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Processing of precursors of 21S ribosomal RNA from yeast mitochondria

(intron/electron microscopy/R-loops/RNA transfer/hybridization/petite mutants)

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ABSTRACT **The transcription and processing of mitochondrial 21S rRNA in a petite strain of *Saccharomyces cerevisiae* has been examined by electron microscopic analysis of R-loop hybrids and by hybridization of labeled mitochondrial DNA probes to RNA transferred to diazobenzyloxymethyl paper. We have shown the presence of a large [5.1- to 5.4-kilobase (kb)] transcript that appears to be a precursor of mitochondrial 21S rRNA. This transcript contains sequences homologous to those of the mature 21S rRNA, to the intervening sequence present in the gene, and to additional sequences at the 3′ end of the molecule. Our data suggest that this precursor of 21S rRNA is processed in two steps. The intron sequence is usually excised first, followed by removal of the extra 3′ sequences. In some cases, however, the 3′ extension is first removed and the intron sequence is then excised. Both pathways appear to lead to formation of the 3.1-kb mature 21S rRNA and a stable 1.2-kb intron transcript. Similar results were obtained with grande MH41-7B mitochondrial RNA by RNA transfer hybridization. We have also observed a number of additional transcripts that may be normal processing intermediates or may result from faulty cleavage–ligation during excision of the intervening sequence.**

The 70- to 75-kilobase (kb) yeast mitochondrial genome and its products have been analyzed extensively. Detailed genetic (e.g., refs. 1 and 2) and restriction endonuclease maps have been derived (e.g., refs. 3–6), regions of the DNA sequence have been determined (e.g., refs. 7–9), and a number of the transcripts have been characterized and mapped (10–15). Yeast mitochondrial DNA (mtDNA) specifies mitochondrial 14S and 21S rRNA, about 25 tRNAs, and seven to nine polypeptides (for review, see ref. 16). Although these gene products account for at most 20–30% of a single-strand DNA equivalent, more than 60% of the mitochondrial genome is transcribed (17). Analysis of mitochondrial transcripts from grande and petite strains by gel electrophoresis has shown that their aggregate molecular weight exceeds the coding capacity of the genome (11, 12). Similarly, transcript mapping with petite yeast strains indicates that multiple RNA species are specified by individual regions of the mitochondrial genome (11, 13). Therefore, large regions of the genome appear to be transcribed, and the products are then processed into mature RNA species. Furthermore, intervening sequences have been demonstrated in the cytochrome *b* (18, 19) and the 21S rRNA (20–22) cistrons and possibly the *OXI 3* region (unpublished results). Transcripts derived from these regions thus require extensive processing. Characterization of RNA processing pathways would appear to be essential for an understanding of the biogenesis of mitochondria.

In this study, we have focused on the analysis of transcripts derived from the 21S rRNA cistron. This gene contains a 1.2-kb intervening sequence (20–22) as well as the genetic locus ω, which has been localized in or near the intervening sequence (intron). The polarity of recombination of chloramphenicol and erythromycin markers is determined by the ω locus. The intron has no known obligatory function, however, because strains lacking the intron respire normally (15).

We have concentrated on an analysis of the 21S rRNA mitochondrial transcripts in the cytoplasmic petite strain F11, a single-deletion mutant. Cytoplasmic petite mutants delete various segments of their mtDNA. The retained sequences are amplified and usually are arranged as tandem repeats (23, 24). Single-deletion petites may be considered natural clones of grande mtDNA. Although mitochondrial protein synthesis is absent in petites, transcription and apparently normal processing of transcripts occur in most strains (11, 13).

The F11 mitochondrial genome has been extensively characterized both genetically and physically (23, 25). The mtDNA is composed of tandem repeats of sequences located between 89 and 9 units on the grande yeast mitochondrial restriction map established by our laboratory (24). The mitochondrial genome of F11 retains the 21S rRNA gene and also codes for a few tRNAs. The strain produces mature 21S rRNA, but because it retains only 15% of the mitochondrial genome, it lacks many other mitochondrial transcripts. Because of the reduced complexity of its genome, the F11 strain is well suited for the study of transcripts of the 21S rRNA region.

