MOLECULAR AND CELLULAR BIOLOGY, Mar. 1981, p. 199-207
0270-7306/81/030199-09$02.00/0

Vol. 1, No. 3

Heat-Sensitive Mutant Strain of *Neurospora crassa*, 4M(t), Conditionally Defective in 25S Ribosomal Ribonucleic Acid Production

MELANIE W. LOO,[1]† NANCY S. SCHRICKER,[2] AND PETER J. RUSSELL[2]*

Department of Genetics, University of Washington, Seattle, Washington 98195,[1] and Biology Department, Reed College, Portland, Oregon 97202[2]

A heat-sensitive mutant strain of *Neurospora crassa*, 4M(t), was studied in an attempt to define its molecular lesion. The mutant strain is inhibited in conidial germination and mycelial extension at the nonpermissive temperature (37°C). Macromolecular synthesis studies showed that both ribonucleic acid (RNA) and protein syntheses are inhibited when 4-h cultures are shifted from 20 to 37°C. Density gradient analysis of ribosomal subunits made at 37°C indicated that strain 4M(t) is deficient in the accumulation of 60S ribosomal subunits in that the ratio of 60S/37S subunits was 0.29:1 compared with 1.6:1 for the parental strain. This phenotype was shown to be the result of a slow rate of processing of, and a deficiency in the amount of, the immediate precursor to 25S ribosomal RNA (the large RNA of the 60S subunit) in the sequence of events constituting the production of mature ribosomal RNAs from the primary transcript of the ribosomal deoxyribonucleic acid, the precursor ribosomal RNA molecule. Analysis of polysomes suggested that the heat-sensitive gene product might function in both the assembly and the function of the 60S ribosomal subunit, since there was a smaller proportion of newly made 60S subunits synthesized at 37°C in the polysome region of the gradients than in the monosome-plus-subunit region. The ribosomal RNA processing defect is apparently responsible for the observed defects in germination and macromolecular synthesis at 37°C, but the precise molecular lesion is not known. On the basis of these results, the heat-sensitive mutant allele in the 4M(t) strain is considered to define the *rip1* (ribosome production) gene locus.

The study of mutant strains in both procaryotes and eucaryotes has been very useful for obtaining information about metabolic processes in the cell. Certain cell processes, such as nucleic acid and protein syntheses, are controlled by indispensable genes, and the only mutations that can be isolated in these genes are conditional mutations, that is, heat-sensitive and cold-sensitive mutants. In this paper we present the results of molecular studies of a heat-sensitive mutant strain of *Neurospora crassa* which is temperature sensitive for conidial (asexual spore) germination and mycelial extension. From studies of molecular labeling patterns, of ribosome and ribonucleic acid (RNA) production, and of polysome distributions, evidence was obtained that the heat-sensitive strain has a conditional defect in ribosomal RNA (rRNA) synthesis in that 25S rRNA is produced in a stoichiometrically lower amount relative to 17S rRNA at the nonpermissive temperature (37°C). As a consequence, the mutant strain exhibits a relative underaccumulation of 60S ribosomal subunits at that temperature compared with the parental strain.

† Present address: Department of Biological Sciences, California State University at Sacramento, Sacramento, CA 95819.

MATERIALS AND METHODS

Strains and culture techniques. The wild-type strain of *N. crassa* was 74A. The inositol-requiring strain was isolation no. 89601 and was stock 497 from the Fungal Genetics Stock Center, Humboldt State University Foundation, Arcata, Calif. Strains heat sensitive for conidial germination and mycelial extension were isolated by ultraviolet light mutagenesis of the *inl* strain followed by the inositolless death enrichment technique (9). One of these heat-sensitive strains, 4M(t), is the subject of this paper. An inositol-independent derivative of 4M(t), called PJ32251, was also used. The heat-sensitive mutation carried by these two strains is *1(t)*. This mutation is considered to lie in the *rip1* locus (*rip* = ribosome production). The locus was named on the basis of the molecular data presented in this paper.

The culture techniques and Vogel's growth media used were those described by Schlitt and Russell (21). Minimal medium supplemented with 50 μg of inositol ml^{-1} (inositol medium) was used for growth of strain 4M(t) and the *inl* strain.

Radioactive labeling of RNA and protein. Continuous and pulse-labeling experiments were done as described by Loo (11). RNA was labeled with [5-^{3}H]uridine or [2-^{14}C]uracil, and protein was labeled with L-[4,5-^{3}H]leucine. In the pulse-labeling experiments, the labeling period was 10 min.

