNA. The processing pathways shown in (A) and (B) are mutually exclusive, showing that the block in processing at A_0–A_2 is not complete in exosome mutants.

Gal::rrp41, *GAl::rrp4* and *mtr3-1*) are shown in Figures 3–5. As a control, a *GAL::U3* strain is shown in Figure 4; depletion of the U3 snoRNA strongly inhibits pre-rRNA processing at sites A_0, A_1 and A_2 (20).

Characteristic pre-rRNA processing defects were seen upon depletion of exosome components. The 35S pre-rRNA was accumulated while the 32S pre-rRNA, the product of A_1 cleavage, was depleted in most mutants. The 20S and $27SA_2$ pre-rRNAs, which are generated by cleavage of the 32S pre-rRNA at site A_2 in ITS1, were also depleted. These results indicate that processing at sites A_1 and A_2 was inhibited in exosome mutants. The level of the 27SB RNA was also reduced, although to a lesser extent. As a consequence the levels of 18S and 25S rRNA were reduced, although not to the same extent in all the exosome mutants (Figs 3–5).

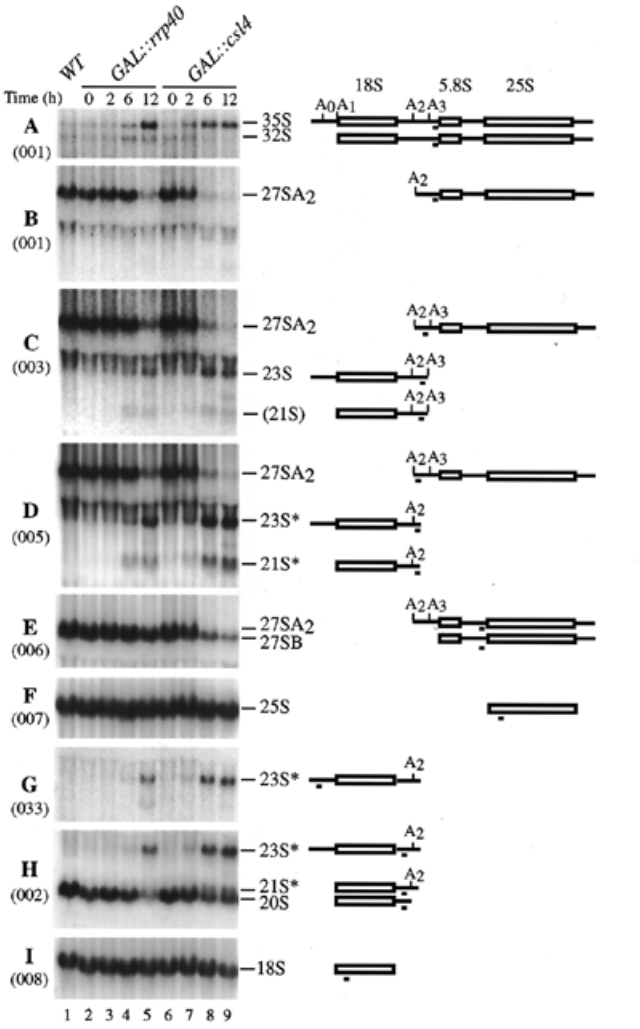

Figure 3. Northern analysis of pre-rRNA processing in exosome mutants. RNA was extracted from *GAL::rrp40* and *GAL::csl4* strains following transfer from permissive, RSG medium to repressive, glucose medium at 30°C for the times indicated. (**A**) and (**B**) Hybridization with probe 001, complementary to ITS1 downstream of site A_3. (**C**) Hybridization with probe 003, complementary to ITS1 upstream of site A_3. (**D**) Hybridization with probe 005, complementary to ITS1 downstream of site A_2. (**E**) Hybridization with probe 006, complementary to ITS2. (**F**) Hybridization with probe 007, complementary to 25S rRNA. (**G**) Hybridization with probe 033, complementary to 5′ETS. (**H**) Hybridization with probe 002, complementary to ITS1 upstream of site A_2. (**I**) Hybridization with probe 008, complementary to 18S rRNA. Probe names are indicated in parentheses on the left. Lane 1, wild-type, 0 h; lanes 2–5, *GAL::rrp40*, 0, 2, 6 and 12 h; lanes 6–9, *GAL::csl4*, 0, 2, 6 and 12 h. The pre-rRNA and rRNA species are schematically represented on the right; rectangles represent the mature rRNA and thin lines the transcribed spacers. The hybridization sites of the probes are indicated on the diagram. The bands labeled 23S* and 21S* are a mixture of the full-length 21S and 23S and the truncated * species, with the truncated forms predominating.