We have previously noted the presence of high-molecular-weight transcripts in petite F11 that were postulated to be precursors of 21S rRNA (11, 13). We have now analyzed these transcripts by electron microscopic analysis of R-loop hybrids and by hybridization to RNA transferred to diazobenzyloxymethyl (DBM) paper, using labeled DNA probes from exon and intron regions of the 21S rRNA cistron. In addition, we have examined transcripts from the grande strain MH41-7B by RNA transfer hybridization. A processing scheme for transcripts of the 21S rRNA cistron is proposed on the basis of the results.

MATERIALS AND METHODS

Yeast (*Saccharomyces cerevisiae*) Strains. A grande strain (ρ^+) MH41-7B (ω^+), and a petite strain, F11, derived from an ω^+ grande strain, were kindly provided by H. Fukuhara and P. Slonimski, respectively (26, 27).

Media and Cultures. For large-scale growth, fresh precultures were prepared from frozen stock in 1% Bacto-peptone/1% yeast extract/1% glucose. Cultures were allowed to grow to mid- or late-logarithmic phase before harvesting.

mtDNA Preparation. Mitochondria were isolated by differential centrifugation from mechanically broken yeast pro-

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Abbreviations: kb, kilobase; mtDNA, mitochondrial DNA; DBM paper, diazobenzyloxymethyl paper.

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toplasts as described by Casey *et al.* (28), and mtDNA was isolated according to Locker *et al.* (23).

Cloning of Intervening Sequence Probe. Restriction endonuclease *Hin*dIII fragments of mtDNA were inserted into the *Hin*dIII site of plasmid pBR322. *Escherichia coli* (strain C600 r^- m^-) was transformed with this DNA, and recombinant plasmids were isolated from the bacteria as described by Berg *et al.* (29). One plasmid with an inserted fragment comigrated on gel electrophoresis with *Hin*dIII fragment 5 (3). This fragment has been shown by Bos *et al.* (20) to be located in the intervening sequence of the 21S rRNA gene. The identity was confirmed by hybridization of complementary RNA prepared from this plasmid to DNA blots (30) of mtDNA cleaved with *Hin*dIII and several other restriction enzymes.

Mitochondrial RNA Preparations. Mitochondria were isolated and purified as described above. Lysis was achieved by suspending of the mitochondrial pellet in 1% Sarkosyl/10 mM Tris·HCl (pH 7.4)/1 mM EDTA, followed by immediate extraction with phenol equilibrated with 10 mM Tris·HCl, pH 7.4 (12). The aqueous phase, which contained the RNA, was placed directly on the gel for electrophoresis (31).

Electrophoresis Gels and Buffer. Nucleic acid samples that were to be transferred to DBM paper were separated by electrophoresis at 100 V for 6 hr on 1.5% agarose/6 M urea gels (31). The gels were stained with ethidium bromide, photographed, and placed in 20 mM sodium phosphate, pH 6.5, buffer for 30 min prior to transfer to the DBM paper.

For the preparative isolation of high molecular weight RNA species for electron microscopy, electrophoresis in 0.6% agarose/6 M urea gels with 40 mM Tris–acetate, pH 8.0/20 mM sodium acetate/1 mM EDTA buffer was used. The gels were run at 100 V for 3–6 hr and stained with ethidium bromide, and the RNA bands were excised under UV illumination. The RNA was extracted and purified as described by Locker (31). A second electrophoresis was performed for further purification when needed.

Electron Microscopy of R-Loops. Hybridizations were performed according to a modification of the procedure described by Thomas *et al.* (32). One hundred microliters of reaction mixture containing 80% (wt/vol) formamide, 0.1 M 1,4-piperazinediethanesulfonic acid (Pipes) buffer at pH 6.7, 10 mM EDTA, 0.3 μg of DNA, and 0.3–0.6 μg of purified RNA was incubated at 35°C for 7–8 hr. The experimentally determined temperature of strand separation for this DNA under these conditions was 37°C.

The resulting hybrids were kept for electron-microscopic analysis for 24–48 hr at 0–4°C. The DNA·RNA hybrids were spread according to a modification of the procedure of Davis *et al.* (33). So that branch migration and melting of the hybrids would be avoided, the 50% formamide hyperphase was kept at 0°C and delivered onto a 23% formamide hypophase maintained at 4°C. The molecules were picked up on a 3.5% parlodion-coated grid and rotary-shadowed. A replica grating was used for calibration.

