Ribonucleic Acid Splicing in *Neurospora* Mitochondria: Secondary Structure of the 35S Ribosomal Precursor Ribonucleic Acid Investigated by Digestion with Ribonuclease III and by Electron Microscopy†

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ABSTRACT: In *Neurospora*, the gene encoding the mitochondrial large (25S) ribosomal ribonucleic acid (rRNA) contains an intervening sequence of approximately 2.3 kilobases (kb). We have identified two temperature-sensitive mutants (289-67 and 299-9) which are defective in a factor encoded by a nuclear gene but required for the splicing of 25S RNA. When grown at the nonpermissive temperature (37 °C), the mutants accumulate a novel 35S RNA (5.2–5.6 kb) which is related to the natural precursor of 25S RNA and which has been shown to be a collinear transcript of the 25S RNA gene including the intervening sequence. In the present work, the secondary structure of 35S RNA was investigated by digestion with ribonuclease III and by electron microscopy of the RNA spread under partially denaturing conditions. Ribonuclease III cleaves 35S RNA predominantly at a central site or sites near the 5′-intron–exon boundary and produces fragments which correspond roughly to half-molecules (2.5–3 kb). Electron microscopy of 35S RNA shows a relatively large, central hairpin (180 ± 45 nucleotides), which presumably corresponds to the central ribonuclease III site, and few other secondary structure features. Both experimental approaches indicate that the large hairpin is not present in 35S RNA. From this finding and from the location of the hairpin near the 5′-intron–exon boundary in 35S RNA, we infer that its formation requires intron sequences. 35S RNA from the mutants can be isolated as a ribonucleoprotein particle associated with almost the full complement of large subunit ribosomal proteins. The 35S RNA in such particles can be cleaved by ribonuclease III at the central site(s), consistent with the idea that the central hairpin is accessible to RNA-processing enzymes in vivo.

Fungal mitochondrial deoxyribonucleic acids (mtDNAs)[1] have been found to contain intervening sequences apparently analogous to those found in eukaryotic chromosomal and viral DNAs (Abelson, 1979; Crick, 1979). In *Neurospora*, the gene encoding the mitochondrial large (25S) ribosomal ribonucleic acid (rRNA) contains an intervening sequence (2.2–2.3 kb) located near the 3′ terminus of the RNA (Figure 1) (Hahn et al., 1979; Mannella et al., 1979a; Heckman & RajBhandary, 1979; de Vries et al., 1979). Studies in our laboratory have shown that the 25S RNA is synthesized via a 35S precursor RNA (5.2–5.6 kb), which is a collinear transcript of the 25S RNA gene including the intervening sequence (Figure 1) (Mannella et al., 1979a; Green et al., 1981). We have also identified two temperature-sensitive mutants [289-67 and 299-9; isolated by Pittenger & West (1979)] which are defective in a factor encoded by a nuclear gene, but required for the splicing of 25S RNA (Mannella et al., 1979a). When the mutants are grown at the nonpermissive temperature (37 °C), the ratio of 25S to 19S RNA is decreased, and the 35S precursor RNA accumulates (Mannella et al., 1979a). The mutants and the large quantities of 35S RNA that can be obtained from them provide unique opportunities to study several aspects of RNA splicing.

At present, there is little information about those structural features of precursor RNAs which are either recognized by the RNA splicing enzymes or play a role in bringing opposite intron–exon boundaries into proximity. Different hypotheses have placed emphasis on RNA primary structure (guide RNA hypothesis) or on RNA secondary and tertiary structure (Abelson, 1979). Thus far, however, direct studies of higher order structure have been possible only for yeast transfer RNA (tRNA) precursors which accumulate in mutant strains and which may be a special case since they contain relatively small introns [14–60 base pairs (bp)] (Knapp et al., 1978; O'Farrell et al., 1978). rRNA and messenger RNA (mRNA) precursors are generally present in low concentrations, and information about their secondary structure has been limited to surveys of base-pairing possibilities inferred from the nucleotide sequences of the corresponding genes [see Abelson (1979)]. Thus far, such studies have provided little insight into secondary structure features which may be relevant to RNA splicing. In addition, studies with *Escherichia coli* rRNAs demonstrate that nucleotide sequence information must be supplemented by direct experimental approaches to identify which of the potential secondary structure features actually occurs in the RNA (Cantor, 1980; Noller, 1980).

In the present work, we have taken advantage of the large quantities of 35S precursor RNA available from the *Neurospora* mutants to directly investigate large secondary structure features by digestion with ribonuclease III and by electron microscopy of the RNA spread under partially denaturing conditions. The most prominent secondary structure feature detected by both approaches is a relatively large hairpin (180 ± 45 nucleotides) located at or near the 5′-intron–exon boundary. This hairpin is not present in 25S RNA, and we infer, therfore, that its formation requires intron sequences.

Materials and Methods

Materials. The wild-type strain was 74OR-23-1*A* (abbreviated 74*A*). The mutant strain, 299-9, was isolated by Pittenger & West (1979). The isolate used in the present study was 299-9 RAC *al-2 a* which has the 74*A*/*a* nuclear back-

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[1] Abbreviations used: DBM, diazobenzyloxymethyl; EDTA, ethylenediaminetetraacetic acid; kb, kilobases or kilobase pairs; mtDNA, mitochondrial deoxyribonucleic acid; $NaDodSO_4$, sodium dodecyl sulfate.

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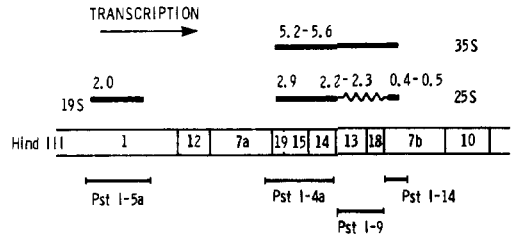

FIGURE 1: Physical map of the rRNA genes of *Neurospora* mtDNA. The *Hin*dIII map is that described by Hahn et al. (1979), Heckman & RajBhandary (1979), and de Vries et al. (1979). Map locations for the *Pst*I fragments were determined by Heckman & RajBhandary (1979). The processing pathway for 25S RNA is as described by Mannella et al. (1979a) and Green et al. (1981). Numbers above RNA species indicate size estimates in kb (Mannella et al., 1979a; Green et al., 1981).

ground and mtDNA of type II according to the nomenclature of Mannella et al. (1979b). Procedures for maintaining strains and preparing conidia were as described by Luck (1963).