Aberrant 23S and 21S RNAs were accumulated in the exosome mutants. These are generated by cleavage at site A_3 in ITS1 in the absence of prior processing at sites A_0–A_2 (21). The 23S RNA extends from the 5′ end of the 35S primary transcript to site A_3 and 21S extends from site A_1 to site A_3 (Fig. 2A). The level of the 33S pre-rRNA, the normal product of cleavage at site A_0, cannot readily be assessed by northern hybridization due to its low abundance and similar size to the 32S pre-rRNA. However, the accumulation of the 35S pre-rRNA and appearance of the 23S RNA indicate that A_0 cleavage is also inhibited. Similar phenotypes were observed for all the essential exosome mutants, as well as for the temperature-sensitive lethal *rrp6*-Δ mutation at non-permissive temperature. Double mutant strains lacking both Rrp6p and Rrp41p have been reported to show stronger phenotypes for some processing activities, such as 3′ end synthesis of snoRNAs (14). However, no significant difference in pre-rRNA processing could be observed in the *GAL::rrp41/rrp6*-Δ double mutant compared to *GAL::rrp41* or *rrp6*-Δ single mutant strains (Fig. 4, lanes 6–13).

We conclude that processing at sites A_0, A_1 and A_2 is inhibited in each of the exosome mutants. It is notable that processing at each of these sites is by endonucleolytic cleavage (22–24). No endonuclease activity was observed to be associated with the purified exosome (7) and its role in these cleavages is very likely to be indirect.

The exosome degrades aberrant pre-rRNA processing intermediates

The 23S RNA has previously been seen in many strains defective in pre-rRNA processing at sites A_0, A_1 and A_2, and the 21S has also been observed. However, in the exosome mutants, truncated versions of these species were detected (designated 23S* and 21S* in Figs 3–5). These give rise to a stronger signal with probe 005 than with probe 003 relative to the $27SA_2$ pre-rRNA, which hybridizes to both probes. Probe 005 is located directly downstream of site A_2 while probe 003 is located 53 nt further 3′, immediately upstream of site A_3 (Fig. 1A), indicating that the 23S* and 21S* RNAs represent short truncations of the 23S and 21S RNAs. Depletion of individual exosome components resulted in variations in the levels of these RNAs. For example, *GAL::rrp4* shows only a partial loss of $27SA_2$ and 20S pre-rRNA (Fig. 5B and G, lanes 6–8) but strongly accumulates 23S* and, in particular, 21S* compared to *mtr3-1* (Fig. 5C, lanes 6–10). In most mutants that affect early cleavages the 23S intermediate is degraded, preventing the synthesis of 18S rRNA (2). This degradation is likely to be carried out by the exosome since all exosome mutants stabilize the truncated 23S* and 21S* RNAs, whereas these RNAs were not detected in other strains defective for the early cleavages. This is shown for the *GAL::U3* strain (Fig. 4, lanes 14–16); the 23S RNA signals obtained with probes 005 and 003 are equivalent when compared to the signal for $27SA_2$.

An additional intermediate, the 17S′ RNA, was seen in some but not all exosome mutants. This appears similar to the 17S′ species mapped in pre-rRNAs carrying mutations at both the A_2 and A_3 sites (25), which extended from the 3′ end of the 5.8S rRNA to heterogeneous sites located within the 5′ region of the 18S rRNA sequence. The same species were observed in strains defective in A_3 cleavage due to mutations in RNase MRP or Rrp5p, and were proposed to result from the activation of a 5′→3′ pre-rRNA degradation pathway (25,26).