Transfer of RNA to DBM Paper and Hybridization of DNA Probes. DBM paper was prepared as described by Alwine *et al.* (34) with *m*-nitrobenzyloxymethylpyridinium chloride obtained from BDH Biochemicals (Poole, England). RNA was transferred from the agarose/urea gels by blotting according to modification of the procedure of Alwine *et al.* (34), in which we eliminated the alkaline treatment of the gel and used potassium phosphate buffer, pH 6.5. ^{32}P-Labeled DNA probes were prepared by nick translation with DNA polymerase I as described by Rigby *et al.* (35). Pretreatment of the DBM paper, hybridization in 10% dextran sulfate, and the washing of the filters were done as described by Wahl *et al.* (36), except that the hybridization buffer contained 30% formamide.

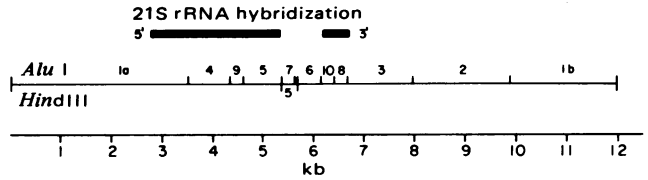

FIG. 1. *Alu* I and *Hin*dIII restriction enzyme maps of F11 mtDNA. The *Alu* I fragments are numbered according to size as they appear in a digest of F11 mtDNA. The *Hin*dIII digest is numbered according to the position of bands in a digest of grande MH41-7B. Both digests are normalized to the single *Xba* I site in F11. The regions to which 21S rRNA fragments hybridize are shown by blocks.

RESULTS

Restriction Enzyme Map of Petite Strain F11 mtDNA. The *Alu* I and *Hin*dIII restriction maps of F11 mtDNA are shown in Fig. 1. The DNA fragments that hybridize with labeled 21S rRNA (unpublished data) are shown by blocks. *Alu* I fragment 4 of F11 was used as a probe for the large exon and the cloned *Hin*dIII fragment 5 of MH41-7B as the probe for intron sequences.

Gel Electrophoresis of Mitochondrial RNA from Grande and Petite F11. Gel electrophoresis in 0.6% agarose/6 M urea of RNA extracted from grande (MH41-7B) and petite F11 mitochondria was carried out as described (12, 31). A prominent 21S band was present in the petite, corresponding in mobility to grande 21S rRNA. Of note are several transcripts having a slower mobility than 21S rRNA in the petite strain. These bands were presumed to be precursors of 21S rRNA (11, 13). A region containing DNA species larger than 21S rRNA was excised from the gels for electron-microscopic examination.

Electron Microscopy. Previous electron-microscopic analysis of the 21S rRNA gene (20–22) has shown the presence of a 1.2-kb intervening sequence located at approximately one-sixth the distance from the 3′ end. When hybrids are formed between 21S rRNA and mtDNA, a complex R-loop structure is observed that consists of three components. Two single-stranded displacement loops corresponding to large and small exons flank a DNA duplex loop corresponding to the intervening sequence (22). A diagrammatic representation is shown in Fig. 2.

When hybrids were made between the purified F11 large molecular weight RNAs and F11 mtDNA, 90% of the observed R-loops had a structure similar to that shown in Fig. 2. One arm of the R-loop in these hybrids always corresponded in size to the 21S 5′ exon, whereas the second arm was variable in length (Fig. 3). Identification of the 5′-3′ orientation of the large hybrids was facilitated by the presence of a characteristic denaturation bubble in the 3′ extension adjacent to the smaller exon. However, a bubble in the same location was not reproducibly observed in DNA duplexes adjacent to smaller R-loop structures. We measured R-loops corresponding in size to the larger (5′) and smaller (3′) exons, as well as a DNA duplex loop of intron size. In total, 135 unambiguous molecules with large R-loops were measured. The 5′ exon, 2.50 ± 0.22 kb (Fig. 4*a*), and