Polysome profiles. The procedure of Mirkes (15) was used, with minor changes as described by Loo (11). In brief, mycelial homogenates were clarified by centrifugation, and samples were applied to linear sucrose gradients in a Beckman SW41 rotor. Gradients were centrifuged for 90 min at 35,000 rpm (150,000 × *g*; r_{av} = 10.93 cm) or for 60 min at 40,000 rpm (196,000 × *g*; r_{av} = 10.93 cm). Gradients were collected by upward displacement on a cushion of sucrose. To obtain absorbancy profiles at 260 nm, the gradients were passed through a flow cell of a Gilford recording spectrophotometer. When necessary, fractions were collected, and the radioactivity in each was determined by liquid scintillation counting. Percentages of monosomes and polysomes were determined as the average of at least two tracings of the chart recordings with a planimeter.

Ribosomal subunit analysis. Mycelial homogenates were prepared as for polysome profiles (see above), and samples were analyzed by centrifugation in linear 5 to 30% (wt/wt) sucrose gradients made in a 0.05 M triethanolamine, pH 7.3, buffer containing 0.1 M KCl. Gradients were centrifuged for 4 h at 40,000 rpm in a Beckman SW41 rotor (196,000 × *g*; r_{av} = 10.93 cm) to resolve the 60S and 37S ribosomal subunits. Fractionation was as described for polysome gradients.

RNA extraction. RNA extraction was done as described by Loo (11) and Mirkes (15). In outline, the samples were extracted with phenol-chloroform-isoamyl alcohol (48:48:4) and saturated with a 0.01 M sodium acetate, pH 6.0, buffer containing 0.1 M Nacl and 0.001 M ethylenediaminetetraacetate, and then the RNA was precipitated with ethanol. The RNA was dissolved in TENS buffer [0.01 M tris(hydroxymethyl)aminomethane-hydrochloride, pH 7.5, containing 0.01 M ethylenediaminetetraacetate, 0.1 M NaCl, and 0.5% (wt/vol) sodium dodecyl sulfate] for density gradient analysis and in TPES buffer (20) [0.036 M tris(hydroxymethyl)aminomethanehydrochloride, pH 7.8, containing 0.03 M NaH_2PO_4, 0.001 M ethylenediaminetetraacetate, and 0.2% (wt/vol) sodium dodecyl sulfate] for electrophoretic analysis.

RNA analysis. (i) Density gradients. Samples of RNA in TENS buffer were layered on a linear, 12-ml, 5 to 20% (wt/wt) sucrose gradient made up in TENS buffer. Centrifugation was at 20°C for 16 h at 22,000 rpm in a Beckman SW41 rotor (59,200 × *g*; r_{av} = 10.93 cm). Gradients were fractionated as described for polysome analysis. Fractions of labeled RNA were precipitated with cold 5% trichloroacetic acid, using 0.1 mg of bovine serum albumin per fraction as a carrier. Radioactivity was determined by liquid scintillation spectrometry.

(ii) Acrylamide gels. High-molecular-weight RNA species were separated in cylindrical 2.4% (wt/vol) agarose and 0.2% (wt/vol) sodium dodecyl sulfate prepared in TPES buffer as described previously (20). The electrophoresis running buffer was TPES buffer. Before the samples were applied, gels were subjected to electrophoresis for 30 min at 5 mA per gel to displace the polymerization catalysts from the gel origin and to introduce sodium dodecyl sulfate into the gel. After this, a 20-μg sample of the purified RNA in TPES buffer was layered on top of the gel, and electrophoresis was carried out at 3 mA per gel for 8 h.

At the conclusion of electrophoresis the gels were removed from the tubes, and, to establish the positions of mature 25S and 17S rRNA's, they were scanned at 260 nm with a recording spectrophotometer. The gels were fractionated into 1-mm slices, using a Mickle gel slicer, 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 by liquid scintillation spectrometry.

RESULTS

Heat-sensitive mutants of *N. crassa* were isolated by mutagenesis of the *inl* strain followed by inositolless death enrichment (9). The heat-sensitive strain, 4M(t), and its *inl*$^{+}$ derivative, PJ32251, show no conidial germination at 35°C or above and a somewhat slower rate of germination compared with the parental *inl* strain at permissive temperatures (25°C or below). Cultures of the heat-sensitive strains initiated at the permissive temperature and shifted to the nonpermissive temperature have an extremely low (almost zero) mycelial growth rate. In comparison, 35°C is the optimal conidial germination and mycelial growth temperature for the parental strain. However, cells of the heat-sensitive strains are not killed by the exposure to the higher temperature since conidial germination or growth proceeds normally when cultures are shifted from 37 to 25°C or below. The biochemical properties of the mutant strain are described below.

Macromolecular synthesis. Continuous-labeling experiments were done with germinating conidial cultures to determine whether strain 4M(t) exhibited a defect in macromolecular synthesis. When incubated directly at 37°C for the first 2 h of germination, conidia of the mutant strain accumulated approximately two times more labeled RNA and protein than at 20°C (Fig. 1). This was very similar to the relative accumulation of these macromolecules observed in the *inl* parental strain (data not shown). In contrast, when a culture of 4M(t) was shifted to 37°C after 4 h of incubation at 20°C, the ratios of labeled macromolecules accumulated at 37 and 20°C at 2 h postshift (i.e., at 6 h) were 1.2:1 for RNA and 1.5:1 for protein (Fig. 1). In comparison, the parental strain accumulated approximately 2.5 times as much RNA and protein in the 2 h after the shift to 37°C than in the 2 h at 20°C.

