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An RNA Conformational Switch Regulates Pre-18S rRNA Cleavage

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Abstract

To produce mature ribosomal RNAs (rRNAs), polycistronic rRNA transcripts are cleaved in an ordered series of events. We have uncovered the molecular basis for the ordering of two essential cleavage steps at the 3'-end of 18S rRNA. Using *in vitro* and *in vivo* structure probing, RNA binding and cleavage experiments, and yeast genetics, we demonstrate that a conserved RNA sequence in the spacer region between the 18S and 5.8S rRNAs base pairs with the decoding site of 18S rRNA in early assembly intermediates. Nucleolar cleavage at site A₂ excises this sequence element, leading to a conformational switch in pre-18S rRNA by which the ribosomal decoding site is formed. This conformational switch positions the nuclease Nob1 for cytoplasmic cleavage at the 3'-end of 18S rRNA and is required for the final maturation step of 18S rRNA *in vivo* and *in vitro*. More generally, our data show that the intrinsic ability of RNA to form stable structural switches is exploited to order and regulate RNA-dependent biological processes.

Keywords

Ribosome Assembly; RNA Conformational Switch; RNA Cleavage; RNP assembly

Introduction

Ribosomes catalyze protein synthesis in all cells. Accordingly, their biosynthesis is tightly regulated. To ensure the structural integrity of mature ribosomes, which is required for their impressive fidelity, ribosome assembly is also expected to be subject to quality-control. Metabolic pathways demonstrate that irreversible steps often provide points of regulation and may play a role in quality control. Pre-rRNAs are produced as polycistronic transcripts that are cleaved in multiple steps to produce the mature rRNAs. Cleavage steps are irreversible, releasing the energy contained in the phosphodiester bond. As such, they represent possible points of regulation and quality control to ensure that only correctly assembled intermediates are further processed. Consistent with this notion, energy-utilizing enzymes are often positioned to regulate cleavage steps; binding of the putative nuclease Rcl1 is promoted by the essential GTPase Bms1¹. Furthermore, RNase MRP-dependent

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cleavage at site A₃ requires the helicase Dbp3², and cleavage at site D requires the activity of the kinases, Rio1 and Rio2, and the ATPase Fap7^{3; 4}.

It has been suggested that Nob1 is the nuclease that generates the 3'-end of 18S rRNA by cleavage at site D in the cytoplasm^{5; 6; 7; 8}. This hypothesis is based on modest accumulation of the 20S precursor and strong depletion of mature 18S rRNA in Nob1 mutants^{5; 6}, Nob1's ability to directly bind to the 3'-end pre-18S rRNA *in vitro* and *in vivo*⁷, and the observation of weak RNA cleavage activity of Nob1 purified from yeast, when a minimal substrate is used⁸. Interestingly, Nob1 localizes to the cytoplasm and nucleolus and is found in both early and late 40S ribosome precursors⁵. These results indicate that while Nob1 is bound to early, nucleolar 40S precursors it remains inactive until after export to the cytoplasm, suggesting that Nob1, the rRNA substrate or both are modified to activate timely or prevent premature cleavage. In that context the Tollervey and Hurt labs have recently described genetic interactions between Nob1 and the DEAD box helicase Prp43, which has a binding site close to Nob1's cleavage site^{8; 9}. A perhaps related observation is the finding that cleavage at site D requires prior cleavage at a downstream site, termed A₂ (reviewed in:¹⁰, see Fig. 1 for a scheme of pre-rRNA processing). The molecular origin for this ordering of cleavage steps is unknown; however, we have speculated previously that it would ensure quality control, as it essentially allows cleavage at site A₂ to be reinspected before allowing further processing¹⁰.

Here we show that in early 40S precursors the top of helix 44 (H44) containing the decoding site is not yet formed. Instead, it is replaced with an alternative duplex between the 3'-strand of H44 and a sequence in ITS1 (internal transcribed spacer 1, "intron"-like RNA between 18S and 5.8S rRNA) immediately downstream of cleavage site A₂. After cleavage at site A₂ this sequence element is removed, which triggers a conformational switch and concomitant formation of the decoding site. This conformational switch is required for correct positioning of Nob1's nuclease domain at cleavage site D, as well as Nob1-dependent D-site cleavage. Furthermore, stabilizing the early structure by mutations in the pre-rRNA delays Nob1-dependent cleavage at site D *in vivo*. Finally, phylogenetic analysis indicates that this conformational switch is conserved throughout eukaryotes.

Results

We have recently described a biochemical system to investigate the interaction between Nob1 and pre-rRNA using *in vitro* transcribed rRNA fragments and recombinant Nob1 protein⁷. These experiments demonstrate that rRNA fragments used herein (Fig. 2A) are folded into specific structures relevant to ribosome assembly *in vivo*, as similar footprinting patterns are observed *in vivo* and *in vitro*⁷. Furthermore, residues adjacent to cleavage site D that are protected by Nob1 *in vitro* also require the presence of Nob1 for protection *in vivo*⁷, suggesting that the modes of Nob1 binding *in vitro* correctly report on Nob1 binding *in vivo*.

Communication between H44 and ITS1 affects Nob1 binding to pre-rRNA

We have shown that Nob1 interacts with the pre-rRNA at helix 44 (H44, light blue segment in Fig. 2A), cleavage site D, as well as the internal transcribed spacer (ITS1) between 18S and 5.8S rRNA (light purple and orange segments in Fig. 2A). Somewhat surprisingly, addition of rRNA downstream of site A₂ (which is not part of Nob1's substrate) increased Nob1 binding 6-fold⁷. To further study this interaction with the distal part of ITS1 (orange segment in Fig. 2A), we compared Nob1 binding to a number of rRNA fragments (Table 1, refer to Fig. 2A for the locations of the ends of these fragments). Extending the rRNA from D to A₂ increases Nob1 binding 14-fold regardless of the presence of H44 (compare top two rows in Table 1). Surprisingly, while addition of H44 contributes ~7-fold to Nob1 binding when rRNAs end at site D or A₂ (Table 1, the effect from addition of H44 is referred to as

K_{H44}), no effect from H44 was observed when additional sequences downstream of site A₂ are included (+278). Furthermore, while adding rRNA 3' to site A₂ increases Nob1 binding 6-fold when H44 is absent, the additional rRNA has no effect when H44 is present. Thus, H44 only contributes to Nob1 binding when rRNA sequences 3' to site A₂ are absent. Likewise, sequences 3' to site A₂ only contribute to Nob1 binding when H44 is absent. These observations suggest that communication between rRNA sequences downstream of site A₂ and H44 affects Nob1 binding.