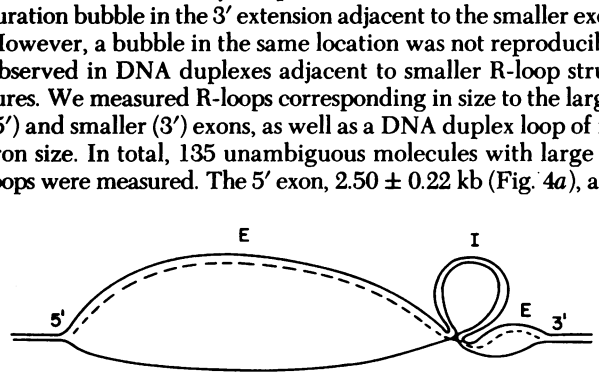

FIG. 2. Diagram of the 21S rRNA R-loop structure (22). DNA strands are designated by solid lines, and the RNA strand by a broken line. The large and small exon loops are designated by E and the intron loop by I. A small denatured region is often observed in the DNA near the small exon loop. The 5′-3′ orientation is based on unpublished data from this laboratory.

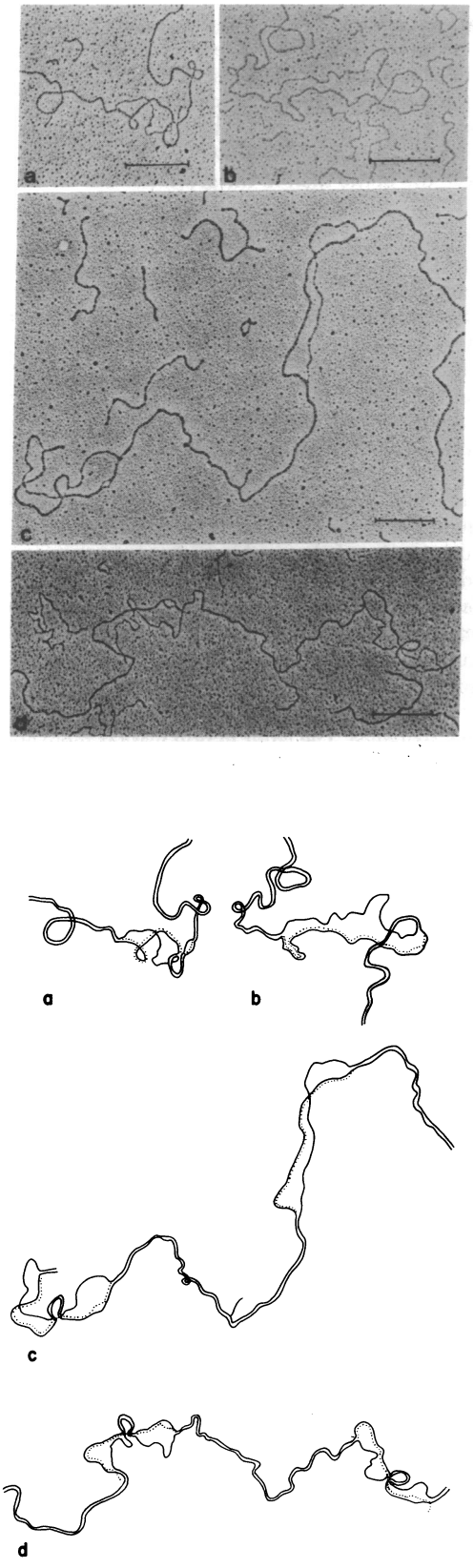

FIG. 3. (*Upper*) Electron micrographs of R-loop structures formed with high molecular weight RNA. (*a*) A 21S rRNA R-loop. (*b*) A single-loop structure. (*c*) An oligomeric molecule containing two R-loop structures; one is a single loop form and the other has an intron loop and an enlarged small exon relative to 21S rRNA. (*d*) An oligomeric molecule containing two R-loop structures, both of which have an enlarged small exon. (*Lower*) Diagrams of R-loop structures shown in *Upper*. DNA strands are represented as solid lines, RNA as dotted lines.