Ribosomal subunit synthesis. Next, the

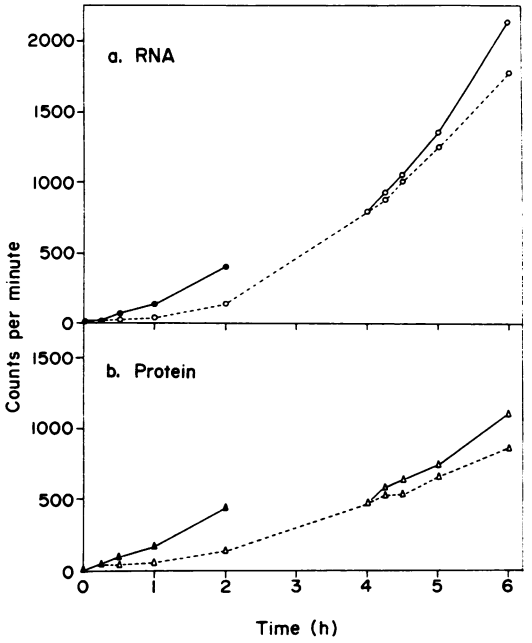

FIG. 1. *RNA (a) and protein (b) synthesis in germinating cultures of strain 4M(t). At time zero, a 40-ml culture was labeled with [^{14}C]uracil (4 μCi; 10 μM) and [^{3}H]leucine (4 μCi; 10 μM). Part of the culture was incubated directly at 37°C; part was shifted to 37°C after 4 h of incubation at 20°C. A control culture was maintained at 20°C throughout. Samples were taken at time intervals to determine the incorporation of label into RNA and protein as described in the text. Symbols: RNA synthesis (●) and protein synthesis (▲) at 37°C; RNA synthesis (○) and protein synthesis (△) during incubation at 37°C after 4 h of preincubation at 20°C; RNA synthesis (○----○) and protein synthesis (△----△) in the control culture incubated at 20°C.*

mutant strain was studied to see whether ribosomal subunit synthesis was normal at 37°C. Logarithmic cultures were shifted up from 20 to 37°C and labeled after the shift with radioactive RNA precursors. Crude cell extracts were centrifuged in gradients under conditions to dissociate ribosomes into subunits and to release messenger RNA. Fractions were collected to determine the size distributions of RNA made at 37°C, and reference sedimentation values were assigned by comparing the profile of labeled RNA with the absorbancy profile. The results showed that the large (60S) ribosomal subunit was labeled to a much lesser degree than the small (37S) subunit in extracts of strain 4M(t) (Fig. 2). After 15 min of labeling at 37°C, the ratio of label in the 60S subunit to that in the 37S subunit was 0.29:1, whereas the ratio for the parental *inl* strain was 1.6:1. When logarithmic cultures were labeled for longer periods of time at 37°C, the ratio of labeled 60S to 37S subunits approached 2.0:1 in the parental strain and 1.4:1 in the mutant strain.

With this distinct molecular phenotype to monitor, the expression of the heat-sensitive mutation in young conidial cultures was investigated. Conidial cultures were labeled for 1 and 2 h at 37°C, and the ribosomal subunits were analyzed by density gradient centrifugation. In the parental strain the ratio of labeled RNAs in the large and small ribosomal subunits was about 1.5:1 after 1 and 2 h of labeling, whereas in the mutant strain the ratio was 0.3 to 0.4:1 in the same labeling period. This indicated that the molecular defect was expressed in early conidial cultures also.

Analysis of RNA. Ribosomal subunit analysis showed that there was no obvious accumulation of any possible precursor to the 60S subunit in the heat-sensitive mutant strain at 37°C (see Fig. 2). To investigate the possibility, then, that 25S rRNA (the large rRNA of the 60S subunit) was synthesized but not assembled into subunits at 37°C, cultures were labeled as for the ribosomal subunit dissociation studies, and whole-cell RNA was extracted. In addition, to eliminate experimental variations and to compare the RNA made by the parental and heat-sensitive strains at 37°C, cultures of the former were labeled with [^{14}C]uracil and cultures of the latter were labeled with [^{3}H]adenine, the cell homogenates were combined, and RNA was extracted and analyzed by density gradient centrifugation. In this way the relative amounts of stable 25S and 17S rRNA made during 2 h at 37°C were determined by measuring absorbancy

