Separate structural elements within internal transcribed spacer 1 of *Saccharomyces cerevisiae* precursor ribosomal RNA direct the formation of 17S and 26S rRNA

Rob W.van Nues, Jeanette M.J.Rientjes, Carine A.F.M.van der Sande[+], Shuraila F.Zerp, Caroline Sluiter, Jaap Venema[§], Rudi J.Planta and Hendrik A.Raué*
Department of Biochemistry and Molecular Biology, Institute for Molecular Biological Sciences. BioCentrum Amsterdam, Vrije Universiteit, De Boelelaan 1083, 1081 HV Amsterdam, The Netherlands

ABSTRACT

Structural features of Internal Transcribed Spacer 1 (ITS1) that direct its removal from *Saccharomyces cerevisiae* pre-rRNA during processing were identified by an initial phylogenetic approach followed by *in vivo* mutational analysis of specific structural elements. We found that *S.cerevisiae* ITS1 can functionally be replaced by the corresponding regions from the yeasts *Torulaspora delbrueckii*, *Kluyveromyces lactis* and *Hansenula wingei*, indicating that structural elements required in *cis* for processing are evolutionarily conserved. Despite large differences in size, all ITS1 regions conform to the secondary structure proposed by Yeh et al. [Biochemistry 29 (1990) 5911 – 5918], showing five domains (I – V; 5′→3′) of which three harbour an evolutionarily highly conserved element. Removal of most of domain II, including its highly conserved element, did not affect processing. In contrast, highly conserved nucleotides directly downstream of processing site A2 in domain III play a major role in production of 17S, but not 26S rRNA. Domain IV and V are dispensable for 17S rRNA formation although an alternative, albeit inefficient, processing route to mature 17S rRNA may be mediated by a conserved region in domain IV. Each of these two domains is individually sufficient for efficient production of 26S rRNA, suggesting two independent processing pathways. We conclude that ITS1 is organized into two functionally and structurally distinct halves.

INTRODUCTION

In *Saccharomyces cerevisiae*, as in other eukaryotes, the genes encoded by 17S (18S), 5.8S and 26S (28S) rRNA are transcribed by RNA polymerase I as a single large precursor that contains additional stretches of nucleotides at both ends as well as separating the mature rRNA sequences (Fig. 1A). Subsequently, these external and internal transcribed spacers, designated 5′ETS, ITS1, ITS2 and 3′ETS, are removed in a number of ordered processing steps, that occur concomitantly with the assembly of the ribosomal proteins, to yield the three mature rRNA molecules (reviewed in references 1 – 3; see Fig. 1B). Recently, several *trans*-acting factors involved in yeast pre-rRNA processing have been identified, including the U3, U14, snR10 and snR30 small nucleolar ribonucleoprotein particles (snoRNPs; see references 4 – 6 for recent reviews), RNase MRP (7, 8), the 5′ → 3′ exonucleases encoded by the *RAT1* (9) and *XRN1* (10) genes and the product of the *NSR1* gene which is structurally related to mammalian nucleolin (11 – 13). With the exception of a site in the 5′ETS that appears to be recognized directly by U3 snRNP through base-pairing (14), however, there is so far little information on the nature of the *cis*-acting elements within pre-rRNA that are important for correct and efficient formation of the mature rRNA species.

Characterization of *cis*-acting processing elements to a large extent depends upon mutational analysis. However, because of the lack of an *in vitro* processing system for yeast pre-rRNA, such mutational analysis can at present only be carried out *in vivo*, in which case one has to circumvent the problem posed by the presence of 150 – 200 chromosomal rDNA units producing large amounts of wild-type rRNA. The so-called 'tagged

ribosome' system recently developed in our laboratory (15) provides a means of solving this problem. This system consists of a centromeric plasmid carrying a complete yeast rDNA unit in which the genes encoding 17S and 26S rRNA each contain a small unique oligonucleotide insertion. These insertions, which themselves do not interfere with either the processing or assembly of the tagged rRNA molecules into ribosomes (15, 16), allow the detection of the transcription products of the plasmid-borne rDNA unit in the presence of the large excess of (pre-)rRNA produced by the chromosomal units. Consequently it is possible to ascertain the effect of mutations in the tagged rDNA unit on the maturation of the individual rRNAs. By means of this system we have previously demonstrated that introduction of relatively large deletions in either the 5'ETS or ITS1 prevent normal production of 17S, but not 26S rRNA, while deletions within ITS2 severely reduce or abolish 26S rRNA production but leave 17S rRNA levels unaffected (16−18). Thus, each of these three spacers contains one or more structural elements essential for the correct and efficient formation of at least one of the high-molecular-weight rRNA species.

In this paper we present the results of a further mutational analysis carried out on ITS1 in order to characterize the *cis*-acting element(s) present in this spacer in more detail. Using a phylogenetic approach we first demonstrated that ITS1 regions from several other, evolutionary more or less closely related, yeast species can functionally replace their *S.cerevisiae* counterpart despite large differences in size and sequence. Subsequent comparison of the various ITS1 regions, taking into account the recently published secondary structure model for *S.cerevisiae* ITS1 (19), revealed the presence of distinct conserved structural features. These were used as a guide in constructing a series of deletion mutants to identify their role in correct and efficient pre-rRNA processing.