the intron, 1.18 ± 0.05 kb (Fig. 4*b*), fell into single size classes. In contrast, the R-loops from the 3′ end were of several size classes (Fig. 4*c*). In the largest class, the 3′ end R-loop measured 1.77 ± 0.18 kb, considerably larger than the 0.58 ± 0.16 kb size of the smaller exon found in hybrids between 21S rRNA and F11 mtDNA. This molecule therefore appears to have an extra 1.2-kb sequence at the 3′ end of the molecule, adjacent to the small exon sequence. We noted another class of molecules with relatively low frequency, in which the 3′ exon R-loop was of intermediate size—i.e., 1.12 ± 0.13 kb (Fig. 4*c*). This class may be the result of *in vitro* degradation of molecules having the larger 3′ extension, or it may represent a second class having a smaller 3′ extension.

We also observed larger single R-loop configurations in about 10% of the hybrids (Fig. 3). These hybrids presumably are formed by transcripts in which intron sequences have not been excised. The size of these loops was consistently larger than the 2.5-kb 5′ exon R-loop (Fig. 4*d*). Three size classes of single-loop hybrids of 3.7 ± 0.2 kb, 4.4 ± 0.2 kb, and 5.4 ± 0.25 kb were observed. The characteristic denaturation bubble was noted in the largest one-loop hybrids, confirming the relationship between these hybrids and the R-loops described above.

We propose that the largest size class (5.4 kb) represents molecules containing the 1.2-kb 3′ extension as well as exon and intron sequences, and that the 4.4-kb class has had the 3′ extension excised. The 3.7-kb class may represent molecules with 5′ exon and intron sequences, but with the 3′-exon sequences removed.

RNA Transfer Hybridization. DNA segments from within the intervening sequence and within the large exon were used for identification of intermediates in the processing of mitochondrial 21S rRNA. The 0.430-kb cloned *Hin*dIII fragment 5 from MH41-7B from within the intervening sequence was used as the intron probe, and the *Alu* I fragment 4 (0.800 kb) from F11, purified from agarose gels, was used as a large exon probe (Fig. 1). These nick-translated fragments were hybridized to F11 and MH41-7B mitochondrial RNAs bound to DBM paper (Fig. 5). Approximate molecular weights of transcripts were obtained with the 14S and 21S rRNAs used as markers of 1.6 kb and 3.1 kb, respectively (values obtained by electron-microscopic measurement).

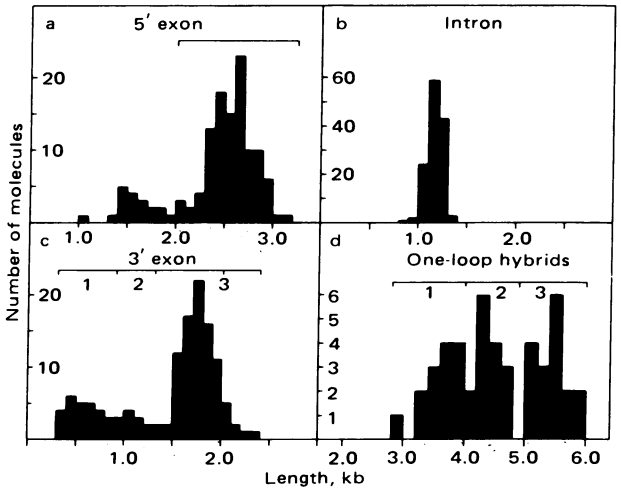

FIG. 4. Length analysis of R-loop regions. (*a*) The large (5′) exon loop measured 2.5 ± 0.22 kb (mean ± SD; n = 106). The distribution was arbitrarily defined (bracket), and smaller molecules were presumed to represent degraded RNA. (*b*) The intron loop measured 1.18 ± 0.05 kb (n = 107). (*c*) The length distribution for the small (3′) exon loop was divided into three regions: 1, 0.58 ± 0.16 kb (n = 27); 2, 1.12 ± 0.13 kb (n = 13); and 3, 1.77 ± 0.18 kb (n = 87). (*d*) The length distribution for single R-loop molecules was divided into three regions: 1, 3.70 ± 0.30 kb (n = 14); 2, 4.43 ± 0.20 kb (n = 15); and 3, 5.43 ± 0.26 kb (n = 17).