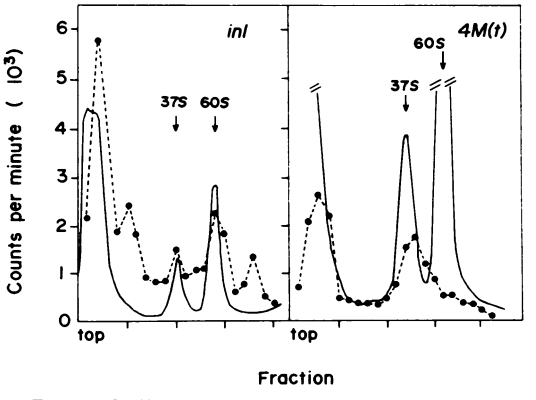

FIG. 2. *Sedimentation profiles of 60S and 37S ribosomal subunits synthesized in logarithmic cultures of the inl strain and strain 4M(t) in 15 min after a shift from 20 to 37°C. Cultures were labeled with [^{3}H] uridine (0.4 μCi ml^{-1}; 29 Ci mmol^{-1}) at the time of the shift. Solid lines represent absorbancy tracing, and dotted lines represent counts per minute in the fractions isolated from the gradients.*

and counts per minute in the fractions, respectively.

The results of these experiments were similar to those of the ribosomal subunit dissociation experiments. There was a significant deficiency of labeled 25S rRNA made at 37°C in early conidial cultures (Fig. 3). In logarithmic cultures there was also a deficiency in labeled 25S rRNA made at 37°C, but it was not as extreme as in conidial cultures (data not shown). No labeled RNA suggestive of any precursor RNA was apparent in the gradient analysis of RNA extracted from either strain.

In view of these results, the kinetics of labeling of 25S and 17S rRNA in the heat-sensitive strain at 25 and 37°C were examined in an attempt to define the RNA synthesis defect in more detail. In eucaryotes, the rRNA genes are transcribed into a high-molecular-weight ribosomal precursor RNA (pre-rRNA) which is then cleaved to produce all of the mature rRNA's except 5S rRNA (see, for example, 13, 18). In wild-type *N. crassa* the synthesis and processing of pre-rRNA is similar to that observed in other eucaryotes (20). From continuous-labeling and pulse-chase experiments it was shown that the first RNA molecule to be synthesized is a 2.4-mega-dalton (Mdal) pre-rRNA, and this is then cleaved to produce 0.7- and 1.4-Mdal RNA species. The former is the mature 17S rRNA of the 37S ribosomal subunit, and the latter is subsequently cleaved to produce the mature 1.27 (25S)- and 0.055 (5.8S)-Mdal rRNA's. As in other eucaryotes, 5S rRNA of *N. crassa* is not transcribed as part of the 2.4-Mdal pre-rRNA molecule.

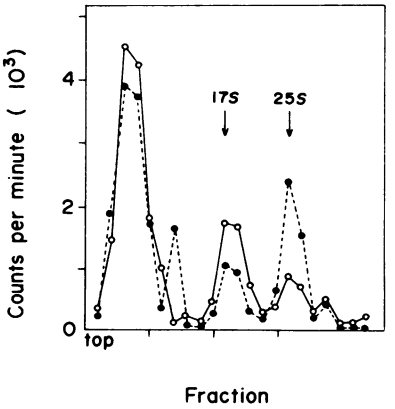

FIG. 3. *Sedimentation profiles of extracted RNA made during 2 h of conidial germination at 37°C by the inl strain (●----●) and strain 4M(t) (○). The inl strain was labeled with [^{14}C]uracil (2 μCi ml^{-1}; 60 mCi mmol^{-1}), and strain 4M(t) was labeled with [^{3}H]adenine (10 μCi ml^{-1}; 17 Ci mmol^{-1}). Cell lysates were extracted and centrifuged together in sucrose gradients as described in the text. The positions of 25S and 17S are indicated by arrows.*

The labeling kinetics of the high-molecular-weight RNA species in the non-inositol-requiring heat-sensitive strain was determined by pulse-labeling midlogarithmic cultures with [^{3}H]uridine for various periods of time at 25 and 37°C. The RNA was extracted, purified, and then analyzed by sodium dodecyl sulfate-acrylamide gel electrophoresis. The gels were fractionated into slices to analyze for the presence of radioactively labeled 2.4-, 1.4-, 1.27-, and 0.70-Mdal RNA species. Representative electrophoretograms from the 25°C experiments are shown in Fig. 4. The positions of the mature 25S (1.27-Mdal) and 17S (0.70-Mdal) rRNA species were determined by scanning the gels at 260 nm, and the positions of the 2.4- and 1.4-Mdal RNA species were calculated from the observed 25S and 17S positions. At each time point examined, three radioactivity maxima were seen. By 10 min after labeling commenced, the three labeled RNAs were the 2.4-, 1.4-, and 0.70-Mdal species. At this time very little mature 1.27-Mdal rRNA was present. After 14 min, labeled 2.4- and 0.70-Mdal RNAs were seen again, and the third labeled band migrated to a position intermediate between the 1.4- and 1.27-Mdal locations, with a point of maximum radioactivity corresponding to a weight of approximately 1.35 Mdal. After 18 min of labeling the three peaks of radioactivity indicated the predominance of RNA species with weights of 2.4, 1.27, and 0.70 Mdal.