MATERIALS AND METHODS

Enzymes, strains, transformation procedures and recombinant DNAs

Restriction enzymes were purchased from New England Biolabs (New England, USA), Pharmacia (Uppsala, Sweden) and Boehringer (Mannheim, Germany). Polynucleotide kinase, *Escherichia coli* DNA polymerase I (Klenow fragment), bacteriophage T4 DNA ligase and T4 DNA polymerase were from GIBCO/Bethesda Research Laboratories (Rockville, MD, USA) and Boehringer. Super Taq polymerase was from HT-Biotechnology Ltd. (Cambridge, UK), Zymolyase 100T from Seikagaku Kogyo Co. (Tokyo, Japan) and helicase from Industrie Biologique Française (Clichy, France).

E.coli DH1 (F^-, *recA*1, *endA*1, *gyrA*96, *thi*-1, *hsd*R17, *sup*E44, *rel*A1, λ^-), SURE™ (Stratagene, La Jolla, CA, USA), JM109 and BMH 71-18mutS (Promega, Madison, WI, USA) were used for construction and propagation of plasmid and phagemid DNA. *Saccharomyces cerevisiae* MG34 (*leu*2, *trp*1, *rad*2, *cir*+) was used for transformation of derivatives of the pORCS(17S*+26S*) plasmid containing the doubly tagged *S.cerevisiae* rDNA unit (16, 18) by either the spheroplast (20) or freeze thaw (21) method. Isolation of DNA and RNA from yeast cells, recombinant DNA procedures, Northern hybridization and primer extension analysis were performed essentially as described previously (15, 16). Sequence analysis was carried out on double-stranded DNA (22) by means of a Sequenase® 2.0 kit (USB, Cleveland, Ohio, USA).

Mutations of ITS1 used in this study are listed in Table I. They are referred to by a Roman numeral indicating the structurally altered domain(s) as present in the secondary structure model shown in Fig. 2, followed by the number of nucleotides that was deleted.

Cloning and sequencing of heterologous ITS1 regions

In order to obtain the nucleotide sequence of the ITS1 regions from different yeast species we subcloned 3.5 kb *Xba*I−*Xba*I fragments from *Torulaspora delbrueckii* (CBS817; Centraal Bureau Schimmelcultures, Baarn, The Netherlands) and *Hansenula wingei* (NRLL, strain 21; IL, USA) rDNA as well as 1.1 kb *Eco*RI−*Bam*HI fragments of *Kluyveromyces marxianus* (CBS6556) and *K. lactis* (NRLL, strain Y1140) rDNA (each spanning the ITS1, the 5.8S rRNA gene and ITS2; ref. 23) into pUC19 or pGEM3. Primers annealing to universally conserved parts of 17S rRNA (from A_{-45} to C_{-28}; coordinates relative to the 3'-end of 17S rRNA) or 5.8S rRNA (from G_{46} toegions. The new ITS1 sequences have been deposited in the EMBL Data Library under accession numbers Z29483 (*T.delbrueckii*), Z29384 (*K.lactis*), Z29385 (*K.marxianus*) and Z29383 (*H.wingei*). The *S.cerevisiae* ITS1 sequence (24) is available under accession # J01352.

Construction of ITS1 replacement mutants

Complete replacement of the *S.cerevisiae* ITS1 was effected with the aid of conserved restriction sites in the flanking 17S and 5.8S rRNA genes (Fig. 1A). For *T. delbrueckii* ITS1 these were *Stu*I and *Sph*I, respectively; for the *K. lactis* spacer we used *Bsp*MI and *Cla*I, while for the insertion of ITS1 fro T_{69} with respect to the 5'-end of the short form of 5.8S rRNA) were used for determining the sequence of the various ITS1 rm *H.wingei Bsp*MI and *Sna*BI were taken. These enzymes were chosen such that the mature rRNA sequences were not altered in case of the replacements with *T.delbrueckii* and *K.lactis* ITS1, yielding mutants Td and Kl, respectively. The *H.wingei* ITS1 replacement, yielding mutant Hw, resulted in the introduction of two substitutions into the 5.8S rRNA sequence (Table I).

In order to be able to construct chimeras between portions of *S.cerevisiae* and *T.delbrueckii* ITS1 we introduced a *Pfl*MI site into either sequence by an A→C substitution at position 212 or 152 respectively (see Figs. 2A and B) using the Altered Site Mutagenesis System™ (Promega). Chimeric ITS1 regions were then constructed by exchanging either the *Stu*I−*Pfl*MI or the *Pfl*MI−*Sph*I fragment of *S.cerevisiae* rDNA for its counterpart from *T.delbrueckii* giving rise to mutants 5'Td and 3'Td, respectively. A chimeric ITS1 containing the first three domains from *H.wingei* fused to domains IV and V from *S.cerevisiae* was constructed by means of a two-step PCR procedure. First, the 3' half of *S.cerevisiae* ITS1 was amplified using two primers annealing to nucleotides A_{284}-C_{307} in ITS1 (Fig. 2A) and to the 26S rDNA tag (15; Fig. 1A) respectively. Similarly, we amplified the 5' region of *H. wingei* ITS1 by means of primers annealing to the 17S rDNA tag (16) and to nucleotides C_{88}-A_{106} of *H. wingei* ITS1 (Figs. 1A and 2D). Both purified products were heat-denatured and directly cooled on ice in order to allow annealing of the sense and antisense single strands of the overlapping terminal ten nucleotides (Fig. 2A, A_{284}-A_{292}). The DNA was made double-stranded by means of Taq polymerase. The resulting product was then amplified by PCR using the primers complementary to the 17S and 26S rDNA tags. The chimeric ITS1 was inserted into the tagged rDNA unit by replacement of

the wild-type *StuI—SphI* fragment (Fig. 1A). This mutant is referred to as 5'Hw. Sequencing revealed an additional U→C substitution at position 306 inadvertently introduced by the PCR amplification of the *S.cerevisiae* portion of the spacer.

