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Neurospora crassa Cytoplasmic Ribosomes: Ribosomal Ribonucleic Acid Synthesis in the Wild Type

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The biosynthesis of ribosomal ribonucleic acid (rRNA) in wild-type *Neurospora crassa* growing at 25°C was investigated by continuous-labeling and pulse-chase experiments using [5-^{3}H]uridine. The results of these experiments suggest the following precursor-product relationships: the first RNA molecule to be synthesized in significant quantities is the 2.4 × 10^6-dalton (2.4-Mdal) ribosomal precursor RNA. This RNA is cleaved to produce two species of RNA with weights of 0.7 and 1.4 Mdal. The former is the mature 17*S* rRNA of the 37*S* ribosomal subunit. The 1.4-Mdal RNA is subsequently cleaved to produce the mature 1.27-Mdal (25*S*) and 61,000-dalton (5.8*S*) rRNA's of the 60*S* ribosomal subunit. In the maturation process, approximately 15 to 20% of the 2.4-Mdal ribosomal precursor rRNA molecule is lost. As in other eukaryotes that have been examined, 5*S* rRNA is not derived from this precursor molecule.

In eukaryotes, ribosomal ribonucleic acid (rRNA) synthesis occurs in the nucleolus (3, 16, 18). The rRNA genes are transcribed into a high-molecular-weight ribosomal precursor RNA (pre-rRNA), which is then methylated and cleaved at specific sites to yield a number of discrete intermediates and finally the mature 18*S*, 28*S*, and 5.8*S* rRNA's (1, 4, 7, 17). Assembly with ribosomal proteins and with 5*S* rRNA (which is transcribed from separate rRNA genes) occurs in the nucleolus during pre-rRNA processing. The resultant ribosomal subunits are then released from the nucleus into the cytoplasm.

rRNA maturation has been studied in a number of eukaryotes, including mammalian cells (17, 31, 32), amphibians (6, 14, 31), insects (8), higher plants (14), and yeasts (19, 20, 26-29). Though similar, the pathways observed in the various systems exhibit significant differences. For example, whereas at least four pre-rRNA species have been identified in mammalian cells (31, 32), *Rana pipiens* (6), and the yeast *Saccharomyces carlsbergensis* (19, 20), only two or three pre-rRNA's have been identified in *Xenopus laevis* (14, 31) and the yeast *Saccharomyces cerevisiae* (28, 29). In light of such reported differences in the rRNA maturation pathways of various eukaryotes, we decided to study the synthesis and processing of pre-rRNA's in wild-type *Neurospora crassa* to provide an additional point of reference and to provide a basis for studying conditional mutants with putative defects in rRNA maturation. In this paper we report the results of this study.

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MATERIALS AND METHODS

Strains. The wild-type strain of *N. crassa* used was the St. Lawrence strain 74A further inbred in the laboratory of A. M. Srb (Cornell University, Ithaca, N.Y.) from whom it was obtained. The methionine-requiring strain *met-1* (38706) was obtained from the Fungal Genetics Stock Center, Humboldt State University Foundation, Arcata, Calif.

Culture techniques. The culture techniques and growth media used were those described by Schlitt and Russell (22). For the methionine-requiring strain, the medium was supplemented with 0.3 mM L-methionine.

Conidia were produced in 125-ml Delong flasks containing 30 ml of solid complete medium. The flasks were incubated at 35°C for the first 48 h and at 25°C for the remainder of the growth time (33). Conidia were usually harvested after a total of 7 days of incubation and then stored in water at 4°C.

Buffers and solutions. The following buffers and solutions were used: (i) TEBS, consisting of 0.01 M tris(hydroxymethyl)aminomethane (Tris), 0.05 M ethylenediaminetetraacetate, 0.05% (wt/vol) bentonite, 0.5% (wt/vol) sodium dodecyl sulfate (pH 7.0 at 4°C); (ii) TPES (13), consisting of 0.036 M tris(hydroxymethyl)aminomethane, 0.03 M NaH_2PO_4, 0.001 M ethylenediaminetetraacetate, 0.2% (wt/vol) sodium dodecyl sulfate (pH 7.8 at 25°C); (iii) scintillation fluid, consisting of 0.4% (wt/vol) 2,5-diphenyloxazole, 0.01% (wt/vol) 1,4-bis-2(4-methyl-5-phenyloxazolyl)-benzene, 25% (vol/vol) Triton X-114 (Rohm and Haas, Philadelphia, Pa.),

and 75% (vol/vol) xylenes. The [5-^{3}H]uridine (8 Ci/mmol) and L-[*methyl*-^{3}H]methionine (11 Ci/mmol) used for labeling cultures were obtained from Schwarz/Mann, Orangeburg, N.Y., and New England Nuclear Corp., Boston, Mass., respectively.

