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Defective splicing of mitochondrial rRNA in cytochrome-deficient nuclear mutants of *Neurospora crassa*

(RNA processing/intervening sequence/mitochondrial ribosome/nuclease S1 mapping/Southern hybridization)

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ABSTRACT **Recent studies have shown that the gene encoding the large (25 S) mitochondrial rRNA of *Neurospora crassa* contains an intervening sequence of 2–2.5 kilobases that is not present in the mature 25S mitochondrial rRNA. Earlier studies had provided evidence that mitochondrial rRNAs in *Neurospora* are synthesized via a 32S precursor RNA that contains sequences for both the mature 19S and 25S RNA species. The present work shows that the intervening sequence is not present in 32S RNA. However, we have identified two temperature-sensitive nuclear mutants that fail to excise the intervening sequence at the nonpermissive temperature (37°C). When grown at 37°C, the mutants show decreased ratios of 25S to 19S RNA and accumulate a novel 35S RNA that appears to consist of 25S RNA plus most or all of the intervening sequence. The mutants are allelic but can be distinguished in temperature shift-up experiments, mitochondrial rRNA processing turning off more rapidly in one than in the other. These mutants should provide powerful new tools for studying RNA processing in eukaryotic cells.**

Recent restriction enzyme mapping of *Neurospora crassa* mtDNA suggests that the gene coding for the large (25 S) rRNA contains an intervening sequence of 2–2.5 kilobases (kb) (ref. 1 and ¶; J. Heckman and U. RajBhandary, personal communication). The current map (Fig. 1) differs somewhat from that originally presented by Küntzel.¶ The revisions are based on new data of Küntzel and coworkers (1), with most features independently confirmed by J. Heckman and U. RajBhandary (personal communication) and supported further by the data presented in this paper. The experimental evidence on which the map is based is summarized in the legend of Fig. 1. Although the original version of the map suggested that the intron contains tRNA genes (3, ¶), in the revised map most of the genes in question appear to be located outside of the intron and adjacent to the 25S RNA gene on both sides (hybridization data of ref. 4; J. Heckman and U. RajBhandary, personal communication).

Studies by Kuriyama and Luck (5) suggested that mitochondrial rRNAs in *Neurospora* are synthesized via a 32S precursor RNA that contains sequences for both 19S and 25S RNA and about 20% nonribosomal sequences. The 32S RNA appeared to be cleaved to intermediate species (P19 S and P25 S) that are then processed to give the mature rRNA species (5). The map of Fig. 1, in which the 19S and 25S RNA genes are separated by 5–6 kb, suggests that 32S RNA is not a continuous transcript of 19S and 25S RNA as proposed by Kuriyama and Luck (5). The possibility that 32S RNA is a spliced transcript is discussed below (see *Discussion*).

In the course of studying mutants with defects in mitochondrial ribosome assembly, we identified two temperature-sensitive nuclear mutants that, at the nonpermissive temperature, accumulate a RNA larger than 32S RNA. In the present work, we demonstrate that this large RNA contains 25S RNA plus the intron (corresponding to *Hin*dIII-13 and -18; Fig. 1). The two mutations appear to be allelic but can be distinguished by their response in temperature shift-up experiments, mitochondrial rRNA processing turning off within 30 min of the shift-up in one mutant and more slowly in the other. These mutants should provide powerful new tools for studying RNA biosynthesis in eukaryotic cells.

MATERIALS AND METHODS

Materials. The wild-type strain was Em 5256A (FGSC 626). Mutant strains were 289-67 and 299-9, isolated by Thad Pittenger (Kansas State University) (6). Procedures for maintaining strains and preparing conidia were as described by Luck (7). Enzymes used in the study were: protease (type VI, Sigma), pancreatic RNase A (type XII-A, Sigma), nuclease S1 [prepared by the procedure of Wold *et al.* (8)], *Hin*dIII and *Bam*HI (Miles and Bethesda Research Laboratories, Rockville, MD), and *Eco*RI (prepared as in ref. 9).