The *rrp6*-Δ strain is impaired in growth at all temperatures, and is lethal at 37°C. At the permissive temperature (25°C) (Fig. 4, lane 2) the *rrp6*-Δ strain accumulated the 23S*, 21S* and 17S′ RNAs (Fig. 4D and G), but the levels of the $27SA_2$

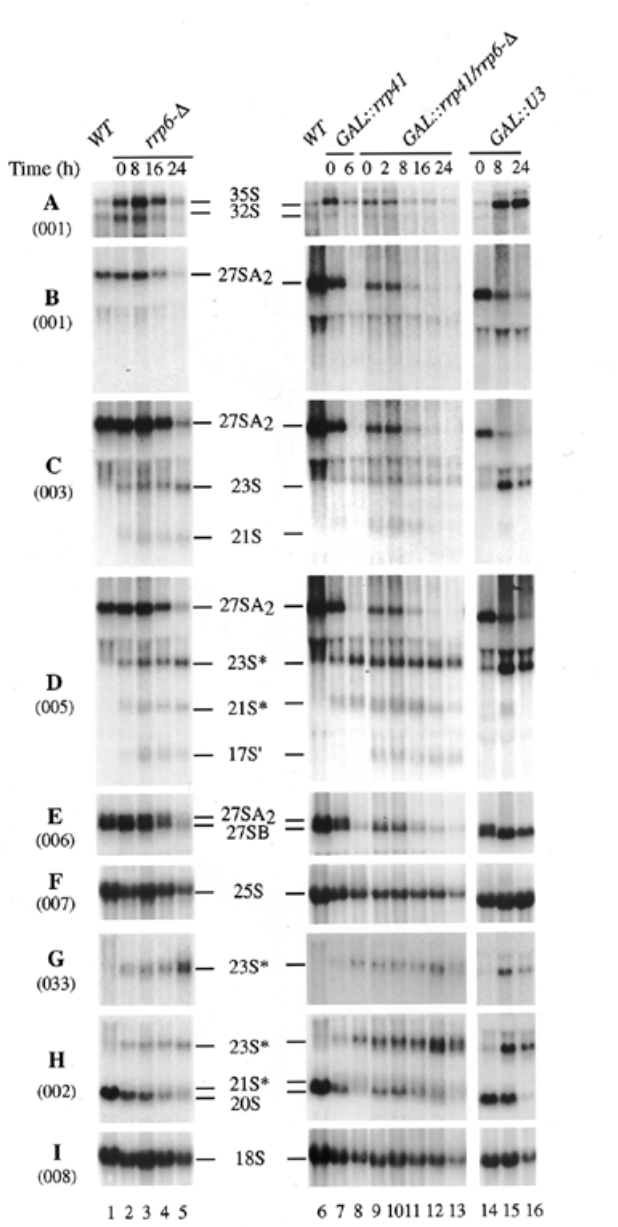

Figure 4. Northern analysis of pre-rRNA processing in single and double mutants. RNA was extracted from the *rrp6*-Δ strain grown in YPD medium after shift from permissive temperature (30°C; 0 h) to non-permissive temperature (37°C) for the times indicated. *GAL::rrp41* strains were grown as described in Figure 2. Probe names are indicated in parentheses. (**A**) and (**B**) Hybridization with probe 001. (**C**) Hybridization with probe 003. (**D**) Hybridization with probe 005. (**E**) Hybridization with probe 006. (**F**) Hybridization with probe 007. (**G**) Hybridization with probe 033. (**H**) Hybridization with probe 002. (**I**) Hybridization with probe 008. Lanes 1 and 6, wild-type; lanes 2–5, *rrp6*-Δ, 0, 8, 16 and 24 h; lanes 7 and 8, *GAL::rrp41*, 0 and 6 h; lanes 8–13, *GAL::rrp41/rrp6*-Δ, 0, 2, 8, 16 and 24 h; lanes 14–16, *GAL::U3*, 0, 8 and 24 h.