A conformational switch in pre-rRNA after cleavage at site A₂

Inspection of rRNA sequences in H44 and 3' to site A₂ reveals the potential for a base pairing interaction (Fig. 2C), thus providing a molecular explanation for the observed communication between H44 and ITS1 downstream of site A₂. This interaction disrupts the top of H44 (indicated by black base pair lines on the right of Fig. 2A), suggesting that it cannot form concomitantly with the mature structure of H44, which contains the decoding site. To test if rRNAs containing both H44 and sequence 3'- to A₂ (shaded in Table 1) adopt a different structure compared to rRNAs lacking at least one of these elements, we performed structure probing experiments. DMS methylates adenosines and cytidines unless they are protected by base pairing and is thus a powerful secondary structure probe. We have used DMS to probe the rRNAs H44/A₂, H45/+278 (each containing one side of the proposed duplex), and H44/+278 (containing both duplex forming elements). The majority of residues have the same reactivity to DMS in all RNAs, suggesting that there are no gross changes in the structures of these RNAs (grey in Fig. 2C, see also Fig. S2). However, a small number of residues exhibit altered accessibility in the H44/+278 rRNA (Fig. 2B, see also Fig. S3A for linescans). These residues are clustered in three main regions of pre-rRNA: around cleavage site A₂ (residues +205–222), in the top of H44 (residues (–35)–(–50)) as well as residues in the proximity of the cleavage site (+22/+23). These residues are highlighted in Figs. 2B and Fig. S3A. All of these residues are more protected in the RNA containing both H44 and RNA 3' to site A₂, as confirmed quantitatively by linescans (Fig. S3A). These data strongly support the existence of two conformations in these RNAs.

Using these protections as a guide, we propose the secondary structures for the H44/+278 and H44/A₂ rRNAs shown in Fig. 2C. Because cleavage at site A₂ removes the sequence element that interacts with H44, we refer to the structures as pre- and post-A₂ cleavage structures, respectively. The proposed secondary structure for the pre-A₂ cleavage RNA is strongly supported by the complete accessibility of residues A(–35)–A(–38) in this structure as well its protection in the mature structure. Further strong support for the proposed secondary structure comes from the accessibility of A(+22) and A(+23) in the pre-A₂ cleavage RNA. These residues are protected in the post-A₂ cleavage RNA, as expected from the proposed secondary structure. In addition, residues A(–47), C(–49) and A(–50) are also even more accessible in the pre-A₂ structure, as expected from the proposed secondary structure. Thus, these results provide support for the proposed pre-A₂ cleavage structure. However, it is also important to note that even if the details of the pre-A₂ structure differ from the proposal in Fig. 2C, the data in Fig. 2B nevertheless provide strong evidence for a conformational switch, as they are independent of the interpretation in Fig. 2C.

To further test if the proposed conformational change occurs as proposed, we perturbed the equilibrium between the two structures and used Nob1 binding and DMS probing to assess conformational switching. Towards this end, we generated two mutants within the pre-rRNA that reduce base pairs expected to be present only in the pre-A₂ cleavage structure (Fig. 3, mutA and mutB). Therefore the mutations should destabilize the proposed pre-A₂ cleavage structure, such that addition of H44 might improve Nob1 binding even in the presence of sequence 3' to site A₂ (H44/+278).

To test this prediction, we compared Nob1's affinity to wild type and mutant H44/+278 and H45/+278 rRNAs (Table 2). While Nob1 binding is essentially unaffected by adding H44 to rRNA ending at position +278 in wild type rRNA, adding H44 in the context of mutA or mutB increases rRNA binding 2.4 or 1.9 fold, respectively. Similarly, H44 has a 3.6 fold effect when the RNA ends at position +228 (Table 1), a truncation that deletes the region altered in mutB altogether (Fig. 2C). By comparison, adding H44 to rRNAs ending at sites A₂ or D increases rRNA binding 7.1 fold (Table 1). Together these data suggest that destabilizing the pre-A₂ structure by point mutations leads to a partial conformational switch. The mutations allow close to half of the intrinsic 7.1 fold effect from H44 to be realized, indicating that the pre-A₂ structure is only formed about half of the time, which suggests that both conformations are similarly stable.

Next, we evaluated the effects from the mutations on the conformational switch by structure probing. DMS probing of the mutant H44/+278 rRNAs should reveal that the structure is at least partially switched to the structure normally only observed in the H44/A₂ or H45/+278 rRNAs. Consistent with this analysis, DMS probing under these conditions shows that a subset of residues uniquely accessible in wild type H44/+278 are protected in the mutant H44/+278 RNA (Fig. S4). Additionally, adenosines or cytidines mutated in place of uridines or guanosines are accessible, as predicted by our model.

Collectively, the preceding data show that pre-rRNAs that contain sequence downstream of site A₂ (*i.e.* prior to cleavage at site A₂) are in a different conformation as compared to pre-rRNAs after cleavage at site A₂. These differences are due to base pairing interactions between one strand at the top of H44 and RNA between cleavage sites A₂ and A₃, which can be perturbed in a predictable manner by mutations. Finally, our data also show that this conformational change sets up the decoding site in the 40S ribosomal subunit.

