The EMBO Journal vol.9 no.12 pp.3989-3996, 1990

Functional analysis of transcribed spacers of yeast ribosomal DNA

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Communicated by R.J.Planta

Making use of an rDNA unit, containing oligonucleotide tags in both the 17S and 26S rRNA gene, we have analyzed the effect of various deletions in the External Transcribed Spacer (ETS) and in one of the Internal Transcribed Spacers 1 (ITS1) on the process of ribosome formation in yeast. By following the fate of the tagged transcripts of this rDNA unit *in vivo* by Northern hybridization we found that deleting various parts of the ETS prevents the accumulation of tagged 17S rRNA and its assembly into 40S subunits, but not the formation of 60S subunits. Deleting the central region of ITS1, including a processing site that is used in an early stage of the maturation process, was also found to prevent the accumulation of functional 40S subunits, whereas no effect on the formation of 60S subunits was detected. The implications of these findings for yeast pre-rRNA processing are discussed.

Key words: ribosomal RNA/yeast/RNA processing

Introduction

Eukaryotic cytoplasmic ribosomes are highly structured particles consisting of four ribosomal RNAs (rRNAs) and ~80 ribosomal proteins (r-proteins). Assembly of these supramolecular complexes takes place largely within the nucleolus. Here the three large rRNA genes are transcribed by RNA polymerase I (Pol I) into a single large rRNA precursor. Even before its synthesis is completed, this precursor starts associating with both ribosomal and non-ribosomal proteins to form a pre-ribosomal particle (Hadjiolov, 1985). Conversion of this particle into mature, functional 40S and 60S ribosomal subunits is an intricate process that encompasses a number of chronologically ordered steps governed by the structure of the ribonucleoprotein complex. While most of the details of this process remain to be elucidated, the processing and modification of the rRNA molecules have been studied extensively (Hadjiolov, 1985; Raué *et al.*, 1988; Klootwijk and Planta, 1989).

Eukaryotic cells may contain up to 1000 copies of each of the rRNA genes, which (except for the one encoding 5S rRNA) are organized in one or a few clusters of tandemly repeated units (Long and Dawid, 1980). Each unit comprises a Pol I operon containing one gene each for 17/18S, 5.8S and 26/28S rRNA, preceded by an External Transcribed Spacer (ETS) and separated by two Internal Transcribed Spacers (ITS1 and ITS2), which are post-transcriptionally removed. The order of the successive events in the major processing pathway in *Saccharomyces cerevisiae* is shown in Figure 1.

For a long time the (pre-)rRNA was primarily looked upon as the structural backbone of the (pre-)ribosome, serving to ensure the correct spatial arrangement of the r-proteins, to which the biological activities of the ribosome were mainly attributed. Although suggestions for a direct involvement of rRNA in the biological activities of the ribosome were put forward by several authors from 1963 onwards (Santer, 1963; Crick, 1968; Woese, 1980), only recently has concrete evidence for such an involvement been obtained, mainly through the use of a number of elegant *in vitro* as well as *in vivo* approaches for unravelling structure-function relationships of prokaryotic rRNA (Dahlberg, 1986; Tapprich and Hill, 1986; Krzyosiak *et al.*, 1987). Together with the discovery of catalytic RNA (Cech and Bass, 1986), these findings have caused the deposition of ribosomal proteins and their replacement by rRNA as the main repository of biological activity of the ribosome. Whereas functional analysis of prokaryotic rRNA is progressing rapidly, similar studies on eukaryotic rRNA have lagged behind, since none of the systems used in prokaryotes is applicable as such to eukaryotes, mainly for two reasons. Firstly, *in vitro* assembly of eukaryotic ribosomes has so far proved to be an intractable problem. Secondly, in eukaryotes the rRNA genes are present in considerably larger numbers than in *Escherichia coli*, thus obscuring any aberrant behavior of the products of the few mutant genes that can be introduced into eukaryotic host cells by transformation. Complete replacement of the many rDNA units in eukaryotes is impossible, with the single exception of the rDNA units in vegetative *Tetrahymena* cells. The peculiar way in which transcriptionally active rDNA is formed in this genus (Yao, 1986) has been elegantly exploited by Sweeney and Yao (1989) to obtain cells that are totally dependent on mutated rRNA genes, and in this way permit the study of the effect of insertions in these genes on *Tetrahymena thermophila* rRNA function. A major disadvantage of this approach, however, is that rDNA mutations causing severe defects in the formation of functional ribosomes can only be scored indirectly, since they will not produce viable transformants.

Recently, we have described a system for the yeast *S. cerevisiae* which allows us to keep track of the transcripts of mutant rDNA units *in vivo*, against a background of as much as a 500-fold excess of wild-type molecules (Musters *et al.*, 1989a). This was achieved by the construction of a plasmid called pORCS (Oligonucleotide-tagged Ribosomal Centromeric DNA of *S.cerevisiae*) carrying a complete yeast rDNA unit in which the 26S rRNA gene was tagged by the insertion of an oligonucleotide (Figure 8). The tagged rDNA unit was shown to be transcribed by RNA polymerase I and the presence of the tag was demonstrated to be a neutral mutation with respect to the formation of functional 60S ribosomal subunits. The effect of rDNA mutations on the

formation and functioning of yeast 60S ribosomal subunits can thus be determined by introducing these mutations into the tagged rDNA unit, and monitoring the fate of the tagged (pre-)rRNA by hybridization with an oligonucleotide that is complementary to the tag.