Next, the labeling kinetics of the high-molecular-weight RNA species of the heat-sensitive strain growing at 37°C were determined in a similar way. The mutant strain was grown to midlogarithmic phase at 25°C, equilibrated to 37°C, and then pulse-labeled and analyzed as before, and electrophoretograms of a representative experiment are shown in Fig. 5. The data were quite similar to those of the 25°C experiments with two exceptions. First, there was a radioactivity maximum at 1.4 Mdal at 10 min, but this species appeared to be processed more slowly at 37 than at 25°C since the radioactivity maximum in that region at 18 min was not at 1.27 Mdal but between 1.4 and 1.27 Mdal. Second, there was a deficiency of radioactivity in the 1.27- to 1.4-Mdal region of the gels compared with the amount of radioactivity to the 0.70-Mdal region. It was concluded that the heat-sensitive mutant strain has a conditional defect in pre-rRNA processing such that at 37°C a lower ratio of 25S/17S rRNA results, compared with 25°C cultures of either the mutant or the wild-type strains. There was no evidence for any unusual accumulation of rRNA precursors or the production of any RNA species not previ-

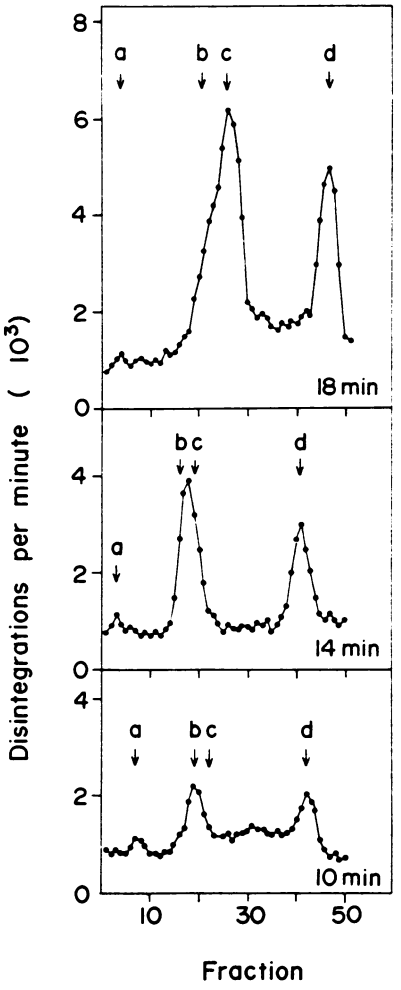

FIG. 4. *Incorporation of [^{3}H]uridine into high-molecular-weight RNA species of heat-sensitive strain PJ32251 at 25°C. A culture of PJ32251 initiated with 5 × 10^6 conidia ml^{-1} was grown to midlogarithmic phase by incubating for 13 h at 25°C, and then [5-^{3}H] uridine was added to a final concentration of 5 μCi ml^{-1}. After the labeling times indicated on the figure, samples of the culture were taken and RNA was extracted. Samples containing 20 μg of RNA were analyzed by electrophoresis as described in the text. The positions of the (a) 2.4-, (b) 1.4-, (c) 1.27-, and (d) 0.70-Mdal RNA species were calculated after the gels were scanned in a spectrophotometer.*

ously determined to be part of the normal pre-rRNA processing scheme (20) in the heat-sensitive strain at either 25 or 37°C.

RNA in polysomes. Since the production of ribosomal subunits requires the association of the pre-rRNA molecule with specific cleavage enzymes, ribosomal proteins, and nonribosomal nucleolar proteins, there were several possible sites for a heat-sensitive defect. To decrease the number of possibilities, studies were made to determine whether or not the ribosomal subunits made at 37°C by strain 4M(t) could function in protein synthesis. Specifically, if the heat-sensitive protein functioned both in producing the 25S rRNA, and hence the 60S subunit, and in translating messenger RNA, then large subunits made at 37°C might appear less frequently in polysomes.