The following enzymes were used in this study: bacterial alkaline phosphatase, T4 polynucleotide kinase, and restriction endonucleases *Pst*I and *Hin*dIII (all obtained from Bethesda Research Laboratories); protease K (Protease XI; Sigma); ribonuclease III (initial batches were obtained from Bethesda Research Laboratories; later batches were obtained from Drs. Victor Shen and David Schlessinger, Washington University School of Medicine, St. Louis, MO).

Recombinant plasmids containing cloned fragments of *Neurospora* mtDNA were obtained from Drs. Joyce E. Heckman and Uttam L. RajBhandary (Massachusetts Institute of Technology, Cambridge, MA) [see Heckman & RajBhandary (1979) and Heckman et al. (1979)]. The plasmids and mtDNA fragments contained in each plasmid were as follows: pHPR9, *Pst*I-9; pHP109, *Pst*I-4a and -9; pHH67, *Hin*dIII-7b.

Growth of Mycelia and Isolation of Mitochondria. Procedures for growth and labeling of cells have been described previously (Lambowitz & Luck, 1976). The wild-type strain was grown for 14 h at 25 °C or 10–12 h at 37 °C. The mutant strain (299-9) was grown for 24 h at 37 °C. Mitochondria were purified by the modified flotation gradient method (Lambowitz, 1979).

Isolation of mtDNA and Mitochondrial RNA. Mitochondrial nucleoprotein pellets were prepared in Ca^{2+}-containing buffer to suppress nuclease activity (Lambowitz & Luck, 1976; Lambowitz, 1979). mtDNA was extracted from such pellets by using the phenol/ribonuclease/protease method (Mannella & Lambowitz, 1978). For RNA isolation, the ribonucleoprotein pellets were rinsed twice with ice-cold distilled water, then dissolved in 2 mL of buffer containing 1% $NaDodSO_4$/100 mM NaCl/2 mM EDTA/100 mM Tris-HCl, pH 8.0, and incubated with protease K (6.2 units, 30 min, 37 °C; protease K was preincubated for 60 min at 37 °C). Aliquots (0.1–0.3 mL) were layered over linear gradients of 6–23% sucrose in 1% $NaDodSO_4$/50 mM NaCl/1 mM EDTA/25 mM Tris-HCl, pH 7.5, and centrifuged in an SW 41 rotor (40000 rpm, 4 h, 20 °C). The gradients were fractionated by monitoring the absorbance at 254 nm by using an ISCO density gradient fractionator. Separated RNA species were ethanol precipitated at least 3 times before use as substrate for ribonuclease III.

Isolation of Ribonucleoprotein Particles. Large subunits of mitochondrial ribosomes and ribonucleoprotein particles containing 35S RNA were isolated as described previously (Lambowitz & Luck, 1976; LaPolla & Lambowitz, 1979). The initial ribonucleoprotein pellets (Lambowitz & Luck, 1976; Lambowitz, 1979) were rinsed with ice-cold distilled water, then dissolved in $HKMTD_{500/25}$ (500 mM KCl/25 mM $MgCl_2$/25 mM Tris-HCl (pH 7.5)/5 mM dithiothreitol), and incubated with 1 mM puromycin (15 min, 35 °C) to dissociate ribosomal subunits. Aliquots (0.3–0.4 mL) were layered over linear gradients of 5–20% sucrose in $HKMTD_{500/25}$ and centrifuged in an SW 41 rotor (40000 rpm, 3 h, 4 °C). Gradients were fractionated as above, pooled fractions were diluted with $HKMTD_{500/25}$, and particles were collected by centrifugation in a Ti 50 rotor (40000 rpm, 12–15 h, 4 °C).

Digestion of RNA with Ribonuclease III and Analysis of Cleavage Products. A 15-μg sample of purified RNA was digested with 1–2 units of ribonuclease III in 50 μL of buffer containing 100 mM NH_4Cl/10 mM $MgCl_2$/20 mM Tris-HCl, pH 7.5, or different NH_4Cl or $MgCl_2$ concentrations as indicated in the figures. Reactions were for 30 min at 37 °C and were terminated by addition of 1.5% $NaDodSO_4$/15 mM EDTA, pH 7.5 (Birenbaum et al., 1979). Reaction products were precipitated with ethanol and analyzed by gel electrophoresis on composite gels containing 2.4% polyacrylamide and 0.8% agarose (Peacock & Dingman, 1968) or denatured with glyoxal and analyzed on 1.4% agarose gels (McMaster & Carmichael, 1977).

Gel Transfer Hybridizations. Southern hybridizations were carried out essentially as described (Southern, 1975). Probes were ribonuclease III fragments of 35S RNA which had been extracted from preparative gels by the method of Kourilsky et al. (1968). The extracted RNA fragments were treated with bacterial alkaline phosphatase and ^{32}P labeled with T4 polynucleotide kinase to specific activities of $(1–2) \times 10^6$ cpm/μg (Silberklang et al., 1979). Hybridizations were carried out in plastic bags at 39 °C for 18–24 h. Aliquots of 1–5 μg of labeled RNA were used per reaction mixture containing 40% formamide/0.3 M NaCl/30 mM sodium citrate and 50 μg of carrier *E. coli* tRNA.

RNA gel transfer hybridizations were carried out as described by Alwine et al. (1977). Probes were recombinant plasmids ^{32}P labeled by nick translation (Mackey et al., 1977) to specific activities of $10^7–10^8$ cpm/μg.

Electron Microscopy. RNA was diluted into 40% formamide, 0.1 mM $MgCl_2$, 100 mM Tris-HCl, pH 8.4, and 50 μg/mL cytochrome *c* and spread onto 10% formamide and 10 mM Tris-HCl, pH 8.4. Monolayers were adsorbed onto parlodion films and stained with uranyl acetate. All full-length molecules with hairpins were photographed. Similar results were obtained for RNA diluted into a solution containing 10 mM EDTA instead of $MgCl_2$.