Here we describe an extension of this system which allows us to study the effect of rDNA mutations on the synthesis and functioning of not only the 60S but also the 40S ribosomal subunits. To that end we have introduced an additional tag into the 17S rRNA gene of the plasmid borne rDNA unit by the insertion of an oligonucleotide in variable region V8 of the gene (nomenclature according to Raué *et al.*, 1989). We show that this tagged 17S rRNA is incorporated into functional 40S ribosomal subunits. Using this doubly tagged plasmid borne rDNA unit we demonstrate that various deletions in the ETS as well as a deletion of the central region of the ITS1 prevents the accumulation of (tagged) 40S subunits, but not that of the 60S subunits. In addition we show that not all the variable regions of the mature rRNA have a structural flexibility, because the insertion of a short oligonucleotide in variable region V3 of the 17S rRNA gene interferes with the formation of 40S subunits.

Results

Construction of a tagged 17S rRNA gene

In order to minimize the possibility that the insertion of the oligonucleotide tag would interfere with either the formation of 40S subunits, or with their functioning in translation, we have chosen a variable region of the 17S rRNA gene as the site of insertion. Variable region V8 (the nomenclature of Raué *et al.*, 1989 will be used throughout this paper for referring to variable regions) of the 17S rRNA gene of *S.cerevisiae* harbors a *Nhe*I site near the region encoding the tip of an RNA stem-loop structure (Figure 3). This site was used for the insertion of a 19 bp synthetic oligonucleotide that was designed to differ as much as possible from the sequence of the *S.cerevisiae* rDNA, given the prerequisites that it should contain a (unique) *Kpn*I site and disturb the proposed secondary structure (Hogan *et al.*, 1984) of the 17S rRNA as little as possible (Figure 2). The tagged 17S rRNA gene was combined with the previously described tagged 26S rRNA gene in a single rDNA unit and the plasmid harboring both oligonucleotide tags was called pORCS(17S*+26S*) to distinguish it from pORCS, which is tagged in the 26S rRNA gene only.

The tag in 17S rRNA is a neutral mutation

Total rRNA isolated from pORCS(17S*+26S*) transformants was analyzed by Northern blot hybridization, using the ^{32}P-labeled oligonucleotide complementary to the 17S rRNA tag as a probe (Figure 3A). A strong hybridization signal was found at a position in the gel corresponding to that of 17S rRNA, whereas no specific hybridization signals were obtained with RNA from either host cells or cells transformed with pORCS. When the same blot was probed with the oligonucleotide complementary to the 26S rRNA tag (Figure 3B), correctly processed, tagged 26S rRNA was found to be present in pORCS(17S*+26S*) transformants. Thus, accumulation of mature rRNAs is not affected by either of the two oligonucleotide insertions.

In an attempt to estimate the ratio between tagged 17S and tagged 26S rRNA in pORCS(17S*+26S*) transformants, an rDNA fragment from pORCS(17S*+26S*) spanning the region that contains both oligonucleotide tags was denatured, glyoxylated and run alongside the RNA samples (Figure 3). Using the hybridization signal obtained with this DNA fragment as a standard, the amounts of tagged 17S and tagged 26S rRNA were found to be approximately equal. Thus these tagged rRNAs accumulate in about an equimolar ratio in cells transformed with pORCS(17S*+26S*).

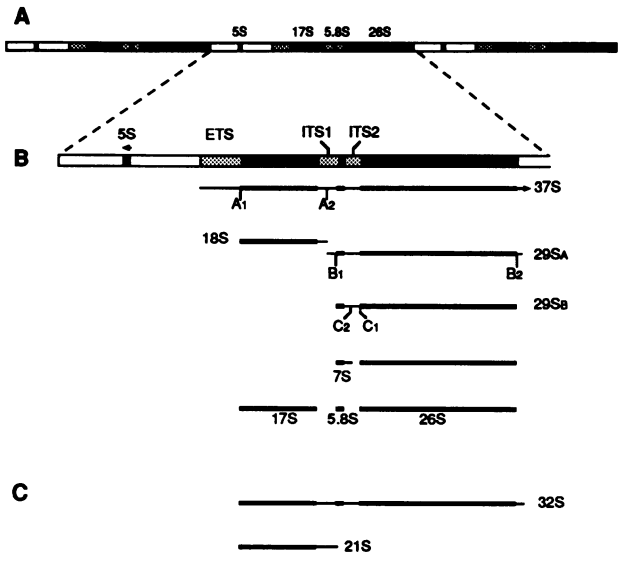

Fig. 1. Genetic organization of rDNA and pre-rRNA processing in *S.cerevisiae*. (**A**) Schematic representation of three tandemly repeated rDNA units. White, shaded and black bars represent non-transcribed spacers, transcribed spacers and rRNA genes, respectively. (**B**) A single rDNA unit aligned with the products of the various steps of the major processing pathway in wild-type cells. Primary transcripts are indicated by arrows. Nomenclature is according to Veldman *et al.* (1981). (**C**) Unusual processing intermediates either found in cells harboring rDNA units from which part of ITS1 has been deleted (32S) or in *snr10.3* strains (21S). Nomenclature of these unusual intermediates is according to Tollervey (1987). See text for details.

The insertion of an oligonucleotide tag in the 17S rRNA gene did not affect the accumulation of 40S ribosomal subunits, nor did it interfere with the competence of these particles to participate in translation. This can be concluded from Figure 4, which shows a Northern blot of RNA isolated from polysomal fractions of yeast cells transformed with pORCS(17S*+26S*), hybridized with an oligonucleotide complementary to either the 17S rRNA tag or the 26S rRNA tag. The polysomal distribution of tagged 17S rRNA was found to be indistinguishable from that of tagged 26S rRNA, which has previously been shown to be the same as that of wild-type rRNA (Musters *et al.*, 1989a). Therefore we can conclude that tagged 17S rRNA is correctly assembled into (pre-)ribosomal particles, which are transported to the cytoplasm and are able to associate with mRNA. A diminished mRNA translation rate of ribosomes containing the tagged 17S rRNA would give rise to different ratios of tagged 17S versus wild-type rRNA in the various polysomal fractions, which was not observed. Thus, 40S ribosomal subunits containing tagged 17S rRNA are not detectably deficient in either the initiation or elongation step of translation.