Growth of Mycelia and Purification of Mitochondria. Growth of mycelia, *in vivo* labeling with [5-^{3}H]uridine and [^{32}P]phosphoric acid, and isolation of mitochondria were carried out as described (10–13). At 25°C, wild-type and mutant cells were harvested after 14 hr of growth. At 37°C, wild-type cells were harvested at 10 hr and mutant cells, at 24 hr.

Isolation of mtDNA and mtRNA. Mitochondrial nucleic acids were isolated from nucleoprotein pellets prepared in Ca^{2+}-containing buffers to suppress nuclease activity (13). mtDNA was extracted from such pellets by using phenol, RNase, and protease treatments as described (14). For mtRNA isolation, mitochondrial nucleoprotein pellets were washed with cold distilled H_2O and suspended in 2 ml of 150 mM NaCl/1 mM EDTA/100 mM Tris·HCl, pH 8.0 (NET buffer) containing 1% sodium dodecyl sulfate ($NaDodSO_4$) (room temperature). The samples were then incubated with 5 units of protease (20 min, 37°C; the protease was preincubated for 60 min at 37°C in 80 μl of NET per unit). In procedure *a*, the treated samples were centrifuged directly through linear gradients of 6–23% sucrose in 1% $NaDodSO_4$/25 mM Tris·HCl, pH 7.5/1 mM EDTA (SW 41 rotor, 40,000 rpm, 4 hr, 20°C). In procedure *b*, the treated pellets (above) were extracted with an equal volume of NET-saturated redistilled phenol. The phenol-extracted

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Abbreviations: kb, kilobase(s); $NaDodSO_4$, sodium dodecyl sulfate.

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¶ Küntzel, H. (1978) in *Neurospora Newsletter*, ed. Marzluf, G. A. (Ohio State Univ., Columbus, OH), Vol. 25, pp. 6–7.

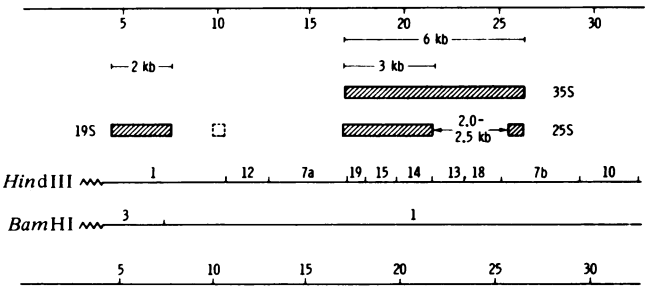

FIG. 1. Restriction enzyme map of *Neurospora* mitochondrial rRNA genes, based primarily on the data of Küntzel and coworkers (1, ¶) with most features independently confirmed by analysis of cloned restriction enzyme fragments (J. Heckman and U. RajBhandary, personal communication). The map positions corresponding to rRNA are based on: (*i*) the hybridization data of Terpstra *et al.* (2) showing that 19S RNA hybridizes to *Hin*dIII-1 and *Bam*HI-3 with slight overlap (less than 0.5 kb) into *Bam*HI-1 and that 25S RNA hybridizes to *Bam*HI-1 and to *Hin*dIII-7, -14, -15, and -19; (*ii*) R-loop mapping experiments (1); (*iii*) the nuclease S1 mapping experiments and size estimates in the present work; and (*iv*) hybridization of gel-purified 19S and 25S RNA to cloned restriction enzyme fragments (J. Heckman and U. RajBhandary, personal communication). Terpstra *et al.* (2) had also reported that 25S RNA hybridizes to *Hin*dIII-1 (500 nucleotide segment indicated by broken lines). However, the R-loop experiments of Küntzel and the hybridization experiments of Heckman and RajBhandary both show that the 0.5-kb segment of 25S RNA is in *Hin*dIII-7b and not in *Hin*dIII-1. The boundaries shown for various RNA species should be considered best estimates and not exact map locations. The scales at the top and bottom indicate map positions according to the convention of Küntzel and coworkers (1, ¶).

