

Base pairing between U3 and the pre-ribosomal RNA is required for 18S rRNA synthesis

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The nucleolus, the site of pre-ribosomal RNA (pre-rRNA) synthesis and processing in eukaryotic cells, contains a number of small nucleolar RNAs (snoRNAs). Yeast U3 snoRNA is required for the processing of 18S rRNA from larger precursors and contains a region complementary to the pre-rRNA. Substitution mutations in the pre-rRNA which disrupt this base pairing potential are lethal and prevent synthesis of 18S rRNA. These mutant pre-rRNAs show defects in processing which closely resemble the effects of genetic depletion of components of the U3 snoRNP. Co-expression of U3 snoRNAs which carry compensatory mutations allows the mutant pre-rRNAs to support viability and synthesize 18S rRNA at high levels. Pre-rRNA processing steps which are blocked by the external transcribed spacer region mutations are largely restored by expression of the compensatory U3 mutants. Pre-rRNA processing therefore requires direct base pairing between snoRNA and the substrate. Base pairing with the substrate is thus a common feature of small RNAs involved in mRNA and rRNA maturation.

Keywords: RNA/RNA processing/ribosomes/small nucleolar RNAs/U3

Introduction

The nucleolus is a specialized compartment of eukaryotic nuclei devoted to the transcription, processing and assembly of rRNA into ribosomal subunits. The small subunit ribosomal RNA, 18S rRNA, and two large subunit rRNAs, 5.8S and 25/28S rRNA, are transcribed by RNA polymerase I (pol I) as a single precursor molecule. In this pre-ribosomal RNA (pre-rRNA) the rRNA coding regions are flanked and separated by non-coding regions called spacers. A complex series of processing reactions are needed to eliminate the 5' and 3' external transcribed spacers (5' ETS and 3' ETS respectively) and the internal transcribed spacers 1 and 2 (ITS1 and ITS2 respectively). We are just starting to define the *cis*-acting elements and *trans*-acting factors involved in this major processing pathway.

A rapidly increasing number of small nucleolar RNAs (snoRNAs) are being characterized (Filipowicz and Kiss, 1993; Fournier and Maxwell, 1993; Sollner-Webb, 1993; Maxwell and Fournier, 1995). Some of these snoRNAs have been shown to be required for pre-rRNA processing,

but very little is known about their mechanism of action (Filipowicz and Kiss, 1993; Fournier and Maxwell, 1993; Sollner-Webb, 1993; Lygerou *et al.*, 1994; Tycowski *et al.*, 1994). Over 20 snoRNAs have been characterized to date in yeast, but only five RNA species (U3, U14, snR10, snR30 and MRP RNA) have been shown to be required for normal pre-rRNA processing (Tollervey, 1987; Li *et al.*, 1990; Hughes and Ares, 1991; Morrissey and Tollervey, 1993; Lygerou *et al.*, 1994). The depletion of any of several snoRNP components (the snoRNAs U3, U14, snR10 and snR30 or the proteins Nop1p, Sof1p and Gar1p) results in a similar defect in pre-rRNA processing. The normal pre-rRNA intermediates in the pathway leading to the synthesis of 18S rRNA are missing, leading to under-accumulation of mature 18S rRNA. In contrast, 5.8S and 25S rRNAs are synthesized normally (Tollervey, 1987; Li *et al.*, 1990; Hughes and Ares, 1991; Tollervey *et al.*, 1991; Girard *et al.*, 1992; Jansen *et al.*, 1993; Morrissey and Tollervey, 1993). The similarities in the phenotypes of the mutants suggest that these snoRNPs are associated in a multi-snoRNP processing complex which cleaves sites A1 and A2 (see Figure 4A) in a coordinated manner.

U3 has also been implicated in pre-rRNA processing in vertebrates. In *Xenopus* oocytes, removal of the 5' third of U3 snoRNA has been reported to interfere with cleavage at the boundary of ITS1 and 5.8S rRNA (Savino and Gerbi, 1990). *In vitro* cleavage of the 5' ETS in mouse and *Xenopus* cell extracts also requires U3; processing is inhibited by depletion of the U3 snoRNP by immunoprecipitation or by oligonucleotide-directed cleavage of the U3 snoRNA (Kass *et al.*, 1990; Mougey *et al.*, 1993b). This processing reaction is associated with the assembly of a large complex in mammalian (Kass and Sollner-Webb, 1990) and *Xenopus* cell extracts (Mougey *et al.*, 1993b). These putative processing complexes can be visualized as terminal balls on nascent RNA pol I transcripts (the so-called Christmas trees) by electron microscopy of chromatin spreads (Mougey *et al.*, 1993a). Terminal knobs have been observed in a multitude of eukaryotes, including yeast (Saffer and Miller, 1986), and their presence is thought to be conserved across eukaryotic evolution (references in Mougey *et al.*, 1993a). However, the level of ETS processing varies greatly between species (Mougey *et al.*, 1993a), suggesting that terminal balls have a conserved function beyond cleavage of the 5' ETS sequences. It may be that such complexes assemble co-transcriptionally on the 5' ETS and then, following transcription termination, function to promote downstream processing events leading to the production of mature rRNAs.