Results

E. coli ribonuclease III cleaves natural substrates as well as eukaryotic RNAs in double-stranded regions, producing discrete fragments which can be resolved by gel electrophoresis (Dunn & Studier, 1973a,b; Schlessinger et al., 1974; Nomoto et al., 1979). In natural substrates, ribonuclease III has been found to be specific for physiological sites at NH_4Cl concentrations between 150 and 300 mM whereas additional sites are recognized at lower salt concentrations (Dunn, 1976). Primary sites in eukaryotic RNAs (e.g., polio virus) have been defined as those recognized at salt concentrations greater than 120 mM and are assumed to include double-stranded regions consisting of 15 or more uninterrupted base pairs (Nomoto et al., 1979).

Ribonuclease III cleavage patterns for 35S and 25S RNA are shown in Figure 2. ^{32}P-labeled RNAs were digested with ribonuclease III in buffers containing 100–200 mM NH_4Cl plus 10 mM $MgCl_2$, and the products were analyzed by gel

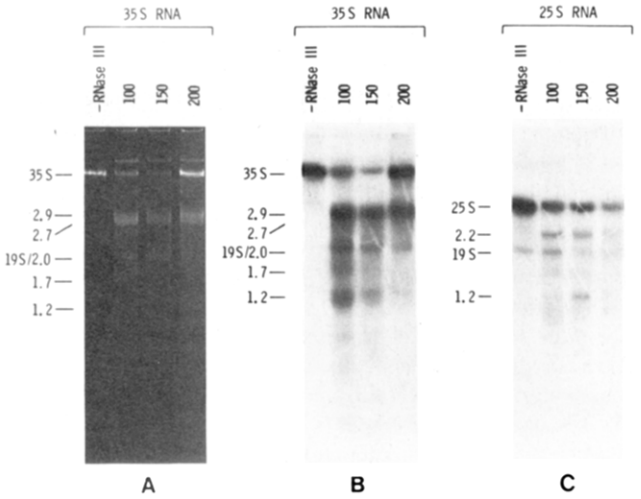

FIGURE 2: Ribonuclease III digests of 35S and 25S RNA. ^{32}P-labeled RNAs were prepared from 2–3 L of cultures, mass labeled with 2 mCi of [^{32}P]orthophosphate in low-phosphate Vogel's minimal medium (0.2 g of KH_2PO_4 per liter) (Lambowitz & Luck, 1976). RNAs were digested with ribonuclease III in buffers containing 100, 150, or 200 mM NH_4Cl (as indicated in the Figure), 10 mM $MgCl_2$, and 20 mM Tris–HCl, pH 7.5. Control slots show RNAs incubated in 100 mM NH_4Cl, 10 mM $MgCl_2$, and 20 mM Tris-HCl, pH 7.5, but without ribonuclease III. Digests were analyzed by electrophoresis on composite gels containing 2.4% polyacrylamide and 0.8% agarose. Size estimates in the figure are based on 35S RNA, 5.5 kb; 25S RNA, 3.3 kb; and 19S RNA, 2 kb. (A) 35S RNA, gel stained with ethidium bromide and photographed under short-wavelength UV light. (B) 35S RNA, autoradiogram of gel in (A). (C) 25S RNA, autoradiogram. Note that the control slot in (A) is loaded with one-half total OD_{260} as the experimental slots. The light band above 35S RNA is mtDNA.

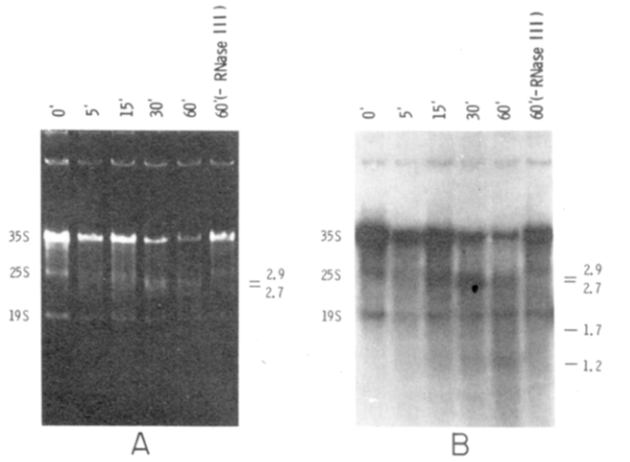

FIGURE 3: Time course of digestion of 35S RNA by ribonuclease III. 35S RNA was digested with ribonuclease III for 0, 5, 15, 30, or 60 min as indicated in the figure in buffer containing 100 mM NH_4Cl, 10 mM $MgCl_2$, and 20 mM Tris-HCl, pH 7.5. The control slot shows 35S RNA incubated in the same buffer for 60 min, but without ribonuclease III. Digests were analyzed by electrophoresis on composite gels containing 2.4% polyacrylamide and 0.8% agarose. (A) Gel stained with ethidium bromide and photographed under short-wavelength UV light. (B) Autoradiogram of gel in (A).

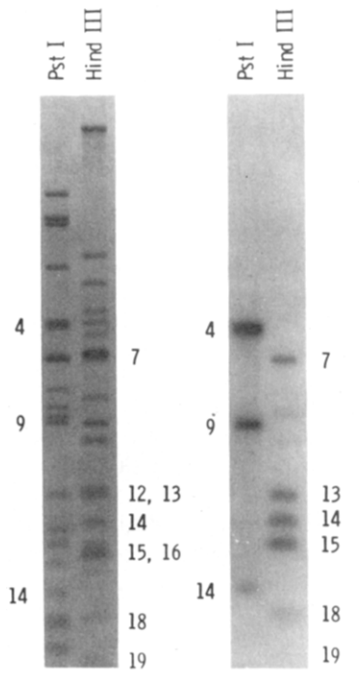

FIGURE 4: Southern hybridization experiments. The 2.9- and 2.7-kb fragments of 35S RNA (see Figure 2) were excised from gels and ^{32}P labeled by treatment with bacterial alkaline phosphatase and T4 polynucleotide kinase. ^{32}P-labeled RNAs were hybridized to separated *Pst*I and Hin*d*III restriction fragments of mtDNA. (Left panel) DNA fragments stained with ethidium bromide and photographed under short-wavelength UV light. (Right panel) Autoradiogram showing DNA fragments which hybridize with ^{32}P-labeled RNAs. The *Pst*I fragments are numbered according to Heckman & RajBhandary (1979).