A 19 bp oligonucleotide insertion in variable region V3 prevents the accumulation of tagged 17S rRNA

Our first attempt to tag the 17S rRNA gene involved the insertion of a 19 bp oligonucleotide in either orientation into

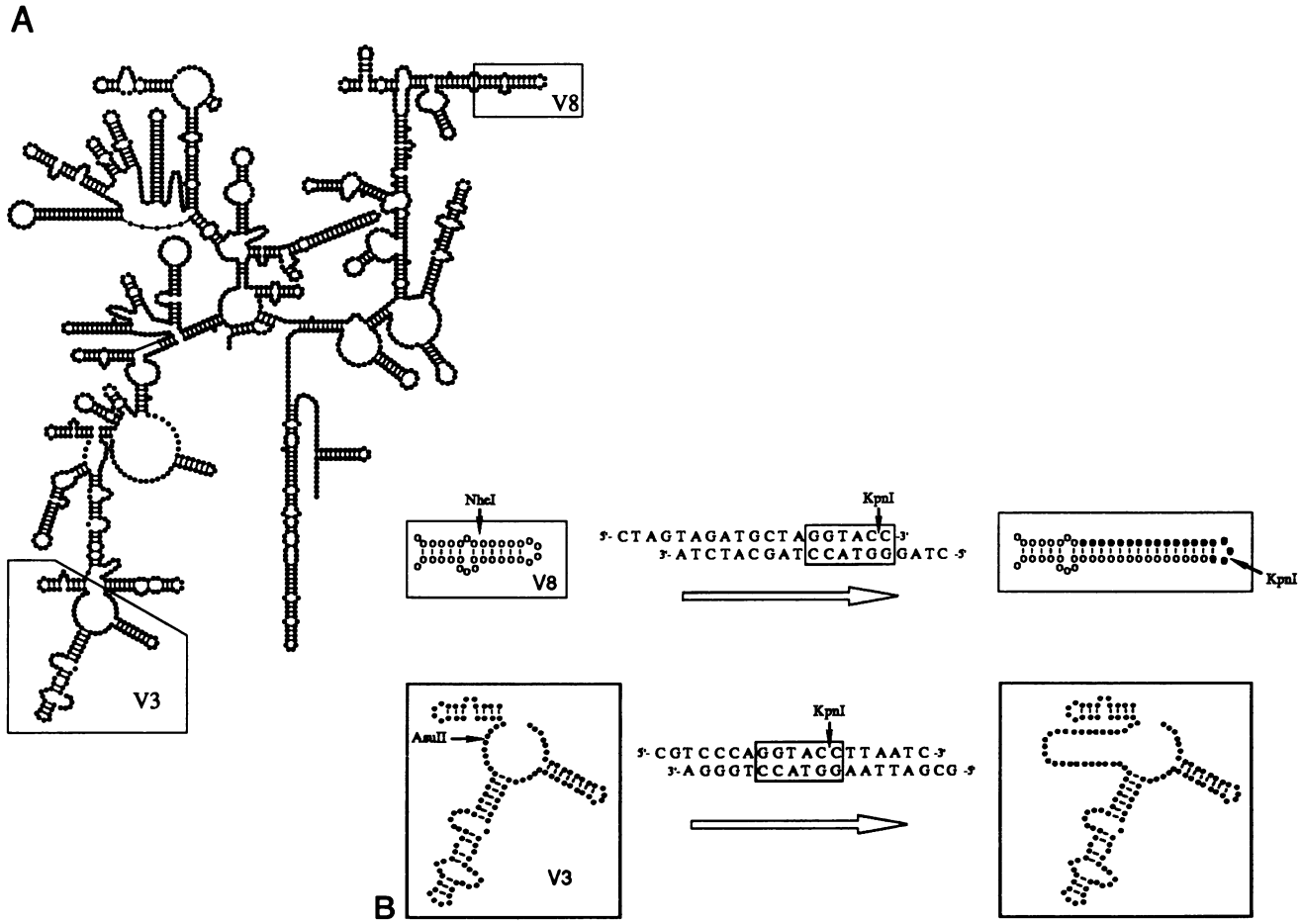

Fig. 2. (**A**) Proposed secondary structure of *S.cerevisiae* 17S rRNA according to the model of Raué *et al.* (1988). Variable regions V3 and V8 are boxed. (**B**) Detailed putative secondary structure of the variable regions before and after insertion of the indicated oligonucleotides. Ribonucleotides encoded by the inserted deoxyoligonucleotides are indicated by filled circles.

the *Asu*II site present in variable region V3 (Figure 2). However, Northern hybridization using probes complementary to the inserted oligonucleotides failed to reveal the presence of any tagged 17S rRNA, whereas 37S pre-rRNA containing the tag could be detected (results not shown). To ascertain that the failure to detect mutant 17S rRNA was a direct consequence of disturbing the integrity of variable region V3, and not a technical artifact related to the composition of the inserted oligonucleotide, the effect of the insertion in the *Asu*II site was also tested in the newly developed tagged 17S rRNA gene of pORCS(17S*+26S*). As can be seen in Figure 3, no tagged 17S rRNA accumulates in pORCS-V3 transformants, whereas tagged 26S rRNA is present at normal levels. Hence, the insertion of the 19 bp long oligonucleotide, in either orientation, into variable region V3 of the 17S rRNA gene interferes with the formation of 40S subunits. The complete absence of any hybridization signal in Figure 3A (lane 4) indicates that defective intermediates in the formation of 40S ribosomal subunits in yeast cells are subjected to rapid degradation, as has formerly been noticed for defective intermediates of 60S subunit formation (Musters *et al.*, 1989a).