Construction of ITS1 deletion mutants

Domain II deletions IIΔ16 and IIΔ154 were obtained by BAL31 digestion of the 1.1 kb *SacI—HpaI* rDNA fragment (cloned into pGEM3) after linearization at the *AflII* site within ITS1 (see Figs. 1A and 2A). Suitable deletions were selected on the basis of size as analyzed by gel electrophoresis. The precise nature of the deletion was determined by dideoxy sequencing (22).

Three small deletions in domain III were constructed by means of T4 DNA polymerase degradation of protruding ends generated by either *PfIMI* (IIIΔ3, IIIΔ4) or *ApaI* (IIIΔ5) digestion followed by religation. Deletion mutant IIIΔ58 was created by cleavage with *PfIMI* and *ApaI* (Figs. 1A and 2A), followed by digestion with T4 DNA polymerase to obtain blunt ends and religation. All domain II and domain III mutants were introduced into the tagged rDNA unit by replacing the wild-type *SacI—SphI* fragment spanning ITS1 with its mutant counterpart.

The deletion removing both domains IV and V (IV−VΔ73) was created via substitution of the *ApaI—SspI* fragment of ITS1 (from C_{274} to U_{357}; Fig. 2A) by an oligonucleotide that restored the wild-type sequence C_{274}-C_{283} except that a U residue was inserted between A_{281} and A_{282}. This insertion introduces an *AccI* restriction site that was subsequently used to construct the domain IV (IVΔ41) and the domain V (VΔ31) deletion mutants by oligonucleotide insertion. For mutant IVΔ41 a synthetic double stranded 32-mer with *AccI* sticky ends was used that essentially restored the wild-type sequence downstream from A_{324} (Table I). Mutant VΔ31 was obtained by inserting a double stranded 42-mer that restored the wild-type sequence from A_{282} to U_{320}. The domain IV and V mutations were introduced into the tagged rDNA unit by replacing the *SacI—XhoI* fragment with its mutant counterpart. All deletion mutants contain the A_{212}→C substitution that creates a *PfIMI* site in domain III (see above and Table I).

RESULTS AND DISCUSSION

In the primary 37S transcript of the *S.cerevisiae* rDNA unit ITS1 is located between the mature 17S and 5.8S rRNA sequences (see Fig. 1A) and thus separates the two portions of the 37S pre-rRNA that become incorporated into the 40S and 60S ribosomal subunits, respectively. ITS1 contains an internal processing site that is cleaved at an early stage of maturation (site A2; see Figs. 1B and 2A) leading to the 20S and 29S$_A$ pre-rRNA species. Subsequent nuclear processing of the latter then generates the mature 5'-end of 5.8S rRNA and removes ITS2 to give the mature 5.8S and 26S rRNA species (Fig. 1B). Formation of the mature 3'-end of 17S rRNA by removal of the remaining portion of ITS1 from the 20S precursor occurs in the cytoplasm (see references 1−3 for recent reviews). Using the tagged ribosome system (15) we have previously shown that deletion of the central 160 nucleotides from ITS1 (positions 112−270; see Fig. 2A), which includes site A2, severely reduces the formation of tagged 17S rRNA but does not detectably affect the level of tagged 26S rRNA (16, 17). This result indicates that site A2 is crucial for correct and efficient formation of the small subunit rRNA. However, when this site is absent an alternative route appears to be available for production of mature 5.8S/26S rRNA. The

Table I. Mutations used in this study

Mutant[a]	Alteration(s) in ITS1[b]
Sc[P]	A_{212}→ C (*PfIMI* site in domain III)
Td	*T. delbrueckii* ITS1 substitution
Td[P]	A_{152} of *T. delbrueckii* ITS1 into C (creates a *PfIMI* site)
5'Td	A_1-G_{219} of Sc[P] substituted by G_1-G_{158} from Td[P]
3'Td	U_{220}-A_{362} of Sc[P] substituted by U_{159}-A_{293} from Td[P]
Kl	*K. lactis* ITS1 substitution
Hw	*H. wingei* ITS1 substitution (U_{34}→ A and A_{54}→ U in 5.8S rRNA)
5'Hw	A_1-C_{283} of Sc replaced by U_1-U_{97} from *H. wingei*; U_{306}→C
IIΔ16	C_{100} ed to G_{117} in Sc[P]
IIΔ154	U_{31} fused to A_{186} in Sc[P]
II-IIIΔ160	G_{111} fused to G_{271} (16)
IIIΔ3	C_{215} fused to G_{219} in Sc[P]
IIIΔ4	A_{214} fused to G_{219} in Sc[P]
IIIΔ58	A_{214} fused to C_{273} in Sc[P]
IIIΔ5	G_{268} fused to C_{274} in Sc[P]
IV-VΔ73	C_{283} fused to A_{358} in Sc[P], $A_{281}A_{282}$→AUA
IVΔ41	A_{282} fused to A_{325} in Sc[P], $A_{281}A_{282}$→AUA, A_{355}→U and U_{357}→C
VΔ31	U_{320} fused to A_{354} in Sc[P], $A_{281}A_{282}$→AUAA, A_{355}→U, U_{357}→C
II-IV-VΔ227	Combination of IIΔ154 and IV-VΔ73