RNA was then fractionated by sucrose gradient centrifugation as in procedure *a* or by gel electrophoresis on composite agarose/acrylamide gels (13, 15). Purified RNAs were eluted from the gels by the procedure of Kourilsky *et al.* (16). Routine analysis of mtRNAs was carried out on composite agarose/acrylamide gels (13, 15). Gels were stained with ethidium bromide (4 μg/ml) and photographed under short-wavelength UV lig' t. Scintillation counting of gel slices was carried out as described (13).

Hybridization. Strip filter hybridization was carried out by the method of Southern (17). Restriction enzyme fragments (4–6 μg per slot) were separated by electrophoresis on 0.8% agarose slab gels (21 cm long, 0.3 cm thick) as described (14). Unless otherwise indicated, mtRNAs for strip filter hybridization were extracted by using procedure *b* and purified by gel electrophoresis. Hybridizations were carried out in a 3.5-ml plexiglass chamber at 40°C for 18–20 hr. Ordinarily, 5–10 μg of labeled RNA (2–5 × 10^4 cpm/μg) was used per reaction mixture containing 40% formamide, 0.3 M NaCl, 30 mM sodium citrate, and 50 μg of carrier (phage Qβ RNA or *Escherichia coli* tRNA). For competition–hybridization experiments, 0.5–1 μg of labeled RNA was added in the presence of a 20-fold excess of competitor RNA with carrier tRNA added to give a total of 100 μg of unlabeled RNA per reaction. Strips were autoradiographed (1–2 weeks) with RP X-Omat x-ray film (Kodak) with Cronex Lightning Plus intensifying screens (Du Pont).

Analysis of RNA Denatured with Glyoxal and Nuclease S1 Mapping. Analysis of glyoxal-denatured RNAs was carried out as described by McMaster and Carmichael (18). Electrophoresis was carried out in 1.4% agarose slab gels containing 5 mM sodium acetate, 40 mM Tris·HCl (pH 7.7), and 1 mM EDTA. Nuclease S1 mapping was essentially as described by Berk and Sharp (19). Hybridization reactions were carried out for 3 hr at 50°C in a final volume of 20 μl containing 50,000–100,000 cpm of ^{32}P-labeled mtDNA (2–5 × 10^4 cpm/μg) and 0.2–0.8 μg of RNA. Neutral gel electrophoresis was on 1.4% agarose gels (as above). Alkaline gel electrophoresis was as described by McDonell *et al.* (20).

RESULTS

Mutants 289-67 and 299-9 were isolated as part of a group of temperature-sensitive mutants that grow rapidly at 25 and slowly at 37°C (6). Mutants defective in mitochondrial function were identified by screening all temperature-sensitive mutants for cyanide-resistant respiration and cytochrome deficiency (6). All mutants deficient in both cytochromes *b* and aa_3 are potentially defective in mitochondrial protein synthesis and were screened by us for defects in mitochondrial ribosome assembly. Of nine mutants examined, six were found to have defects in the assembly of one or the other mitochondrial ribosomal subunit. Genetic experiments carried out in our laboratory (data to be published elsewhere) showed that mutants 289-67 and 299-9 are closely linked on chromosome I, mapping approximately 10 map units from *a1-2* and within 5 map units of *nic-1*. In pairwise crosses, we found no wild-type recombinants among 300 random ascospores. Pittenger and West (6) have provided evidence that both mutations are recessive to the wild-type alleles but fail to complement each other in (unforced) heterokaryons. It seems likely, therefore, that the two mutations are allelic.

Fig. 2 shows gel electrophoretic analysis of mtRNAs from wild-type and mutant strains grown at 25 and 37°C, the permissive and nonpermissive temperatures. At 25°C, all three strains showed major bands corresponding to the 32S, 25S, and 19S RNAs. At 37°C, the wild-type profile was unchanged but the mutants showed: (*i*) a striking accumulation of an apparently larger RNA species designated 35S RNA (based on electrophoretic mobility relative to the other mtRNA species), (*ii*) decreased levels of 32S RNA, and (*iii*) decreased ratios of 25S to 19S RNAs, visible in the gel of Fig. 2 but more pronounced in other experiments with cells grown at slightly higher temperatures (39°C). Both the wild-type and mutant gel profiles showed light bands for RNAs migrating more slowly than the 32S and 35S RNA species.