RNA analysis by acrylamide gel electrophoresis. Total cell RNA was prepared as described by Russell et al. (21). The RNA was dissolved in TPES buffer and then analyzed by acrylamide gel electrophoresis in cylindrical gels that were formed in Plexiglas tubing (100 by 6.5 mm) according to Bishop et al. (2). High-molecular-weight RNA species were separated in 2.6% (wt/vol) acrylamide gels containing 0.5% (wt/vol) agarose for the experiments with the wild type, and in 2.4% (wt/vol) acrylamide gels containing 1.0% agarose for the experiments with strain *met-1*. Low-molecular-weight RNAs were separated in 7.5% (wt/vol) acrylamide gels.

Electrophoresis was carried out at room temperature using the TPES buffer system. The gels were polymerized in electrophoresis buffer without sodium dodecyl sulfate using *N,N,N',N'*-tetramethylethylenediamine (0.033 ml/g of acrylamide) and freshly dissolved ammonium persulfate (10% wt/vol in water, 0.33 ml/g of acrylamide) as polymerization catalysts. Before the samples were applied, gels were subjected to electrophoresis for 30 min at 5 mA/gel to displace the polymerization catalysts from the gel origin and to introduce sodium dodecyl sulfate into the gels. After this, a sample of the purified RNA, containing not more than 25 μg of RNA, was layered on the top of the gel, and electrophoresis was carried out at 5 mA/gel for 3 to 5 h for the dilute gels, and for 3 h in the case of the 7.5% gels.

At the conclusion of electrophoresis the gels were removed from the tubes and scanned at 260 nm using a recording spectrophotometer (Beckman model 25) equipped with a gel scanner accessory. The gels were fractionated into slices, and the RNA in each slice was hydrolyzed in 0.25 ml of 0.3 N Protosol (New England Nuclear Corp.) containing 5% (vol/vol) water. Radioactivity in each slice was determined in the xylene-based scintillation fluid.

RESULTS

Identification of high-molecular-weight RNA species. The ribosomes of *N. crassa* contain two high-molecular-weight RNA species, namely, the 25*S* and 17*S* RNAs found in the 60*S* and 37*S* ribosomal subunits, respectively (5). These RNAs were readily identified in phenol extracts of *Neurospora* mycelia, and their molecular weights were estimated by polyacrylamide gel electrophoresis (12, 13). Using *Escherichia coli* 23*S* (1.08×10^6 daltons [1.08 Mdal]) and 16*S* (0.56 Mdal) rRNA's as standards (24), we calculated that the 25*S* rRNA was 1.27 Mdal and the 17*S* rRNA was 0.70 Mdal.

Before studying the synthesis of these rRNA's in detail, we identified and characterized the putative precursors to these molecules. To identify the major pre-rRNA species, we took advantage of the fact that rRNA's and their precursors are methylated (25) and may be labeled selectively in the presence of [*methyl*-^{3}H]methionine. To ensure that sufficient label would be incorporated into RNA we used a methionine-requiring mutant strain grown under conditions of limiting methionine. A 14-h culture of this strain was incubated with [*methyl*-^{3}H]methionine for 6 min at room temperature, and the RNAs were extracted, purified, and displayed on an acrylamide gel. There are four high-molecular-weight RNA species that become labeled under these conditions (Fig. 1). The two fastest-migrating RNAs are the mature 1.27-Mdal and 0.7-Mdal species. The other two species are 2.4 Mdal and 1.4 Mdal and were tentatively identified as precursors to the mature rRNA's. That these four bands represent distinct species of RNA was shown by the fact that treatment of [5-^{3}H]uridine-labeled RNA (see following section) for 10 min at 60°C, in the presence or absence of 4 M urea, did not cause an alteration in the migration of, nor the disappearance of, any of the bands. (It is interesting to note that this treatment did not result in the dissociation of the 25*S* rRNA into pieces as was described by Lucas and Vanderhoef (15).) None of the four RNAs is degraded by treatment with deoxyribonuclease.