[a]The amount of xx nt deleted from domain Y is abbreviated as YΔxx.
[b]Nucleotides and domains are numbered as in Figure 2.
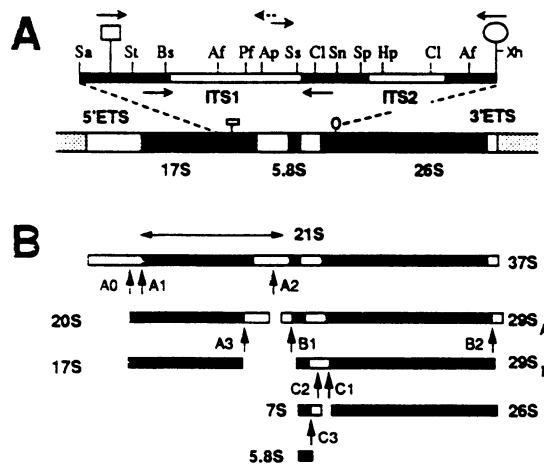

Figure 1. Panel A: Genetic organization of the rDNA unit of *S.cerevisiae*. Mature sequences and spacer regions are shown in black and white, respectively. The 1.4 kb *SacI—XhoI* fragment spanning ITS1 and ITS2 is enlarged to show the restriction sites used for construction of the various ITS1 mutants. Af, *AflII*; Ap, *ApaI*; Bs, *BspMI*; Cl, *ClaI*; Hp, *HpaI*; Pf, *PfIMI*; Sa, *SacI*; Sn, *SnaBI*; Sp, *SphI*; Ss, *SspI*; St, *StuI*; Xh, *XhoI*. Primers used for sequencing or PCR amplification are indicated by arrows below and above the fragment, respectively. The primer annealing to *H. wingei* ITS1 is dashed. The positions of the tags in the 17S and 26S rRNA genes are marked. Panel B: The major pre-rRNA processing pathway of *S.cerevisiae*. Processing sites are indicated by arrows. An aberrant intermediate accumulating in the snR10 mutant strain (25, 26) and discussed in the text is shown above the 37S precursor.

same conclusion was also drawn from experiments in which cleavage at A2 was strongly delayed by inactivating the snR10 snoRNP (25, 26).
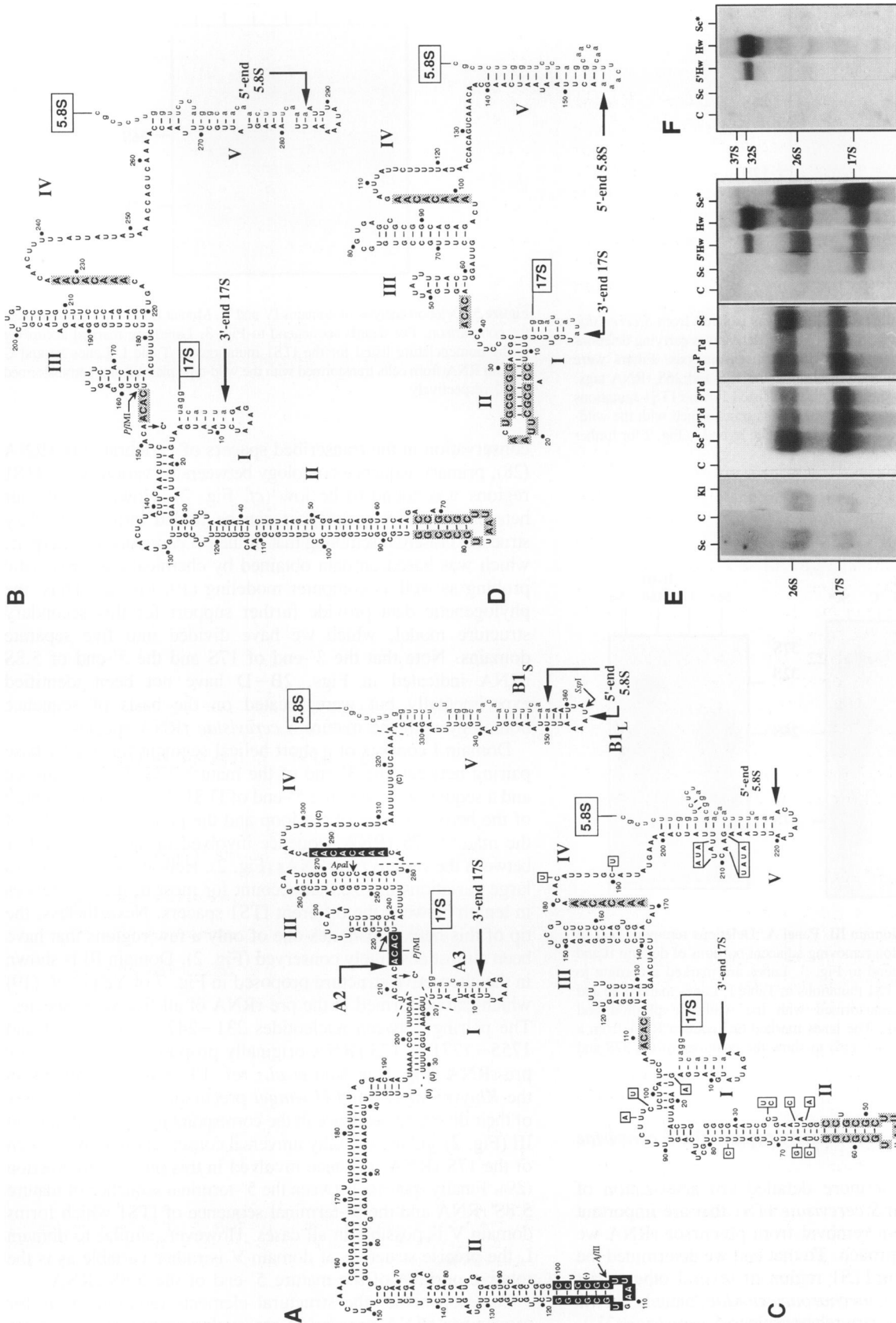
Figure 2. Secondary structure models for the ITS1 regions. Panel **A**: *S.cerevisiae* (19, 24). Panel **B**: *T. delbrueckii*. Panel **C**. *K. lactis* (sequence differences with *K. marxianus* ITS1 are shown in boxes) and Panel **D**: *H. wingei*. Regions of significant primary structure conservation are highlighted. Flanking mature 17S and 5.8S rRNA sequences are shown in lower case. Domains are separated by dotted lines and are indicated by Roman numerals. Nucleotides are numbered with respect to the 5′ end of ITS1. In panel A processing sites present in *S.cerevisiae* ITS1 are shown. Nucleotides described by Yeh *et al.* (19) that are absent from our sequence (24) are shown within brackets. Restriction sites used in construction of mutant ITS1 regions are indicated. This includes the *Pfl*MI site introduced by the A$_{212}$→C substitution. Panel **E**: Effect of complete or partial replacement of *S.cerevisiae* ITS1 by its heterologous counterparts. pORCS(17S* + 26S*) derivatives carrying the altered ITS1 were introduced into *S.cerevisiae* MG34. Total RNA from the transformants was analyzed by Northern blotting by simultaneous use of the probes complementary to the 17S and 26S rRNA tags. Lanes are marked according to the nomenclature listed for the ITS1 mutations in Table I. Lanes Sc contain RNA from transformants carrying the wild-type *S.cerevisiae* ITS1. Lanes C contain RNA from untransformed MG34 cells. Differences in intensity between individual panels are due to differences in the specific activity of the probes used in separate experiments. Equal amounts of RNA (as determined by the A$_{260}$ of the samples) were loaded in all lanes of each individual experiment. Minor differences in intensity within a single panel are caused by slight differences in copy number between individual transformants (15). The lanes marked Sc* contain RNA from a 'high-copy number' Sc transformant (15) to show the positions of the 37S and 32S pre-rRNA. Panel **F**: The same filter as shown at the far right of panel E was hybridized with a probe complementary to the *H. wingei* ITS1 sequence (C$_{88}$·A$_{106}$; *cf.* Fig. 2D).
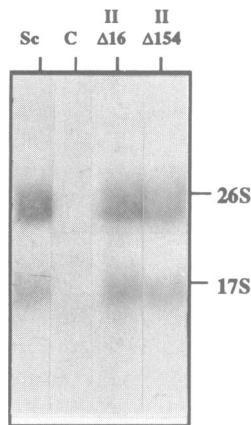
Figure 3. Deletion analysis of domain II. RNA was isolated from *S.cerevisiae* MG34 cells transformed with pORCS(17S* + 26S*) derivatives carrying deletions within domain II of ITS1 and subjected to Northern analysis. Filters were simultaneously hybridized to probes annealing to the 17S and 26S rRNA tags. Lanes are marked according to the nomenclature listed for the ITS1 mutations in Table I. Lanes Sc and C contain RNA from cells transformed with the wild-type plasmid and untransformed cells, respectively. See legend to Fig. 2 for further details.
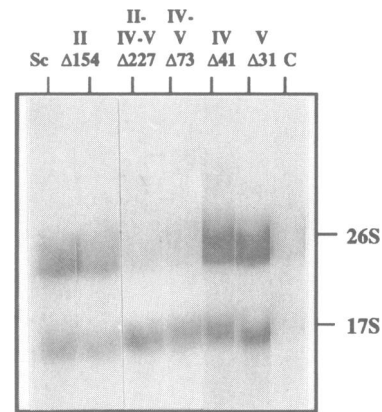