The structure of the novel 35S RNA from the mutants was compared with that of 32S RNA by Southern hybridizations of the ^{32}P-labeled RNA species to mtDNA *Hin*dIII restriction fragments (Fig. 3). For the purpose of these experiments, we adopt the conservative view that 32S RNA is a hybridization probe containing the sequences of both 19S and 25S RNA (data from ref. 5). No further assumptions are required. The two sets of hybridizations (strips 1–3 and 4–6) were for mutants 289-67 and 299-9, respectively. For both strains, 32S RNA showed hybridization to *Hin*dIII fragments 1, 7, 14, and 15 (strips 1 and 4), as expected from the map in Fig. 1. Strips 2 and 5 show that 35S RNA from both mutants hybridized to these same fragments (however, hybridization to *Hin*dIII-1 was weak; see below) and also to *Hin*dIII-13 corresponding to the 25S RNA

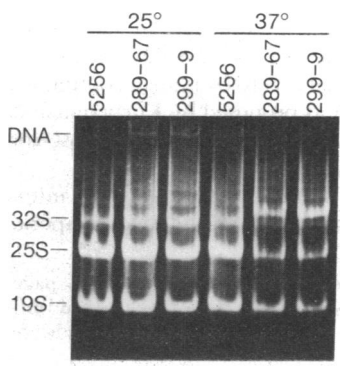

FIG. 2. Gel electrophoretic analysis of mtRNA from wild-type Em 5256A and mutants 289-67 and 299-9. MtRNA was extracted by procedure *b* and analyzed on composite agarose/acrylamide gels. The direction of electrophoresis was from top to bottom.

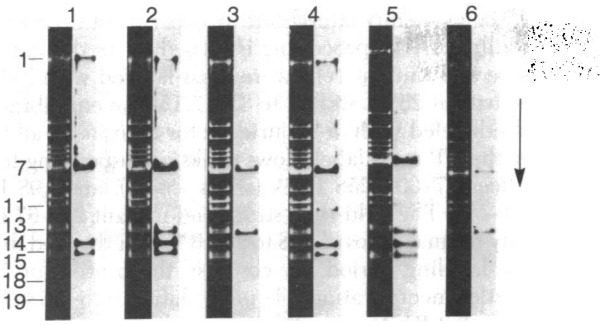

FIG. 3. Strip filter hybridization of ^{32}P-labeled RNAs to *Neurospora* mtDNA *Hin*dIII fragments. Strips: 1, 289-67 32S RNA; 2, 289-67 35S RNA; 3, 289-67 35S RNA in the presence of excess unlabeled 32S RNA; 4, 299-9 32S RNA; 5, 299-9 35S RNA; 6, 299-9 35S RNA in the presence of excess unlabeled 32S RNA. The 32S and 35S RNAs were obtained from mutants grown at 25°C and 37°C, respectively. MtRNA was extracted by procedure *b* and individual species were purified by gel electrophoresis. Arrow indicates direction of electrophoresis.

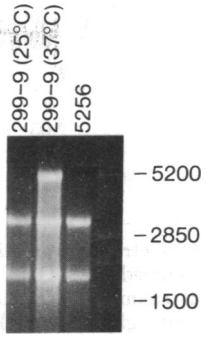

FIG. 5. Gel electrophoretic analysis of mitochondrial rRNAs after denaturation with glyoxal. Procedure *b* was used to isolate mtRNAs from wild-type and mutant strains grown at 25 and 37°C. Glyoxalation reactions and gel analysis were carried out as described by McMaster and Carmichael (18). Lane at far right shows adenovirus 2 *Sma* I DNA fragments used as molecular weight standards. The lengths of fragments *Sma* I-3, -5, and -8 are indicated (ref. 19 and unpublished maps of adenovirus 2 DNA). Direction of electrophoresis is from top to bottom.

intron. Strips 3 and 6 show competition experiments in which ^{32}P-labeled 35S RNA was hybridized to the *Hin*dIII fragments in the presence of excess unlabeled 32S RNA. Only *Hin*dIII-7 and -13, corresponding to the intervening sequence, showed hybridization. The results show that 35S RNA contains most or all of the intervening sequence and that this sequence is absent in the 32S RNA probe.

