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Neurospora crassa Temperature-Sensitive Mutant Apparently Defective in Protein Synthesis

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A temperature-sensitive mutant of *Neurospora* was isolated which appeared to be defective in the initiation of protein synthesis. The defect in mutant *34Cts* was apparently due to a single gene mutation, and was recessive in heterokaryons. Conidial germination was normal and hyphal growth was nearly so in the mutant at 20 C, but both were greatly inhibited at 35 C. After 15 min at 35 C there was a reduced rate of protein synthesis, followed by decreases in ribonucleic acid and deoxyribonucleic acid synthesis. The percentage of ribosomes in polysomes declined at 35 C and the average size of polysomes decreased. Because messenger ribonucleic acid synthesis and energy metabolism were affected after the decrease in protein synthesis, it was believed that some part of the translational system may be affected by the mutation. Mutant *34Cts* was given the designation *psi-1*.

Temperature-sensitive (ts) mutants have afforded the study of many indispensable functions and developmental pathways (2, 6, 19). We had hoped to dissect the process of conidial (vegetative spore) germination in *Neurospora* by isolating a set of ts mutants which would not germinate at 35 C, but would do so at 20 C. After screening over 50 ts mutants, we found that none were specifically defective in conidial germination. That is, those that did not germinate at 35 C were defective in functions required throughout the vegetative life cycle.

One of the nongerminating ts mutants was studied in more detail. It was recessive in its effect on macroscopic growth. By macromolecular labeling patterns and polysome distributions, it appeared to be defective in the initiation of protein synthesis.

Evidence for this defect and its relation to germination are presented in this paper.

MATERIALS AND METHODS

Strains. Temperature-sensitive mutants were isolated using the inositol-less death enrichment technique (10). Macroconidia from an *inos a* strain (FGSC #89601) were mutagenized with sufficient ultraviolet (UV) light to kill 50 to 80% of the conidia. Treated conidia were plated on minimal medium (containing sorbose to restrict colony size) and incubated for 3 to 5 days at 33 C. Plates were then supplemented with inositol to a final concentration of 50 μg/ml, and shifted to 20 C. Resulting colonies were isolated and maintained on minimal medium containing inositol.

Media. Vegetative stocks were maintained on Vogel minimal medium (Vogel, personal communication, see Microb. Genet. Bull. 13:42-43) supplemented with 50 μg of inositol per ml and 1.5% agar. This is hereafter called inos medium. Large cultures of conidia were grown in 125-ml Erlenmeyer flasks on 25 ml of inos medium. Labeling and growth studies were done in inos medium without agar. For colony counts, cells were plated on inos sorbose (1.5% sorbose, 0.2% glucose) medium (10), which restricts colony size. Crosses were carried out on synthetic crossing medium (22).

Dry weight and viability tests. Dry weights were taken by filtering 10-ml samples onto preweighed filter paper disks after given incubation times and temperatures. Samples were dried to constant weight for at least 12 h at 60 C, and appropriate corrections were made for loss of filter paper weight.

For viability tests, cultures at given times and temperatures were diluted and plated on inos sorbose medium. Colonies could be counted after 3 to 4 days of incubation at 20 C.

Genetic techniques. Heterokaryons were made by mixing suspensions of conidia of different ts mutants in overdrops on plates, and allowing fusion to occur at 20 C for 12 h. Plates were then incubated at 37 C, and monitored for colony formation.

Radioactive labeling studies. Conidia from 6- to 14-day-old flask cultures were harvested by agitation with sterile water or inos medium, and filtered through several layers of glass wool. They were suspended in inos medium at concentrations of 10^6 to 10×10^6 cells/ml. Cultures were incubated on a rotary shaker at 20 or 35 C. In continuous labeling experiments, label was added to the cultures at time 0, and 2-ml samples were withdrawn at appropriate times into trichloroacetic acid (final concentration 5%) or NaOH (final concentration 1.0 M). Concentrations and specific activities are given in the figure legends. Isotopes used were L-[4,5-^{3}H]leucine, specific activity

10 Ci/mmol (Amersham/Searle); [2-^{14}C]uracil, 52 Ci/mol (Schwarz BioResearch); [8-^{3}H]adenine, 17 Ci/mmol (Schwarz BioResearch); [5-^{3}H]uridine, 2 Ci/mmol (Schwarz BioResearch); and D-[^{14}C]glucose, 8 mCi/mmol (Schwarz BioResearch).