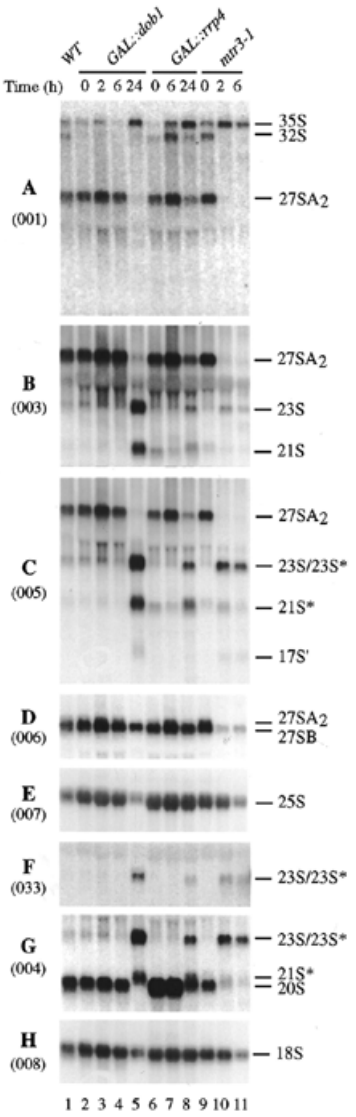

Figure 5. Depletion of Dob1p or exosome components has similar effects on pre-rRNA processing. Growth of *GAL*-regulated and ts mutants was as described in Figures 2 and 3. Probes are located as indicated in Figure 2. (**A**) Hybridization with probe 001. (**B**) Hybridization with probe 003. (**C**) Hybridization with probe 005. (**D**) Hybridization with probe 006. (**E**) Hybridization with probe 007. (**F**) Hybridization with probe 033. (**G**) Hybridization with probe 004. (**H**) Hybridization with probe 008. Lane 1, wild-type; lanes 2–5, *GAL::dob1*, 0, 2, 6 and 24 h; lanes 6–8, *GAL::rrp4*, 0, 6 and 24 h; lanes 9–11, *mtr3-1*, 0, 2 and 6 h at 37°C.

and 27SB pre-rRNAs were unaltered. Clear depletion of these pre-rRNAs (Fig. 4D and E) and the 18S and 25S rRNA (Fig. 4F and I) was observed at late times after transfer to 37°C (Fig. 4, lane 5). This is, however, unlikely to be the cause of the lethality in *rrp6*-Δ strains since growth is strongly inhibited before substantial depletion of the pre-rRNA or mature rRNA occurs. These data indicate that the requirements for Rrp6p in pre-rRNA degradation and processing are at least partially separable.

The putative RNA helicase Dob1p functions with the exosome in pre-rRNA processing and 23S degradation

3′ end processing of the 5.8S rRNA, as well as degradation of the excised 5′-ETS region, requires a member of the DEAD-box family of putative RNA helicases Mtr4p/Dob1p (Fig. 5, lane 5) (16). A *GAL::dob1* strain genetically depleted of Dob1p strongly accumulated the 35S pre-rRNA, as well as the 23S, 21S and 17S′ RNAs (Fig. 5, lane 5), while the 32S, 20S and $27SA_2$ pre-rRNAs were depleted. As a consequence the levels of mature 18S and 25S rRNA are reduced (Fig. 5E and H). Notably, the 23S and 21S RNAs were accumulated on depletion of Dob1p, rather than the truncated 23S* or 21S* intermediates, since the signals obtained with probes adjacent to sites A_2 (005) and A_3 (003) are equivalent when compared to the signal obtained for $27SA_2$. We conclude that depletion of Dob1p has a stronger stabilizing effect on the A_3-cleaved RNAs than does depletion of individual components of the exosome. This is similar to the relative effects of depletion of Dob1p and exosome components on the processing of the 7S pre-rRNA and excised 5′-ETS-A_0 fragment; in each case the full-length RNA predominates on depletion of Dob1p while partially truncated fragments predominate on depletion of exosome components (14,16). It is not clear whether the primary role of Dob1p is to unfold the pre-rRNA secondary structures or to target the exosome to its substrates.

The exosome is required for efficient processing at site A_3

The $27SA_3$ pre-rRNA is not normally detected by northern hybridization due to its very low abundance. The exosome mutants were therefore all analyzed by primer extension from oligo 013, which hybridizes within the 5′ region of ITS2 (Fig. 1A). Primer extension results for some mutants are shown in Figure 6. The primer extension stop at site A_3 was strongly reduced in most of the exosome mutants (Fig. 6B), indicating a reduced level of the $27SA_3$ pre-rRNA. We conclude that, despite the appearance of the 23S and 21S RNAs, cleavage at site A_3 is actually inhibited in the exosome mutants (Fig. 2B). The stabilization of the 23S* and 21S* RNAs is therefore likely to be greater than it appears from their steady-state levels.