Two small fragments (*Hin*dIII-18 and -19; 650 and 450 base pairs, respectively) mapped to the region of the intron and the 25S rRNA, respectively (Fig. 1). Small fragments are poorly transferred by the Southern procedure (17) and hybridization to these fragments does not show up in the photograph. In the autoradiogram, however, the strips for both 32S and 35S RNAs show "light" hybridization to *Hin*dIII-19 and the strips for 35S RNA show, in addition, "light" hybridization to *Hin*dIII-18. In the competition experiment for 299-9 (strip 6), all of the hybridizations are "light" so we cannot make a definite statement about the presence or absence of *Hin*dIII-18. In the competition experiment for 289-67, however, hybridization to *Hin*dIII-18 is visible in the autoradiogram, consistent with the map position of *Hin*dIII-18 in the intron.

To define further the structure of 35S RNA, we carried out Southern hybridizations to the separated *Bam*HI fragments (Fig. 4). Strips 1 and 2 corresponding to 32S and 35S RNA, respectively, show hybridization to *Bam*HI-1 and -3. However, competition experiments (strips 3 and 4) show that 35S RNA almost completely outcompeted ^{32}P-labeled 32S or 19S RNA from *Bam*HI-1 but not from *Bam*HI-3. These results suggest that 35S RNA contains all sequences present in 25S RNA (*Bam*HI-1) but is missing sequences present in 19S RNA (*Bam*HI-3). The competition results leave open the possibility that 35S RNA includes the small segment of 19S RNA present in *Bam*HI-1 (see legend, Fig. 1).

Considered together, the hybridization experiments lead to the conclusion that 35S RNA consists of 25S RNA plus the intervening sequence corresponding to *Hin*dIII-13, -18 and -7b. The working model is shown in Fig. 1. In constructing the model, we attached greater weight to the competition experiments which are less vulnerable to contamination of the RNA probes than are direct hybridizations. The hybridization of 35S RNA to *Hin*dIII-1 and *Bam*HI-3 has not been indicated because, at this point, it could merely reflect contamination of the 35S RNA preparations by 32S RNA which migrates closely in the gels (see Fig. 2). The alternative possibility is that the weak hybridization of 35S RNA to *Hin*dIII-1 is real and reflects an additional splicing event involving the excision of *Hin*dIII-7a and -12. The second possibility could account for formation of the 32S RNA.

The exact boundaries of the RNA molecules in the model should be considered tentative, in particular the position of 25S RNA sequences in *Hin*dIII-7a and -7b. In addition, it is possible that precursor RNAs contain nonribosomal sequences at their 5′ or 3′ ends which could bear on the interpretation of the hybridization data. Both 32S and 35S RNAs show strong hybridization to *Hin*dIII-7 (Fig. 3). This hybridization could include a contribution from nonribosomal sequences in *Hin*dIII-7a or

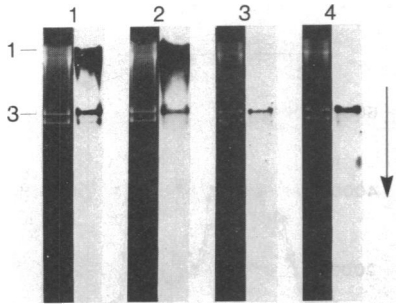

FIG. 4. Strip filter hybridization of ^{32}P-labeled RNAs from 299-9 to *Neurospora* mtDNA *Bam*HI fragments. Strips: 1, 32S RNA; 2, 35S RNA; 3, 32S RNA in the presence of excess unlabeled 35S RNA; 4, 19S RNA in the presence of excess unlabeled 35S RNA. Highly purified 19S RNA was obtained from 299-9, grown at 25°C, by phenol extraction of sucrose gradient-purified mitochondrial small subunits. Other procedures were as in Fig. 3. Arrow indicates the direction of electrophoresis.