electrophoresis. The cleavage patterns are relatively simple. For 35S RNA, the predominant products at high salt concentration (200 mM NH_4Cl) appear to be two closely spaced fragments, ca. 2.9 and 2.7 kb, presumably reflecting a ribonuclease III site near the center of the molecule (stained gel, Figure 2A; autoradiogram, Figure 2B). There are also minor fragments which become more prominent when reactions are carried out at lower salt concentrations (1.2 and 1.7 kb and probably at 2.0 kb, comigrating with contaminating 19S RNA; autoradiogram, Figure 2B). Additional experiments were carried out to test other reaction conditions: cleavage patterns at 35 and 70 mM NH_4Cl were essentially the same as those at 100 mM NH_4Cl, except for somewhat decreased yields of the 2.9-kb fragment (data not shown). At 200 mM NH_4Cl, the same cleavage patterns were obtained at 5, 10, and 25 mM $MgCl_2$, and the reaction was inhibited at 0 and 1 mM $MgCl_2$ (data not shown). Finally, phenol extraction of 35S RNA had no apparent effect on the sensitivity of the central site to ribonuclease III cleavage (data not shown). In the case of 25S RNA, ribonuclease III cleavage patterns under standard reactions conditions (100–200 mM NH_4Cl plus 10 mM $MgCl_2$) show light fragments at 2.2 and 1.2 kb (autoradiogram, Figure 2C). It should be noted that the efficiency of ribonuclease III cleavage at the central ribonuclease III site in 35S RNA is higher than that at any other site in 35S RNA or 25S RNA.

The central ribonuclease III site is of particular interest with respect to RNA splicing for two reasons: (i) the fragment lengths indicate that it must be located at or near the 5′-intron–exon boundary (Figure 1) and (ii) it corresponds to a structural feature which is present in 35S RNA, but not in 25S RNA, as judged by the sizes of fragments for 25S RNA and other evidence (see Discussion). The following additional experiments support the existence of the central ribonuclease III site.

(a) Time course experiments (Figure 3) show that the 2.9- and 2.7-kb fragments appear together at early times, as expected if they are generated by a single ribonuclease III cleavage. The 1.7- and 1.2-kb fragments just begin to appear at later times.

(b) Southern hybridization experiments were carried out in which the 2.9- and 2.7-kb fragments were excised together from gels, ^{32}P labeled by using T4 polynucleotide kinase, and hybridized to separated *Pst*I and Hin*d*III restriction fragments

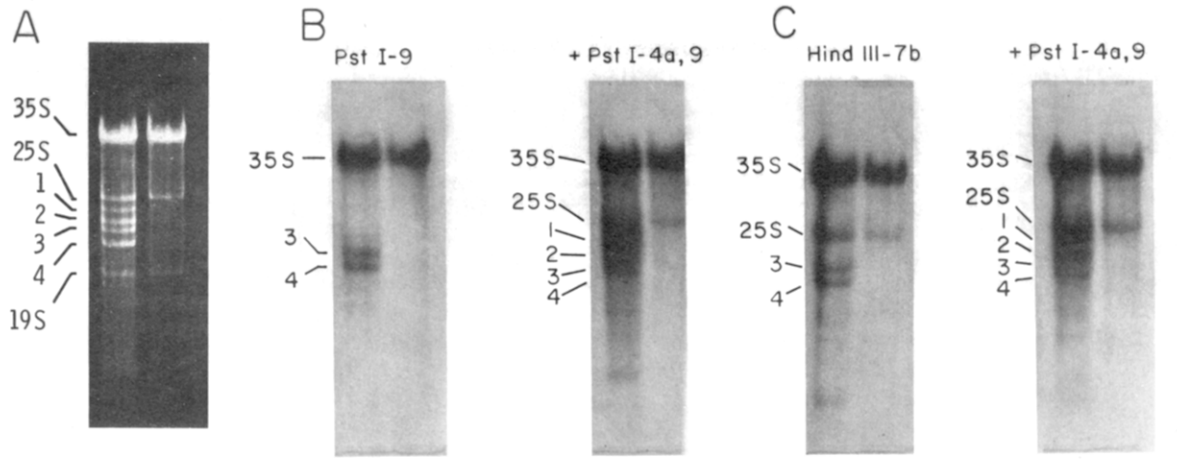

FIGURE 5: Ribonuclease III digests of 35S RNA. 35S RNA was digested with ribonuclease III in buffer containing 100 mM NH_4Cl, 10 mM $MgCl_2$, and 20 mM Tris-HCl, pH 7.5. Products were denatured with glyoxal and analyzed by electrophoresis on 1.4% agarose gels. (A) Gel stained with ethidium bromide and photographed under short-wavelength UV light. Slots 1 and 2 show 35S RNA incubated with and without ribonuclease III. (B and C) RNA gel transfer hybridization analysis of ribonuclease III fragments. Gel patterns similar to the pair in (A) were transferred to DBM paper strips and hybridized with ^{32}P-labeled DNA probes. (B) Strips hybridized with ^{32}P-labeled *Pst*I-9 (plasmid pHPR9) and then rehybridized with ^{32}P-labeled *Pst*I-4a, -9 (plasmid pHP109). (C) Strips hybridized with ^{32}P-labeled *Hin*dIII-7b (plasmid pHH67) and then rehybridized with *Pst*I-4a, -9 (plasmid pHP109).

of mtDNA. Figure 4 shows that hybridization is detected to all restriction fragments expected for 35S RNA (*Pst*I-4a, -9, and -14 and *Hin*dIII-7, -13, -14, -15, -18, and -19), consistent with the idea that the 2.9- and 2.7-kb fragments are half-molecules which contain all 35S RNA sequences.