Figure 5. Deletion analysis of domains IV and V. Mutant IIΔ154 has been included for comparison. For details see legend to Fig. 3. Lanes are marked according to the nomenclature listed for the ITS1 mutations in Table I. Lanes Sc and C contain RNA from cells transformed with the wild-type plasmid and untransformed cells, respectively.
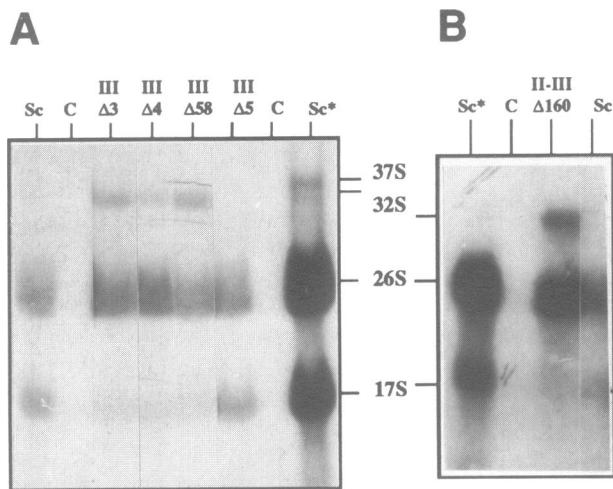

Figure 4. Deletion analysis of domain III. Panel A: Deletions removing part of domain III only. Panel B: Deletion removing adjacent portions of domain II and III (16, 17). For details see legend to Fig. 3. Lanes are marked according to the nomenclature listed for the ITS1 mutations in Table I. Lanes marked Sc and C contain RNA from cells transformed with the wild-type plasmid and untransformed cells, respectively. The lanes marked Sc* contain RNA from a 'high-copy number' Sc transformant (15) to show the positions of the 37S and 32S pre-rRNA.