Various deletions in the ETS interfere with the production of tagged 17S rRNA but not with that of tagged 26S rRNA

In all rRNA operons analyzed so far, the small subunit (SSU) rRNA gene is preceded by an external transcribed spacer

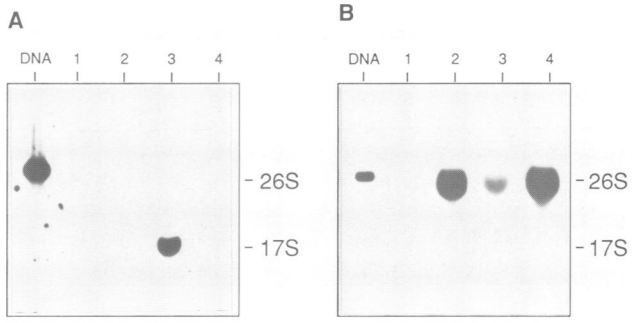

Fig. 3. Production of tagged rRNA by pORCS(17S*+26S*) and pORCS-V3 transformants. (**A**) Total RNA (20 μg) from MG34 host cells (lane 1), MG34 cells transformed with either pORCS (lane 2), pORCS(17S*+26S*) (lane 3) or pORCS-V3 (lane 4) was fractionated on a 1% agarose gel, blotted onto Hybond N and hybridized with the ^{32}P-labeled oligonucleotide complementary to the tag in region V8 of 17S rRNA. In the lane marked DNA 50 ng of an *Xba*I digest of pORCS(17S*+26S*) DNA was run alongside the RNA samples, after being heat-denatured and glyoxylated. The hybridization signal is caused by a 3.6 kb fragment that contains both oligonucleotide insertions. (**B**) Analysis of the same filter with the ^{32}P-labeled oligonucleotide complementary to the tag in 26S rRNA.

(ETS). The length of this element varies widely among different species, from several hundred bp in prokaryotes to ~4000 bp in mammals. In *S.cerevisiae* the ETS has a length of 699 bp (Klootwijk and Planta, 1989). Several

observations suggest that the ETS is not merely a dispensable element of the primary ribosomal transcript, but that it might have a function in the processing and assembly of the precursor rRNA. For instance, the first steps in the processing of mammalian rRNA involve cleavages within the ETS, rather than a complete removal of the ETS (Miller and Sollner-Webb, 1981; Gurney, 1984; Kass *et al.*, 1987). This suggests that the ETS in these organisms plays a role in the early stages of (pre-)ribosome assembly. Moreover, the fact that the ETS in some organisms was found to be associated with proteins (Herrera and Olsen, 1986; Jordan, 1987), also indicates that this element, despite its rapid removal from the primary transcript in yeast (Veinot-Drebot, 1988), might serve an important role in the maturation of the preribosome. To investigate whether the ETS does indeed contribute to the process of ribosome formation in yeast we constructed four deletion mutants. Three of these lack various parts of the ETS, while in the fourth mutant almost the complete ETS was deleted except for 20 bp at its 5′-end (required for promoter activity; see Musters *et al.*, 1989b) and 46 bp directly flanking the 17S rRNA gene (Figure 5). When placed in an rDNA unit carrying the tagged 17S and 26S rRNA genes, neither of these deletions was found to affect the production of tagged 26S rRNA, as demonstrated by Northern analysis of total RNA derived from the pertinent transformants with an oligonucleotide complementary to the tag in 26S rRNA (Figure 6). Moreover, Northern analysis of polysomal RNA fractions from cells that harbor the tagged operon lacking the complete ETS, demonstrated that 60S subunits carrying the tagged 26S rRNA were fully functional (data not shown). Thus, the presence in *cis* of the ETS is not required for the formation of functional 60S subunits *in vivo* in *S.cerevisiae*.

The formation of tagged 17S rRNA, however, was affected by the ETS deletions. When a twin blot of the one used in Figure 6A was analyzed with the oligonucleotide complementary to the 17S rRNA tag, tagged 17S rRNA could only be detected in pORCS(17S*+26S*) transformants (Figure 6B, lane 2), whereas the level of tagged 26S rRNA was even higher for the other transformants used in this experiment (Figure 6A). Thus, each of the deletions in the ETS prevents the accumulation of tagged 17S rRNA, from which we infer that these deletions interfere with the correct formation of 40S subunits.

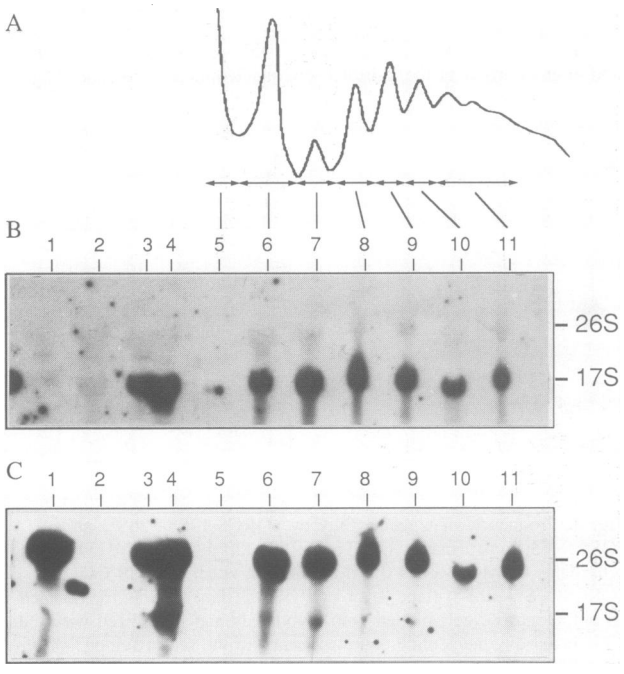

Fig. 4. Distribution of tagged rRNA in polysomal fractions of pORCS(17S*+26S*) transformants. (**A**) Fractionation profile of isolated polysomes on a 10–40% sucrose gradient. Fractions were pooled as indicated and the RNA isolated from each fraction was electrophoresed on a 1.0% agarose gel, blotted onto Hybond N and hybridized with the ^{32}P-labeled oligonucleotide complementary to either the 17S rRNA tag (**B**) or the 26S rRNA tag (**C**). Lane 1: 20 μg total RNA from a pORCS transformant. Lane 2: 20 μg total RNA from untransformed MG34 host cells. Lanes 3 and 4: 10 and 20 μg, respectively, of total RNA from a pORCS(17S*+26S*) transformant.