Many snoRNAs, including U3, contain stretches of nucleotides complementary to sequences in precursor or mature rRNAs, which are therefore potential targets for

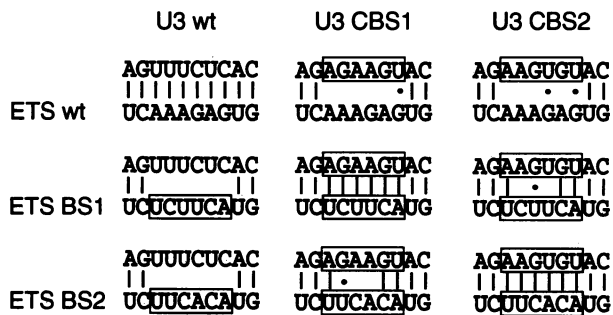


Fig. 1. Base-pairing potential between U3 and pre-rRNA in the 5' ETS. Wild-type and mutated pre-rRNA sequences (ETS wt, ETS BS1 and ETS BS2, nt +470 to +479), shown 5'→3', are aligned with wild-type and mutant U3 snoRNAs (U3 wt, U3 CBS1 and U3 CBS2, nt +39 to +48), shown 3'→5'. GC and AU base pairs are marked by vertical bars; dots indicate GU base pairs. Mutant 5' ETS and U3 sequences are boxed. Each multiple base substitution in the 5' ETS abolishes complementarity between pre-rRNA and U3 in the central 6 nt. Each compensatory mutation in U3 completely restores Watson–Crick base pairing; U3 CBS1 is fully complementary to ETS BS1, as U3 CBS2 is to ETS BS2. A variable degree of complementarity is shown in the other combinations. *In vivo* psoralen cross-linking between the two molecules was shown in this region, providing physical support for the proposed base-pairing interaction (Beltrame and Tollervey, 1992)

processing/assembly reactions. In several organisms (rat and human cells, *Saccharomyces cerevisiae* and *Trypanosoma brucei*) U3 snoRNA has been cross-linked *in vivo* to the 5' ETS of pre-rRNA (Maser and Calvet, 1989; Stroke and Weiner, 1989; Beltrame and Tollervey, 1992; T.Hartshorne, personal communication). In yeast, one *in vivo* cross-linking site corresponds to a 5' ETS region showing 10 nucleotides (nt) of perfect complementarity to a mainly single-stranded region of the U3 snoRNA. We have shown that deletion of these 10 nt in the 5' ETS reproduces *in cis* all the effects of U3 depletion *in trans* (Beltrame *et al.*, 1994). In this report we demonstrate that Watson–Crick base pairing between U3 and the 5' ETS is indeed crucial for pre-rRNA processing.

Results

Base substitutions in the 5' ETS region complementary to U3 are lethal

The deletion of the nucleotides complementary to U3 in the 5' ETS blocks 18S rRNA synthesis, suggesting that a base pairing interaction between U3 and the 5' ETS is required for pre-rRNA processing. We directly tested this hypothesis by creating multiple base substitutions in the 5' ETS, which disrupt the complementarity to U3 (Figure 1). The U3 sequence complementary to the 5' ETS is mainly, but not totally, single-stranded (Ségault *et al.*, 1992). In particular, the central 6 nt were shown to be single-stranded, whereas the remaining 4 nt were shown to be engaged in intramolecular base pairing in the two stems flanking the single-stranded region of U3. To avoid potential problems with the stability of U3, which might be caused by base substitutions disturbing the stems, we restricted mutagenesis to the central 6 nt of the complementarity region on the two molecules. Two different base substitutions (BS1 and BS2) were constructed in the 5' ETS, both of which alter the central 6 nt of the 10

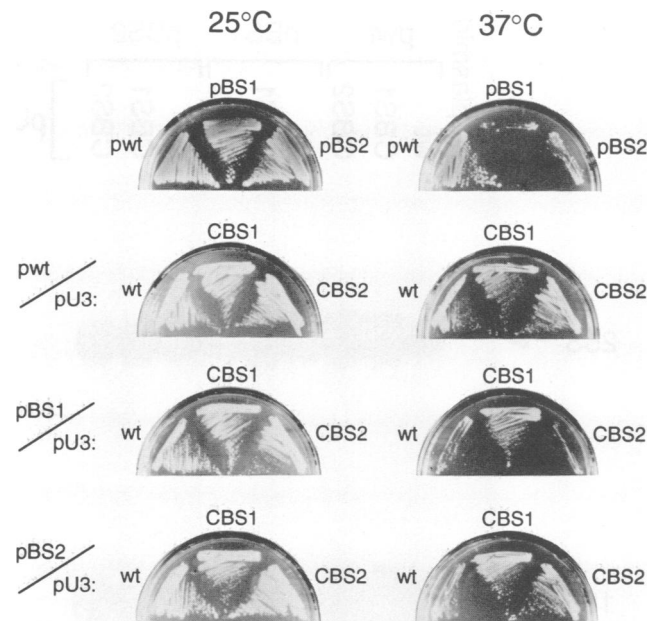


Fig. 2. Suppression of pre-rRNA mutations by compensatory changes in U3 snoRNA. A yeast strain carrying a *ts* mutation in RNA pol I was transformed with a plasmid containing one rDNA repeat under the control of the *GAL7* promoter and (where indicated) with a second plasmid carrying an intron-less U3 gene. The 5' ETS sequence on the plasmid-borne rDNA copy was either wild-type (pwt) or mutated (pBS1 or pBS2); the U3 copy on the plasmid was also either wt (pU3-wt) or mutated (pU3-CBS1 or pU3-CBS2). The sequences of the mutants are shown in Figure 1. Growth on galactose plates at 25 and 37°C is shown for each strain. Cells carrying pBS1 or pBS2 are unable to grow at 37°C, whereas cells carrying the pwt control grow at both 25 and 37°C. The growth defect of the pBS1 strain is suppressed by transformation with pU3-CBS1, but not by transformation with pU3-wt or pU3-CBS2. The growth defect of the pBS2 strain is suppressed by transformation with pU3-CBS2 or pU3-CBS1, but not by transformation with pU3-wt. Panels with the pwt strain transformed with pU3-wt, pU3-CBS1 and pU3-CBS2 are shown as controls.

bp of complementarity, strongly reducing the potential for base pairing between U3 and the pre-rRNA.