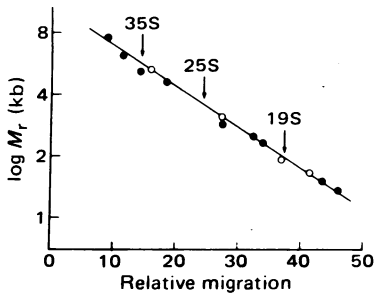

FIG. 6. Semilogarithmic plot of molecular weight versus electrophoretic mobility of glyoxal-denatured RNAs and DNAs. ●, Adenovirus 2 DNA *Sma* I fragments (ref. 19 and unpublished maps of adenovirus 2 DNA); ○, *E. coli* 16S and 23S RNA and human 18S and 28S RNA (18, 21–23). Arrows indicate the electrophoretic mobilities of 19S, 25S, and 35S mtRNAs.

Table 1. Length of RNA determined by three methods

RNA	Length, kb		
	Method 1	Method 2	Method 3
19S	1.9	2.05	2.05
25S	3.6	3.4	3.05
		(2.9)	
35S	5.7	5.5	5.5

The methods were: 1, gel electrophoresis of RNAs denatured with glyoxal; 2, nuclease S1-trimmed RNA·DNA hybrids analyzed on neutral gels; 3, nuclease S1-trimmed RNA·DNA hybrids analyzed on alkaline gels. Methods 1 and 2 give the length of the RNA species. Method 3 gives the length of continuous transcripts (19). The 19S and 35S RNAs are substantially continuous transcripts whereas the 25S RNA is spliced, the longest continuous transcript being about 3000 nucleotides. Method 2 also showed a 2900-nucleotide fragment (indicated in parentheses) which is presumed to be due to the "clipping phenomenon" (i.e., partial S1 digestion at the splice point) described by Berk and Sharp (19) and thus provides additional evidence for the splice in 25S RNA. Molecular weights for method 1 were interpolated from the calibration curve of Fig. 6; those for methods 2 and 3 were determined from similar curves with adenovirus 2 DNA restriction fragments as standards.

-7b or both in addition to the 0.5 kb segment of 25S RNA in *Hin*dIII-7b (see Fig. 1). The same point is pertinent to the interpretation of the competition hybridization experiments. In particular, the data of Fig. 3 show that 35S RNA contains sequences in *Hin*dIII-7 that are not present in the 32S RNA. Although it seems likely that the additional sequences include a segment of the intron in *Hin*dIII-7b, we cannot exclude the possibility that they also include segments at the ends of the 35S RNA molecule (i.e., in *Hin*dIII-7a or -7b).

Length estimates of the different RNA species were obtained by gel electrophoresis of the RNAs after denaturation with glyoxal (Figs. 5 and 6). Table 1 compares these length estimates with the lengths of continuous transcripts determined by the nuclease S1 mapping procedure of Berk and Sharp (19). The data show that 19S RNA is a continuous 2-kb transcript whereas the 25S RNA is spliced, the longest continuous transcript being 3 kb of the total 3.5 kb. The 32S RNA could not be detected in glyoxal-denatured RNA preparations from either wild-type or mutant cells grown at 25°C (Fig. 5), indicating that 32S RNA is not a continuous polynucleotide chain, a possibility specifically left open in the study of Kuriyama and Luck (5). For 35S RNA, the data suggest a continuous 5.5-kb transcript, in good agreement with the value expected from the hybridization experiments (5.5–6 kb) and making use of the known molecular weights of the *Hin*d III fragments. Considered together, the data provide strong supporting evidence for the model in Fig. 1.