Further analysis led to some refinement of this conclusion by showing that the 2.9- and 2.7-kb bands each consist of two closely related RNA species which differ slightly in size. Figure 5A shows gel electrophoretic analysis of ribonuclease III digests of 35S RNA on 1.4% agarose gels following denaturation of the digests with glyoxal. The 2.7- and 2.9-kb fragments previously detected on Peacock–Dingman gels (Figure 2) are now resolved into four fragments with apparent molecular weights (in the new gel system) ranging from 2.5 to 3 kb (Figure 5A). These fragments were characterized by RNA gel transfer hybridization, with cloned restriction fragment probes corresponding to different parts of the 25S RNA gene. As shown in Figure 5B,C, the two smallest fragments hybridize with both *Pst*I-9 and *Hin*dIII-7b (3′ half of 35S RNA; Figure 1) whereas the two largest fragments hybridize with *Pst*I-4a (5′ half of 35S RNA; Figure 1). There are three possible explanations for these results: (a) that there are two closely spaced structures containing double-stranded stems, (b) that there is a single structure (e.g., a large hairpin) which is cleaved at different positions by ribonuclease III, or (c) that there is a single ribonuclease III cleavage site, but two classes of 35S RNA molecules which differ in having short (100 nucleotides) 5′- and 3′-end extensions. The resolution of additional bands in the gel of Figure 5 could be attributed either to better separation of RNA fragments in this gel system or to dissociation of nicked RNAs under fully denaturing conditions. It should be noted that we detect no ribonuclease III fragment which corresponds to the excised intron (i.e., a fragment which hybridizes only with *Pst*I-9).

Electron microscopic examination of 35S RNA spread under partially denaturing conditions suggests that the central ribonuclease III site(s) correspond(s) to a 180 ± 45 nucleotide central hairpin. Under the spreading conditions described under Materials and Methods, about half of the 35S RNA molecules examined contained hairpins. Twenty-four hairpin-containing molecules were chosen at random, photographed, and measured. Representative electron micrographs are shown in Figure 6. The data from the measurements

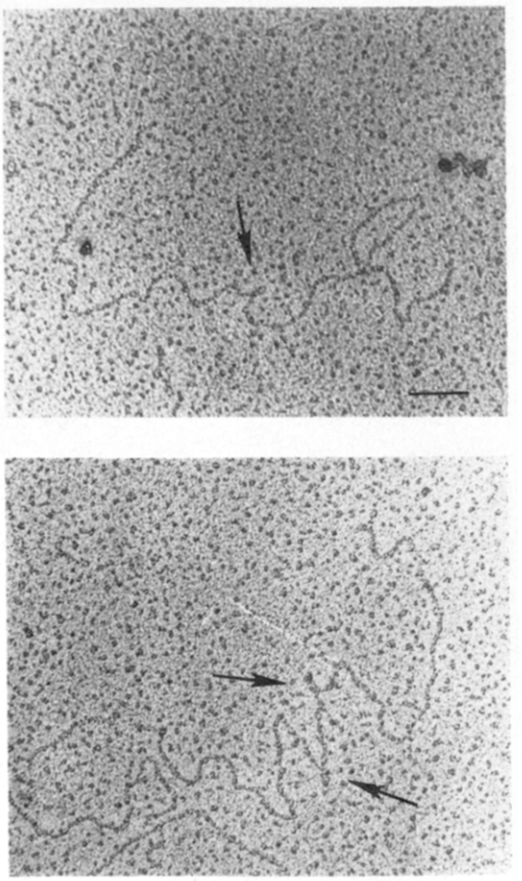

FIGURE 6: Electron micrographs of 35S RNA molecules which contain hairpins. (Top) Molecule containing central hairpin; (bottom) molecule containing central hairpin and one additional hairpin. The bar indicates a length of 0.1 μm.

(Figures 7 and 8) show that 19 of the 24 molecules contain a central hairpin of 180 ± 45 nucleotides and that the lengths of the arms measured from this hairpin to the ends of the molecules are 0.906 ● 0.058 and 0.820 ± 0.067 μm. With the length of 35S RNA being 5.5 kb, the measured arm lengths correspond to 2.80 and 2.52 kb, in close agreement with the lengths expected from the ribonuclease III data. The correlation between the central hairpin and the central ribo-

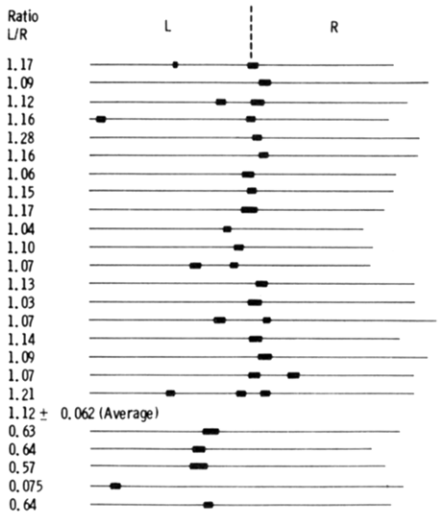

FIGURE 7: Gallery of 35S RNA molecules containing hairpins. The figure shows 24 molecules containing hairpins which were photographed at random. Molecules containing the central hairpin have been oriented with the longer arm toward the left. The heavier bars indicate the size and position of each hairpin.

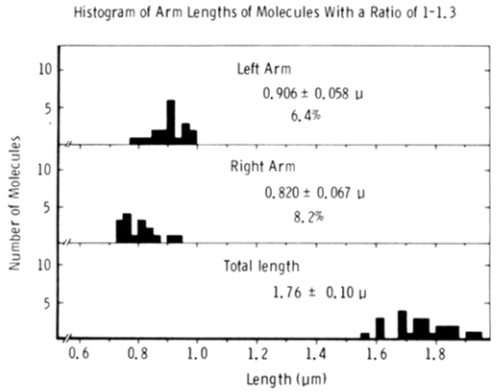

FIGURE 8: Histogram showing the lengths of arms for molecules containing the central hairpin (i.e., molecules having arm ratios of 1–1.3). Where an additional hairpin was found, its length was included in the length of the arm. The total lengths of all molecules are plotted at the bottom. Each histogram shows the mean ± standard deviation for the length measurements.

nuclease III site(s) is strengthened by the paucity of other secondary structure features detected in 35S RNA. Figure 7 shows that the central hairpin is the only structural feature detectable in nearly half of the molecules and that the remaining molecules contain only one or two additional hairpins. It is also noted that four of the five molecules which lack the central hairpin contain another hairpin ca. 2.1 kb from one end (Figure 7). In the case of 25S RNA, fewer than 20% of all molecules contained hairpins, and these were smaller and more randomly distributed than the large central hairpin in 35S RNA (data not shown). The electron microscopy provides no indication of large loop structures, either under the spreading conditions described under Materials and Methods or under less denaturing conditions with lower concentrations of formamide and/or higher concentrations of $MgCl_2$.