23S pre-rRNA is in the pre-A₂ cleavage structure *in vivo*

While the use of these small RNAs had been extensively validated and shows sensitivity to the same perturbations *in vivo* and *in vitro*⁷, we wanted to provide *in vivo* evidence for the proposed early pre-rRNA structure. Since 23S rRNA extends beyond site A₂ to site A₃ (Fig. 1, pathway B), our model predicts that 23S rRNA is in the pre-A₂ cleavage structure. We accumulated 23S rRNA by Bms1 depletion [Fig. S5^{11; 12}], and used *in vivo* DMS probing to test whether the modification pattern resembled that of the pre- or post-A₂ structure *in vitro*.

Fig. 2D shows that *in vivo* the signature residues for the pre-A₂ cleavage structure in H44 and around cleavage site D [A(−35)–A(−38); A(+22,23)] are accessible and that their modification pattern is akin to the *in vitro* probing data obtained with the pre-A₂ structure (H44/+278, compare Figs. 2B and D). This finding demonstrates that the pre-A₂ structure exists *in vivo*, as 23S rRNA is found in this structure, thereby validating the results obtained with *in vitro* transcripts.

Nob1 interacts differently with pre-rRNAs prior to and after A₂ cleavage

The different contributions from H44 to Nob1 binding when RNAs with and without sequence 3' to site A₂ are used might reflect the use of Nob1-binding energy to facilitate switching between the RNA structures, with identical modes of Nob1-interaction with pre- and post-A₂ cleavage RNAs. Alternatively, Nob1 might establish different interactions with these RNAs. To address this question, we compared the DMS protection profiles of pre-rRNAs in the pre- and post-A₂ cleavage structures under conditions in which Nob1 binding is saturated (Fig. 4). Many Nob1-dependent protections of rRNA are observed both in the pre- and post-A₂ cleavage structures. However, full protections around the Nob1-dependent

cleavage site D are only observed in the post-A₂ cleavage structure, while several residues remain fully or partially accessible in the pre-A₂ conformation (see also linescans in Fig. S6). These protections are specific to the post-A₂ cleavage structure arise from Nob1's PIN domain and are also observed *in vivo*⁷. Thus, when Nob1 binds to early pre-ribosomes prior to cleavage at site A₂, it is not positioned correctly for cleavage at site D. These data indicate that Nob1-dependent cleavage at site D requires the pre-rRNA to be in the post-A₂ cleavage structure.

Nob1 preferentially cleaves pre-rRNAs in the post-A₂ cleavage structure

To directly test whether Nob1 cleaves pre-rRNAs at site D, we added Mn²⁺ to the Nob1-rRNA complexes. Mn²⁺ is required for the activity of several PIN domains, and recent work with Nob1 has shown that in the presence of Mn²⁺ it can cleave a small model substrate, albeit with very poor efficiency⁸. As expected, in the presence of Mn²⁺, Nob1 cleaves 3'-end labeled H44/A₂ pre-rRNAs at site D (Fig. 5) to generate the labeled D/A₂ 3'-product. Similarly, a 5'-end labeled H44/A₂ transcript produces the labeled H44/D product when incubated with Nob1 (Fig. S7A). Furthermore, a body-labeled transcript produces both the H44/D and the D/A₂ products (Fig. S7B). In all cases cleavage requires wild-type Nob1 protein and is not observed with the lethal D92N mutant, in which the Mn²⁺ binding site is obliterated (Figs. 5 and S7), indicating that cleavage is carried out by Nob1 and not a contaminating protein. Finally, mutation of U(-1) to G, which inhibits cleavage *in vivo*¹³, also inhibits cleavage *in vitro* (Fig. S7C), although some miscleavage at a downstream site is observed, as previously shown⁸. These data provide additional support that Nob1 is the nuclease for cleavage at site D and demonstrate that our *in vitro* system shows the same behavior as D-site cleavage *in vivo*.

Next, we wanted to determine whether the observed cleavage at site D requires pre-rRNA to be in the post-A₂ cleavage structure, as suggested by our model, or if it could also proceed in the pre-A₂ structure. Indeed, H44/+278 rRNA, which is in the pre-A₂ cleavage structure, is not cleaved by Nob1 when physiological concentrations (0.2 μM; cellular concentration ~ 0.12 μM¹⁴) are employed (Fig. 5, 13±2 and 3±1 % of H44/A₂ and H44/+278 rRNAs cleaved, respectively). The absence of activity in the longer RNA is not due to a detrimental effect from the additional rRNA element between A₂ and +278, as H45/+278 rRNA (which is in the post-A₂ cleavage structure) is efficiently cleaved (Fig. S7D). These data strongly suggest that the difference in the RNA structure is responsible for the observed lack of cleavage of the pre-A₂ rRNA. These results demonstrate that productive Nob1 binding and cleavage of pre-18S rRNA requires prior cleavage at site A₂ and release of the intervening RNA between A₂ and A₃.

Trapping the pre-A₂ cleavage structure slows 18S rRNA maturation *in vivo*

To test directly whether the observed conformational switch is required for Nob1-dependent cleavage *in vivo*, we stabilized the pre-A₂ cleavage structure by converting an internal loop in the pre-A₂ structure into Watson-Crick base pairs (mutS, see Fig. 3). To maintain the function of mature 18S rRNA, mutations were made in ITS1, as the complementary region of H44 comprises the universally conserved decoding site. These mutations will not affect the post-A₂ cleavage structure, as the changed nucleotides are downstream of site A₂ (Fig. 3). Mutated rDNAs were encoded on a plasmid under the control of a galactose promoter¹⁵. This plasmid was transformed into yeast strain NOY504, which carries a deletion in the PolI subunit Rpa12, rendering it temperature sensitive¹⁶. Thus, only plasmid-encoded rRNA is produced at 37 °C. If the switch is essential for Nob1-dependent rRNA cleavage at site D, one would expect accumulation of the 20S precursor (Nob1's substrate) in strains where the switch is delayed by strengthening of the pre-A₂ cleavage structure.