Labeling kinetics of high-molecular-weight

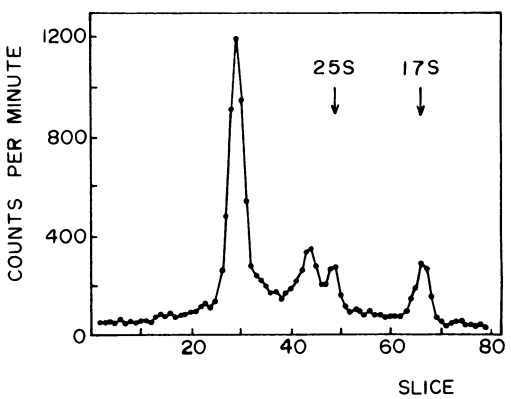

FIG. 1. *Incorporation of [methyl-^{3}H]methionine into high-molecular-weight RNAs. A culture of the met-1 strain was grown for 14 h at 25°C, and then [methyl-^{3}H]methionine was added to a final concentration of 20 μCi/ml. After 6 min at 25°C, the culture was harvested, and the RNAs were extracted. A sample containing 25 μg of RNA was analyzed on a 2.4% acrylamide-1% agarose gel by electrophoresis for 3.25 h at 5 mA/gel at room temperature as described. The gel was scanned at 260 nm to determine the positions of the mature 25S and 17S rRNA's, fractionated into 1.1-mm-thick slices, and processed for counting.*

rRNA species. The labeling kinetics of the high-molecular-weight RNA species were examined by pulse-labeling 14-h cultures of the wild type for various periods of time at 25°C with [5-^{3}H]uridine. The RNAs were extracted, purified, and analyzed by polyacrylamide gel electrophoresis. By 6 min after labeling commenced, three species of RNA are labeled (Fig. 2). One of these migrates coincidentally with mature 0.70-Mdal rRNA, whereas the other two correspond to the 2.4-Mdal and 1.4-Mdal RNA species described previously. At this time very little labeled mature 1.27-Mdal rRNA is present. After 12 and 18 min of labeling, all four large RNA species are labeled, with most of the label in the 1.4-, 1.27-, and 0.70-Mdal species. After 24 min, most of the label is present in the mature 1.27 and 0.70 Mdal rRNA's.

The kinetics of labeling of the four large RNAs of wild-type *Neurospora* were analyzed by summing the tritium counts of the three to five gel fractions around each peak, normalizing the values for each time point to the quantity of RNA applied to the gel, and graphing the values obtained against time (Fig. 3). It is apparent that the 2.4-Mdal RNA is the first to become labeled, and maximal radioactivity for this species is reached after 9 to 10 min. The 1.4- and 0.70-Mdal RNAs are the next species to become labeled. Their labeling kinetics are similar for the first 12 min, showing a low level of radioactivity during the next 9 min. The radioactivity in 1.4-Mdal RNA reaches a maximum at 12 min, whereas the amount of label incorporated into the 0.70-Mdal RNA continues to increase. The 1.27-Mdal RNA shows an initial lag of at least 6 min during which little radioactivity is incorporated. After that time the labeling of this RNA is rapid, and is approximately linear after 12 min of labeling. In addition, during the course of this experiment, the radioactivity in the culture medium was mea-