Heterologous spacers can functionally replace the *S.cerevisiae* ITS1

As a first step towards a more detailed characterization of structural elements within *S.cerevisiae* ITS1 that are important for its correct and efficient removal from precursor rRNA we applied a phylogenetic approach. To that end we determined the nucleotide sequence of the ITS1 region of several other yeast species from the subfamily *Saccharomycetoidae*, namely [in the order of decreasing evolutionary relationship to *S.cerevisiae* (27)]: *Torulaspora delbrueckii*, *Kluyveromyces lactis*, *K. marxianus* and *Hansenula wingei*. In accordance with the limited structural

conservation in the transcribed spacers of vertebrate pre-rRNA (28), primary sequence homology between the various yeast ITS1 regions was found to be low (*cf.* Fig. 2). However, all four heterologous ITS1 sequences can be folded into a secondary structure closely resembling that of their *S.cerevisiae* counterpart, which was based on data obtained by chemical and enzymatic probing as well as computer modeling (19; Fig. 2). Thus, the phylogenetic data provide further support for this secondary structure model, which we have divided into five separate domains. Note that the 3'-end of 17S and the 5'-end of 5.8S rRNA indicated in Figs. 2B−D have not been identified experimentally but were located on the basis of sequence homology with the mature *S.cerevisiae* rRNA species.

Domain I consists of a short helical segment formed by base pairing between the 3'-end of the mature 17S rRNA sequence and a sequence close to the 5'-end of ITS1. However, the length of the helix, the size of the loop and the precise nucleotides of the mature 17S rRNA sequence involved in the pairing differ between the various pre-rRNAs (Fig. 2). Helical domain II shows large variations in size that account for most of the differences in length between the different ITS1 spacers. Nevertheless, the tip of this helix constitutes one of only a few regions that have been almost absolutely conserved (Fig. 2). Domain III is shown in the alternative structure proposed in Fig. 7 of Yeh *et al.* (19) which can be formed in the pre-rRNA of all five yeast species. The pairing between nucleotides 231−247 of domain III and 1755−1771 of 17S rRNA originally proposed for *S.cerevisiae* pre-rRNA (Fig. 6 in Yeh *et al.*; ref. 19) is highly unlikely in the *Kluyveromyces* and *H.wingei* precursor molecules in view of their divergent sequence in the corresponding region of domain III (Fig. 2) and the virtually universal conservation of the portion of the 17S rRNA sequence involved in this putative interaction (29). Finally, pairing between the 5'-terminal sequence of mature 5.8S rRNA and the 3'-terminal sequence of ITS1 which forms domain V is possible in all cases. However, similar to domain I, the precise structure of domain V is rather variable as is the relative position of the mature 5'-end of the 5.8S rRNA.

To test whether the structural elements required in *cis* for proper pre-rRNA processing are evolutionarily conserved we partially or completely replaced *S.cerevisiae* ITS1 (362 nt; ref. 24) with its counterparts from three other yeast species. Analysis

of the steady state levels of tagged mature 17S and 26S rRNA by Northern analysis (Fig. 2E) demonstrates that both the *T.delbrueckii* (293 nt; lane Td) and *K.lactis* (227 nt; lane Kl) ITS1 are functional when inserted into the tagged *S.cerevisiae* rDNA unit, although the steady state level of tagged 26S rRNA produced from the former substitution mutant may be somewhat reduced. In contrast to our observations on ITS2 (18), even chimeras between the *S.cerevisiae* and *T.delbrueckii* ITS1 regions (Fig. 2E; lanes 5'Td and 3'Td) remain functional. The A→C substitution shortly upstream from processing site A2 that was introduced to create the *Pfl*MI site used in the construction of these chimeras (Fig. 2A). is also neutral with respect to processing (Fig. 2E; lanes Sc^P and Td^P).

The most divergent of the three heterologous spacers, the *H.wingei* ITS1 (153 nt), still supports efficient formation of tagged mature 17S and 26S rRNA. However, in this case increased levels of a 32S-like precursor rRNA containing the *H.wingei* ITS1 are detected (Figs. 2E and F, lane Hw) indicating a decrease in the rate of processing at site A2. Replacement of only domains I through III by the corresponding region from *H. wingei* ITS1 also causes retardation of cleavage at A2 (Figs. 2E and F, lane 5'Hw). However, as judged from the lower amount of the 32S precursor relative to 17S and 26S rRNA, the effect is less severe than seen in the complete replacement. These data indicate a predominant role for elements contained within domains I–III in determining the efficiency of A2 cleavage. The presence of a severely truncated form of domain II in *H. wingei* ITS1 suggests that most of this domain is of minor importance for pre-rRNA processing.

The data reported above support the secondary structure model derived for *S.cerevisiae* ITS1 by Yeh *et al.* (19). More importantly, they show that, despite large variations in primary structure, the elements required in *cis* for the correct and efficient processing of *S.cerevisiae* pre-rRNA have largely been conserved over a relatively wide evolutionary range. This is in sharp contrast to the ITS2 spacer, which can be functionally replaced only by its counterpart from the closely related *T. delbrueckii* but not by that from either *K.lactis* or *H.wingei* (2, 18).

The preservation of function even by the chimeric spacers indicates the absence of extensive higher order interactions between the 5'- and 3'-terminal portions of ITS1. This again contrasts sharply with the situation for ITS2 which appears to be folded into a compact conformation (2, 18; RWvN, unpublished).

Three of the five domains discernable in ITS1 contain significant regions of sequence conservation (Fig. 2) that are plausible candidates for *cis*-acting processing elements. In order to ascertain the role of these sequences as well as that of specific conserved secondary structure elements we undertook a systematic deletion analysis of the individual domains of ITS1.