RNA was extracted from strains grown at 37 °C in YP-galactose and analyzed by Northern blotting to quantitate 20S rRNA levels. As shown in Fig. 6A, residual transcription from the endogenous promoters is observed leading to a 20S signal in the strain containing an empty plasmid. However, because this signal is substantially below the signal observed for strains containing wild type plasmid and all mutants, the 20S levels can still be interpreted. 18S rRNA produced from the plasmid was visualized using a probe specific to a sequence tag engineered into the plasmid-encoded rRNA (18Stag). The splicesomal RNA U2 was used as a loading control for normalization (e.g. 20S/U2 ratio). In addition, growth was monitored. As expected from our model, stabilizing the pre-A₂ cleavage structure by mutating base pairs in ITS1 (mutS) leads to a 2.3 fold accumulation of 20S rRNA relative to strains with wild type rRNA (Fig. 6A). Correspondingly, a small, but reproducible growth phenotype is observed (Fig. 6B), manifested by differences in colony size and numbers among the wild type and mutS samples.

To rule out the possibilities that the accumulation of 20S rRNA arises from misfolding of 20S rRNA or the disruption of interactions with additional processing factors due to nucleotide changes, we carried out two control experiments. First, we introduced a second set of complementary mutations in H44 (α -mutS1 and α -mutS2, see Fig. 3). When combined with mutS these mutations rescue the stabilization of the pre-A₂ cleavage structure by breaking the additional base pairs. Importantly, combining these mutations (mutS/ α -mutS1 and mutS/ α -mutS2) also rescues 20S rRNA accumulation, as expected if the mutS changes reduce 20S cleavage due to changes in the stability of the pre-A₂ structure. Despite the positive effect on pre-rRNA processing, RNA mutations in H44 are lethal (Fig. 6B) because they disrupt the mature 18S rRNA structure in the universally conserved decoding site, leading to nonfunctional mature rRNA. This loss of function also results in degradation of the resulting 18S rRNA (Fig. 6A), likely via the no-go rRNA decay pathway¹⁷. Because 20S rRNA is also subject to no-go decay¹⁷, this degradative pathway could be responsible for at least some of the reduction in 20S rRNA observed with the H44 mutants (although this seems unlikely as unprocessed wild type 20S rRNA is poorly functional and therefore equally degraded in that pathway¹⁸). Nevertheless, we performed an additional control experiment and introduced a second set of neutral mutations (mutN) into ITS1 (Fig. 3). The mutN changes involve the same nucleotides as in mutS; however, they were designed *not* to affect the stability of the pre-A₂ (or post-A₂) cleavage structure. As expected, these mutations do not affect pre-rRNA processing and have very small effects on cell growth, further demonstrating that the deleterious effects of the mutS mutation on 20S processing are the consequence of stabilizing the pre-A₂ cleavage structure and not of changes in the nucleotide sequence. Together, our data show that delaying the conformational switch by stabilizing the pre-A₂ cleavage structure inhibits 20S rRNA processing, strongly indicating that the switch is required for Nob1-dependent cleavage of 20S rRNA to generate the mature 3'-end of 18S rRNA.

The ability to form the pre-A₂ cleavage structure is phylogenetically conserved

To determine if the conformational switch is conserved in other eukaryotes, we used the program rnabob¹⁹ to search for a conserved element that can interact with the top of H44. rnabob searches a given sequence for a defined secondary structure: in this case a duplex whose 5'-element is the highly conserved sequence in H44 that base pairs with ITS1 in the yeast *S. cerevisiae*. A sequence that can form a duplex with the top of H44 was identified in ITS1 in a variety of model organisms (yellow in Fig. S9). To confirm that the computationally identified sequences are located between sites A₂ and A₃, we needed to identify these cleavage sites, which are not mapped in other organisms and not conserved strongly enough to be recognized by sequence alone. To use the additional information from conserved structural elements, we first aligned ITS1 sequences from yeasts very closely

related to *S. cerevisiae*²⁰. Even among these species, ITS1 diverges in length (137–402 nt) and sequence (Fig. S8A, Table S1). However, several structural elements are conserved (going 3' to 5', Fig. S8B): a stem-loop (SL) directly preceding the 5'-end of 5.8S rRNA (SL3), stem-loops that follow (SL2) and precede (SL1) cleavage site A₃ (CA-rich), cleavage site A₂ (CA-rich), and finally a stem with a conserved UUAANT loop, where we have observed Nob1-dependent footprints⁷. Notably, the H44-interacting elements (regions A and B) that were mutated to destabilize the pre-A₂ cleavage structure are conserved and located between cleavage sites A₂ and A₃, providing evidence that all yeasts undergo the conformational switch described herein. We then analyzed m-fold predictions for ITS1 structures from several model organisms for these structural “landmarks” to identify cleavage sites A₂ and A₃ and mapped the sequences identified by rnabob relative to the predicted sites A₂ and A₃. In all cases, the sequence predicted to interact with H44 is located between the predicted cleavage sites A₂ and A₃ (Fig. S9A), suggesting that the conformational change we have observed is conserved throughout eukaryotes.