The effects of these mutations on the processing of pre-rRNA were tested by expression within the full-length pre-rRNA in a genetic background in which the mutated rDNA is the only transcribed rDNA repeat. A yeast strain carrying a temperature-sensitive (*ts*) mutation in RNA pol I was transformed with a plasmid containing an rDNA repeat under the control of the *GAL7* promoter, which is transcribed by RNA pol II (Nogi *et al.*, 1991, 1993; Beltrame *et al.*, 1994; Henry *et al.*, 1994). At 37°C on galactose-containing medium this becomes the only active rDNA repeat, because chromosomal rDNA transcription is inhibited. Plasmids expressing the wild-type pre-rRNA (pwt) are able to support growth at 37°C, whereas plasmids expressing pre-rRNA containing the BS1 or BS2 mutations (pBS1 and pBS2) are unable to support growth at 37°C (Figure 2, upper panels). All strains grow at 25°C, at which temperature the chromosomal rDNA is transcribed. This suggests that functional ribosomes are not made from the mutant pre-rRNAs.

Northern analysis was used to determine whether the mutant pre-rRNAs are processed to mature rRNAs (Figure 3). The rDNA repeat in which the 5' ETS mutations were tested also carries neutral tag sequences in the 18S and

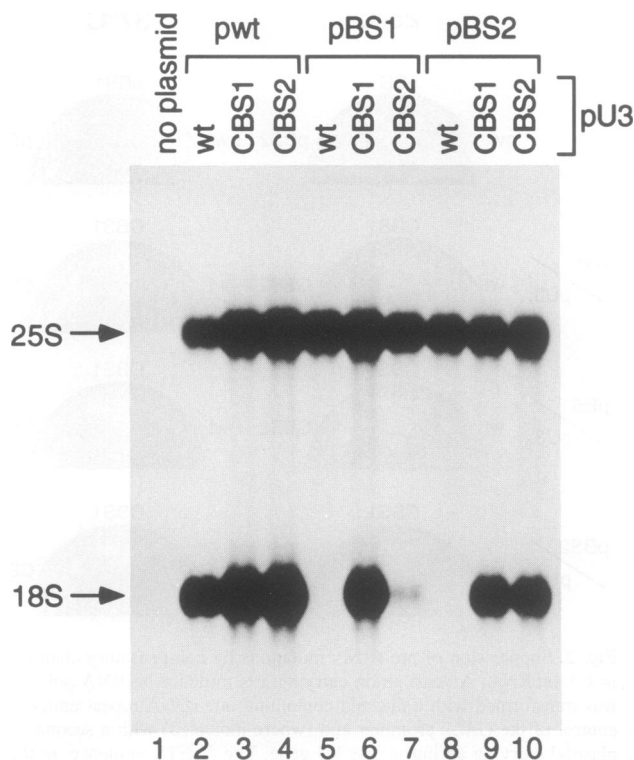


Fig. 3. 18S rRNA synthesis is inhibited by 5' ETS mutations and restored by compensatory mutations in U3. An untransformed control shows no background from chromosomal rRNA (lane 1). When transformed with a wild-type rDNA repeat under the control of the *GAL7* promoter, cells synthesize both 18S and 25S rRNAs in the presence of either wt or mutated U3 (lanes 2–4). The BS1 and BS2 mutations in the 5' ETS inhibit 18S rRNA synthesis in the presence of wt U3 snoRNA (lanes 5 and 8). Synthesis of 18S rRNA is restored at different levels in the presence of the counter-mutated CBS1 and CBS2 U3 snoRNAs (lanes 6, 7, 9 and 10). Total RNA was extracted from a yeast strain carrying a *ts* mutation in RNA pol I following incubation for 6 h at 37°C in galactose medium. RNA was separated on a 1.2% agarose–formaldehyde gel and transferred to a nylon membrane. Oligonucleotides complementary to short tags inserted in the 18 and 25S rRNA sequences in the plasmid-borne rDNA repeat were used as probes for Northern hybridization.

25S rRNAs, allowing identification of mature rRNA molecules which have been synthesized from the plasmid-borne rDNAs. Both 18S and 25S rRNAs are synthesized from the plasmid expressing the wild-type pre-rRNA (Figure 3, lane 2). In contrast, strains carrying base substitution mutations in the 5' ETS (pBS1 and pBS2) do not accumulate 18S rRNA at 37°C, whereas 25S rRNA is normally synthesized (Figure 3, lanes 5 and 8). It therefore appears that the BS1 and BS2 mutant pre-rRNAs do not support growth, because they cannot be processed to yield mature 18S rRNA.

Compensatory mutations in U3 restore viability and 18S rRNA synthesis

The question arises whether the mutant pre-rRNAs could be processed normally if complementarity to U3 were restored. We therefore generated compensatory base changes in U3 snoRNA (CBS1 and CBS2), which potentially restore perfect complementarity with the BS1 and BS2 pre-rRNAs respectively, allowing the same number of hydrogen bonds to the mutated 5' ETS sequences as are present in the wild-type interaction (Figure 1). The

U3 sequence that we mutated was shown to be single-stranded in snoRNA (Ségault *et al.*, 1992) and we therefore expected that the mutations would not disrupt U3 secondary structure. The two yeast U3 genes each contain an intron (Myslinski *et al.*, 1990). To avoid potential effects of the mutations on splicing efficiency, the base changes were introduced into an intron-less construct expressed from the natural promoter and terminator regions (pU3-wt, see Materials and methods). Plasmids expressing U3 with the compensatory base changes in U3 are designated pU3-CBS1 and pU3-CBS2.