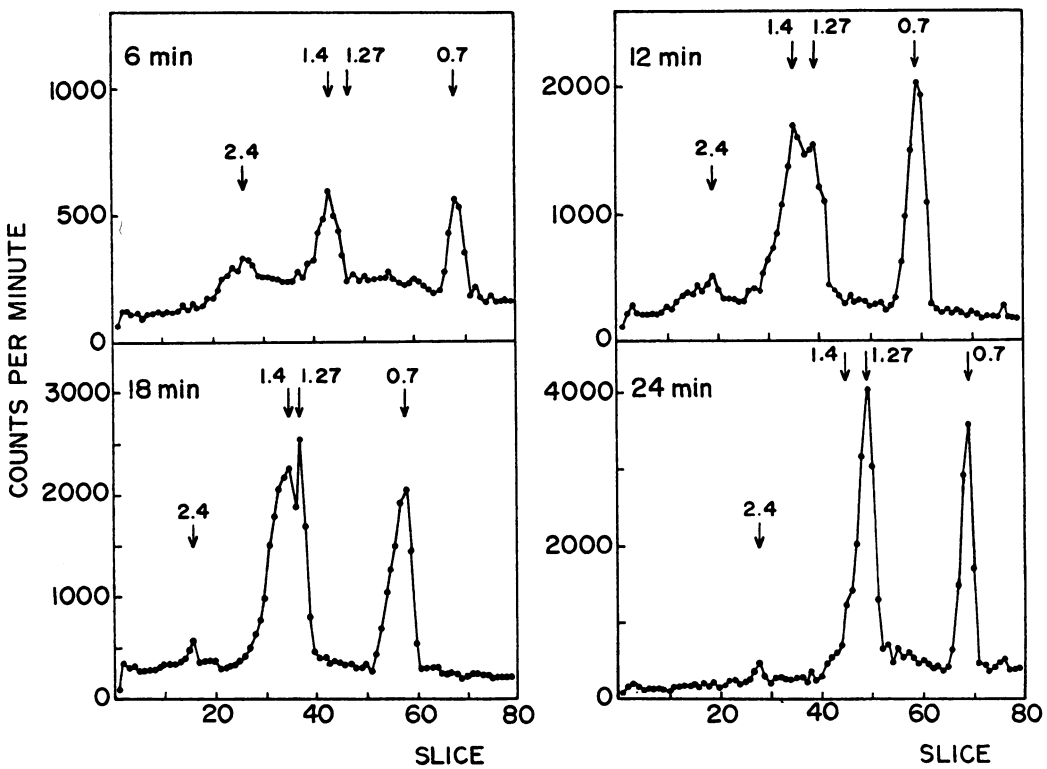

FIG. 2. *Incorporation of [5-^{3}H]uridine into high-molecular-weight RNAs of wild type at 25°C. Cultures of the wild type were grown for 14.5 h at 25°C, and then [5-^{3}H]uridine was added to a final concentration of 4 μCi/ml. After various labeling times, the cultures were harvested and the RNAs were extracted. Samples (containing up to 25 μg of RNA) were analyzed on 2.6% acrylamide-0.5% agarose gels by electrophoresis for 5 h at 5 mA/gel at room temperature as described. The gels were scanned at 260 nm to determine the positions of the mature 25S and 17S rRNA's, fractionated into 0.95-mm slices, and processed for counting. The positions of the 2.4-, 1.4-, 1.27-, and 0.7-Mdal RNA species are indicated by arrows.*

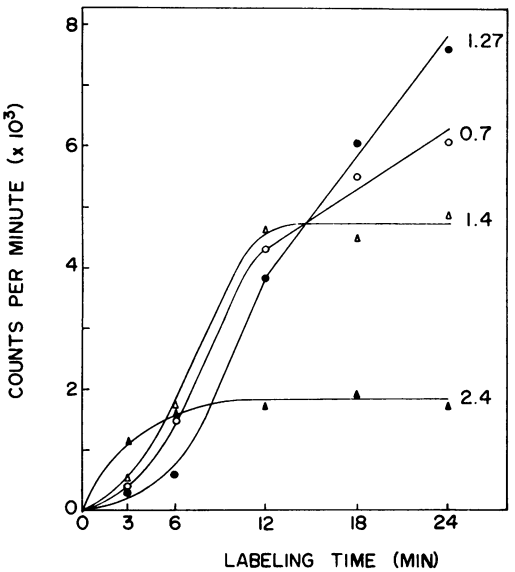

FIG. 3. *Summary of kinetics of synthesis of precursor and mature high-molecular-weight RNAs of wild type at 25°C. Using the data from Fig. 2, and additional time points not shown, the amount of radioactivity in each of the RNA species was determined. The values were normalized with respect to the amount of total RNA applied to the gels.*

sured. The data obtained indicated that there was an excess of the labeled precursor present throughout the labeling period, and thus exhaustion of the endogenous tritiated uridine triphosphate pool was not a factor in this experiment.

These results suggest that the 2.4-Mdal RNA is the precursor to both 1.27- and 0.70-Mdal RNA. The close similarity in the kinetics of labeling of the 1.4- and 0.70-Mdal RNAs, and the relatively long lag period before rapid labeling kinetics are seen for the 1.27-Mdal RNA, suggests the following pathway for synthesis of mature rRNA's in *Neurospora*. The first pre-rRNA to become labeled in detectable quantities is the 2.4-Mdal molecule, which is then cleaved to produce simultaneously the 0.70- and 1.4-Mdal species. The former is the mature 17*S* RNA found in the 37*S* cytoplasmic ribosomal subunit. Subsequent cleavage of the 1.4-Mdal RNA produces the 1.27-Mdal RNA, which is the mature 25*S* rRNA of the 60*S* ribosomal subunit.

Precursor-product relationships of high-molecular-weight RNAs determined by pulse-chase experiments. To verify the proposed precursor-product relationships between 2.4-Mdal RNA and the mature rRNA's, pulse-chase experiments were done. Electrophoretograms of a representative pulse-chase experiment are shown in Fig. 4. The kinetics of labeling of the four large RNAs were analyzed as for the pulse-labeling experiments (Fig. 5). Although the procedures used did not eliminate the incorporation of label into newly synthesized RNA during the chase, the results do permit meaningful conclusions to be made. The results show that, as the amount of radioactivity in the 2.4-Mdal RNA decreases, there is a rapid increase in the radioactivity found in the 1.4- and 0.70-Mdal RNAs. After approximately 6 min of chase, the amount of radioactivity in the 1.4-Mdal species reaches a maximum. This occurs at the same time as the amount of radioactivity in the 1.27-Mdal RNA starts to increase rapidly. After the 6-min time point, the amount of radioactivity in the 1.4-Mdal RNA begins to decline and only the mature rRNA's show an increase in the amount of radioactivity incorporated. These results reinforce the hypothesis presented earlier that the 2.4-Mdal RNA is the precursor to the mature 1.27- and 0.70-Mdal rRNA's. Production of the 1.27-Mdal RNA proceeds via a 1.4-Mdal intermediate, whereas the 0.70 is apparently a direct cleavage product.