Previous studies showed that 35S RNA can be isolated from the mutants in a ribonucleoprotein particle associated with almost the full complement of large subunit ribosomal proteins (LaPolla & Lambowitz, 1979). Experiments were carried out to determine whether the 35S RNA in such particles is susceptible to cleavage by ribonuclease III. A fraction enriched in 35S RNA particles, but also containing mature large subunits, was isolated from the mutants by sucrose-gradient

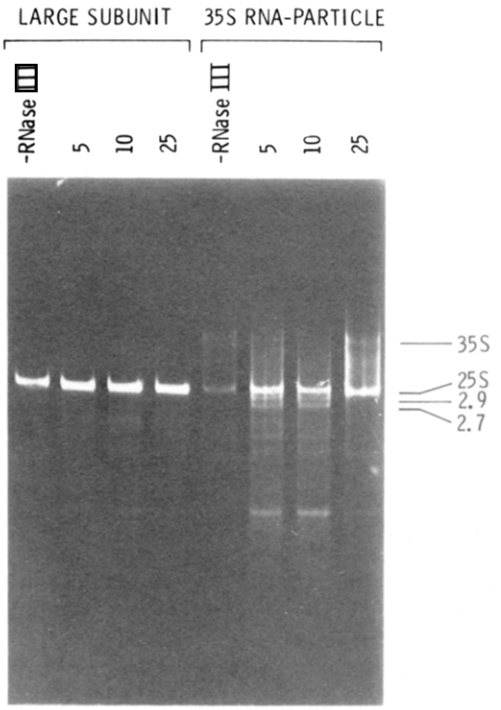

FIGURE 9: Ribonuclease III digests of ribonucleoprotein particles. Large (50S) ribosomal subunits were isolated from 37 °C grown wild-type 74*A*. A particle fraction containing 35S RNA particles and large subunits was isolated from 37 °C grown mutant 299-9. Particles were digested with ribonuclease III in buffers containing 200 mM NH_4Cl, 5, 10, or 25 mM $MgCl_2$ (as indicated in the figure), and 20 mM Tris-HCl, pH 7.5. RNAs were extracted by the Na-$DodSO_4$/diethyl pyrocarbonate procedure (Lambowitz & Luck, 1976; Lambowitz, 1979) and analyzed by electrophoresis on composite gels containing 2.4% polyacrylamide and 0.8% agarose. Control slots show ribonucleoprotein particles incubated in 200 mM NH_4Cl, 10 mM $MgCl_2$, and 20 mM Tris-HCl, pH 7.5, but without ribonuclease III.

centrifugation. The particles were treated with ribonuclease III in buffers containing different $MgCl_2$ concentrations, and RNAs were extracted and analyzed by gel electrophoresis. The results are shown in Figure 9. The most important finding is that treatment of the mutant particle preparations with ribonuclease III at 5 and 10 mM $MgCl_2$ results in production of the 2.9- and 2.7-kb fragments; the reaction is inhibited at 25 mM $MgCl_2$, presumably because the particles become so tightly folded that 35S RNA is no longer accessible to the enzyme. Control slots show that the 2.9- and 2.7-kb fragments do not appear when wild-type large subunits are treated with ribonuclease III nor when mutant particle preparations are incubated without ribonuclease III. Control experiments in the previous study (LaPolla & Lambowitz, 1979) showed that the mutant particle fractions do not contain substantial amounts of *free* 35S RNA which might give rise to the 2.9- and 2.7-kb fragments. We conclude, therefore, that the 35S RNA in the ribonucleoprotein particles can be cleaved by ribonuclease III at the central site(s). This finding is consistent with the idea that the central site(s) is (are) accessible to RNA-processing enzymes in vivo.

Discussion

Figure 10 is a schematic diagram summarizing the major secondary structure features of 35S RNA detected by ribonuclease III digestion and by electron microscopy. Ribonuclease III cleaves 35S RNA predominantly at a central site or sites [labeled (1)] which we assume corresponds to a central hairpin of 180 ± 45 nucleotides visualized by electron microscopy. Additional ribonuclease III fragments are produced at lower yield. Some of these minor fragments can be ac-

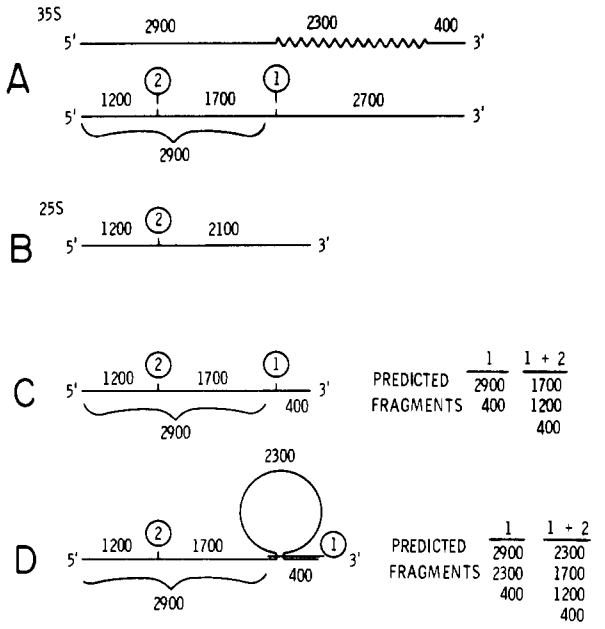

FIGURE 10: Schematic diagrams showing (A and B) secondary structure features detected in 35S or 25S RNA and (C and D) ribonuclease III fragments predicted for hypothetical secondary structures. (A) 35S RNA. (1) indicates the central ribonuclease III site(s) which is (are) assumed to correspond to the 180 ± 45 nucleotide central hairpin. (2) indicates an additional ribonuclease III site which gives rise to minor fragments in digests of 25S and 35S RNA (see Figure 2). (B) 25S RNA. (2) indicates the same ribonuclease III site as in 35S RNA. (C) Ribonuclease III fragments predicted for 25S RNA if the central hairpin [i.e., site (1) of 35S RNA] were retained in 25S RNA. (D) Ribonuclease III fragments predicted for 35S RNA if 35S RNA contains an intron loop and if ribonuclease III cuts at the stem of the loop.

counted for by assuming that there is a second site [labeled (2)] located in the coding segment, 1.2 kb from the 5′ end of 35S RNA or 25S RNA. [Site (2), however, cannot be correlated with a structural feature detected by electron microscopy.] The ribonuclease III cleavage sites are presumed to correspond to structural features which contain double-stranded stems 15 bp or longer, but the approaches used provide no further insight into structural details. Features identified as hairpins by electron microscopy could include conventional hairpins, stem–loop structures, or more complex configurations with double-stranded branches. It should be noted that the approaches used in our study do not detect any large secondary structure feature near the 3′-intron–exon boundary. An important qualification, however, is that neither ribonuclease III digestion nor electron microscopy would detect short hairpins which almost certainly exist in the RNA.