The wild-type and mutant U3 constructs were introduced into strains expressing the pre-rRNAs with base substitutions in the 5' ETS and growth was examined at 37°C (Figure 2). The compensatory mutations in U3 that fully restore complementarity to the mutated 5' ETS sequences in pre-rRNA suppress the lethality of the base substitution mutations. Yeast cells expressing the BS1 mutation in pre-rRNA are viable at 37°C when transformed with pU3-CBS1, as is the case for BS2 mutants transformed with pU3-CBS2 (Figure 2). When base pairing is only partially restored (as in the case of BS1/CBS2 or BS2/CBS1; see Figure 1) the situation is somewhat different. Cells expressing the BS2 mutant pre-rRNA are viable at 37°C when transformed with either pU3-CBS1 or pU3-CBS2, whereas only pU3-CBS1 can restore viability to cells expressing the BS1 mutant pre-rRNA (Figure 2).

The growth phenotypes strictly correlate with the level of 18S rRNA synthesized in the different strains. The introduction of pU3-CBS1 allows synthesis of wild-type levels of 18S rRNA from pre-rRNA containing the 5' ETS BS1 mutation (Figure 3, lane 6); transformation with pU3-CBS2 leads to production of only a trace level of 18S rRNA (Figure 3, lane 7). A substantial amount of 18S rRNA is produced from pre-rRNA carrying the 5' ETS BS2 mutation in the presence of either pU3-CBS1 or pU3-CBS2 (Figure 3, lanes 9 and 10). It should be noted that although the CBS1 mutation in U3 does not restore full complementarity with the BS2 pre-rRNA, the base-pairing potential is greater than between wild-type U3 and BS2 (Figure 1).

Cleavage at all U3-dependent processing sites is blocked by mutation of the pre-rRNA and restored by the compensatory mutations

The wild-type yeast pre-rRNA processing pathway is shown in Figure 4B. The 35S primary transcript is normally cleaved at sites A0 (in the 5' ETS), A1 (at the 5' end of 18S rRNA) and A2 (in ITS1). This generates the 20S pre-rRNA, precursor to 18S rRNA, and the 27SA pre-rRNA, the precursor to 5.8S and 25S rRNAs. In wild-type strains the 27SA pre-rRNA is cleaved by RNase MRP at site A3 in ITS1, generating the 27SA' pre-rRNA (Henry *et al.*, 1994; Lygerou *et al.*, 1994). The cleavages at sites A0, A1 and A2 are inhibited in strains which are genetically depleted of U3 snoRNA or associated proteins (Hughes and Ares, 1991; Beltrame *et al.*, 1994). In the absence of these cleavages, the 35S pre-rRNA accumulates and can be cleaved directly at site A3. As shown in Figure 4C, this generates the 27SA' pre-rRNA and a 23S pre-rRNA which is present only at very low levels in wild-type strains of yeast. In U3 snoRNP mutant strains the 27SA' pre-rRNA is processed normally to 5.8S and 25S rRNA