The experiments to be discussed were done in

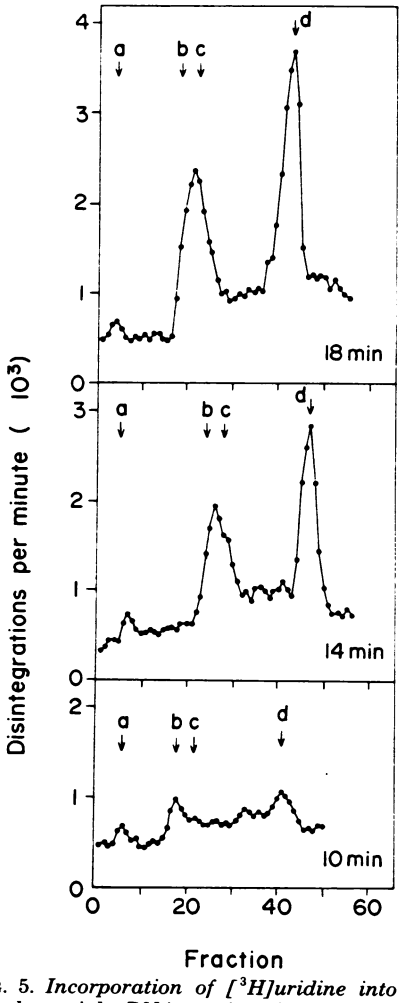

FIG. 5. *Incorporation of [^{3}H]uridine into high-molecular-weight RNA species of the heat-sensitive strain PJ32251 at 37°C. A culture of PJ32251 initiated with 5 × 10^6 conidia ml^{-1} was grown for 9 h at 25°C and then shifted to 37°C. After equilibrating for 60 min at that temperature, [5-^{3}H]uridine was added to a final concentration of 5 μCi ml^{-1}, and RNA was extracted and analyzed as described in the legend to Fig. 4. (a) 2.4-, (b) 1.4-, (c) 1.27-, and (d) 0.70-Mdal RNA positions.*

the following way. Cultures were labeled as in the previously described sedimentation analysis experiments, and whole-cell extracts were centrifuged in density gradients to display polysomes. The gradients were fractionated, and the absorbancy of each fraction was determined. The fractions were pooled into two larger fractions: one contained the material sedimenting as monosomes or smaller, and the other contained the material sedimenting as polysomes. RNA was extracted from each fraction and centrifuged in gradients to determine the ratio of labeled 25S to 17S rRNA.

The polysome absorbancy profiles of extracts from logarithmic cultures of the *inl* strain and strain 4M(t) grown at 20°C and then shifted to 37°C and labeled for 30 min were examined. The mutant strain showed no deficiency in polysomes, although the size distribution of polysomes was slightly larger than was seen for the parental strain. The mutant strain also contained relatively larger amounts of subunits, particularly small subunits.

When extracted RNA from the pooled polysome and monosome-plus-subunit fractions was analyzed on density gradients to determine the distribution of rRNA's (and hence of ribosomal subunits) made at 37°C, the results shown in Table 1 were obtained. In logarithmic cultures, the 25S/17S rRNA ratio in polysomes was significantly lower in strain 4M(t) than in the *inl* strain, whereas the ratio in monosomes plus subunits was the same for both strains. Conidial cultures labeled at 37°C for the first 2 h of incubation were analyzed in the same way. Here, the 25S/17S rRNA ratio was much lower in both fractions in the heat-sensitive mutant strain compared with the parental *inl* strain. Thus, it appeared that in strain 4M(t) at 37°C there were more old small subunits in the subunit region (contributing to the absorbancy) and fewer new large subunits in the polysome region.

TABLE 1. *Ratio of 25S/17S rRNA's in polysomes and in monosomes plus subunits of the heat-sensitive strain, 4M(t), and the parental inl strain, grown under two culture conditions*

Gradient fraction	25S/17S rRNA ratio			
	Logarithmic culture of:		Early conidial culture of:	
	4M(t)	*inl* strain	4M(t)	*inl* strain
Polysomes	0.3:1	1.3:1	0.3:1	0.9:1
Monosomes plus subunits	1.3:1	1.3:1	0.55:1	1.25:1

DISCUSSION

In this paper we have studied a variety of molecular events in a heat-sensitive strain of *N. crassa* that is inhibited in conidial germination and mycelial growth at the nonpermissive temperature. Macromolecular synthesis studies showed that both RNA and protein syntheses were inhibited in the mutant when 4-h cultures were shifted from 20 to 37°C. Pulse-labeling experiments suggested that the biochemical defect in the heat-sensitive strain affected RNA synthesis before protein synthesis (data not presented). Messenger RNA synthesis and the synthesis of at least some proteins persisted at 37°C in the mutant strain, since the degradation of tryptophan to kynureninase could be induced at similar rates in the heat-sensitive and parental strains at 37°C (data not shown).

The major class of RNA transcribed in the cells is rRNA, and hence we compared the ribosomal subunits and the rRNA species made in the heat-sensitive and parental strains at 37°C. Density gradient analysis of ribosomal subunits made at 37°C by logarithmic cultures showed that the mutant strain was deficient in the accumulation of 60S subunits in that the 60S/37S ratio was 0.29:1 compared with 1.6:1 for the parental strain after a 15-min labeling period. Similar results were obtained with early conidial cultures. When subunits were analyzed from logarithmic cultures labeled for longer periods, there was still a significant difference in the subunit ratios between the mutant and the parental strains, but the deficiency in 60S subunits in the mutant was not as extreme as in the cultures labeled for a short time. This tendency toward a more normal subunit ratio after longer labeling periods in the mutant at 37°C might be explained if the transient inhibition of total rRNA transcription and processing impeded the accumulation of labeled small subunits. In any case, these ribosomal subunit results suggested the possibility that the heat-sensitive mutant strain might be synthesizing 25S rRNA (the high-molecular-weight rRNA species of the 60S subunit) normally but its assembly with ribosomal proteins to produce 60S subunits was inhibited such that relatively few such subunits were matured. Analysis of 37°C labeled whole-cell RNA on sucrose gradients gave results similar to those with the subunit analysis in that the 25S/17S rRNA ratio was significantly lower in the mutant strain. No radioactivity maxima that were possible rRNA precursors were seen in the gradients, although with the resolution possible with these techniques, significant amounts of such molecules would have been required for them to be detected.