The central hairpin in 35S RNA is of particular interest with respect to RNA splicing because it is located at or near the 5′-intron–exon boundary. The following evidence indicates that this hairpin is not present in 25S RNA: (a) The sizes of the ribonuclease III fragments for 25S RNA are not consistent with retention of the hairpin near the splice junction. More specifically, Figure 10C shows that if ribonuclease III site (1) were retained in 25S RNA, the ribonuclease III fragments produced would have been clearly distinguishable from those actually observed. (b) The efficiency of ribonuclease III cleavage at the central site in 35S RNA [site (1)] was found to be higher than that at any other site in 35S RNA or 25S RNA. (c) Electron microscopy does not detect any large, reproducible secondary structure feature in 25S RNA under conditions where the central hairpin is reproducibly observed in 35S RNA. (Under our spreading conditions, the central hairpin was observed in 40% of all 35S RNA molecules examined and in 80% of those molecules which showed any secondary structure feature.) From the finding that the hairpin is not present in 25S RNA and from the position of the hairpin near the 5′-intron–exon boundary, we infer that its formation requires intron sequences.

Three findings draw particular attention to the potential significance of the central hairpin: (a) It is the most prominent secondary structure feature detected in 35S RNA amidst a paucity of other secondary structure features, (b) it is located at or near the 5′-intron–exon boundary, and (c) in the 35S RNA particles which contain almost the full complement of large subunit ribosomal proteins, the 35S RNA can still be cleaved by ribonuclease III at the central site(s). The latter finding suggests that the central hairpin would be accessible to other RNA-processing enzymes and is consistent with the possibility that the central hairpin contributes to the structure recognized by the RNA-splicing enzyme.

We emphasize that the significance of the central hairpin is still a matter of conjecture and that there is no direct evidence that it plays a role in RNA splicing or any other process. In addition, since the exact location of the hairpin relative to the splice junction remains to be determined, it is still possible that the presence of the hairpin is fortuitous and that the critical element recognized by the RNA-splicing enzyme is some specific nucleotide sequence or some small, as yet undetected secondary structure feature.

Recently, Yin et al. (1980) presented preliminary data on DNA sequences in the rRNA–tRNA region of *Neurospora* mtDNA. These authors identified a repetitive 18-nucleotide palindromic sequence which contains two cleavage sites for restriction endonuclease *Pst*I. This sequence was found at several locations which could correspond to RNA-processing sites, specifically, between some tRNA genes in *Hin*dIII-7b and within the 25S RNA gene, near both the 5′- and 3′-intron–exon boundaries (i.e., at the boundaries of *Pst*I-9; Figure 1). It is possible that the 5′-*Pst*I palindrome identified by Yin et al. (1980) forms part of the large central hairpin identified in our study, but this point can only be decided when more complete base-sequence information is available. It is noteworthy that the approaches used in our study do not detect any obvious secondary structure feature near the 3′-*Pst*I palindrome nor do they provide evidence for a large intron-containing loop formed by base pairing between *Pst*I palindromes at opposite intron–exon boundaries (see Figure 10D). These additional secondary structure features may be present in the RNA, but undetected under the conditions used in our study, or they may be present in the RNA in vivo, perhaps stabilized by binding of ribosomal proteins.

DNA sequences have been determined for intron–exon boundaries in the large rRNA genes of *Tetrahymena* (Wild & Sommer, 1980), *Chlamydomonas* chloroplasts (Allet & Rochaix, 1979), and yeast mitochondria (Dujon, 1980; Bos et al., 1980) as well as other genes in eukaryotic chromosomal and viral DNAs [see review by Abelson (1979)]. Hairpin structures have been the focus of attention in only a few of these studies. The DNA sequence corresponding to the precursor of adenovirus 2 hexon mRNA shows a potential hairpin of 30 bp, consisting of intron and coding sequences at the 3′-intron–exon boundary (Akusjarvi & Pettersson, 1978). Zain et al. (1979) have carried out a detailed computer analysis of regions of adenovirus 2 DNA which contain the leader sequences for late mRNAs. Their analysis showed that intron–exon boundaries could consistently be drawn so that the splice point is contained within the single-stranded loop of a stem–loop structure. The authors suggested that such struc-

tures are relevant to RNA splicing.

Binding of ribosomal proteins can strongly affect RNA secondary and tertiary structure and may facilitate the formation of structures required for RNA splicing. Work with many organisms has shown that ribosomal proteins can bind rapidly to ribosomal precursor RNAs in vivo (e.g., Warner & Soeiro, 1967; Liau & Perry, 1969). Since all known rRNA introns are located near the 3′ end of the RNAs (Glover & Hogness, 1977; Pellegrini et al., 1977; Wellauer & Dawid, 1977; White & Hogness, 1977; Rochaix & Malnoe, 1978; Wild & Gall, 1979; Merten et al., 1980; Bos et al., 1980; Dujon, 1980; Green et al., 1981), it is likely that at least some ribosomal proteins bind to precursor RNAs prior to RNA splicing. In future studies, it may be possible to gain insight into the role of ribosomal proteins by more detailed analysis of their effect on the conformation of 35S RNA in ribonucleoprotein particles.