A large portion of domain II is dispensable for processing

As mentioned above, the results of the replacement experiments described in the previous section already suggest that most of domain II is devoid of structural elements essential for pre-rRNA processing. Nevertheless, in all ITS1 spacers depicted in Fig. 2 this domain contains the same structural element at its apical end. To test whether this element plays an essential role in pre-rRNA processing we removed sixteen of its nineteen conserved nucleotides (positions 101–116). However, this deletion did not significantly affect the steady-state level of either 17S or 26

rRNA, as shown by the Northern analysis depicted in Fig. 3 (lane IIΔ16). Alterations in the size of the stem or sequence of the loop of this conserved element also had no effect on production of the mature rRNA species (data not shown).

In order to obtain direct evidence for the lack of important *cis*-acting elements in the rest of domain II we further reduced its size by a deletion of 154 nucleotides (positions 32–185) which maintains the helical structure of the remaining portion. This deletion also failed to have any noticeable effect on processing as judged from the results of the Northern analysis shown in Fig. 3 (lane IIΔ154).

These data constitute experimental support for the presence of domain II as a distinct secondary structural feature of ITS1 in yeast pre-rRNA. At least the distal two-thirds of this domain are dispensable for normally efficient pre-rRNA processing despite the fact that this region contains an evolutionarily highly conserved element. However, the central part of domain II does seem to play a role in some other aspect of ribosome biogenesis. Using a system in which yeast cells can be made completely dependent upon the mutant rDNA units (JV & HAR, manuscript in preparation) we have found that the IIΔ154 deletion causes a significant reduction in growth rate relative to control cells expressing wild-type rDNA that is not observed for the IIΔ16 deletion (RWvN, unpublished experiments). Since removal of domain II is a very late step in the maturation of 17S rRNA occurring in the cytoplasm, possible functions that could be envisaged are a role in the assembly of the 20S precursor rRNA into the 43S pre-ribosomal subunit or in transport of this RNP particle over the nuclear envelope.

The possible involvement of the portion of domain II directly adjacent to domains I and III remains to be investigated. It should be noted, however, that there is no significant sequence conservation within this region and even secondary structure is poorly conserved, in particular in the *K.lactis* ITS1 that, nevertheless, supports a normal rate of processing (Fig. 2E).

Deletions in domain III affect 17S maturation

We next turned our attention to domain III, previously shown to contain crucial information for efficient production of mature 17S including site A2 (16, 17, 25, 26). Reverse transcription analysis locates processing site A2 in the middle of a single-stranded region of the secondary structure model just in front of the sequence ACAC (30, 31; JV unpublished results) four nucleotides downstream from the previously mapped 3'-end of the 20S pre-rRNA (32, 33). The ACAC sequence element is absolutely conserved in the corresponding region of all four heterologous yeast ITS1 spacers, whereas the sequence preceding A2 shows considerable divergence. Deletions removing either the two or the three 3'-terminal nucleotides of the ACAC element (plus the U$_{218}$ residue; positions 216–218 or 215–218) severely reduce the formation of 17S rRNA and retard processing of the 32S precursor (Fig. 4A, lanes IIIΔ3 and IIIΔ4). Accumulation of 26S rRNA appears to be unaffected. These results clearly indicate that the evolutionarily highly conserved ACAC sequence forms part of the recognition site for the processing factors operating at A2. The sequence directly upstream from A2 seems to be of lesser importance, since as already mentioned, substitution of A$_{212}$ located two nucleotides upstream of A2 by a C residue has no noticeable effect on processing (Fig. 3, lane Sc^P). Furthermore, as discussed above, the *H.wingei* ITS1, which contains no significant sequence

homology with its *S.cerevisiae* counterpart in the region upstream of the ACAC element (Fig. 2), still supports relatively efficient processing.

That the conserved ACAC element may in fact be sufficient for directing cleavage at A2 is further suggested by the finding that a longer deletion (positions 215−272) which removes almost all of domain III does not have a more severe effect on processing than its smaller IIIΔ4 counterpart (Fig. 4A, *cf.* lane IIIΔ58 to lane IIIΔ4). Moreover, disruption of the second helical segment in domain III by deletion of nt 269−273 does not disturb formation of either 17S or 26S rRNA (Fig. 4A, lane IIIΔ5). Finally, a deletion removing the portion of domain III downstream from position 229, as well as all of domain IV, has been found to cause only a slight delay in processing at A2 (Y. Henry and D. Tollervey, pers. commun.). Since deletion of domain IV alone has no detectable effect on the formation of 17S rRNA (see below), it appears that the downstream two-thirds of domain III is dispensable for cleavage at A2, although it may contain element(s) that increase the efficiency of this processing event.