The production of mature ribosomal subunits is complex and involves many proteins and localized interactions. In higher eucaryotes, rRNA synthesis and ribosome assembly occur in the nucleolus (3). The rRNA genes are transcribed into a high-molecular-weight pre-rRNA which is methylated at specific sites soon after transcription (27). The modified pre-rRNA then associates with ribosomal proteins and with 5S rRNA which has been transcribed elsewhere in the nucleus. Production of mature ribosomal subunits from this ribonucleoprotein complex involves specific cleavages of the pre-rRNA, resulting in the removal of nonmature rRNA sequences and the formation of specific protein-nucleic acid and protein-protein alignments (for reviews, see 4, 12, 13, 18, 29). Pre-rRNA synthesis and processing, and ribosomal subunit production in the fungi, occur by essentially the same process as in higher eucaryotes (20a), as has been shown by experiments with the yeasts *Saccharomyces cerevisiae* (17, 26, 27) and *Saccharomyces carlsbergensis* (2, 7, 25) and with the fungus being studied here, *N. crassa* (20). The details of the pre-rRNA processing in *Neurospora* have already been presented in Results.

Nearly all of the pre-rRNA processing steps seem to be vulnerable to temperature-sensitive defects. A mutation in the rRNA cistrons per se seems unlikely for strain 4M(t) because (i) most of the known heat-sensitive mutations are expressed via protein gene products, (ii) the rRNA cistrons in eucaryotes, including *N. crassa* (5, 8), are highly reiterated, so that a mutation in one of these cistrons is not expected to have appreciable consequences, and (iii) the heat-sensitive mutation is located in linkage group IIR whereas the rRNA cistrons for 17S, 5.8S, and 25S rRNA's are located in linkage group V.

A specific RNA polymerase transcribing pre-rRNA could be heat sensitive in strain 4M(t). In all eucaryotes for which it has been analyzed, including fungi, the sequence of mature rRNA sequences in the pre-rRNA molecule has been shown to be 5'-17S-5.8S-25S-3' (6, 10, 14, 28). In *Neurospora* this information has not been obtained, but it is assumed that the pre-rRNA is organized as in other eucaryotes. With this in mind, a heat-sensitive RNA polymerase would have to complete transcription of 17S rRNA at nearly normal levels before expressing itself. This would be possible if the enzyme fell off or was blocked midway in its transit down the ribosomal deoxyribonucleic acid transcriptional unit. Incomplete transcription of the pre-rRNA might also result from some obstacle to the polymerase. There is some precedence for this; for example, the drug cordycepin is believed to inhibit 28S rRNA synthesis in HeLa cells in that way (22). Thus, since the 25S (or 28S) rRNA gene is further from the initiation of transcription, it may be transcribed to a relatively lesser extent when an agent impeding transcription is present in low doses. These possibilities would be eliminated if a precursor to 25S rRNA was observed in the mutant strain at the nonpermissive temperature. In other systems where a heat-sensitive mutant has been found which similarly underaccumulates the 60S ribosomal subunit, precursors to the 25S (28S) rRNA have been found. For example, a heat-sensitive mutant strain of the hamster cell line BHK-21 is unable to synthesize 28S rRNA and hence large ribosomal subunits at 39°C (24). At that nonpermissive temperature the 45S pre-rRNA is transcribed but there is a block in the processing of 32S RNA, the immediate precursor to 28S rRNA, such that very little 28S rRNA results (23). In yeast, a heat-sensitive mutant has been described which fails to produce 60S ribosomal subunits at 36°C (1). In that strain, the 35S pre-rRNA is transcribed and is cleaved to produce the 20S and 27S precursor RNAs. The former is cleaved correctly to produce 18S rRNA, but the 27S RNA precursor is not cleaved; thus, the 25S and 5.8S rRNA's are not formed.