Labeling kinetics of low-molecular-weight RNAs. In addition to 1.27-Mdal RNA, the 60*S* ribosomal subunit of eukaryotes also contains two low-molecular-weight rRNA's, termed 5.8*S* and 5*S*. The 5.8*S* RNA sequence has been shown to be part of the primary transcript of the rRNA genes for all eukaryotes that have been examined. During the processing events, 5.8*S* RNA is released and is found associated with the large RNA of the 60*S* subunit. By contrast, 5*S* RNA is transcribed from genes that are not usually near the genes for the other rRNA's, and becomes associated with preribosomal particles in the nucleolus. Thus it was of interest to compare the kinetics of labeling of the 5.8*S* and 5*S* RNAs with those of the mature rRNA's in *Neurospora*.

Recently Lucas and Vanderhoef (15) showed that 5.8*S* RNA is hydrogen bonded to 25*S* rRNA in *Neurospora* and that it can be released by heat treatment. We have found that treatment of RNA samples for 10 min at 60°C in the presence of of 4 M urea is an effective method for releasing 5.8*S* RNA. Thus, RNA samples prepared from the pulse-labeled cultures described earlier (Fig. 2 and 3) were treated under these conditions, and the low-molecular-weight RNAs (5.8*S*, 5*S*, and 4*S* transfer RNA) were analyzed by electrophoresis on 7.5% acrylamide gels (Fig. 6). Assuming molecular weights of 25,000 and 40,000 for *Neurospora* 4*S* and 5*S* RNA, respectively (5), we

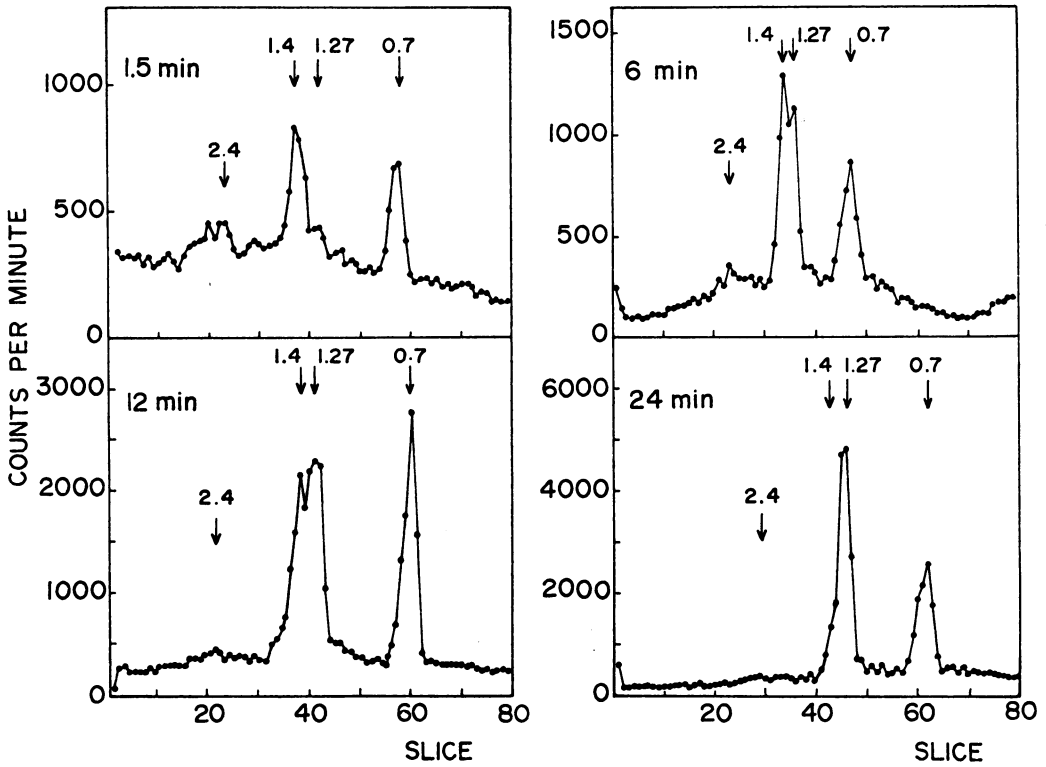

FIG. 4. *Precursor-product relationships of the high-molecular-weight RNAs of the wild type examined by pulse-chase at 25°C. Cultures of the wild type were grown for 14.5 h at 25°C, and then [5-³H]uridine was added to a final concentration of 10 μCi/ml. After 4.5 min of labeling, the cultures were collected on filters, washed rapidly and extensively first with minimal medium and then with minimal medium containing 460 μg of unlabeled uridine per ml, and then resuspended in minimal medium containing 460 μg of unlabeled uridine per ml. This represented a 1,500-fold excess of unlabeled uridine over the amount of uridine present during the labeling period. Cultures were harvested after various chase times, the RNAs were extracted, and samples were analyzed by electrophoresis as described in the legend for Fig. 2.*

