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Modification of rRNA as a 'quality control mechanism' in ribosome biogenesis

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Abstract An efficiently expressed rDNA plasmid was used to quantitatively analyze the effect of base changes in modified positions associated with the peptidyl transferase center of the 25S rRNA from the yeast *Schizosaccharomyces pombe*. The results show that, unlike normal RNA and relative to a less conserved modified position outside the center, these mutant RNAs are highly unstable and rapidly degraded with little or no effect on cell growth. These results provide direct evidence that the positions of modification can be critical sites for nuclease attack. Taken together with previous genetic analyses of rRNA modification, they raise the possibility that rRNA modification may act, at least in part, as a quality control mechanism to help ensure that only functional RNA is incorporated into active ribosomes. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: rRNA processing; RNA modification; Nucleolus; Ribosome; snoRNA

1. Introduction

The eukaryotic ribosome is assembled in the nucleolus where the pre-rRNA precursor is synthesized, integrated with ribosomal proteins and processed into mature rRNAs. In the course of RNA processing the precursor molecule is cleaved, but also modified with the conversion of some uridines to pseudouridines and the addition of methyl groups to specific nucleotides. The vast majority of the methylations are at the 2′-*o*-position of the ribose moiety with others on the bases (see [1,2]). Although first characterized more than three decades ago (e.g. [3,4]), the role of these modifications has remained surprisingly unclear. The modifications occur on the nascent pre-rRNA, most of them even before the transcript is completed; all, apparently, are limited to the universally conserved cores of the mature rRNAs. Such observations have led many to speculate that they must be involved in fundamental aspects of ribosome biogenesis or function. Until recently, little was also known about the modification processes themselves, but studies on the role of the small nucleolar RNAs have now provided exciting information about the mechanisms which underlie many of the site-specific modifications. Initially, sequence complementarities between one family of fibrillarin-associated snoRNAs and the mature rRNAs [5,6] were demonstrated to be essential for rRNA methylation. More recently, complementary between a second family of snoRNAs and the mature rRNAs also were shown to be essential for the site-specific synthesis of pseudouridine [7].

Despite this rapid progress in our understanding of the modification processes, the role of most modifications remains speculative. Many roles have been suggested, including influences on ribosome maturation (e.g. [8,9]), rRNA conformation [10,11] and ribosomal function [12]. In bacterial ribosomes, which contain fewer ribose methylations but more base methylations, at least some of the modifications have been shown to confer resistance to toxic antibiotics (see [13]) and even a role in the heat shock response [14]. Early occurring ribose methylation could fine-tune the folding of elongating pre-rRNA and modulate ribosomal protein binding. Indeed, altered processing has been reported [15,16] but the inhibition of methylation also has been shown not to be critical for pre-rRNA maturation [17]. Even more surprising [18,19], yeast cells lacking most of the snoRNAs grow normally, and even Ψ residues which appeared to be good candidates for direct involvement in the peptidyl transferase function [12] again appear not to be essential [7].

In view of the limited information about the role of modified nucleotides, we examined the effect of genetic change in modified positions by using in vivo mutagenesis analysis and an expression system which forces mutated RNA transcripts to compete with normal transcripts. As previously described [20–22], by using efficiently expressed rDNA plasmids with suitable RNA 'tags', normally growing transformants can be prepared in which more than half the rRNA is plasmid-derived and easily quantified. Under these competitive conditions, we demonstrate that changes in modified positions within the core of the peptidyl transferase center can dramatically alter the stability of the mature rRNA.

2. Materials and methods

2.1. Construction and expression of mutant rRNA genes

Changes in the 25S rRNA sequence of the *Schizosaccharomyces pombe* rDNA were introduced using pFL20/Sp5.8i4, a previously constructed [23] pFL20 yeast shuttle vector recombinant containing one complete *S. pombe* rDNA transcriptional unit which was 'tagged' with a four base insert in the 5.8S rRNA sequence. Mutant sequences were prepared by a two-step PCR amplification strategy and substituted for the normal sequences by standard cloning techniques as previously described [24]. Recombinants containing the mutant sequences were amplified in *Escherichia coli* strain C490, and this DNA was used to verify the constructions by DNA sequencing [25] and to transform *S. pombe* strain h^- leu1-32 ura4-D18 by the method of Prentice [26]. The

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presence of the recombinant DNA sequence was confirmed by PCR amplification directly using an isolated transformed colony as a template and an initial 10 min denaturation step. Of the two primers, one was specific for the rDNA sequence and the other was specific for the plasmid polylinker sequence. All subsequent analyses were performed on transformed cells grown at 30°C in minimal medium No. 2 [27].