Discussion

A model for regulation of 18S rRNA 3'-end formation

Our data indicate that an RNA element located immediately downstream of cleavage site A₂ forms a duplex with the 3'-end of H44 (shown in red in Fig. 7, I), thereby preventing the formation of the top of H44 in early 40S precursors. Although Nob1 (shown in green) binds to this early precursor, as suggested by ultracentrifugation⁵ and data herein, our data show that its binding site does not center around cleavage site D in this early precursor. Cleavage at site A₂ (Fig. 7, II) and subsequent dissociation of the 3'-element that starts at A₂ (either as the A₂-A₃ piece or the longer A₂-B₁, Fig. 7, III), then allows for the conformational switch. The conformational switch sets up the decoding site at the top of H44 and allows for productive binding of Nob1's PIN domain at cleavage site D (Fig. 7, IV), resulting in Nob1-dependent cleavage at site D (Fig. 7, V). Phylogenetic analysis indicates that the ability to form the pre-A₂ cleavage structure is conserved throughout eukaryotes, indicating its importance for proper ribosome assembly. Thus, we have identified a conserved conformational switch that regulates the final cleavage step in the production of 18S rRNA in eukaryotes. *In vivo* the A₂-A₃ piece is degraded by both the nuclear exonuclease Rat1 as well as its cytoplasmic ortholog Xrn1²¹, suggesting that the conformational switch promoted by release of the A₂-A₃ piece might occur in a manner that is closely related to nuclear export, perhaps at the nuclear periphery or during export.

While our data strongly support the existence of two different pre-rRNA structures, they provide no direct evidence for the formation of the suggested base pairs (which could only come from NMR or X-ray crystallography experiments). Nevertheless, we do believe that we have isolated the rRNA sequences involved in base pairing in the pre-A₂ cleavage structure, as the DMS probing, the Nob1 binding experiments, the *in vitro* and *in vivo* mutagenesis, and the phylogenetic analysis all implicate the same region within ITS1 and H44. However, because of the large internal loop, which makes secondary structure predictions harder, the exact base pairs formed could differ from the proposed model.

It is tempting to speculate that cleavage at site A₂ provides an entry point for a dedicated RNA helicase that catalyzes dissociation of the inhibitory RNA element. Prp43 and its co-factor Pfa1 have been recently shown to have genetic interactions with Nob1⁸. In addition, Prp43 has binding sites near the base pairs in H44 involved in the switch⁹. These interactions between Nob1, the helicase Prp43, and the conformational switch described herein make Prp43 a candidate for effecting the proposed switch *in vivo*. However, it has also been shown that Prp43 removes box C/D snoRNAs^{9; 22; 23}. The snoRNA snR70 binds adjacent to the Prp43 binding site and is accumulated in the presence of Prp43 mutants^{9; 23}.

Furthermore, because snR70 binding is expected to prevent the formation of the post-A₂ cleavage structure, but not the pre-A₂ cleavage structure (Figure S10), a second plausible model would be that Prp43 is required to remove snR70. Mutations in Prp43 or Pfa1 would then lead to accumulation of snR70 and concomitant stabilization of the pre-A₂ cleavage structure. These considerations suggest that further research is required to link Prp43 or any helicase to the switch described herein.

The conformational switch model in Fig. 7 explains the previously mysterious observation that the 23S and 22S intermediates, which accumulate to high levels in strains where cleavage at sites A₀, A₁, and A₂ is disrupted, are not substrates for cleavage at site D and thus are not converted to 18S rRNA (reviewed in ref. ¹⁰). In contrast to the 20S intermediate, which ends at site A₂, 23S and 22S rRNAs extend to site A₃ (Fig. 1). As expected from our model, *in vivo* DMS probing herein shows that 23S rRNA is in the pre-A₂ cleavage structure, which would inhibit further processing at site D. Interestingly, a novel 22.5S intermediate that accumulates in the partially functional Mrd1GFP strain is a substrate for Nob1, whereas the canonical 23S intermediate is not ²⁴. Thus, 23S levels are unaffected in the Mrd1GFP/Nob1 depletion, while 22.5 levels increase. Although 23S and 22.5S RNAs appear to have the same 5'-end (including the entire 5'-ETS), the 3'-end of the 22.5S intermediate ends 5–12 nucleotides beyond site D, which would leave this RNA in the post-A₂ cleavage structure, competent for Nob1-dependent cleavage.

Several strains with deletions around cleavage site A₂ have been described, in which cleavage at site A₂ does not proceed yet 18S rRNA is made ^{15; 25}, seemingly contradicting the importance of cleavage at site A₂ for processing of pre-rRNA *in vivo*. However, it is important to point out that these deletions also abolish the ability to form the pre-A₂ cleavage structure, such that rRNA starts in the post-A₂ cleavage structure. Furthermore, data in the literature describe two mutant forms of the RNA binding protein Rrp5, in which the three C-terminal (out of 12) S1 RNA binding domains are deleted ^{26; 27}. In these strains cleavage at site A₂ is entirely abolished, yet 18S production and growth are normal or near normal [reviewed in: ¹⁰]. While these data apparently contradict the present model, they can be reconciled by a model in which the deleted RNA binding domains are stabilizing the pre-A₂ cleavage structure. Indeed prior data in the literature show that while deletions of the C-terminal S1 domains lead to loss of cleavage at site A₂, deletions of the N-terminal S1 domains result in loss of cleavage at site A₃ ²⁸. This finding indicates that Rrp5 is bound between cleavage sites A₂ and A₃, which is consistent with *in vitro* binding experiments ²⁹ and corroborated by quantitative experiments with recombinant Rrp5 (Young, C.L. & K.K., unpublished data). These data indicate that Rrp5 might play an important role in stabilizing the pre-A₂ structure.

Regulation of Nob1's activity

Our data provide evidence for a conformational switch in 40S ribosome assembly that serves to order cleavages at sites A and D, thereby temporally regulating cleavage at site D. If removal of the A₂-A₃ piece is also coupled to export, as discussed before, this could also help to spatially regulate Nob1-dependent pre-18S cleavage.