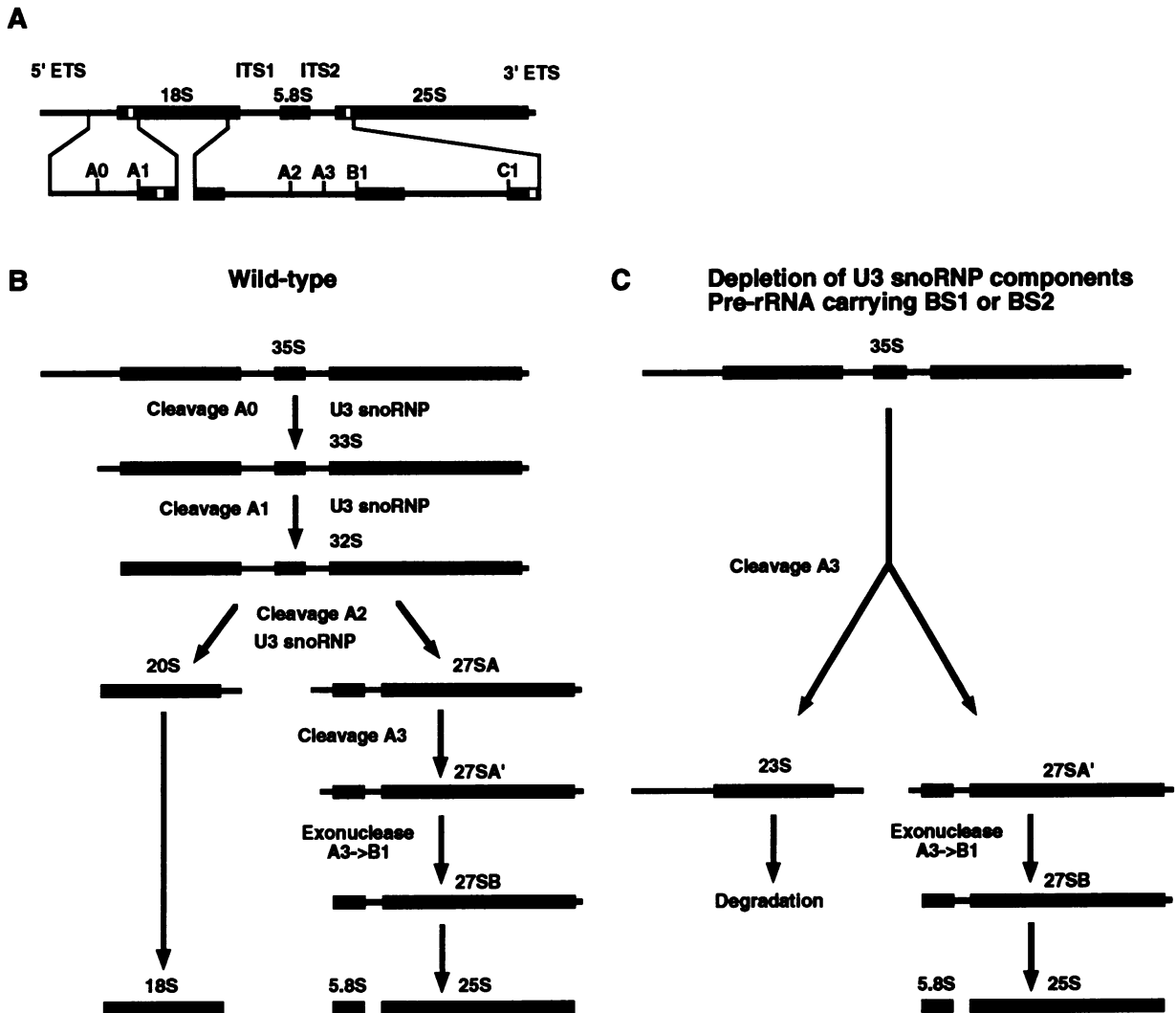


Fig. 4. Structure of pre-rRNA and the pre-rRNA processing pathway. (A) Structure of the yeast pre-rRNA showing major cleavage sites. Thick lines indicate the mature rRNA domains; open boxes within these indicate the sites of insertion of the tags. (B) The major pre-rRNA processing pathway in wild-type strains of yeast. U3 snoRNP-dependent processing steps are indicated. For simplicity the alternative processing pathway which generates a minor, 5' extended form of the 27SB pre-rRNA and 5.8S rRNA (Henry *et al.*, 1994) is not shown. (C) The processing pathway observed for pre-rRNA carrying the BS1 or BS2 mutations in the 5' ETS and in strains genetically depleted of U3 snoRNA or associated proteins.

and processing at sites B1 (the 5' end of the mature 5.8S rRNA region) and C1 (the 5' end of the mature 25S rRNA region) is unaffected. In contrast, the 23S pre-rRNA is rapidly degraded, preventing the synthesis of 18S rRNA. As will be demonstrated below, the BS1 and BS2 mutations in the pre-rRNA reproduce the processing pathway shown in Figure 4C, while the compensatory mutations largely restore the wild-type pre-rRNA processing pathway.

Sites A0 and A2 are the 5' ends of the 33S and 27SA pre-rRNA species respectively. Primer extension analysis shows that formation of RNA species with 5' ends at sites A0 (Figure 5A) and A2 (Figure 5C) is strongly inhibited by the BS1 or BS2 mutations (Figure 5, compare the pBS1 and pBS2 samples in lanes 5 and 8 with pwt in lane 2). For the BS1 mutant, processing at sites A0 and A2 is restored by the CBS1 compensatory mutation in U3 (compare pBS1 expressing wild-type U3, lane 5, with pBS1 expressing the CBS1 U3, lane 6). For the pBS2 mutation, processing at sites A0 and A2 is restored by expression of either the CBS1 or CBS2 U3 mutants (compare pBS2 expressing wild-type U3, lane 8, with

pBS2 expressing CBS1 or CBS2 U3, lanes 9 and 10). Sites A3 and B1 are the 5' ends of the 27SA' and 27SB pre-rRNAs respectively. Formation of RNA species processed at sites A3 (Figure 5C) and B1 (Figure 5C) is not affected by the BS1 or BS2 mutations. Consistent with the results of Northern hybridization, the level of 18S rRNA shown by the primer extension stop at site A1 is dramatically reduced by either the BS1 or the BS2 mutation in the pre-rRNA (Figure 5B, lanes 5 and 8) and restored by the compensatory mutations (Figure 5B, lanes 6, 9 and 10). A very faint band at A1 is seen in the strain carrying BS1 and CBS2 (Figure 5B, lane 7). The level of 25S rRNA shown by the primer extension stop at site C1 is not altered by the pre-rRNA mutations. All residual cleavages in the BS1 and BS2 mutants and all cleavages restored by the compensatory mutations in U3 are accurate at the nucleotide level.

These results are supported by Northern hybridization (Figure 6). The base substitution mutations in the 5' ETS prevent synthesis of normal 20S and 27SA pre-rRNAs and lead to accumulation of the 35S and 23S pre-rRNAs