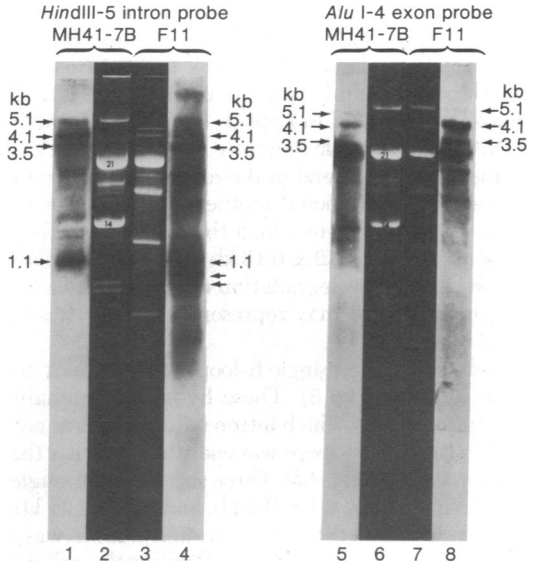

FIG. 5. RNA transfer hybridizations with grande MH41-7B and petite F11 mitochondrial RNA. Total mitochondrial nucleic acids were electrophoresed in 1.5% agarose/6 M urea gels and transferred to DBM paper (34). Lanes 2 and 6 are MH41-7B mitochondrial RNA and lanes 3 and 7 are F11 mitochondrial RNA stained with ethidium bromide. The intron probe, cloned *Hin*dIII fragment 5 of MH41-7B, and the exon probe, *Alu* I fragment 4 of F11, were ^{32}P-labeled by nick translation. Lanes 1 and 4 show hybridization with the intron probe and lanes 5 and 8 with the exon probe. Molecular weight calibrations are based on electron microscopic measurements of 21S and 14S mitochondrial rRNA (marked 21 and 14 in lanes 2 and 6) of 3.1 and 1.6 kb, respectively.

With the intron probe, no hybridization to 21S rRNA was observed, but hybridization occurred to three bands above the 21S rRNA region, estimated at approximately 5.1, 4.1, and 3.5 kb. The largest (5.1 kb) band probably represents 21S rRNA molecules that contain the intron and the 3′ extension. The 4.1-kb band corresponds most closely to the 21S rRNA + intron sequence, whereas the 3.5-kb band corresponds to the large exon + intron sequence. In the grande strain, the 4.1-kb band hybridizes more intensely than the 3.5-kb band; the opposite is true in F11. A transcript of 1.1 kb, which corresponds to the size of the intervening sequence, is observed in both grande and petite mitochondrial RNA. The intron probe also hybridizes to 3.0-kb and 2.7-kb transcripts. With F11 mitochondrial RNA, there is hybridization to two additional bands of 1.0 and 0.9 kb below the intron band. These additional bands in the lower region of the gel may represent the intervening sequence in a second conformational form such as a circle, or they may be due to further processing of this RNA species.

The exon probe hybridizes to the 21S rRNA and to three larger bands of 5.1, 4.2, and 3.5 kb. The 3.5-kb band is more abundant in the petite than in the grande strain.

DISCUSSION

An interpretation of the RNA transfer hybridization and electron-microscopic data is shown in Table 1. The measurements obtained by the two procedures give similar results. The largest rRNA transcript (5.1–5.4 kb) observed was found in low concentration by both techniques. RNA transfer hybridization shows that this transcript contains sequences homologous to those of the exon and intron, whereas electron microscopy demonstrates that it also contains an extra sequence at the 3′ end. The postulated structure of this transcript is shown in Table 1, together with the size derived from electron-microscopic measurements of its individual components (5′ exon, intron, 3′ exon, and extension. It is possible that this transcript is itself processed from a larger, undetected, RNA species.

A second size class of RNA molecules (4.1–4.4 kb), detected by both methods, appears to be composed of two different types of molecules. By electron microscopy, RNA hybrids of 4.4 kb are found that have conserved the intron and lost the 3′-end extension, or that have retained the 3′-end extension but not the intervening sequence. The latter hybrid was far more abundant than the former. Similarly, RNA transfer hybridization shows hybridization of the intron probe to a 4.1-kb RNA band and a much stronger hybridization to an RNA species of similar size (4.2 kb) with the 5′-exon probe. The two types of 4.4-kb RNA species observed by electron microscopy are not resolved by gel electrophoresis.