Fig. 7 shows a temperature shift-up experiment to determine how rapidly mtRNA processing is turned off in the mutants. Wild-type and mutant cells were mass-labeled with [^{32}P]orthophosphate at 25°C, shifted to 37°C (15-min equilibration), and pulse-labeled with [5-^{3}H]uridine for 30 min. In all three profiles, the ^{32}P mass-label shows peaks corresponding to 32S RNA (slices 27–30), 25S RNA (slices 38–39), and 19S RNA (slices 51–52). The wild-type strain and mutant 289-67 show essentially normal ratios of 19S to 25S RNA synthesized during the pulse-labeling period. By contrast, the profile for 299-9 shows little incorporation of pulse label into 25S RNA, suggesting that RNA processing is turned off rapidly after the temperature shift-up. In other experiments with a 30-min 37°C equilibration period, we were able to demonstrate that 35S RNA begins to accumulate in 299-9 before it does in 289-67 (data not shown). Thus, although the two mutants appear to be allelic, their phenotypes can be distinguished by the more rapid turn-off of RNA processing in 299-9 than in 289-67.

DISCUSSION

The present report describes two nuclear mutants of *Neurospora* that have a temperature-sensitive defect in the processing of mitochondrial rRNA. Nuclease S1 mapping experiments confirm the essential features of the model of Küntzel and colleagues (1, ¶), showing in particular that the 19S rRNA gene is continuous and that the 25S RNA gene contains a 2- to 2.5-kb intron located ≈0.5 kb from one end. Our results show that the intron is not present in 32S RNA but that excision is defective in mutants 289-67 and 299-9 grown at the nonpermissive temperature (37°C). The mutants grown at 37°C show decreased ratios of 25S to 19S RNA and accumulate a novel 35S RNA species that appears to be a continuous transcript of the 25S RNA gene including the intervening sequence. At this point, it is not known whether 35S RNA is part of the normal processing pathway or an abnormal product found only in the mutants. However, the results are consistent with those from other experimental systems, showing that genes containing intervening sequences are transcribed continuously as long precursor RNAs from which the intervening sequences are removed by splicing events (24–28). Recent studies show that 35S RNA is associated with almost the full complement of large subunit proteins (unpublished data).

At present, nothing is known about the location of promotor sites for the mitochondrial rRNAs or the nature of the primary transcript(s). The concept of Kuriyama and Luck (5) that the 32S RNA is a tandem transcript of 19S and 25S RNAs is clearly incorrect because, according to the map of Fig. 1, the genes encoding 19S and 25S RNAs are separated by at least 5 kb. In addition, the glyoxal denaturation experiments (Fig. 5) show

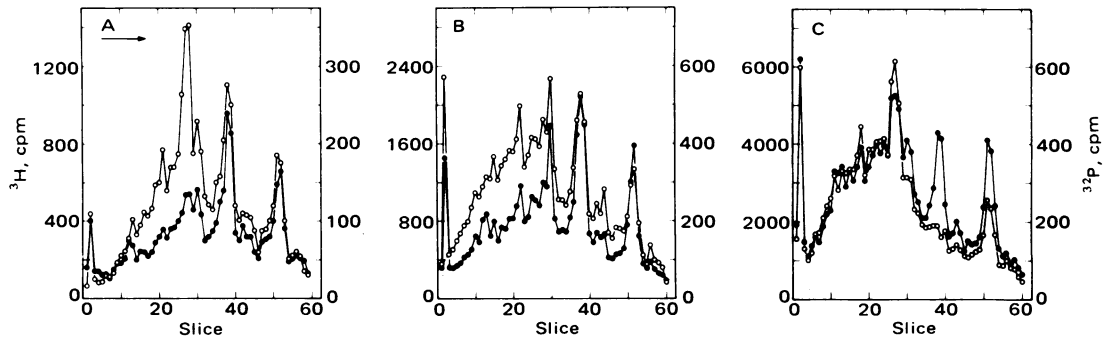

FIG. 7. MtRNA synthesis in wild-type and mutant strains after a shift in growth temperature from 25 to 37°C. Cells were grown at 25°C for 14 hr in the presence of [^{32}P]orthophosphate, transferred to fresh medium at 37°C, equilibrated for 15 min, and pulse-labeled with [5-^{3}H]uridine for 30 min. MtRNAs were extracted by procedure *b* and analyzed on composite agarose/acrylamide gels. The three strains were run in parallel in the same experiments and the experiment was carried out three times with the same results. ○, ^{3}H radioactivity; ●, ^{32}P radioactivity. Arrow, direction of electrophoresis. (*A*) Wild-type, 5256; (*B*) mutant 289-67; (*C*) mutant 299-9.