Heterogeneous levels of the primer extension stops at sites B_{1L} and B_{1S} were observed (Fig. 6A). Primer extension detects both the 27SB pre-rRNAs and 3′ extended forms of the 5.8S rRNA, since these have the same 5′ ends. The observed alterations presumably reflect the combination of reduced 27SB levels in the mutants (Figs 3–5; the same relative amounts of RNA were used for northern hybridization and primer extension) and the accumulation of 3′ extended 5.8S rRNA seen in all exosome mutants.

A primer extension stop at site A_2 was observed in the *rrp6*-Δ mutant, consistent with the unaffected level of the $27SA_2$ pre-rRNA in this strain at permissive temperature (Fig. 4). More unexpected was the detection of a strong primer extension stop at site A_2 in the *GAL::csl4* strain (Fig. 6A). Clear primer extension stops were also observed in the *GAL::rrp4*, *GAL::rrp41*, *GAL::rrp44* and *GAL::rrp45* strains (data not shown). In other experiments, somewhat stronger primer extension stops at A_2 were seen for the *GAL::rrp40* and *GAL::dob1* strains than in Figure 6A (data not shown). The A_2 primer extension data appeared inconsistent with the loss of the $27SA_2$ pre-rRNA detected by northern hybridization (Figs 3–5). The primer extension signal could be accounted for if an RNA cleaved at site A_2 but shorter than the $27SA_2$ pre-rRNA was accumulated. No such RNA was detected in our northern analysis of high molecular weight RNAs, prompting us to re-examine low molecular weight RNAs in search of such a species (Fig. 7). The mutants in which the A_2 primer extension stop persisted, *GAL::rrp4*, *GAL::rrp40*, *GAL::rrp41*, *GAL::rrp44*, *GAL::rrp45* and *GAL::csl4*, but not other exosome mutants, accumulated a series of discrete RNA species larger than the 7S pre-rRNAs (shown for *GAL::csl4* in Fig. 7). As described previously, the Csl4p-depleted strain showed an accumulation of 3′ extended forms of the 5.8S rRNA that extended in a ladder up to the 7S pre-rRNA at site C_2 (Fig. 7A), a characteristic defect in exosome mutants (8). The RNAs larger than 7S could be detected with a probe 3′ to site A_2 (probe 005; Fig. 7D), but not with a probe 5′ to site A_2 (probe 002; Fig. 7E). They were also not detected with a probe hybridizing 3′ to site C_2 (data not shown). From their electrophoretic mobilities and hybridization patterns, we conclude that the largest species (A_2–C_2 in Fig. 7) extends from site A_2 to C_2 while the shorter RNAs (A_2–C_2* in Fig. 7) extend from A_2 to sites between the 3′ end of 5.8S rRNA and C_2, most likely terminating at the same sites as the 3′ extended forms of 5.8S rRNA seen in the exosome mutants (Fig. 7A). Consistent with this interpretation, the strain depleted of Dob1p showed some accumulation of the full-length A_2–C_2 RNA but not the A_2–C_2* species (data not shown) and also accumulated 5.8S that was 3′ extended to site C_2, rather than to intermediate sites (14,16). We conclude that

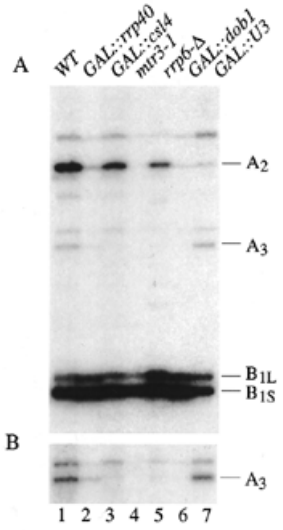

Figure 6. Primer extension analysis through ITS1 in exosome mutants. Primer extension was performed using an oligonucleotide (033) which hybridizes within ITS2. (**A**) Primer extension stops at sites A_2, A_3, B_{1S} and B_{1L}. (**B**) Stronger exposure of primer extension stop at site A_3. RNA extracted from GAL-regulated constructs or ts mutants, were collected after transfer to repressive glucose medium or 37°C, respectively, for the following lengths of time: lane 1, wild-type, 0 h; lane 2, *GAL::rrp40*, 12 h; lane 3, *GAL::csl4*, 12 h; lane 4, *mtr3-1*, 6 h at 37°C; lane 5, *rrp6*-Δ at 25°C; lane 6, *GAL::dob1*, 24 h; lane 7, *GAL::U3*, 24 h.