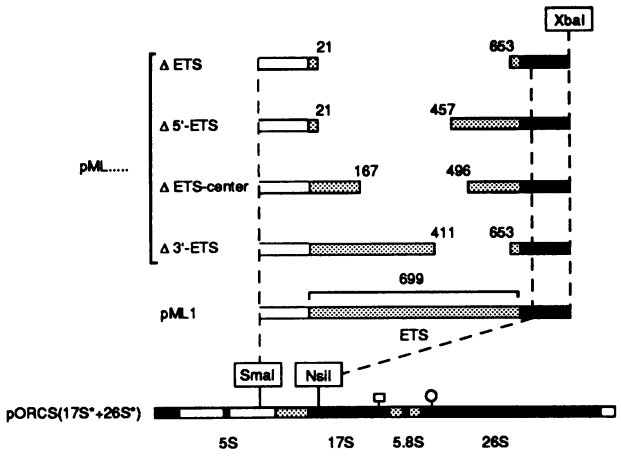

Fig. 5. Tagged rDNA units carrying an ETS deletion. A subclone comprising the ETS (pML1) was used to create four different ETS deletion mutants, which were transferred back into the doubly tagged rDNA unit of pORCS(17S*+26S*). Numbers indicate the end points of the deletions, the 5′-end of the ETS being position 1. For all plasmids only the rDNA moieties are shown (black, shaded and white bars representing rRNA genes, transcribed spacers and non-transcribed spacers, respectively). The tags in the genes for 17S (▽) and 26S rRNA (♀) are indicated.

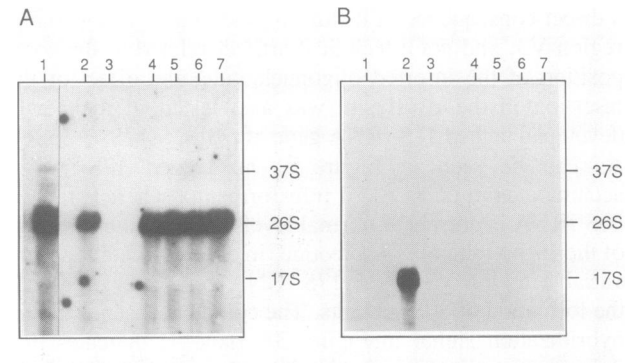

Fig. 6. Effect of deletions in the ETS on the levels of tagged rRNAs. (**A**) 20 μg of total RNA from untransformed MG34 host cells (lane 3) or cells transformed with either pORCS (lane 1), pORCS(17S*+26S*) (lane 2), pORCSΔETS (lane 4), pORCSΔ5′-ETS (lane 5), pORCSΔETS-center (lane 6) or pORCSΔ3′-ETS (lane 7) was fractionated on a 1% agarose gel, blotted onto Hybond N and hybridized with the ^{32}P-labeled oligonucleotide complementary to the 26S rRNA tag. (**B**) a twin blot of the one shown in panel A, but now hybridized with the ^{32}P-labeled oligonucleotide complementary to the tag in the 17S rRNA gene.

A deletion comprising the central part of ITS1 prevents the accumulation of tagged 17S rRNA, but not tagged 26S rRNA

ITS1 is located between the 3′-end of the 17S rRNA gene and the 5′-end of the gene for 5.8S rRNA (Figure 1), and thus separates the part of the 37S pre-rRNA destined to become incorporated in 40S ribosomal subunits from that ending up in 60S ribosomal subunits. In *S.cerevisiae* ITS1 has a length of 362 bp (Veldman *et al.*, 1980) and contains an internal processing site that is cleaved at an early stage of rRNA maturation (site A2, *cf.* Figure 1). Subsequent processing events, also occurring in the nucleus, generate the mature 5′- and 3′-ends of 5.8S and 26S rRNA. Formation of the mature 3′-end of 17S rRNA, however, does not take place until the 40S subunits have reached the cytoplasm (Udem and Warner, 1973; Trapman and Planta, 1976). Since processing sites homologous to site A2 have not been reported for other eukaryotes (Bowman *et al.*, 1981; Gerbi, 1985), we wanted to investigate the significance of this processing event for yeast ribosome formation. To this end a region of 160 bp, spanning processing site A2, was deleted from the doubly tagged rDNA unit, yielding pORCSΔITS1 (Figure 7A). Northern analysis of RNA from pORCSΔITS1 transformants demonstrated the presence of about the same amount of tagged 26S rRNA as in the (high-copy-number) pORCS transformant, whereas the amount of tagged 17S rRNA was found to be much lower than in the (low-copy-number) pORCS(17S*+26S*) transformant (Figure 7). Moreover, a 32S precursor was found to accumulate in pORCSΔITS1 transformants, that was not detected in cells containing the unmutated tagged operon (Figure 7). Apparently, the deletion of processing site A2 has altered the sequence of processing events for the tagged, mutated pre-rRNA in such a way that the formation of 60S subunits is unaffected, whereas the formation of 40S subunits is blocked.