Proposed structures for these two RNA transcripts are shown in Table 1. The two molecules derive from different orders of processing: one in which the intron is excised as a primary step, and another in which the 3′ end of the molecule is excised first. In the petite strain F11, excision of the intron appears to be the predominant first step, but either order could lead to the production of a mature 3.1-kb 21S rRNA.

Stable intron-sized transcripts are detected by hybridization of the intron-specific probe to RNA transfers. In F11, three species are detected, ranging in size from 0.9 to 1.1 kb, but in the grande strain MH41-7B only a single intron transcript of 1.1 kb is detected.

Table 1. Comparison of transfer hybridization and electron-microscopic data

Transfer hybridization				Electron microscopy of R-loops			
Intron probe		5′ exon probe					
Size, kb	Intensity	Size, kb	Intensity	R-loop structure observed	Length, kb	Relative frequency	Interpretation
5.2	+	5.1	+		5.4 ± 0.3	+	5′ E I E Ext. 3′
—	—	4.2	++		4.3 ± 0.3	++	5′ E E Ext. 3′
4.1	+				4.4 ± 0.2	+	5′ E I E 3′
3.5	++	3.5	++		3.7 ± 0.3	+	5′ E I 3′
—	—	3.1	++++		3.1 ± 0.4	++++	5′ E E 3′
3.0	+	—	—	Not investigated	—	—	5′ I E Ext. 3′
2.7	+	2.8	+	Not investigated	—	—	5′ E 3′
1.1	++	—	—	Not investigated	—	—	5′ I 3′
1.0	++						
0.9	+						

R-Loops are drawn as in Fig. 2. Lengths of R-loops are given as mean ± SD. In the right-hand column: E, exon; I, intron; Ext., extension.

We also observed a 3.7-kb transcript by both electron microscopy and RNA transfer hybridization (Table 1). It contains sequences homologous to both the intron and the 5′-exon probes and has a continuous R-loop structure by electron microscopy. The 3.7-kb size suggests that the structure contains only the 5′-exon and the intron sequences, but hybridization to a 3′-exon-specific probe is necessary to fully characterize this species. Such a species may be an intermediate that occurs during the process of intron excision and splicing, or it may result from misprocessing or alternative processing of larger RNA transcripts at the level of intron excision. If misprocessing does occur, a series of faulty products may be generated. We observed a variety of transcripts of appropriate size by using transfer hybridization (Table 1). Additional species containing the 3′ exon are not detected by our 5′-exon and intron probes. Furthermore, with our R-loop hybridizations to date we have analyzed only RNA larger than 21S rRNA (>3.1 kb). Analysis of smaller transcripts by electron microscopy and analysis of RNA transfers with hybridization probes specific for the 3′ exon and the 3′ extension are not yet complete.

On the basis of these results, we propose the following model for the processing of the 21S rRNA precursor in petite strain F11. The largest rRNA precursor characteristically contains mature 21S rRNA sequences (5′ + 3′ exon), sequences homologous to those of the intron, and an extra RNA sequence at the 3′ end. In the petite, the intron is usually excised first and a 4.4-kb RNA molecule is obtained. This molecule is further cleaved at the 3′ end to generate the 3.1-kb mature 21S rRNA. Less frequently, intron excision and 3′-terminal cleavage occur in reverse order. These observations suggest that the two processing steps (intron excision and 3′-end removal) do not occur in a rigid sequence. Intron excision, however, is carried out at a faster rate; 3′ excision therefore appears to constitute the rate-limiting step for the processing of the 21S rRNA precursors in petite strain F11. The RNA transfer hybridization experiments indicate that similar processing steps occur in the grande.

Several differences between petite and grande hybridizations were observed. The 3.7-kb transcript that presumably represents the 5′-exon plus intron sequences was more prominent in the petite. Furthermore, the 1.0- and 0.9-kb intron sequences were noted only in the petite, whereas the 1.1-kb transcript was present in both. The accumulation of intermediates in the petite may be due to differences in the levels of processing enzymes relative to the concentration of the transcripts.

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