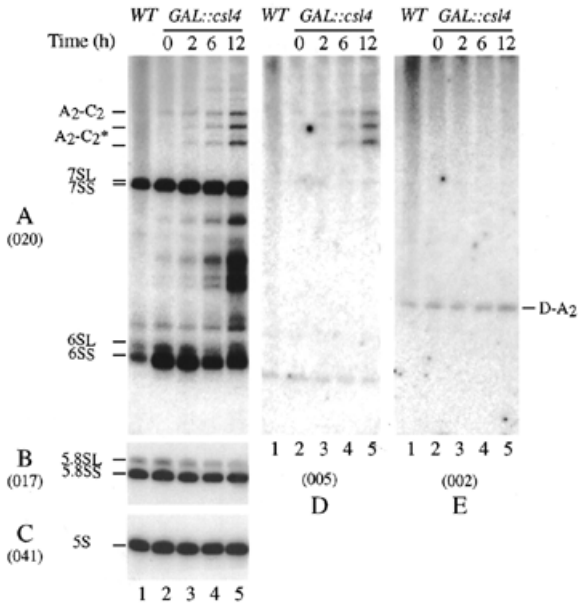

Figure 7. Aberrant A_2–C_2 pre-rRNAs accumulate in exosome mutants. RNA was extracted from the *GAL::csl4* strain grown on RSG medium (0 h) and after transfer to repressive glucose medium for various lengths of time, and run on a 6% polyacrylamide gel for analysis of low molecular weight RNA. Lane 1, wild-type, 0 h; lanes 2–5, *GAL::csl4* for 0, 2, 6 and 12 h. (**A**) Hybridization with probe 020. (**B**) Hybridization with probe 017. (**C**) Hybridization with probe 041. (**D**) Hybridization with probe 005. (**E**) Hybridization with probe 002. The weak band visible in all lanes in (D) probably represents cross-hybridization to the mature 5.8S rRNA.

the presence of the A_2–C_2 and A_2–C_2* species was responsible for the strong primer extension stop at site A_2 detected in *GAL::csl4* and other strains depleted of exosome components (Fig. 6).

Forms of the 5.8S rRNA that are 5′ extended to site A_2 were previously observed in strains defective in A_3 cleavage due to mutations in either the RNA or protein components of RNase MRP (27–31). We conclude that in strains depleted of components of the exosome or Dob1p, processing of the pre-rRNA at site A_3 is inhibited, leading to the observed reduction in the $27SA_3$ and $27SB_S$ pre-rRNAs. The residual $27SA_2$ pre-rRNA is cleaved at site C_2 in ITS2, generating the A_2–C_2 fragment, which is itself a substrate for the exosome and Dob1p.

DISCUSSION

We have previously reported that the exosome is required during ribosome synthesis for maturation of the 3′ end of the 5.8S rRNA. Here we show that all 10 essential components of the complex are also required for the early steps of pre-rRNA processing, at sites A_0, A_1, A_2 and A_3 as was recently reported for Rrp43p (17). No direct substrate for the exosome is apparent in these processing reactions, which involve only endonucleolytic cleavage (21–24). The exosome is involved in the synthesis of many snoRNAs (14,15), including species required for these cleavages, but defects in the processing of known snoRNAs do not account for the pre-rRNA processing inhibition.

It is notable that many mutations that affect synthesis of the 5.8S and 25S rRNAs and the 60S ribosomal subunit also affect the synthesis of 18S rRNA (32) (reviewed in 1,2). It appears probable that the requirement for many of these factors, including the exosome, is indirect. The assembly of the 60S synthesis factors is likely to be monitored as part of a quality control mechanism that ensures that only correctly processed and assembled pre-rRNAs are matured to ribosomal subunits. In wild-type cells this presumably functions only to transiently delay processing until the missing factor has bound, but in strains genetically depleted of processing factors results in the partial or complete inhibition of processing. It is clear that there is a high degree of integration between different steps in ribosome synthesis. Mutations in the 5′-ETS, 3′-ETS or ITS2 regions were each shown to inhibit processing in ITS1 (19,33,34), leading to the proposal that the pre-rRNA processing machinery might exist as a single large complex (33,35).