2.2. Preparation and analysis of ribosomal RNAs and precursors

For all analyses, RNA was isolated from whole cells by sodium dodecyl sulfate/phenol extraction as previously described [22], either directly or after cell disruption, by vortexing with an equal volume of glass beads. For 5.8S rRNA analyses, the RNA was fractionated on 8% polyacrylamide gels and visualized by methylene blue staining [20]. For Northern blot analyses, 10 μg of RNA was fractionated on 1.5% agarose/formaldehyde gels, capillary-transferred to nylon membranes (Zetabind, CUNO, Meriden, CT, USA), hybridized with a ^{32}P-labeled ITS2-derived DNA fragment and washed as previously described [22,28]. Membranes were stained with methylene blue to confirm that equal amounts of RNA were transferred [29].

3. Results and discussion

As illustrated in Fig. 1, targeted mutations were introduced into the 25S rRNA sequence by a two-step PCR-based strategy [20,24]. To allow us to track the plasmid-derived pre-rRNA transcripts and to determine the levels of plasmid-derived rRNAs, a previously well-characterized neutral marker was used, a four base insertion in the 5.8S rRNA sequence [23]. When genes containing this marker were expressed in vivo, about 50–60% of the ribosomal RNA was mutant and easily detected by an altered electrophoretic mobility for the 5.8S rRNA (e.g. Fig. 2). The 25S rRNA substitution mutations were constructed using mutagenic primers to amplify overlapping 'cassette' sequences (Fig. 1a), which were substituted in the normal rDNA by restriction enzyme digestion and cloning (Fig. 1b). In each case the mutant sequence and plasmid construct were verified by DNA sequencing before being introduced into yeast cells. The presence of the mutant sequence in the yeast cells was also confirmed by PCR amplification.

As also indicated in Fig. 1, the core of the peptidyl transferase center contains six Ψs and four 2′-*o*-methylated sites which are conserved in yeast, flies, mouse and humans (see [30]) but were found to be dispensable when the snoRNAs, essential to their synthesis, were eliminated [7]. One of the sites (nucleotides 3015–3017) is hypermodified with both a pseudouridylic acid residue and two 2′-*o*-methylated nucleotides (UmGmΨp). In the present study the sequence at four modified positions was altered, both the Ψ and 2′-*o*-methylated residues in the hypermethylated site and the Am residue in the second highly modified region (nucleotides 3038–3042). In addition, a change was also made in an adjoining region, a 2′-*o*-methylated site (nucleotides 3135–3136) in most of the studied organisms but not yeast. As shown in Fig. 2, all the changes had dramatic effects on the amount of plasmid-derived 5.8S rRNA with only trace amounts of tagged mature RNA being detected when sites were changed in the peptidyl transferase center. In contrast to the mutations in the highly conserved modified sites, the mutations in the third less con-