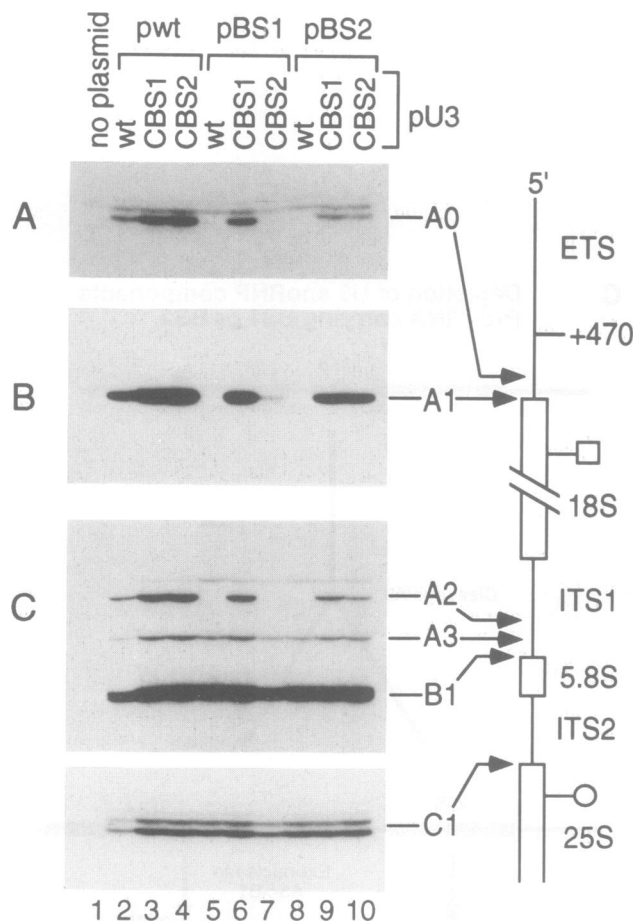


Fig. 5. Primer extension analysis of cleavage sites in pre-rRNA processing. Cells were grown and RNA was extracted as described for Figure 3. Primer extensions were run alongside dideoxynucleotide sequences on denaturing 6% polyacrylamide gels; the positions of cleavage sites are indicated. The drawing on the right sketches part of the pre-rRNA primary transcript; spacer regions are drawn as simple lines, mature rRNA regions as blocks. The positions of the 18 and 25S tags are indicated by a square and a circle respectively; the major processing sites are indicated by arrows. The +470 sign marks the site of *in vivo* cross-linking to U3 in the 5' ETS, adjacent to the BS1 and BS2 mutations. Lanes 1–10 are primer extensions with: lane 1, RNA from an untransformed strain; lanes 2–4, RNA from cells carrying pwt and pU3-wt, CBS1 or CBS2; lanes 5–7, RNA from cells carrying pBS1 and pU3-wt, CBS1 or CBS2; lanes 8–10, RNA from cells carrying pBS2 and pU3-wt, CBS1 or CBS2. An oligonucleotide complementary to the 18S/ETS boundary was used to analyze site A0 (A); the faint band 1 nt 5' of A0 is due to residual chromosomal rDNA transcription (Beltrame *et al.*, 1994). Oligonucleotides complementary to the 18 and 25S tags were used for the experiments shown in (B) and (C) respectively. Different exposures of the same gel are used to show primer extension stops of different intensities in (C). The untransformed control shows the background from chromosomal pre-rRNA (lane 1).

(Figure 6; compare the pBS1 and pBS2 samples in lanes 5 and 8 with the pwt sample in lane 2). In this strain background the steady-state level of the 32S pre-rRNA is low and this species could not be detected even in control samples. The level of 27SB pre-rRNA is not altered by the BS1 or BS2 mutations. The 33S and 27SA' pre-rRNAs cannot be detected by Northern hybridization in wild-type strains. When compensatory base changes are introduced into U3, synthesis of the 20S and 27SA pre-rRNAs is restored (Figure 5, lanes 6, 9, 10); processing of BS1 pre-rRNA is restored by CBS1, processing of BS2 pre-rRNA

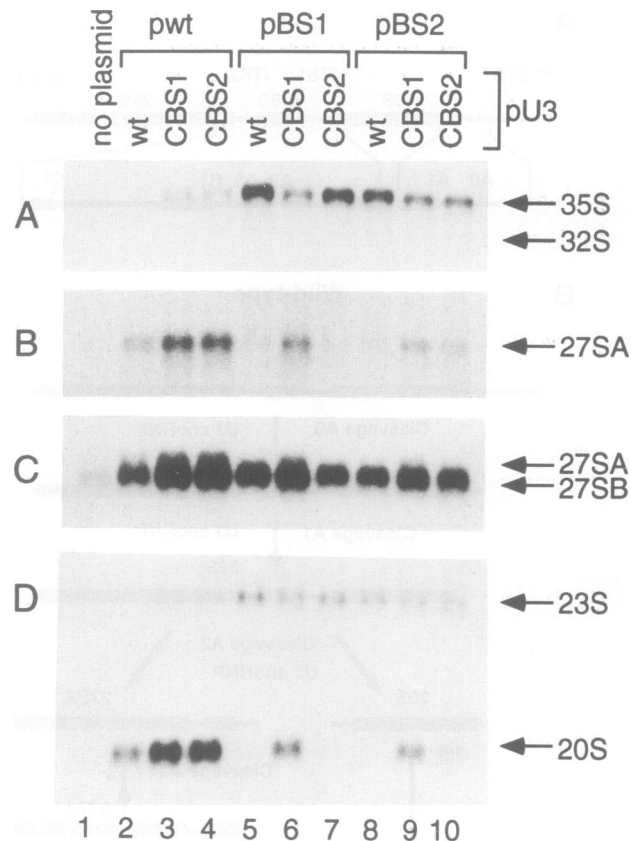


Fig. 6. Northern analysis of pre-rRNA processing intermediates. Filters were prepared as for Figure 3 and hybridized with oligonucleotide probes complementary to: (A and D) the 5' region of ITS1; (B) the 3' region of ITS1; (C) ITS2. Lane 1, RNA from an untransformed strain; lanes 2–4, RNA from cells carrying pwt and pU3-wt, CBS1 or CBS2; lanes 5–7, RNA from cells carrying pBS1 and pU3-wt, CBS1 or CBS2; lanes 8–10, RNA from cells carrying pBS2 and pU3-wt, CBS1 or CBS2. Only relevant parts of each Northern blot are shown and the positions of the major pre-rRNAs are indicated. The untransformed control shows the background from chromosomal pre-rRNA (lane 1).