While we believe that this switch is essential for ribosome assembly (stabilizing the early structure slows D-site cleavage and 23S pre-rRNA is not processed to 18S rRNA), it seems unlikely that this is the only event that regulates Nob1 cleavage *in vivo*. Cytoplasmic 20S rRNA is exceptionally stable with a half-life time of minutes *in vivo*, suggesting that D-site cleavage is the rate-limiting step *in vivo*. While we cannot rule out the possibility that this 20S intermediate is in the pre-A₂ cleavage structure, we have no evidence for the presence of the A₂-A₃ piece in 43S ribosomes (data not shown), making this scenario highly unlikely. Furthermore, two kinases, Rio1 and Rio2, as well as the ATPase Fap7 are required for

cytoplasmic cleavage by Nob1 and it is possible if not likely that at least one of them acts on Nob1, either by phosphorylating Nob1 or by repositioning Nob1, Rps14, or even Rps5³⁰; ³¹. Such a model is also consistent with the observation that recombinant Nob1, as well as Nob1 purified from yeast⁸, are poor enzymes, with very slow turnover.

Tollervey and colleagues have recently suggested that Nob1 is mispositioned on 43S pre-ribosomes. This suggestion is based on the lack of full Nob1-dependent protection from DMS modification of cleavage site D in the steady state³². While such a proposal would be consistent with the model presented herein, it conflicts with our previous data, where we have shown that Nob1 does protect the cleavage site⁷. As far as we can tell the only difference between the two data sets is the presence of an HA-tag in the sample from the Tollervey lab. Previous Northern analysis with that strain shows that overexpressing the HA-tagged Nob1 under a galactose-promoter produces a substantial accumulation of 20S rRNA⁵. No such 20S accumulation is observed when Nob1 is overexpressed without an HA-tag (data not shown), suggesting that the 20S accumulation arises from the HA-tag. Epitope tags, even small ones like the HA-tag, can change protein localization, stability, as well as their interaction with other protein partners, as observed for a C-terminal tag on Nob1³². We thus believe that the lack of full protection of the cleavage site in the presence of Nob1 is due to the tag and not to inherent positioning of Nob1 distant from the cleavage site. This conclusion remains consistent with the proposed conformational switch because in the steady-state 20S accumulates in 43S pre-ribosomes, which are likely in the post-A₂ structure, as no A₂-A₃ piece can be detected in these particles (data not shown).

Why are rRNA processing steps ordered?

The conformational switch we have uncovered explains the ordering of cleavage at sites A₂ and D. Prior data indicate similar ordering for cleavages at sites A₁ and A₂, as no mutations have been described that lead to cleavage at site A₂ without prior cleavage at site A₁ [see¹⁰; ³³ and references therein]. Similarly, processing at site B_{1S} to give the 5'- end of 5.8S_S rRNA requires prior cleavage at site A₃ [reviewed in:¹⁰]. What is the rationale for these ordered processing steps? Although there is a hierarchy in binding of ribosomal proteins³⁴; ³⁵ as well as assembly factors³⁶; ³⁷, there are also parallel pathways by which ribosomal proteins and assembly factors bind (*e.g.*³⁶; ³⁸). Perhaps the strictly ordered cleavage steps are introduced to reconvert parallel pathways into singular intermediates. This is important because cleavage steps are irreversible, energy-releasing steps that have the potential to shift the energetic landscape of assembly towards the product³⁹. Thus, they should be carefully controlled and only occur if the assembly intermediate is correct. Surveying a single intermediate is much easier than inspecting the many intermediates possible from non-converging pathways. We suggest that pathways converge prior to important irreversible steps to assist quality control of ribosome assembly.

A conformational switch after Dim1/KsgA methylation

Prior data indicate that a conformational change may follow the activity/dissociation of the methyltransferase KsgA, the bacterial homolog of Dim1, which methylates the conserved adenosines in H45. KsgA does not methylate native 30S subunits but only an alternative conformer induced by low temperature or magnesium concentrations⁴⁰. The structures of mature 30S subunits show that A1518 and A1519 (A1781 and 1782 in yeast) make tertiary contacts with two subsequent base pairs in H44 (shown as olive lines in Fig. 2C), which could render them inaccessible. Interestingly, these docking sites are not present in the pre-A₂ structure, indicating that these conformational switches might be related.

RNA conformational switches are used to impose hierarchy and regulation onto RNA dependent processes

A large body of work on the assembly of bacterial ribosomal subunits has shown that the hierarchical order in assembly of primary, secondary, and tertiary binding ribosomal proteins is due to conformational switches in the rRNA induced by r-protein binding. Here, we show that the hierarchy in rRNA cleavage steps is also explained by rRNA conformational switches. Further extending the comparison, several snRNA-mRNA or snRNA-snRNA interactions are remodeled during spliceosome assembly^{41; 42}, and it seems likely that RNA switches also impose this hierarchy.

In addition, RNA conformational switches are also often slow, which can introduce points of regulation, as has been well-described for the case of pre-mRNA splicing^{41; 42}. Perhaps nature has found a way to exploit RNA's "Achilles' heel" - its propensity to form stable, alternative secondary structures that often lead to misfolding - to stall important biological processes, thereby allowing for quality control and regulation.

Experimental Procedures

Protein purification

Nob1 and D92N/Nob1 were cloned and purified as previously described⁷.

In vitro transcription

In vitro transcription was carried out as previously described⁷.

Mutagenesis

rRNA mutants for *in vivo* use were generated in pWL160 (a generous gift from M. Fournier) via the Quikchange method using PfuUltra II Fusion HS DNA polymerase (Stratagene). To prepare rRNA mutant templates for *in vitro* transcription, mutagenesis was carried out in the pUC-based plasmids described before⁷.

RNA binding

RNA binding experiments were performed by gel-shift analysis as previously described⁷.

RNA cleavage

Body-labeled rRNAs were transcribed *in vitro* as described before⁷. For 3'-end labeling, RNA was labeled with ³²P-pCp and RNA ligase (NEB) as per manufacturer's instructions. For 5'-end labeling, RNA was dephosphorylated using antarctic phosphatase (NEB) as per manufacturer's instructions and labeled with γ -³²P ATP using T4 polynucleotide kinase (NEB). These RNAs were gel-purified and eluted as described for *in vitro* transcriptions. Experiments were performed as for RNA binding with the following modifications: After folding in 10 mM MgCl₂, RNAs and protein were incubated for 1 hr at 30 °C in the presence of 2 mM MgCl₂ and 8 mM MnCl₂. 0.2 μ M Nob1 was used with trace amounts of RNA in Fig. 5 as well as with U(-1)G H44/A₂ while 5 μ M Nob1 was used with the other RNAs. After the reaction, RNA was separated for analysis using phenol:chloroform extractions and precipitated. Samples were resuspended in formamide and run on a 6% denaturing acrylamide gel, which was dried and analyzed by phosphorimager.