In the experiments described in this paper, the kinetics of labeling of the mature high-molecular-weight rRNA's in the heat-sensitive strain were examined to see whether a precursor to 25S rRNA was produced. Acrylamide gel electrophoresis was used to analyze the RNA in order to obtain better resolution than is possible with sucrose density gradients. At 25°C, the kinetics of pre-rRNA synthesis and processing in the mutant strain were essentially the same as has been shown previously for wild-type *N. crassa* (20). That is, a 2.4- and 1.4-Mdal precursor RNAs were apparent among the RNAs extracted from cultures labeled for a relatively short time. The ratio of mature 25S/17S rRNA produced after 18 min of labeling was very similar to that of the wild type. There was one difference in pre-rRNA processing in the mutant strain at 25°C compared with the wild-type scheme described previously (20). That was that discrete 1.4- and 1.27-Mdal radioactivity were not seen in the mutant studies. This point may be interpreted in the following way. At each time point, there appears to be a heterogeneous population of molecules in the 1.27- to 1.4-Mdal range, and at early times there is a predominance of the 1.4-Mdal species, whereas at the later times the 1.27-Mdal RNA predominates. Nonetheless, this does not alter the conclusion that pre-rRNA processing appears to be normal in the mutant strain at 25°C.

In contrast to the 25°C results, differences

were seen in the processing events in the mutant strain shifted to 37°C. Again, 2.4- and 1.4-Mdal RNAs were apparent, thus resolving the question of whether a precursor to 25S rRNA is produced in the mutant at the nonpermissive temperature. Significantly, though, the processing of the 1.4-Mdal RNA to the 1.27-Mdal (25S) mature rRNA appeared to be inhibited. Further, there was much less of the 1.4-Mdal species produced relative to the 0.70-Mdal (17S) RNA in the mutant strain at 37°C compared with 25°C. From these data it was concluded that the strain has a conditional defect in pre-rRNA processing and that this is responsible for the relative underaccumulation of 60S ribosomal subunits at 37°C. The heat-sensitive defect is not an absolute block in processing and, again, this was expected since the mutant had to survive incubation at the nonpermissive temperature. In addition, the partial block in RNA synthesis was observed at 37°C in the macromolecular synthesis experiments. The later inhibition (again incomplete) of protein synthesis in the same studies was presumably the result of the disproportionality of ribosomal subunits, and possibly a defect in the translational ability of the 60S subunit (see below).

Concerning the observed conditional defect in pre-rRNA processing, it is possible that the heat-sensitive gene product is a protein interacting with pre-rRNA after it is transcribed. Correct processing of the pre-rRNA is dependent upon specific methylation of that molecule; thus, if the 25S RNA sequence is methylated to a subnormal level in the precursor molecule at 37°C, then it would be susceptible to degradation and 25S rRNA would be produced in subnormal amounts relative to the 17S rRNA. In this case, the heat-sensitive mutation would be in a gene coding for a methylation enzyme that is specific for the 25S RNA end of the pre-rRNA transcript. On the other hand, a heat-sensitive cleavage enzyme might also cause the destabilization of 25S rRNA and its immediate precursor. Here, the differential effect on 17S and 25S rRNA could result from either a heat-sensitive protein which interacts specifically with precursors to 25S rRNA or the fact that these precursors have a higher probability of interacting with a general processing enzyme, which is defective at some low frequency.

Finally, a heat-sensitive ribosomal protein might destabilize the 1.4-Mdal precursor at 25S rRNA in the precursor ribosomal particles so that fewer 60S subunits are produced than 37S subunits at 37°C. A relatively lower amount of large subunits made at 37°C was observed in polysomes than in monosomes-plus-subunits in the mutant strain. This suggested that the heat-sensitive gene product might be a ribosomal protein functioning in both the assembly and the function of the 60S ribosomal subunit. However, it was also apparent from profiles of labeled RNA in polysome gradients that new subunits did not function immediately in protein synthesis. That is, the specific radioactivity of RNA in the monosome and subunit regions was much higher than in the polysome region. It is possible, then, that subunits must mature before participating in translation. If such maturation takes place in separate subunit pools, it is further possible that the release of large subunits from their pool is defective in the mutant. On the other hand, a heat-sensitive defect interfering with precursor ribosomal particle processing and the release of completed subunits might have the same effect.

In conclusion, the heat-sensitive mutant strain studied has a conditional defect in the production of 60S ribosomal subunits. Since rRNA is synthesized, assembled into ribosomes, and incorporated into polysomes at a high rate during conidial germination (16), the observed defect in ribosome production may be responsible for the absence of conidial germination in the mutant strain at 37°C. Alternatively, it is possible that essential proteins that are synthesized during that period are not made rapidly enough owing to the disproportionality of subunits. The ribosome assembly defect is apparently also the basis for the observed macromolecular synthesis differences from the parental strain. The precise molecular lesion in the heat-sensitive strain remains to be determined.

ACKNOWLEDGMENTS

We thank Jenny Russell for excellent technical assistance.

This research was supported by grants from the Public Health Service National Institute of General Medical Sciences (GM22488), the National Science Foundation (PCM76-21478), and the Medical Research Foundation of Oregon to P.J.R. Some equipment used in this project was purchased with funds from Public Health Service Biomedical Research Support grant RR07168 and from a National Science Foundation, Special Equipment Program (grant PRM-7919955).

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