The aberrant pre-rRNAs that arise from processing inhibition, the 23S, 21S and A_2–C_2 fragments, are themselves degraded by the exosome complex, with truncated forms accumulating in the exosome mutant strains. The 23S RNA is present at very low levels in many strains and may be a normal substrate for the exosome. The putative DEAD-box RNA helicase Dob1p is required for the function of the exosome in the 3′ processing of the 5.8S rRNA and degradation of the 5′-ETS region of the pre-rRNA (16), and also appears to be required for degradation of the 23S, 21S and A_2–C_2 RNAs.

An obvious question is why the 23S* and 21S* RNAs are predominately accumulated in the exosome mutants, rather than the full-length fragments or shorter intermediates. The end points have not been mapped but the migration of these species is not visibly different from the 21S and 23S RNAs, indicating that they have only short truncations. Oligo 003, that does hybridize to the truncated species, extends precisely to site A_3 so even a very short truncation would prevent hybridization. Site A_3 is predicted to be single stranded but is located a few nucleotides downstream of a strong predicted stem–loop structure (36). It may be that only the short single-stranded tail is removed from the 23S and 21S RNAs in the exosome mutants.

In most strains that are defective in processing at sites A_0–A_2, the 23S pre-rRNA is rapidly degraded without detectable intermediates. This shows that the degradative enzymes are able to degrade the 18S rRNA region, which is highly structured and is presumably bound by many ribosomal proteins, with high processivity. The putative RNA helicase, Dob1p, may well play a key role in opening the RNP structure of the pre-rRNA during this degradation. The pre-rRNA region from A_2–C_2 was also accumulated in the exosome mutants, probably as a consequence of the inhibition of processing at site A_3. The fragment from A_2 to the 3′ end of the 5.8S rRNA is observed in strains defective in A_3 cleavage due to mutations in RNase MRP (27–31). We assume that in these mutants the exosome plus Dob1p digests the 3′ end of the A_2–C_2 back to the 3′ end of the 5.8S rRNA.

There are differences in the fates of the 23S RNA and 5′-ETS region, which are completely degraded by the exosome, and substrates such as the precursors to the 5.8S rRNA and U3 snoRNA, which are processed to products of discrete length. In

the case of the U3 snoRNA, short 3′ extended pre-snoRNA species are specifically protected from degradation by binding of the Lhp1p protein (the yeast homolog of human La) (37; J.Kufel, C.Allmang and D.Tollervey, unpublished data). It may be that an RNA binding protein is specifically required to stall the exosome complex ~8 nt 3′ to the mature 3′ end of the 5.8S rRNA, generating the 6S pre-rRNA and allowing slower final trimming to the mature 5.8S rRNA. Alternatively, the 23S and 5′-ETS may be targeted for degradation such that the exosome complex that assembles on these RNAs is more processive than the form of the complex that engages in processing of the 7S pre-rRNA. Initial experiments indicate that multiple activated forms of the exosome can be biochemically fractionated (P.Mitchell, unpublished data).

ACKNOWLEDGEMENTS

We would like to thank J. Kufel and C. Bousquet-Antonelli for critical reading of the manuscript. This work was supported by the Wellcome Trust.