DMS probing

In vivo and *in vitro* DMS probing of rRNAs were performed as previously described⁷.

Growth analysis of rRNA mutant strains

Yeast strain NOY504 (a generous gift from M. Nomura) lacks the Rpa12 subunit of PolI and is therefore temperature sensitive¹⁶. This strain was transformed with pWL160¹⁵ encoding wild type rRNA or fully sequenced mutant derivatives described above. pWL160-encoded 18S and 25S rRNAs also contain a unique sequence tag by which they can be distinguished from endogenous rRNA¹⁵. These were incubated on YP-galactose plates either for 3 days at 30 °C or for 5 days at 37 °C.

Northern analysis of rRNA mutant strains

Yeast cells were grown at 37 °C to deplete endogenous PolI as described⁴³. Briefly, 100 ml of yeast strain NOY504 with plasmids encoding wild type rRNA¹⁵, mutant rRNA, or no rRNA were grown to an OD ~ 0.06 at 30 °C in YP galactose prior to shift to 37 °C for 6 h. Cells were harvested by centrifugation, and RNA was extracted using the hot phenol method. 10 µg total RNA was loaded onto a 1.25% agarose gel containing 6.7% formaldehyde and run for 12h with circulating MESA buffer at 95 V. The RNA was transferred overnight onto a Hybond N membrane by passive transfer. After blocking, RNA was probed for pre-rRNAs⁴⁴ or the tag in 18S rRNA¹⁵. Normalization was performed by probing against the snRNA U2⁴⁵. Analysis of counts in 20S rRNA relative to U2 in the negative control show that ~80% of the rRNA precursors are from the plasmids and not the genomic rDNA locus.

Phylogenetic Analysis

In addition to *S. cerevisiae*, other rDNA sequences were downloaded from Genbank as follows: *S. paradoxus* gi:2627120, *C. glabrata* gi:28793968, *C. albicans* gi:454300, *S. pombe* gi:288694, *D. melanogaster* gi:158246, *X. laevis* gi:65056, *C. elegans* gi:6830, *R. norvegicus* gi:2624399, and *H. sapiens* gi:555853. These organisms range in ITS1 length from 137 to 1095 nts (Table S2). The program rnabob 2.1¹⁹ was used to search for a “helical” structure in these sequences that would be complementary to the proximal region of helix 44, defined as UAGAGGAANUARAAGUCGUAACAAGGU. (For *C. elegans*, this region of helix 44 was defined as UGACCGGGUAAAAGUCGUAACAAGUU.) Because rnabob does not account for random bulges in the helix, a mismatch factor of 8–9 was used, as found in *S. cerevisiae*. Sequence alignments of the ITS1 region were performed using ClustalW and refined by hand. Structure predictions of the ITS1 region were performed using M-fold to further refine the alignment and refine the H44-interacting region in *X. laevis* and *C. elegans*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Cartoon of pre-rRNA cleavage pathways. For simplicity 60S processing pathways are not indicated. Cleavage sites discussed herein are shown in bold. Pathway A shows cleavage at sites A₀, A₁, and A₂ yielding 20S pre-rRNA, before cleavages at D and A₃, resulting in production of mature 18S and 25S rRNA. Alternatively, cleavage at site A₃ without prior cleavage at sites A₀-A₂ (pathway B) produces mature 25S rRNA and the 23S pre-rRNA, which is not converted into 18S rRNA.

Figure 2. pre-rRNAs prior to and after cleavage at site A₂ are in different conformations. (A) Schematic showing pre-rRNA constructs used in this study, comprising the 3' minor domain of 18S and the 5' end of ITS1. Green arrows represent 5'-ends, and red arrows represent 3'-ends of constructs. Color-coded schematic of the RNA conformational change after A₂-cleavage is included for clarity. (B) Examples of *in vitro* DMS probing data by primer extension. H44/+278 rRNAs are in the pre-A₂ cleavage structure, while H44/A₂ and H45/+278 rRNAs are in the post-A₂ cleavage structure. Reverse transcriptions were carried out with oligos d (left) and b (right panel). Note that reverse transcriptase stops prior to the modified nucleotide while ddNTP incorporation leads to a stop after the incorporated nucleotide. Thus, the sequencing ladder is upshifted 1 nt relative to the DMS lane. Labeled residues show increased accessibility in the pre-A₂ cleavage structure. (C) Mapping of the data in (B) onto secondary structure models for the two pre-rRNA conformers: pre-A₂ cleavage (H44/+278) and post-A₂ cleavage (H44/A₂). Residues equally accessible to DMS are shown by gray circles, residues more accessible in the pre-A₂ cleavage structure are shown in orange triangles. Open circles show mild accessibility as compared to filled circles. The olive line indicates a tertiary interaction observed in the 30S crystal structures. This interaction is expected to be disrupted in the pre-A₂ structure, as indicated by the strike-through. Arrows represent the ends of *in vitro* transcribed RNAs used herein and shown in (A). All numbering is relative to site D. See Figs. S2 and S3A for full DMS probing gels and line scan quantitations. (D) The 23S pre-rRNA is in the pre-A₂ cleavage conformation. *In vivo* DMS probing of 23S pre-rRNA. Yeast strain YKK37 has the *bms1*

gene under the control of a galactose-inducible promoter and was grown in YP-glucose overnight at 30 °C to deplete Bms1. Primer extension using oligo b is shown. Compare to pre-A₂/+DMS lane in (B), right panel. The conserved, Dim1-methylated adenosines in H45 (–18/–19) are indicated. Native methylation of these residues in 20S rRNA leads to a strong reverse transcription stop even in the absence of DMS⁷, which is not present in 23S, consistent with Dim1-dependent methylation occurring late in the cytoplasm⁴⁶. Lack of primer extension above the Dim1 site in 20S rRNA also means that all readings above that region arise from the 23S rRNA in the sample obtained in the absence of Bms1, even though there is substantial amount of 20S remaining in our sample (46%). Refer to Figure S5 for the full DMS gel and Northern analysis of the rRNA from these strains. Figure S3B has linescan quantitations.