that 32S RNA is not a continuous polynucleotide chain. However, reexamination of the data of Kuriyama and Luck (5), even in the most critical light, makes it seem clear that: (*i*) RNA molecules with electrophoretic mobility equivalent to 32S are present in gels under nondenaturing conditions; (*ii*) these molecules contain roughly equal concentrations of 19S and 25S RNA sequences; and (*iii*) at least some are precursors as judged by pulse and pulse-chase experiments. In addition, mitochondrial ribosomal precursor particles that appear to consist of 32S RNA associated with newly synthesized small and large subunit proteins have been identified on sucrose gradients (29). The apparent association of 32S RNA with pulse-labeled proteins argues against the possibility that 32S RNA is merely a nonspecific aggregate of mature 19S and 25S RNAs. There are three other possibilities: (*i*) that there are separate 32S precursors for 19S and 25S RNAs, (*ii*) that 32S RNA is a specific complex of 19S and 25S RNA precursors that must form during assembly, and (*iii*) that 32S RNA is produced via a second splicing event that would involve excision of an intron consisting of *Hin*dIII-7a and -12 (Fig. 1).

Recently, Bos *et al.* (30) showed that the yeast mtDNA gene coding for the large (21 S) mitochondrial rRNA contains a 1100-nucleotide intron located 400–500 nucleotides from one end of the gene. In the case of yeast, however, the intervening sequence is found only in some yeast strains and appears to be correlated with the ω^+ allele of the ω locus associated with polar recombination events (30).

The only previously described RNA splicing mutant is the yeast strain ts136 (31). When grown at the nonpermissive temperature, this mutant accumulates several nuclear precursor tRNAs that contain intervening sequences of 10–20 nucleotides (25, 26). In addition, the synthesis of mRNAs appears to be affected in ts136 and the mutant may also accumulate rRNA precursors (31, 32). These findings support the suggestion made by Hutchison *et al.* (31) that ts136 is primarily defective in RNA transport from nucleus to cytoplasm, possibly related to a defect in RNA processing (splicing) (25, 26, 32). With respect to *Neurospora*, we were surprised to find two mutants defective in mitochondrial rRNA splicing in the present rather small collection of temperature-sensitive mutants. Although the sample size is admittedly limited, it is possible that high temperature sensitivity is actually a selection for such processing mutants.

Many of the enzymes of mitochondrial nucleic acid metabolism are known to be encoded by nuclear genes (33) so it is not surprising to find that nuclear gene mutations affect mitochondrial rRNA splicing. Of course, the full range of splicing defects in the mutants—whether other mitochondrial genes or nuclear genes are also affected—remains to be determined. Although the two mutants appear to be allelic (see also ref. 6), they can be distinguished by their response in temperature shift-up experiments, mitochondrial rRNA processing turning off more rapidly in 299-9 than in 289-67. These results suggest that the mutations affect the same component but confer different inactivation kinetics at the nonpermissive temperature. Because the mutations are nuclear, the primary defect probably involves either the RNA splicing enzyme itself or some component that binds to or modifies the mitochondrial rRNAs for splicing to occur. The most obvious candidates in the second class are the mitochondrial ribosomal proteins. However, we could find no alteration in mitochondrial ribosomal proteins from the mutants as judged by comigration of mutant and wild-type proteins on two-dimensional gels (unpublished data). The mutants provide attractive possibilities for future experiments; in particular, the 35S RNA can be obtained in large quantities for structural studies and for use as a substrate in the isolation of the mitochondrial rRNA splicing enzymes.

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