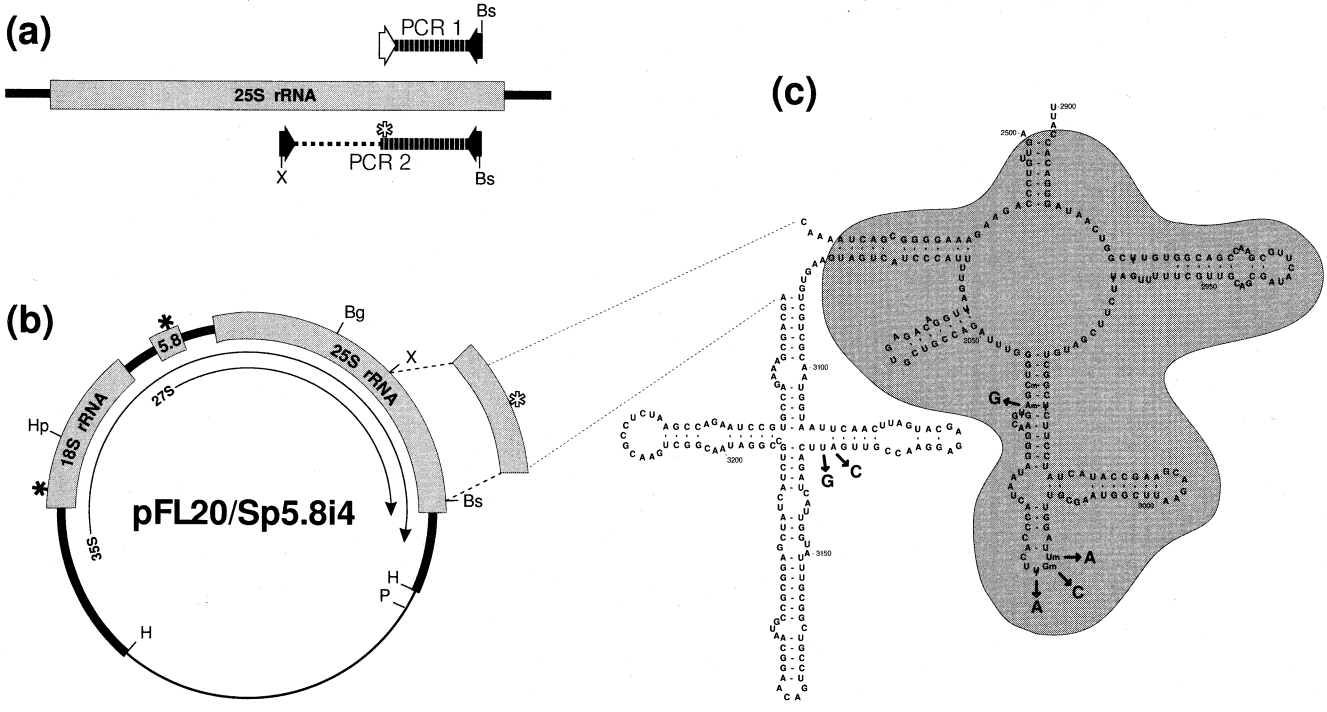

Fig. 1. PCR-mediated changes in modified positions of the 25S rRNA sequence in *S. pombe* rDNA. a: The core of the peptidyl transferase center sequence was PCR-amplified (PCR1) using a mutagenic primer (open arrow) and a 25S rRNA-specific primer preceding a *Bst*BI (Bs) restriction site. The mutated (open star) sequence was extended in a 5′-end direction (PCR2) using the product of the first reaction and a second 25S rRNA-specific primer preceding an *Xho*I (X) restriction site. b: After *Bst*BI and *Xho*I endonuclease digestion the second product was substituted for the normal rDNA sequence by standard cloning techniques and a pFL20-derived yeast shuttle vector containing an *S. pombe* rDNA transcriptional unit with a 'tag' (closed star) in the 5.8S rRNA [23]. Recombinants were picked randomly and confirmed by restriction enzyme digestion and DNA sequencing. Purified plasmid DNA was used to transform *S. pombe* strain h$^-$ leu1-32 ura4-D18 as previously described [20]. c: Modified nucleotides in the core of the peptidyl transferase center from the *S. pombe* 25S rRNA. The arrows indicate nucleotide substitutes which were introduced in the core (shaded) or adjacent region.

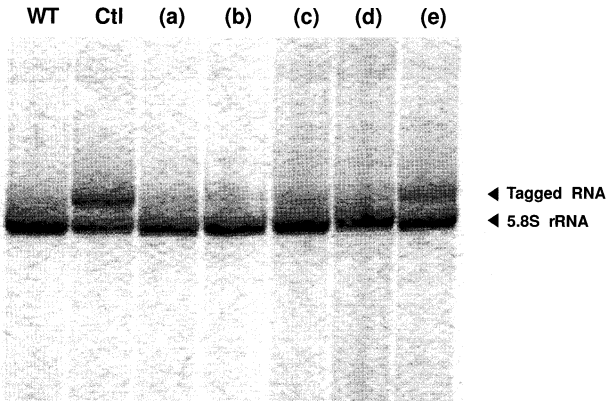

Fig. 2. Mature 5.8S RNA from ribosomes of *S. pombe* cells transformed with rDNA containing changes in modified positions of the 25S rRNA sequence. Whole cell RNA was prepared from normal *S. pombe* (WT) and cells transformed with pFL20/Sp5.8i4 (Ctl), pFL20/Sp25A$_{3015}$–A$_{3017}$ (a), pFL20/Sp25A$_{3017}$ (b), pFL20/Sp25G$_{3040}$-C$_{3059}$ (c), pFL20/Sp25G$_{3040}$ (d) and pFL20/Sp25C$_{3135}$G$_{3136}$ (e), fractionated on a 12% polyacrylamide gel and stained with methylene blue to visualize the RNA bands.