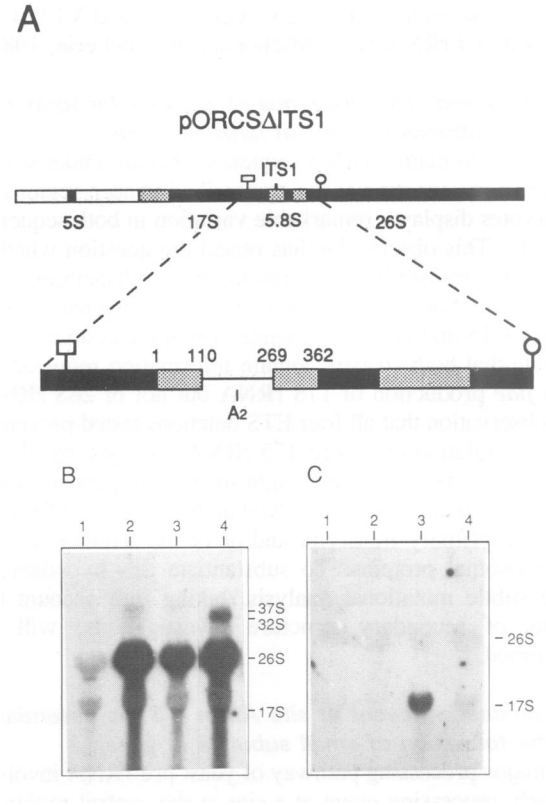

Fig. 7. Effect of a deletion in ITS1 on the levels of tagged rRNAs. (**A**) Schematic representation of an rDNA unit harboring the tags in 17S rRNA (♀) and 26S rRNA (♀), and carrying a 160 bp deletion in ITS1 that removes processing site A2. The lower line is an enlargement showing the position of the deletion in ITS1 (thin line). Numbering starts at the 5′-end of ITS1, and the indicated numbers refer to first and last base pairs of the deletion. 20 μg of total RNA from untransformed MG34 host cells (lane 1) or cells transformed with either pORCS (lane 2), pORCS(17S*+26S*) (lane 3) or pORCSΔITS1 (lane 4) was fractionated on a 1% agarose gel, blotted onto Hybond N and hybridized with the ^{32}P-labeled oligonucleotide complementary to either the 26S rRNA tag (**B**) or the 17S rRNA tag (**C**).

Discussion

A neutral tag for the 17S rRNA gene

The successful tagging of the 26S rRNA gene of pORCS has enabled us to study the effect of yeast rDNA mutations on the formation of 60S subunits *in vivo* (Musters *et al.*, 1989a). The same tagged gene can also be used to identify the features of 26S rRNA essential for ribosome function. In this paper we describe a similar tagging of the 17S rRNA gene by insertion of a 19mer in variable region V8, which makes it possible to extend this type of analysis to the 40S subunits. Insertion of this tag is shown to be a silent mutation with respect to processing, assembly and transport of the ribosomal subunits, as well as to their translational activities. Though we cannot rule out the possibility that the presence of the tag in 17S rRNA affects the kinetics of the maturation process, the polysomal distribution of tagged 17S rRNA was found to be identical to that of wild-type rRNA. Therefore, this system is suitable for the identification of *cis*-acting elements in the rDNA that are involved in the process of ribosome formation, as well as those parts of 17S- and 26S rRNA that are essential to the translational activities

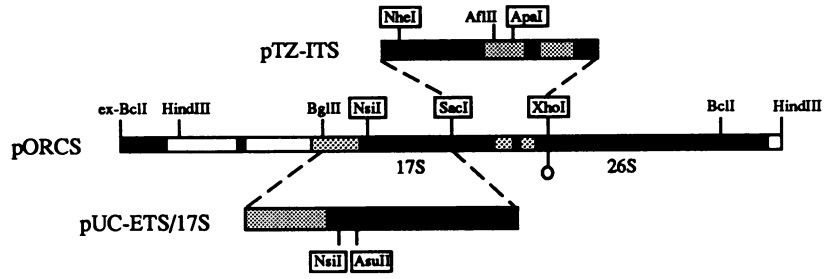

Fig. 8. Schematic representation of the yeast rDNA moiety of pORCS and derived plasmids. pORCS, a plasmid containing a complete rDNA unit with a tagged 26S rRNA gene has been described previously (Musters *et al.*, 1989a). pTZ-ITS and pUC-ETS/17S are intermediate plasmids, used in constructing pORCS(17S*+26S*), pORCSΔITS1 and pORCS-V3. Restriction enzyme sites which are unique within a specific plasmid are boxed and the tag in the 26S rRNA gene is represented by (♀). The tag in the 17S rRNA gene was inserted into the *Nhe*I site (*cf.* Fig. 2).

of the yeast ribosome. The only other system so far available for *in vivo* mutational analysis of eukaryotic rDNA has recently been described by Yao and coworkers (Sweeney and Yao, 1989; Yao and Yao, 1989), and is based upon transformation of conjugating *T.thermophila* cells with a mutant rDNA unit. Such transformation completely replaces the trancriptionally active wild-type rDNA units of the developing macronucleus with mutuated units. The *Tetrahymena* system has the advantage that it can not only monitor the overall effect of an rDNA mutation on the formation and translational activity of the ribosomes, but also allows analysis of the kinetics of these processes, since the cells are completely dependent on ribosomes containing the mutated rRNA. On the other hand, mutations that prevent the formation of functional ribosomes cannot be studied in this system, since they will not give rise to viable transformants. The latter type of mutation, however, can be analyzed in our system, since the presence of wild-type rRNA derived from the chromosomal genes ensures the continuing ability of the transformed cells to grow. Thus, the two different approaches can be used to complement each other in the identification of the functional regions in eukaryotic rDNA.