calculated the molecular weight of *Neurospora* 5.8S RNA to be 61,000.

The kinetics of labeling of the low-molecular-weight RNAs were analyzed as before, and are summarized in Fig. 7. The results indicate that there is a lag of approximately 3 min before rapid synthesis of labeled transfer RNA occurs. The results also show that, whereas the synthesis of labeled 5S RNA begins immediately, there is a lag of 6 to 12 min in the appearance of labeled 5.8S RNA. This lag is comparable to that observed for the appearance of mature 25S rRNA (Fig. 3). These results are consistent with the hypothesis that the 25S and 5.8S rRNA's have a common precursor.

DISCUSSION

Pulse-labeling and pulse-chase experiments were done to study the synthesis of mature rRNA's in wild-type *N. crassa* growing exponentially at 25°C. [5-³H]uridine rather than [*methyl*-³H]methionine was used as the radioactive precursor for the following reasons: (i) we wanted to compare the kinetics of synthesis of the large RNAs with those of the small RNAs and, if the situation is the same in *Neurospora* as in yeast, 5S and 5.8S RNAs are unmethylated (26, 30); (ii) newly synthesized RNAs are often effectively labeled with [*methyl*-³H]methionine by growing methionine-requiring strains under conditions of limiting methionine (10, 19, 20, 28). We wished to study rRNA synthesis under normal physiological conditions, and it is clear that methionine starvation can cause gross physiological changes. In particular, Somberg et al. (23) have shown that, in a *met*⁻ strain of *N. crassa* starved for methionine, there is extensive degradation of RNA in addition to the cessation of RNA and protein synthesis.

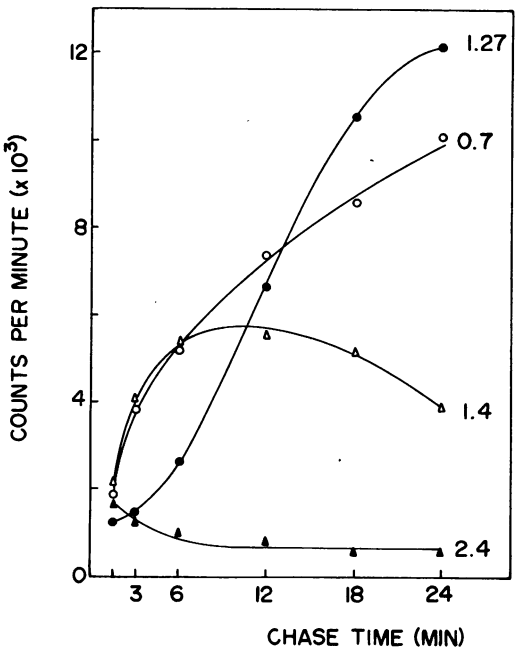

FIG. 5. *Summary of the [5-³H]uridine pulse-chase experiment. The data were derived from the pulse-chase experiment (Fig. 4 and additional time points not shown) as described in the legend to Fig. 3.*

The data obtained from the labeling experiments with [5-³H]uridine suggest that the sequence of steps shown in Fig. 8 occurs in the biosynthesis of rRNA's in wild-type *Neurospora* growing exponentially at 25°C. The topography of the mature rRNA sequences in the 2.4-Mdal pre-rRNA is presented as 5'-17*S*-25*S*-3' by analogy with the arrangement demonstrated in, for example, *S. carlsbergensis* (26) and Novikoff rat tumor cells (11). The first pre-rRNA to become labeled in detectable quantities in *Neurospora* is the 2.4-Mdal molecule, which is cleaved to produce the 0.7- and 1.4-Mdal RNAs. The former is the 17*S* rRNA of the 37*S* ribosomal subunit, and the latter is a precursor to the 1.27-Mdal (25*S*) and 61,000-dalton (5.8*S*) RNAs of the 60*S* ribosomal subunit. By analogy with yeast (27), these processing events are presumably occurring in the nucleolus in complex ribonucleoprotein particles. During the processing of the 2.4-Mdal pre-rRNA to produce the mature rRNA's, approximately 15 to 20% of the molecule is nonconserved. This value is similar to that found for pre-rRNA processing in *S. cerevisiae* (28), *Neurospora* mitochondria (9), *Xenopus* and higher plants (14), and the housefly (8).