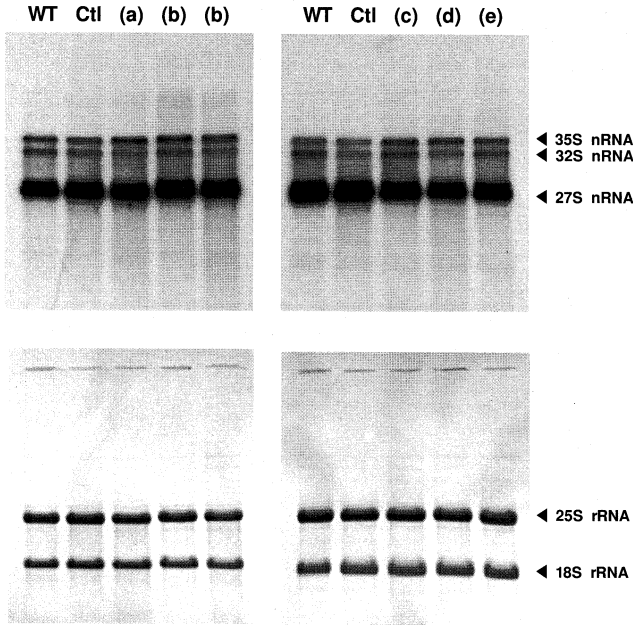

Fig. 3. Expression of *S. pombe* rDNA containing changes in modified positions of the 25S rRNA sequence. Whole cell RNA was prepared from normal *S. pombe* (WT) and cells transformed with pFL20/Sp5.8i4 (Ctl), pFL20/Sp25A$_{3015}$–A$_{2017}$ (b), pFL20/Sp25G$_{3040}$-C$_{3059}$ (c), pFL20/Sp25G$_{3040}$ (d) and pFL20/Sp25C$_{3135}$G$_{3136}$ (e), and fractionated on an 0.8% agarose gel. For precursor analyses (upper panel), the RNA was transferred to a nylon membrane and hybridized with a ^{32}P-labeled ITS2 sequence-specific probe [22,28]. Membranes were stained with methylene blue (lower panel) to confirm that approximately equal amounts of RNA were transferred.

served site (nucleotides 3135 and 3136) clearly were less critical, resulting in significant levels of plasmid-derived RNA. As shown in Fig. 2 (lane e), the amount of plasmid-derived 5.8S rRNA was reduced but still easily detected. As indicated in Table 1, in this mutant the plasmid-derived RNA level was reduced by about 50%.

The dramatic changes which were observed in Fig. 2 could be the result of three very different effects: an inhibition in rRNA expression, an inhibition in rRNA processing or a highly unstable rRNA population. To test these alternate possibilities nascent RNA transcripts were investigated by Northern blot hybridization analyses. As shown in Fig. 3 (top), when whole cell RNA was prepared from the transformed cells and probed with an ITS2-specific DNA fragment, comparable levels of expression and processing were observed in all cell types. In untransformed cells and cells transformed with normal or mutant rDNAs, equal amounts of the 35S rRNA transcript as well as 32S and 27S rRNA intermediates were evident. There was neither a reduced level of precursor consistent with a reduced expression, nor was there an increase in any precursor which would indicate a defective step, as previously observed with other mutants (e.g. [21]). To ensure equal extraction and RNA transfers, the membranes were also stained with methylene blue, revealing essentially equal amounts of mature 18S and 25S rRNAs (Fig. 3, bottom). Taken together, these results indicate that a cleavage step is not adversely affected and RNAs with mutations in the modified positions are simply highly unstable and degrade very rapidly, resulting in an rRNA population which is almost entirely chromosome-derived. Despite these striking differences in the amounts of plasmid-derived RNA and RNA stability, as summarized in Table 1, all the transformants grew with essentially equal growth rates.

Since the plasmid-derived rRNA normally represents about 50–60% of the mature ribosomal RNA population, and little or no plasmid-derived RNA was observed with the mutant genes, clearly about half the transcribed RNA was degraded without an effect on the growth rate. This was somewhat

Table 1
Growth rate of *S. pombe* cells transformed with rDNA containing changes in modified positions of the 25S rRNA sequence

Plasmid	Modification[a]	Plasmid-derived[b] 5.8S rRNA	Doubling time[c]
Untransformed		0	4.7
pFL20/Sp5.8i4		49.7 ± 2.3	4.9
pFL20/Sp25A$_{3015}$–A$_{3017}$	UmGmΨ	Trace	4.9
pFL20/Sp25A$_{3017}$	Ψ	Trace	4.7
pFL20/Sp25G$_{3040}$C$_{3059}$	Am	Trace	4.8
pFL20/SpG$_{3040}$	Am	Trace	4.8
pFL20/SpC$_{3135}$G$_{3136}$	GmUm	25.8 ± 1.9	4.6