The function of variable regions of rRNA

The absence of any detectable deleterious effect resulting from the insertion of a 19mer in variable region V8 of the 17S rRNA gene demonstrates that the precise structure of this region is not of critical importance for either ribosome formation or the biological function of the 40S subunits. This observation constitutes the third example of a eukaryotic variable region where insertion of foreign sequences does not interfere with the formation of functional ribosomes *in vivo*. Earlier reports concerned an 18 nucleotide (nt) insertion in variable region V2 of yeast 26S rRNA (Musters *et al.*, 1989a) and a 119 nt insertion in region V18 of *T.thermophila* 26S rRNA (Sweeney and Yao, 1989). Similarly, replacement of variable region V9 of yeast 26S rRNA with its equivalent from other organisms, or deletion of almost all of this region, was found to have no deleterious effect on the formation of functional ribosomes *in vivo* (this laboratory, unpublished results). Thus, a number of variable regions display a considerable degree of structural flexibility in both yeast and *T.thermophila*. These observations lend support to the notion that variable regions may be the remnants of either ancient internal transcribed spacers (Boer and Gray, 1988; Dover, 1988) or mobile elements (Ware *et al.*, 1985), the presence of which is tolerated merely because they do not interfere wih ribosome formation and function. This would account for the variability of these regions in both size and sequence between different organisms (Dams *et al.*, 1988; Raué *et al.*, 1988). However, in a number of cases insertions in variable regions have been found to exert a negative effect on the formation of functional ribosomes *in vivo*. Attempts to transform *T.thermophila* cells with rDNA units containing insertions of 2.3 knt in variable region V18 or 119 nt in variable region V13 of the 26S rRNA gene, were unsuccessful (Sweeney and Yao, 1989). Since the insertion of the same 119 nt sequence in V18 did not affect the formation of functional ribosomes (Sweeney and Yao, 1989), the effect of its insertion in V13 cannot be due to the introduction of spurious processing or transcription termination signals, as could be argued in the case of the 2.3 knt V18 insertion. Also, as we have shown here, two different oligonucleotide insertions in region V3 of the yeast 17S rRNA are both deleterious to the formation of small ribosomal subunits, whereas the production of 37S pre-rRNA and large ribosomal subunits is unaffected. Thus, the most plausible explanation is that the insertions in *T.thermophila* 26S rRNA V13 and yeast 17S rRNA V3 interfere with some stage in the assembly of pre-ribosomal particles. Hence, whereas some variable regions show considerable structural flexibility, others appear to be subject to fairly strict structural constraints. Such constraints may be a reflection of the recruitment of these regions for lineage specific functions, as has been proposed for regions V3 and V13 of the large subunit rRNA gene (Michot and Bacchellerie, 1987).

Both ETS and ITS1 are required in yeast for formation of small subunits but not of large subunits

In contrast to mature rRNA sequences that are under strong evolutionary constraint, the transcribed spacer regions in eukaryotes display a remarkable variation in both sequence and size. This observation has raised the question whether eukaryotic transcribed spacers might be dispensable for ribosome formation altogether. Our studies using partial deletions of ETS and ITS1 in the tagged ribosome system clearly indicate that both spacers contain information required for *bona fide* production of 17S rRNA but not of 26S rRNA. The observation that all four ETS deletions tested prevented the accumulation of tagged 17S rRNA suggests that (long-range) secondary structure might form an important aspect of the *cis*-acting elements involved in the removal of the ETS from the primary transcript and/or of the binding site for pre-ribosomal proteins. To substantiate this hypothesis a more subtle mutational analysis, taking into account the results of secondary structure investigations, will be performed.

The processing event at site A2 in ITS1 is essential for the formation of small subunits in yeast

The major processing pathway of yeast pre-rRNA involves an early processing event at a site in the central region of ITS1 (site A2, *cf.* Figure 1). The processing pathways reported for higher eukaryotes (Bowman *et al.*, 1981), do not comprise a similar step. However, the recent discovery that in hamster cells transfected with mouse rDNA a site in an internal region of mouse ITS1 is cleaved at an early stage of rRNA maturation (Raziuddin *et al.*, 1989), might indicate that processing at a comparable site does occur in higher eukaryotes, but has hitherto gone undetected. In yeast, processing at site A2 appears to take place simultaneously with the processing event that generates the mature 5′-end of 17S rRNA, which resembles the generation of mature 16S rRNA in prokaryotes (Abelson, 1979). The accumulation of the unusual 32S precursor in cells containing pORCSΔITS1 suggests that processing at site A1 can proceed independently from processing at A2, as opposed to what has previously been suggested (Veldman *et al.*, 1981). Evidence has recently become available for the role of at least two snRNAs in this stage of yeast pre-rRNA processing. The snR128 (U14) species appears to be required for 37S pre-rRNA processing because its depletion causes severe under-accumulation of mature 17S rRNA and its 18S precursor (Li *et al.*, 1990). Most likely snR128 plays a role in processing at A1 or A2 although an indirect effect of this factor on rRNA maturation cannot be excluded. In addition

snR10 RNA seems to assist in processing at sites A1 and A2 (Tollervey, 1987). In *snR10.3* mutants an alternative processing pathway, using site B1 at an earlier stage, was demonstrated to be employed with strongly increased efficiency. Thus the 32S precursor most likely is cut at this site, generating 29SB and a precursor named 21S by Tollervey (Tollervey, 1987, *cf.* Figure 1C). Our results suggest that subsequently 29SB is used efficiently for the formation of 60S subunits, since removal of processing site A2 does not affect the accumulation of 26S rRNA. This is not surprising because the same precursor also is an intermediate in the major processing pathway in wild-type cells. However, the small subunit rRNA precursor generated by this pathway (21S) did not give rise to the formation of 40S subunits in our experiments, as opposed to results obtained with *snr10.3* mutants (Tollervey, 1987). Thus, our results suggest that the cold sensitivity of *snr10.3* mutants is due to a defect in the assembly of small ribosomal subunits.