is restored by CBS1 or CBS2. The pre-rRNA processing which is restored by the compensatory mutations is not, however, as efficient as in the wild-type; the levels of 35S and 23S pre-rRNAs, although reduced by the presence of the compensatory mutations in U3, are still elevated. Moreover, processing of wild-type pre-rRNA is mildly impaired by expression of either mutant U3 snoRNA (Figure 5 and data not shown).

Together the Northern hybridization and primer extension results show that the BS1 and BS2 mutations inhibit pre-rRNA cleavage at A0, A1 and A2, preventing synthesis of the 33S, 32S, 27SA and 20S pre-rRNAs and the 18S rRNA; cleavage at all sites is restored by the compensatory mutations in the U3 snoRNA.

Discussion

We have generated 6 nt base-substitution mutations (BS1 and BS2) in the 5' ETS of pre-rRNA and functionally tested them in an experimental system in which only the mutant rDNA repeat is transcribed. The pre-rRNA mutations lie in a region which was initially identified as a U3 binding site by *in vivo* cross-linking (Beltrame and Tollervey, 1992) and is complementary to the U3 snoRNA

(Figure 1). The BS1 and BS2 mutations disrupt the potential base pairing between the pre-rRNA and U3. This results in lethality (Figure 2), due to a very strong inhibition of 18S rRNA synthesis (Figure 3). In contrast, production of 25S rRNA is not affected. As judged by both primer extension and Northern blot analyses, cleavage of the U3-dependent processing sites A0, A1 and A2 (in the 5' ETS, at the 5' end of 18S rRNA and in ITS1 respectively) is specifically blocked (Figures 5 and 6). In contrast, subsequent processing at sites A3, B1 and C1 are unaffected. The base substitutions changing the central 6 nt of the region in the 5' ETS complementary to U3 exactly mimic depletion of the U3 snoRNA itself. This strongly supports the hypothesis that Watson-Crick base pairing in this region between U3 and its substrate is crucial for subsequent processing steps.

To demonstrate that this is indeed the case we introduced counter-mutations in U3 (CBS1 and CBS2) which restore complementarity to the mutant pre-rRNA molecules. The growth defect caused by each base substitution in the 5' ETS can be compensated for by the presence of a plasmid expressing the corresponding counter-mutated U3. The compensatory mutations in U3 show a remarkable degree of suppression of the pre-rRNA processing defects; 18S rRNA is normally synthesized and cleavage at the A0, A1 and A2 processing sites is efficiently restored and is accurate at the nucleotide level. Processing intermediates abolished by the 5' ETS mutations (the 33S, 27SA and 20S pre-rRNAs) are restored by the compensatory mutations in U3.

The simultaneous expression of wild-type and mutated U3 snoRNAs in the cell appears to be slightly detrimental to the efficiency of pre-rRNA processing, as attested by the elevated level of 23S pre-rRNA in strains expressing the wild-type rDNA repeat and either mutated U3. Although the chromosomal wild-type U3 snoRNA supports cleavage at sites A0, A1 and A2, processing at these sites is slowed, perhaps because the mutant U3 snoRNAs can still assemble with snoRNP factors to form inactive particles unable to bind to the 35S pre-rRNA. The primary transcript would then be cleaved at site A3, before cleavage at sites A0, A1 and A2, generating the 23S species.

Unexpectedly, the two base-substitution mutants behave differently. The BS1 mutation is fully suppressed only by the counter-mutation in U3 which completely restores the potential to form 10 bp with the pre-rRNA. In contrast, the BS2 mutation can be suppressed by either the CBS2 or CBS1 mutation in U3, which fully or partially restore complementarity. Suppression of BS2 by either CBS2 or CBS1 is, however, somewhat less efficient than suppression of the BS1 mutation by CBS1. This indicates that base pairing between the two molecules is crucial, but that a more limited number of hydrogen bonds than in the wild-type interaction can be sufficient to allow binding of U3 to the pre-rRNA. The secondary structure of the regions of complementarity in the two molecules and/or additional factors stabilizing the interaction may explain these results.

It is notable that binding of U3 to the 5' ETS is required for processing reactions in the pre-rRNA which occur up to 2 kb downstream, at site A2 in ITS1. We speculate that spacer regions which are far apart in the primary structure are brought into close proximity by processing complexes

involving multiple snoRNPs. The U3 snoRNP appears to bind to the pre-rRNA at an early stage of pre-rRNA processing, potentially directing the assembly of such a multi-snoRNP complex.

Several small nuclear RNAs (snRNAs) have been shown to play essential roles in pre-mRNA maturation involving direct base pairing with their RNA substrates. An ordered series of base-pairing interactions between U1, U2, U5, U6 snRNAs and the pre-mRNA takes place during spliceosome assembly and catalysis (Nilsen, 1994). Likewise, the U7 snRNP acts in 3' end processing of animal histone pre-mRNA through a base-pairing interaction between the 5' end of the U7 snRNA and the purine-rich downstream element of the pre-mRNA (Schaufele *et al.*, 1986; Bond *et al.*, 1991). This is the first demonstration that direct base pairing between a snoRNA and the substrate is also required for pre-rRNA processing. It is notable that the degree of complementarity involved, 10 perfect base pairs, is substantially greater than shown between snRNAs and their pre-mRNA substrates (Madhani and Guthrie, 1994). Many other snoRNAs also contain extended stretches of perfect complementarity to sequences in precursor or mature rRNAs (Maxwell and Fournier, 1995), which are being interpreted as potential targets for processing/folding reactions. Our data suggest that base pairing with the substrate is a common feature of small RNAs involved in mRNA and rRNA maturation.