[a]Modified nucleotide(s) which is changed in the plasmid rDNA.
[b]Whole cell RNA was fractionated as described in Fig. 2. The stained gels were dried, the images were captured and the relative amount of tagged 5.8S rRNA as a percentage of the total 5.8S rRNA was determined using Molecular Analyst software (Bio-Rad Laboratories, Richmond, CA, USA).
[c]Hour/doubling for logarithmically growing cells in selective medium as determined by the absorbancy at 550 nm. Values are averages for two to three replicate experiments.

surprising but entirely consistent with past studies of highly unstable rRNA products. For example, in earlier studies on mutations in the 5.8Ss rRNA structure [21,22] or the transcribed spacers regions [22,32], similar highly unstable rRNA products that also represented 50–60% of the rRNA transcripts were not present as mature ribosomal RNA. It is clear that yeast cells are able to dramatically overproduce ribosomal RNA with little effect on the growth, assuming that growth is otherwise well-supported. Similar absences of effects were also reported in previous genetic analyses [7,19,20] when mediating snoRNAs were eliminated with little or no change in the growth rate. Clearly, in those instances, an unmethylated RNA was incorporated into functional ribosomes and the RNA was sufficiently stable to be processed and assembled into mature subunits. As in the present study, it is attractive to speculate that in the snoRNA-compromised cells a more rapid turnover in rRNA may have been compensated for by an overproduction of the rRNA transcripts. Whatever the case, in the present study the absence of plasmid-derived mature RNAs or elevated precursor levels strongly suggest that the altered RNAs cannot be properly assembled into pre-ribosomal particles because of a reduced affinity for cognate proteins. The competition with normal RNA in the present study may exaggerate this difference and lead essentially to the complete absence of mutant rRNA in the mature ribosomes.

Despite the apparent ability of cells to easily overexpress rRNA as needed, this would represent a wasteful use of nucleotides and a stabilization of the rRNA by the modifications, clearly, would represent significant conservation. Since they interact with other cellular components and are likely to be more accessible, sites such as the peptidyl transferase center are also likely to be more open to nuclease attack, and the presence of specific modifications that minimize the attacks would be entirely consistent. Again, a reduced affinity for a ribosomal protein which actually protects the RNA from degradation similarly could be the primary cause of RNA degradation.

A second perhaps more important role for the modifications can also be suggested based on our past studies of rRNA synthesis in *S. pombe*. Although originally thought not to exist, interdependencies in the processing of ribosomal RNAs have been demonstrated in many respects. For example, an extended hairpin structure in the 3′ expressed sequence tag (EST) has been shown to be critical, not only to the processing of the 3′-end in the large subunit rRNA, but also to the maturation of the 5.8S rRNA, some 3000 nucleotides upstream of the 3′EST [31], and, to a lesser extent, even the level of the 18S rRNA [22]. Similarly, mutations in the 5′EST are critical to the maturation of the 18S rRNA but also severely affect the 5.8S and 25S rRNAs [32]. Observations such as these suggest that the formation of the 80–90S nucleolar precursor particle and the subsequent cleavages of the nascent rRNA transcripts represent, at least in part, a quality control mechanism which helps ensure that only functional rRNA is incorporated into cytoplasmic ribosomes. This is very important to cell growth and viability because ribosomes carry out protein synthesis in polysomes and a single defective ribosome can easily inactivate or greatly slow all the ribosomes in a polysomal chain. Such a feature greatly enhances the potential negative consequences of mutations.

In view of the present study, it seems reasonable to speculate that the rRNA modifications may play a similar role. As already indicated, site-specific rRNA modifications are dependent on snoRNAs. These contain long sequence complementarities to the mature rRNAs and pair directly with the complementary rRNA sequences in order to guide the modification processes [6,7]. As a result, such interactions effectively proof-read portions of the nascent rRNA sequence, as altered sequences may not pair effectively and, therefore, a modification would not occur. If the unmodified RNA was unstable or less stable it would subsequently be eliminated from the nascent RNA population. Since the results in this study indicate that nucleotide positions which are modified actually can be critical for nuclease attack and lead to an elimination of the mutant RNAs we believe these results, together with the above noted sequence scanning hypothesis, provide for a second quality control mechanism which, again, helps ensure that only stable and functional rRNAs are incorporated into mature ribosomes.

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