Materials and methods

Enzymes and strains

Restriction enzymes, polynucleotide kinase, *E.coli* DNA polymerase I (Klenow fragment) and T4 DNA ligase were purchased from Bethesda Research Laboratories (Gaithersburg, MD). *Asu*II was from Promega (Madison, WI) and *Afl*II was from New England Biolabs (Beverly, MA). Zymolyase-100T was obtained from Seikagaku Kogyo Co. (Tokyo, Japan), helicase from Industrie Biologique Française (Clichy, France) and calf intestine alkaline phosphatase from Boehringer (Mannheim, Germany). Oligonucleotides were synthesized using an Applied Biosystems 381A DNA synthesizer.

E.coli DH1 (F$^-$*rec*A1 *end*A1 *gyr*A96 *thi*-1 *hsd*R17 *sup*E44 *rel*A1 λ$^-$) was used for constructions and propagation of plasmids. *S.cerevisiae* MG34 (*leu2 trp1 rad2 cir*$^+$) was used for expression of pORCS and related plasmids.

Tagging the 17S rRNA gene

The 1.4 kb *Sac*I−*Xho*I fragment of pORCS (Musters *et al.*, 1989a), comprising the 3′-end of the 17S rRNA gene, ITS1, the 5.8S rRNA gene, ITS2 and the 5′-end of the tagged 26S rRNA gene was inserted in pTZ18U (USB, Cleveland, OH), in which the *Kpn*I site had been disrupted by the insertion of a 18 bp *Xho*I linker. The resulting plasmid (pTZ-ITS, Figure 8) was linearized with *Nhe*I, dephosphorylated by treatment with calf intestine alkaline phosphatase and recircularized in the presence of a 5-fold molar excess of a 19 bp long oligonucleotide containing a *Kpn*I site (Figure 2), that had been phosphorylated at the *Nhe*I-compatible cohesive ends. Oligonucleotide-containing pTZ-ITS was linearized by extensive digestion with *Kpn*I to ensure that only one copy of the oligo would be present in the final construct, purified by gel electrophoresis and electroelution, and recircularized. The *Sac*I−*Xho*I insert of the resulting plasmid was used to replace the analogous fragment of the original pORCS plasmid, yielding pORCS(17s*+26S*) (*cf.* Figure 8).

Construction of mutants

(i) pORCS-V3: insertion of a 19 bp oligonucleotide in variable region V3. The 1.8 kb *Bgl*II−*Sac*I fragment of pORCS, comprising large parts of both the ETS and the 17S rRNA gene, was inserted between the *Bam*HI and *Sac*I sites of pUC19. The resulting plasmid, pUC-ETS-17S (Figure 8), was linearized with *Asu*II, dephosphorylated and recircularized in the presence of a 5-fold molar excess of a 19 bp long oligonucleotide containing a *Kpn*I site (Figure 2), that had been phosphorylated at the *Asu*II-compatible cohesive ends. Oligonucleotide-containing plasmids were linearized by extensive *Kpn*I digestion to ensure insertion of only a single copy of the oligo, purified by gel electrophoresis and electroelution and recircularized. The 1.0 kb *Nsi*I−*Sac*I fragment of the resulting plasmid was used to replace the analogous fragment of pORCS(17S*+26S*), yielding pORCS V3.

(ii) Construction of deletions in the ETS. The construction of pML1, a subclone of the region encompassing the yeast Pol I promoter and the complete ETS in pSP65 (Promega, Madison, WI), as well as the insertion of *Bam*HI linkers at random positions in this plasmid have been described previously (Musters *et al.*, 1989a). Several clones of the collection of plasmids that harbor the linker insertion within the ETS, have been sequenced using appropriate oligonucleotide primers, to determine the exact position of the linker. Suitable clones were cut with *Bam*HI and *Bgl*I, the fragments were purified by gel electrophoresis and electroelution, and used to construct pMLΔETS, pMLΔ5′-ETS, pMLΔETS-center and pMLΔ3′-ETS (Figure 5). The *Sma*I−*Nsi*I fragment of these plasmids was used to replace the analogous fragment of pORCS(17S*+26S*), yielding pORCSΔETS, pORCSΔ5′-ETS, pORCSΔETS-center and pORCSΔ3′-ETS, respectively.

(iii) pORCSΔITS1: a deletion in ITS1. The 160 bp *Afl*II−*Apa*I fragment of pTZ-ITS was replaced with a single-stranded oligonucleotide with a length of 8 nt (5′-TTAAGGCC-3′) that fuses the cohesive ends generated by these enzymes. Digestion with *Stu*I was used to screen against transformants harboring plasmids in which multiple copies of the oligonucleotide had been inserted, since this would create an additional *Stu*I site. The 1.2 kb *Sac*I−*Xho*I insert of the resulting plasmid was used to replace the analogous fragment of pORCS(17S*+26S*), yielding pORCSΔITS1 (Figure 7).

Miscellaneous techniques

Yeast transformation was performed by the method of Beggs (1978) with minor modifications. RNA from transformed cells was isolated essentially according to Kraig *et al.* (1982). Polysome isolation, labelling of oligonucleotides and blotting techniques have been described previously (Musters *et al.*, 1989a).

Acknowledgements

The authors wish to thank Professor Dr H.A.Raué for fruitful discussions and critical reading of the manuscript. This study was supported in part by the Netherlands Foundation for Chemical Reserch (S.O.N.) with financial aid from the Netherlands Organization for Scientific Research (N.W.O.).

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Received on July 25, 1990; revised on September 3, 1990