Concerning the low-molecular-weight RNA species, our results suggest that, as in other eukaryotes that have been examined, 5*S* rRNA is not transcribed as part of the pre-rRNA molecule. That this RNA is not covalently bound to the other RNAs of the 60*S* subunit is shown by the fact that it is released from the ribosome during the RNA extraction treatment. 5.8*S* rRNA is known to be noncovalently bound to 25*S* rRNA in the 60*S* ribosomal subunit of *Neurospora* (15). We have calculated its molecular weight to be 61,000. This value is very similar to that reported for yeast 5.8*S* rRNA (28), but differs from the 55,000 value reported by Lucas and Vanderhoef (15) for *Neurospora* 5.8*S* rRNA. Differences in the electrophoresis conditions may be responsible for this discrepancy.

In the continuous labeling experiment, the labeling of the 2.4-Mdal and 1.4-Mdal precursors reached maximal values. When these values are corrected for the molecular weights of the two RNAs, the relative pool sizes of the two precursors may be determined (Table 1). These data show that the pool size of 1.4-Mdal RNA is approximately 4.5 times that of the 2.4-Mdal precursor. Similar calculations for the possibly homologous RNAs in *S. carlsbergensis* protoplasts growing at 15°C (26) and in *S. cerevisiae* protoplasts growing at 23°C (28) give pool size ratios of approximately 2 and 3, respectively. The differences in the relative pool sizes may be the result of either different physiological states of the three organisms under the growth conditions used, or different rate constants of cleavage at different points in the processing pathway, or perhaps a combination of these two factors. Alternatively, the RNAs in question may not be homologous.

Altogether, it is apparent that the synthesis and processing of pre-rRNA in wild-type *N. crassa* is similar, in general, to those events in other eukaryotic organisms studied to date, including mammalian cells (17, 31, 32), *Xenopus* (14, 31), *Rana* (6), insects (8), and yeasts (26, 28, 29). In addition, the production of the rRNA's of *Neurospora* cytoplasmic ribosomes is very similar to the production of *Neurospora* mitochondria rRNA's (9). However, it is noteworthy that no immediate precursor of 17*S* rRNA is observed in *Neurospora* under the growth conditions used. A precursor to the mature rRNA of the small ribosomal subunit has been reported in yeast (26, 28, 29) and in other eukaryotic systems, for example, the housefly (8) and HeLa cells (31, 32). However, such a precursor has not been seen in mouse L cells (31, 32), in *Xenopus* (14, 31), or in plants (14). At least in mammalian cells, the presence or absence of the precursor to 18*S* rRNA may re-