As shown in Fig. 4A, a small but significant amount of 17S rRNA is still produced from the IIIΔ3, IIIΔ4 and IIIΔ58 mutant rDNA units. In fact, we find that even the previously described ITS1 deletion (positions 112−270; refs. 16, 17) which completely removes A2 still supports the formation of a small amount of 17S rRNA (Fig. 4B). Moreover, yeast strains that were made dependent upon rDNA units containing any of these four deletions turned out to be viable, and thus must produce functional 40S ribosomal subunits, although they grow at a reduced rate (RWvN, unpublished data). Similar results have been obtained by Lindahl and coworkers (L. Lindahl, pers. commun.). Finally, affecting A2 cleavage by inactivation of snR10 causes the appearance of an aberrant 20S-like intermediate (called 21S; see Fig. 1B), having its 3'-end between A2 and B1, which may still give rise to a small amount of mature 17S rRNA (25, 26). Together these data indicate the existence of an alternative — though less efficient — processing route leading to the formation of mature 17S rRNA which remains available when cleavage at A2 is impaired. Strikingly, domain IV harbours a single-stranded stretch of eight highly conserved nucleotides, also containing the sequence ACAC (Fig. 2). Possibly, therefore, the alternative processing route is mediated by this conserved region of domain IV leading to a slightly extended 20S-like intermediate that can still be processed to 17S rRNA. The low efficiency of this alternative route may be due to the sequence context of the ACAC element as compared to that of the normal A2 site (*cf.* the reduction in processing efficiency at A2 in the *H.wingei* ITS1; Fig. 2E). The fact that we do not detect the alternative 20S-like intermediate in the tagged ribosome system is likely to be due to metabolic instability (25). During the preparation of this manuscript we learned of direct experimental support from the Tollervey laboratory for the occurrence of a hitherto undetected cleavage event within the conserved region of domain IV as part of the normal processing pathway leading to 5.8S/26S rRNA (Y. Henry and D. Tollervey, pers. commun.).

Domains IV and V can independently support normal 26S rRNA formation

In order to analyze the role of domains IV and V in formation of the mature rRNA species we first constructed deletion mutant IV-VΔ73 which lacks both these domains (positions 284−357;

Fig. 2A). As shown in Fig. 5 this deletion does not detectably affect the steady state level of tagged 17S rRNA but causes a considerable reduction in the amount of tagged 26S rRNA. No accumulation of 32S rRNA can be observed. From these data we conclude that domains IV and V are dispensable for processing at A2 but play an important role in the production of normal steady state amounts of mature 26S rRNA.

Next, we assessed the role of each of the two domains separately using deletion mutants IVΔ41 (lacking nucleotides 283−324) and VΔ31 (lacking nucleotides 321−353), which were both obtained by insertion of the appropriate oligonucleotide into mutant IV-VΔ73. To our surprise normal steady state levels of tagged 26S rRNA were restored in both cases (Fig. 5). Reverse transcription analysis of the tagged 26S rRNA showed the presence of the correct 5'-end in all mutants (data not shown).

Because domains IV and V do not share any obvious primary or secondary structural elements these data suggest the existence of two independent pathways for processing of the 29S_A precursor into the mature large subunit rRNA species. This conclusion is in agreement with genetic data obtained with yeast strains containing a mutation in either the *RAT1* or the *NME1* gene, that respectively encode a 5'→3' exonuclease (9, 34) and the RNA component of the MRP RNase (7, 8, 30, 31). Inactivation of either of the two gene products or depletion of the latter resulted in a differential effect on the formation of the two forms of 5.8S rRNA normally present in yeast ribosomes (35). While the level of the major form, having its 5'-end at B1_S (Fig. 2A), was significantly reduced, the amount of the minor form ending at B1_L seven nucleotides farther upstream remained virtually the same, suggesting that B1_S and B1_L are the result of independent processing events on the 29S_A pre-rRNA (8). Recent experiments carried out by Tollervey and coworkers provide further support for the existence of such independent pathways (Y. Henry and D. Tollervey, pers. commun.). Unfortunately, the tagged ribosome system used in our experiments does not allow us to assess the nature of the 5.8S rRNA produced by the domain IV and V deletion mutants. Experiments using a different system are now in progress to answer the question whether these two domains indeed mediate independent B1_L and B1_S processing routes.

CONCLUSIONS

The data presented in this paper clearly indicate that the ITS1 spacer of yeast pre-rRNA can be divided into two functionally distinct parts. The 5'-terminal portion, consisting of domains I−III, contains element(s) that are crucial for production of normal levels of mature 17S rRNA, but is largely, if not completely, dispensable for normally efficient 26S rRNA formation. Conversely, the 3'-terminally located domains IV and V play a major role in 26S rRNA formation but their presence is immaterial for 17S rRNA production.

Within the 5'-terminal portion the information necessary and sufficient to direct the processing machinery to site A2 in domain III is likely to be limited to the sequence immediately surrounding this site, with a predominant role for the highly conserved ACAC element directly downstream from A2 (Figs. 3 and 4). The inessentiality of most of domain II, as well as domains IV and V, for efficient 17S rRNA formation is further underscored by the analysis of a final mutant in which deletions IIΔ154 and IV-VΔ73 were combined. This deletion, which removes 227 of the

362 residues of ITS1, has no significant effect of the level of tagged 17S rRNA (Fig. 5, *cf.* lane II−IV-VΔ227 to lanes Sc and IIΔ154). The remainder of domain III also is not essential but may contain element(s) that improve the efficiency of cleavage at A2. Furthermore, the data also indicate that processing at A3, which generates the mature 3'-end of 17S rRNA (Fig. 2A), is directed predominantly by the immediate sequence context of this site.

Either domain IV or domain V of the 3'-terminal portion of ITS1 is necessary and sufficient for the production of normal steady state levels of mature 26S rRNA, providing a further argument in support of the existence of two independent pathways for processing of the $29S_A$ precursor (8).

The functional distinction between the two parts of ITS1 is not an absolute one. A deletion that removes A2 still allows some production of mature 17S rRNA by an alternative processing route, probably involving cleavage within domain IV. Similarly, a deletion removing both domain IV and V does not completely abolish 26S rRNA formation indicating that the region of domain III downstream of A2 contains sufficient information to sustain at least some processing of the $29S_A$ precursor. This functional redundancy, as well as the relative insensitivity of ITS1 function to structural alterations stands in sharp contrast to the situation observed for ITS2, the function of which is subject to much tighter structural constraints (2, 18; RWvN, JV, RJP & HAR, manuscript in preparation).

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