Materials and methods

Yeast strains, plasmids and media

Yeast strain NOY504 (MATA, *rrn4::LEU2, ade2-101, ura3-1, trp1-1, leu2-3,112, can1-100*), generously provided by Dr M.Nomura, was used for all the experiments described. This strain carries a disrupted *rrn4* (*rpa12*) gene and shows a ts phenotype due to inactivation of RNA pol I (Nogi *et al.*, 1993). It can be kept alive at 37°C by a *GAL7-rDNA* hybrid construct, transcribed by RNA pol II. We used a modified version of plasmid pNOY102 (Nogi *et al.*, 1991), carrying two small tags in the 18S and 25S coding sequences (Beltrame *et al.*, 1994). This plasmid (pwt) carries a 2 μ origin of replication and a *URA3* marker.

Extra copies of U3 were introduced into the cell in an intron-less construct carrying the natural promoter and terminator regions (pU3-wt), cloned in plasmid pASZ11 (Stotz and Linder, 1990). A *PstI-EcoRI* snR17A genomic fragment was mutagenized to eliminate the intron from the U3 coding sequence and cloned into pASZ11 (R.Fournier and C.Branlant, personal communication). We modified this construct (generously provided by Dr C.Branlant) by the insertion of an *EcoRI* site 70 bp downstream of the 3' end of the U3 coding region; genomic sequences downstream of this site were deleted. The pU3-wt construct is an *ARS-CEN* plasmid carrying an *ADE2* marker.

Galactose medium contained 2% galactose, 0.67% yeast nitrogen base without amino acids (Difco) supplemented with adenine, leucine and tryptophan. Adenine was omitted when cells were transformed with pU3 plasmids. For plates, agar was included at a 2% final concentration.

Construction of 5' ETS and U3 mutations

Mutagenesis in the 5' ETS region was carried out by PCR as described (Beltrame *et al.*, 1994). A tagged rDNA plasmid (pBSrDNA2XtagURA3-6; see Beltrame and Tollervey, 1992) was used as template DNA. Each mutation was created using two external oligonucleotides (corresponding to the ETS sequence +102/+120 from the transcription start site and to the 18S template DNA strand +56/+34) and a single mutagenic oligonucleotide (5'-GATTAGAGGAAACTCTTTCATGCTATGGTATGGTGACG-3' for BS1 and 5'-GATTAGAGGAAACTCTTCACATGCTATGGTATGGTGACG-3' for BS2). A restriction fragment containing the mutation was cloned into the tagged *GAL7-rDNA* plasmid (pwt), replacing the wt fragment.

The mutations in U3 were introduced by PCR, using the pU3-wt plasmid as template DNA. Two oligonucleotides complementary to the 5' and 3' U3 flanking regions (5'-AACTGCAGCATAACAGCGCC-

TTAAGG-3' and 5'-GGAATTCTAATCCAATTTCTTAACTG-3') and a mutagenic oligonucleotide (5'-TTCTATAGGAATCGTCATGAAGAGACTCTTCAAAAAGAGC-3' for CBS1 and 5'-TTCTATAGGAATCGTCATGTGAAGACTCTTCAAAAAGAGC-3' for CBS2) were used for each mutation. A *SalI-EcoRI* fragment from the PCR product was used to replace the wt fragment in pU3-wt. The region was fully sequenced to discard random mutations introduced by Taq polymerase.

Northern hybridization

Total RNA was extracted from a yeast strain carrying a *ts* mutation in RNA pol I following incubation for 6 h at 37°C in galactose medium, as described (Beltrame *et al.*, 1994). RNA was separated on a 1.2% agarose-formaldehyde gel and transferred to a nylon membrane. RNA extracted from 0.4 OD₆₀₀ units of cells was loaded for each sample.

Northern hybridization was with labelled oligonucleotides complementary to short tags inserted in the 18S and 25S rRNA sequences in the plasmid-borne rDNA repeat (Figure 3) or with oligonucleotides specific for pre-rRNA species (Figure 6).

Oligonucleotides were: 5'-CGAGGATCCAGGCTTT-3' for the 18S tag; 5'-ACTCGAGAGCTTCAGTAC-3' for the 25S tag; 5'-CGTTTTT-AATTGTCTTA-3' for the 5' region of ITS1; 5'-ATGAAAACCCAC-AGTG-3' for the 3' region of ITS1; 5'-GGCCAGCAATTTCAAGTTA-3' for ITS2.

Primer extension

Cells were grown and RNA was extracted as for Northern hybridization; total RNA extracted from 0.4 OD₆₀₀ units of cells was used for each sample. Primer extensions were performed as described (Beltrame and Tollervey, 1992) using oligonucleotides complementary to the 18S/ETS boundary (Figure 5A) (5'-CCAAATAACTATCTTAAAAG-3') or to the 18S and 25S tags (Figure 5B and C respectively). Although not shown in the figures, the same oligonucleotides were also 5' phosphorylated with unlabelled ATP and used to generate dideoxy sequence ladders from plasmid DNA templates, which were run alongside the primer extension samples.

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