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References

Abelson, J. (1979) *Annu. Rev. Biochem. 48*, 1035–1069.
Akusjarvi, G., & Pettersson, U. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 5822–5826.
Allet, B., & Rochaix, J. D. (1979) *Cell (Cambridge, Mass.) 18*, 55–60.
Alwine, J. C., Kemp, D. J., & Stark, G. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5350–5354.
Birenbaum, M., Shen, V., Nikolaev, N., & Schlessinger, D. (1979) *Methods Enzymol. 59*, 824–837.
Bos, J. L., Osinga, K. A., Van der Horst, G., Hecht, N. B., Tabak, H. F., Van Ommen, G. J. B., & Borst, P. (1980) *Cell (Cambridge, Mass.) 20*, 207–214.
Cantor, C. R. (1980) in *Ribosomes: Structure, Function and Genetics* (Chambliss, G., Craven, G. R., Davies, J., Davis, K., Kahan, L., & Nomura, M., Eds.) pp 23–49, University Park Press, Baltimore, MD.
Crick, F. (1979) *Science (Washington, D.C.) 204*, 264–271.
de Vries, H., de Jonge, J. C., Bakker, H., Meurs, H., & Kroon, A. (1979) *Nucleic Acids Res. 6*, 1791–1804.
Dujon, B. (1980) *Cell (Cambridge, Mass.) 20*, 185–197.
Dunn, J. J. (1976) *J. Biol. Chem. 251*, 3807–3814.
Dunn, J. J., & Studier, F. W. (1973a) *Proc. Natl. Acad. Sci. U.S.A. 70*, 1559–1563.
Dunn, J. J., & Studier, F. W. (1973b) *Proc. Natl. Acad. Sci. U.S.A. 70*, 3296–3300.
Glover, D. M., & Hogness, D. S. (1977) *Cell (Cambridge, Mass.) 10*, 167–176.
Green, M. R., Grimm, M. F., Goewert, R. R., Collins, R. A., Cole, M. D., Lambowitz, A. M., Heckman, J. E., Yin, S., & RajBhandary, U. L. (1981) *J. Biol. Chem. 256*, 2027–2034.
Hahn, U., Lazarus, C. M., Lunsdorf, H., & Kuntzel, H. (1979) *Cell (Cambridge, Mass.) 17*, 191–200.
Heckman, J. E., & RajBhandary, U. L. (1979) *Cell (Cambridge, Mass.) 17*, 583–595.
Heckman, J. E., Yin, S., Alzner-DeWeerd, B., & RajBhandary, U. L. (1979) *J. Biol. Chem. 254*, 12694–12700.
Knapp, G., Beckmann, J. S., Johnson, P. F., Fuhrman, S. A., & Abelson, J. (1978) *Cell (Cambridge, Mass.) 14*, 221–236.
Kourilsky, P., Marcaud, L., Sheldrick, P., Luzzati, D., & Gros, F. (1968) *Proc. Natl. Acad. Sci. U.S.A. 61*, 1013–1020.
Lambowitz, A. M. (1979) *Methods Enzymol. 59*, 421–433.
Lambowitz, A. M., & Luck, D. J. L. (1976) *J. Biol. Chem. 251*, 3081–3095.
LaPolla, R. J., & Lambowitz, A. M. (1979) *J. Biol. Chem. 254*, 11746–11750.
Liau, M. C., & Perry, R. P. (1969) *J. Cell Biol. 42*, 272–283.
Luck, D. J. L. (1963) *J. Cell Biol. 16*, 483–499.
Mackey, J. K., Brackmann, K. H., Green, M. R., & Green, M. (1977) *Biochemistry 16*, 4478–4483.
Mannella, C. A., & Lambowitz, A. M. (1978) *Biochem. Biophys. Res. Commun. 80*, 673–679.
Mannella, C. A., Collins, R. A., Green, M. R., & Lambowitz, A. M. (1979a) *Proc. Natl. Acad. Sci. U.S.A. 76*, 2635–2639.
Mannella, C. A., Pittenger, T. H., & Lambowitz, A. M. (1979b) *J. Bacteriol. 137*, 1449–1451.
McMaster, G. K., & Carmichael, G. G. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 4835–4838.
Merten, S., Synenki, R. M., Locker, J., Christianson, T., & Rabinowitz, M. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 1417–1421.
Noller, H. F. (1980) in *Ribosomes: Structure, Function and Genetics* (Chambliss, G., Craven, G. R., Davies, J., Davis, K., Kahan, L., & Nomura, M., Eds.) pp 3–22, University Park Press, Baltimore, MD.
Nomoto, A., Lee, Y. F., Babich, A., Jacobson, A., Dunn, J. J., & Wimmer, E. (1979) *J. Mol. Biol. 128*, 165–177.
O'Farrell, P. Z., Cordell, B., Valenzuela, P., Rutter, W. J., & Goodman, H. M. (1978) *Nature (London) 274*, 438–445.
Peacock, A. C., & Dingman, C. W. (1968) *Biochemistry 7*, 668–674.
Pellegrini, M., Manning, J., & Davidson, N. (1977) *Cell (Cambridge, Mass.) 10*, 213–224.
Pittenger, T. H., & West, D. J. (1979) *Genetics 93*, 539–555.
Rochaix, J. D., & Malnoe, P. (1978) *Cell (Cambridge, Mass.) 15*, 661–670.
Schlessinger, D., Ono, M., Nikolaev, N., & Silengo, L. (1974) *Biochemistry 13*, 4268–4271.
Silberklang, M., Gillum, A. M., & RajBhandary, U. L. (1979) *Methods Enzymol. 59*, 58–109.
Southern, E. M. (1975) *J. Mol. Biol. 98*, 503–517.
Warner, J. R., & Soeiro, R. (1967) *Proc. Natl. Acad. Sci. U.S.A. 58*, 1984–1990.
Wellauer, P. K., & Dawid, I. B. (1977) *Cell (Cambridge, Mass.) 10*, 193–212.
White, R. L., & Hogness, D. S. (1977) *Cell (Cambridge, Mass.) 10*, 177–192.
Wild, M. A., & Gall, J. G. (1979) *Cell (Cambridge, Mass.) 16*, 565–573.
Wild, M. A., & Sommer, R. (1980) *Nature (London) 283*, 693–694.
Yin, S., Heckman, J. E., Sarnoff, J., & RajBhandary, U. L. (1980) in *The Organization and Expression of the Mitochondrial Genome* (Kroon, A. M., & Saccone, C., Eds.) pp 307–310, Elsevier/North-Holland Biomedical Press, Amsterdam.
Zain, S., Gingeras, T. R., Bullock, P., Wong, G., & Gelinas, R. E. (1979) *J. Mol. Biol. 135*, 413–433.