Figure 3.

pre-rRNA mutations used in this study. Highlighted residues are changed to the indicated sequence. Mut A and B destabilize the inhibitory structure. Mut S stabilizes the inhibitory structure. Mut α S1 and α S2 in combination with mut S remove the stabilization. Mut N have no effect on the inhibitory or mature structure, but mutate the same residues mutated in Mut S.

Figure 4.

Nob1 interacts differently with pre-rRNAs prior to and after cleavage at site A₂. (A) Examples of DMS probing data by primer extension with oligo b, as described in Figure 2. Blue circles indicate residues more accessible in the pre-A₂ (than in the post-A₂) structure, and green circles represent residues that are more accessible in the post-A₂ (than in the pre-A₂) structure. (B) Mapping of the data in (A) onto the secondary structure models for the two pre-rRNA conformers: H44/+278 (pre-A₂) and H44/A₂ (post-A₂), as in Figure 2. See Fig. S2 for full DMS probing gels, and Figure S6 for linescan quantitations.

Figure 5.

Nob1 only cleaves RNA in the post-A₂ structure. 0.2 μM Nob1, D92N/Nob1, or no protein was incubated with 3'-end labeled H44/A₂ (post-A₂ structure) or H44/+278 (pre-A₂ structure). RNAs corresponding to the 3' products (D/A₂, D/+278) were used as sizing standards.

Figure 6.

Stabilizing pre-rRNA in the pre-A₂ cleavage structure delays 20S rRNA processing and slows yeast growth. Wild-type (+), mutated, or empty (-) plasmids carrying the rDNA gene under a galactose-inducible promoter were transformed into the NOY504 strain, in which a deletion of PolII-subunit Rpa12 renders PolII-driven expression of the endogenous rDNA temperature sensitive. At 37 °C, PolII-driven, plasmid-encoded rDNA represents the only source of rRNA in these yeast. (A) rRNA was purified from yeast grown in YP-galactose at 37 °C and subjected to Northern analysis. Probes specific for 20S rRNA (oligo e), plasmid-derived 18S rRNA (oligo f, 18S_{tag} refers to a unique silent mutation in the 18S of the plasmid-encoded rDNA), and U2 (oligo g, a splicesomal RNA used as a loading control for normalization) were analyzed. RNA mutations are described in Figure 3. S α S1 and S α S2 represent the presence of both the S and α S mutations. Bar graph represents quantitation of 20S signal from 5 different experiments, normalized for equal loading using the signal from the U2 probe. Standard error is displayed. (B) Yeast growth on YP-galactose plates at 37 °C, grown for 5 days, or at 30 °C, grown for 3 days. Strains are indicated at left, and each spot from left to right represents a 10-fold dilution of the inoculating yeast culture, with 10⁵ cells in the most concentrated spot.

Figure 7.

Model for the regulation of cleavage at the 3'-end of 18S rRNA. H44 is shown in blue, H45 in dark blue, rRNA participating in inhibitory interactions in red, Nob1 in green, and the remainder of the pre-40S particle in yellow, with sites D and A₂ as marked. Relevant RNA helices are shown as cylinders, the connection of H44 to the rest of rRNA as a dotted line. Early rRNA precursors containing RNA 3'- to site A₂ are in the pre-A₂ cleavage conformation, in which the decoding site in H44 is not yet formed and elements immediately 3' to site A₂ form base pairs with the 5'-end of H44 (shown as a red cylinder-duplex in I). Cleavage at site A₂ (II) and removal of the inhibitory RNA piece (III) lead to the conformational switch (IV). This switch leads to formation of the decoding site and repositioning of Nob1 (green) to bind cleavage site D, leading to Nob1-dependent cleavage at that site (V).

Table 1

Relative Nob1 Binding Affinities to Different rRNAs

3'-end	$K_{d,rel}$		
	−H44	+H44	K_{H44}
D (0)	89	12.5	7.1
A₂ (+212)	6.4	0.9	7.1
+228	1.8	0.5	3.6
+278	1	1.2	0.8

All binding affinities were obtained at 30 °C, 50 mM HEPES, 100 mM KCl and are the averages of at least three experiments.

−H44 RNAs start at −31 (H45), while +H44 RNAs start at −164 (H44, see Fig. 2A). K_{H44} is the contribution from H44, obtained by dividing the $K_{d,rel}$ values in the presence and absence of H44. See Fig. S1 for binding graphs. RNAs in the shaded regions are in the pre-A₂ cleavage structure.

Table 2
Stabilizing the Post-A₂ Cleavage Structure Allows for H44 Interaction with Nob1 when A₂-A₃ RNA Is Present.

	Wild-type	Mutant A	Mutant B
H45/+278	0.28 ± 0.05	0.33 ± 0.08	0.21 ± 0.06
H44/+278	0.35 ± 0.05	0.14 ± 0.01	0.11 ± 0.01
K_{H44}	0.8	2.4	1.9

All binding dissociation constants (K_d , μM) were obtained at 30 °C, 50 mM HEPES, 100 mM KCl and are the averages of at least three experiments. K_{H44} is the binding contribution of H44, determined by dividing the K_d values in the presence (H44/+278) and absence (H45/+278) of H44.