REFERENCES

1. Kressler,D., Linder,P. and de La Cruz,J. (1999) *Mol. Cell. Biol.*, **19**, 7897–7912.
2. Venema,J. and Tollervey,D. (1999) *Annu. Rev. Gen.*, **33**, 261–311.
3. Moritz,M., Paulovich,A.G., Tsay,Y.-F. and Woolford,J.L.J. (1990) *J. Cell Biol.*, **111**, 2261–2274.
4. Rotenberg,M.O., Moritz,M. and Woolford,J.L.J. (1988) *Genes Dev.*, **2**, 160–172.
5. Lafontaine,D.L., Preiss,T. and Tollervey,D. (1998) *Mol. Cell. Biol.*, **18**, 2360–2370.
6. Mitchell,P., Petfalski,E. and Tollervey,D. (1996) *Genes Dev.*, **10**, 502–513.
7. Mitchell,P., Petfalski,E., Shevchenko,A., Mann,M. and Tollervey,D. (1997) *Cell*, **91**, 457–466.
8. Allmang,C., Petfalski,E., Podtelejnikov,A., Mann,M., Tollervey,D. and Mitchell,P. (1999) *Genes Dev.*, **13**, 2148–2158.
9. Burkard,K.T. and Butler,J.S. (2000) *Mol. Cell. Biol.*, **20**, 604–616.
10. van Hoof,A. and Parker,R. (1999) *Cell*, **99**, 347–350.
11. Mian,I.S. (1997) *Nucleic Acids Res.*, **25**, 3187–3195.
12. Briggs,M.W., Burkard,K.T. and Butler,J.S. (1998) *J. Biol. Chem.*, **273**, 13255–13263.
13. Anderson,J.S.J. and Parker,R.P. (1998) *EMBO J.*, **17**, 1497–1506.
14. Allmang,C., Kufel,J., Chanfreau,G., Mitchell,P., Petfalski,E. and Tollervey,D. (1999) *EMBO J.*, **18**, 5399–5410.
15. van Hoof,A., Lennertz,P. and Parker,R. (2000) *Mol. Cell. Biol.*, **20**, 441–452.
16. de la Cruz,J., Kressler,D., Tollervey,D. and Linder,P. (1998) *EMBO J.*, **17**, 1128–1140.
17. Zanchin,N.I. and Goldfarb,D.S. (1999) *Nucleic Acids Res.*, **27**, 1283–1288.
18. Tollervey,D. and Mattaj,I.W. (1987) *EMBO J.*, **6**, 469–476.
19. Beltrame,M. and Tollervey,D. (1992) *EMBO J.*, **11**, 1531–1542.
20. Hughes,J.M.X. and Ares,M.J. (1991) *EMBO J.*, **10**, 4231–4239.
21. Henry,Y., Wood,H., Morrissey,J.P., Petfalski,E., Kearsey,S. and Tollervey,D. (1994) *EMBO J.*, **13**, 2452–2463.
22. Veldman,G.M., Brand,R.C., Klootwijk,J. and Planta,R.J. (1980) *Nucleic Acids Res.*, **8**, 2907–2920.
23. Veldman,G.M., Klootwijk,J., van Heerihuizen,H. and Planta,R.J. (1981) *Nucleic Acids Res.*, **9**, 4847–4862.
24. Venema,J., Henry,Y. and Tollervey,D. (1995) *EMBO J.*, **14**, 4883–4892.
25. Allmang,C., Henry,Y., Morrissey,J.P., Wood,H., Petfalski,E. and Tollervey,D. (1996) *RNA*, **2**, 63–73.
26. Venema,J. and Tollervey,D. (1996) *EMBO J.*, **15**, 5701–5714.
27. Shuai,K. and Warner,J.W. (1991) *Nucleic Acids Res.*, **19**, 5059–5064.
28. Lindahl,L., Archer,R.H. and Zengel,J.M. (1992) *Nucleic Acids Res.*, **20**, 295–301.
29. Lygerou,Z., Mitchell,P., Petfalski,E., Séraphin,B. and Tollervey,D. (1994) *Genes Dev.*, **8**, 1423–1433.
30. Dichtl,B. and Tollervey,D. (1997) *EMBO J.*, **16**, 417–429.
31. Chu,S., Zengel,J.M. and Lindahl,L. (1997) *RNA*, **3**, 382–391.
32. Zanchin,N.I., Roberts,P., DeSilva,A., Sherman,F. and Goldfarb,D.S. (1997) *Mol. Cell. Biol.*, **17**, 5001–5015.
33. Allmang,C. and Tollervey,D. (1998) *J. Mol. Biol.*, **278**, 67–78.
34. Van Nues,R.W., Rientjes,J.M.J., Morré,S.A., Mollee,E., Planta,R.J., Venema,J. and Raué,H.A. (1995) *J. Mol. Biol.*, **250**, 24–36.
35. Morrissey,J.P. and Tollervey,D. (1995) *Trends Biochem. Sci.*, **20**, 78–82.
36. Yeh,L.-C.C., Thweatt,R. and Lee,J.C. (1990) *Biochemistry*, **29**, 5911–5918.
37. Yoo,C.J. and Wolin,S.L. (1997) *Cell*, **89**, 393–402.
38. Lafontaine,D. and Tollervey,D. (1996) *Nucleic Acids Res.*, **24**, 3469–3472.
39. Kadowaki,T., Schneiter,R., Hitomi,M. and Tartakoff,A.M. (1995) *Mol. Biol. Cell*, **6**, 1103–1110.