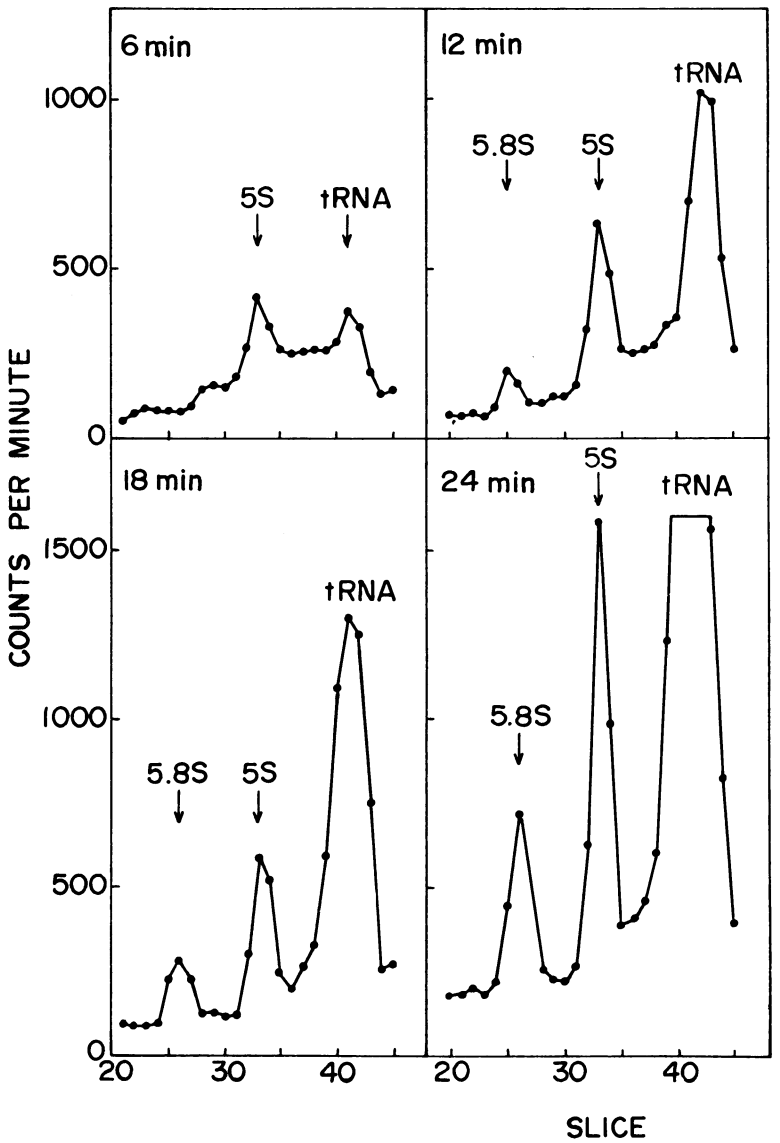

FIG. 6. *Incorporation of [5-^{3}H]uridine into low-molecular-weight RNAs of wild type at 25°C. Samples of RNA from the experiment described in the legend to Fig. 2 were brought to 4 M urea and analyzed on 7.5% acrylamide gels by electrophoresis for 3 h at 5 mA/gel at room temperature as described. The gels were scanned at 260 nm to determine the positions of mature 4S, 5S, and 5.8S RNAs, fractionated into 1.3-mm slices, and processed for counting.*

flect alternate rRNA maturation pathways resulting from different physiological states (32). Therefore, the apparent absence of an immediate precursor to 17S rRNA in *Neurospora* under the growth conditions used does not preclude the existence of such an RNA, either in small amounts under these conditions, or in large amounts in different physiological conditions.

In conclusion, we have demonstrated that the synthesis and processing of pre-rRNA in wild-type *N. crassa* growing exponentially at 25°C occurs by a pathway similar to that shown for other eukaryotic organisms. The elucidation of these steps now makes it possible to examine mutant strains with conditional lesions in rRNA synthesis for defects in the processing pathway.

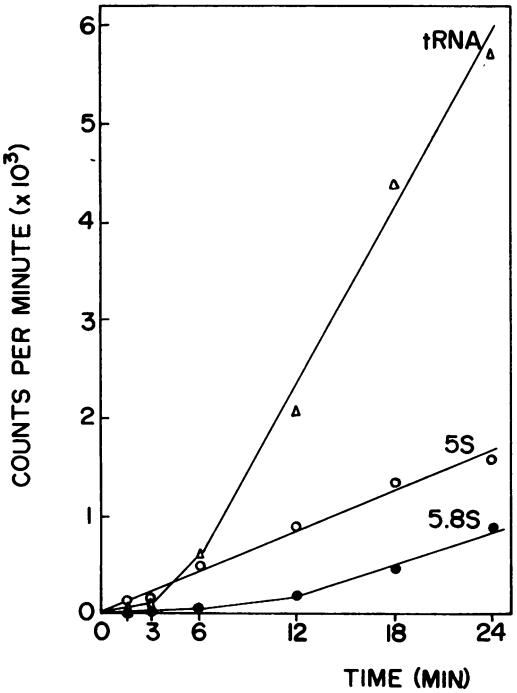

FIG. 7. *Summary of kinetics of synthesis of low-molecular-weight RNAs of wild type at 25°C. The amount of radioactivity in 4S, 5S, and 5.8S RNA at the various time points was determined as described in the legend to Fig. 3.*

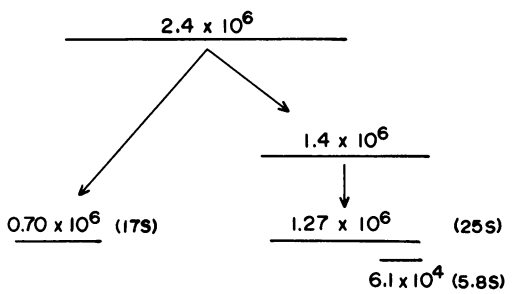

FIG. 8. *Proposed scheme for rRNA synthesis in wild-type N. crassa growing at 25°C.*

TABLE 1. *Relative cellular amounts of 2.4-Mdal and 1.4-Mdal precursor RNAs*

RNA species	Amt of radioactivity (cpm)[a]	Relative cellular amt[b]
2.4-Mdal	1,800	1
1.4-Mdal	4,700	4.5

[a] The plateau values of labeling of the two RNAs were derived from the data presented in Fig. 3.

[b] To obtain the relative pool sizes of the two precursors, the values in the second column were corrected for the molecular weights of the two RNA species relative to the molecular weight of 2.4×10^6 RNA.

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