In pulse-labeling studies, 2-ml samples from unlabeled cultures were withdrawn at stated times into 0.2 μCi of [^{3}H]leucine (0.25 μM/μCi) or 0.08 μCi of [^{14}C]uracil (100 μM/μCi). A 10- or 15-min labeling period was terminated by the addition of cold trichloroacetic acid to a final concentration of 5%.

In all labeling experiments, protein, nucleic acids, and cell wall were precipitated in 5% trichloroacetic acid at 4 C for at least 1 h. Since there is no specific label for deoxyribonucleic acid (DNA) in *Neurospora*, labeled DNA was determined by treating uracil-labeled cells with 1.0 M NaOH at 35 C for 2 h before neutralization and precipitation with trichloroacetic acid. Longer periods of hydrolysis did not reduce the alkali-resistant, acid-precipitable counts. Samples were filtered onto glass fiber filters, washed twice with 15 ml of cold 5% trichloroacetic acid, twice with 10 ml of cold 95% ethanol, and dried under a heat lamp. Dried filters were placed in glass vials containing toluene-based scintillation fluid (Omnifluor), and counted for 5 min. All labeling experiments were repeated at least once.

Polysome profiles. The procedure of Mirkes (13) was used. Samples of 10^{7} to 10^{8} cells were filtered from liquid cultures at given times and temperatures. The pad of cells was scraped into a chilled mortar. Twice the volume of washed sea sand and a few drops of cold buffer (0.01 M K^{+}, 0.01 M Mg^{+2}, 0.03 M triethanolamine) were added to the pad. The pad was ground for 45 s. Three more milliliters of cold buffer was added, and grinding was continued until a uniform orange paste was obtained. The homogenate was poured into chilled Corex tubes and centrifuged in a refrigerated Sorvall centrifuge for 10 min at 12,100 $\times$ *g*. A 1-ml amount of the supernatant fluid was layered on a linear, 11-ml, 10 to 40% (wt/wt) sucrose gradient made up in the grinding buffer. Gradients were centrifuged in a Spinco SW41 rotor for 90 min at 35,000 rpm (160,000 $\times$ *g*) or 60 min at 40,000 rpm (200,000 $\times$ *g*). Gradients were collected by pumping 50% sucrose into the bottom of each tube at a constant rate to force the gradient through the flow cell of a Gilford recording spectrophotometer. When necessary, fractions were collected from the flow cell with a Gilson microfractionator. Percentages of monosomes and polysomes were determined as the average of at least two tracings of the chart recordings with a planimeter. All glassware was heat-treated for 5 h before use to minimize nuclease contamination.

RNA extraction. Whole cell ribonucleic acid (RNA) was extracted from supernatants prepared as for polysome profiles. Polysomal RNA was prepared from pooled polysome fractions of gradients, or from cell fractions sedimenting through 0.5 ml of 40% sucrose after 12 h at 39,000 rpm in a Spinco 40Ti rotor (190,000 $\times$ *g*). Extraction was carried out as described by Mirkes (13), with chloroform:phenol:isoamyl alcohol (48:48:4), saturated with ethylenediaminetetraacetic acid (EDTA) buffer (0.01 M sodium acetate [pH 6.0] containing 0.1 M NaCl and 0.001 M EDTA). After precipitation with 2.5 volumes of absolute ethanol, the extracted RNA was dissolved in a buffer of 0.01 M tris(hydroxymethyl)aminomethane (Tris), 0.1 M NaCl, 0.001 M EDTA, and 0.5% sodium dodecyl sulfate (SDS). This was layered on a linear, 12-ml, 5 to 20% sucrose (wt/wt) gradient, made up in Tris-NaCl-EDTA-SDS buffer. Centrifugation was carried out at 20 C for 16 h at 22,000 rpm in a Spinco SW41 rotor (55,000 $\times$ *g*). Fractionation was as described for polysome gradients. Fractions of labeled RNA were precipitated with 0.1 mg of bovine serum albumin as a carrier, and cold 5% trichloroacetic acid.

Polyadenylic acid-containing RNA was determined by gravity filtration over glass fiber filters to which polyuridylic acid [poly(U)] had been bound. The complete procedure is described by Sheldon et al. (18). The method poly(U) sepharose chromatography is described in full by McDonnell and Gallas (personal communication; manuscript in preparation).

RESULTS

Genetic properties. Mutants *25Cts* and *34Cts* were isolated in the same mutant selection experiment by their survival at 33 C, under conditions in which growth would have led to death by inositol starvation. In subsequent tests of growth properties they behaved nearly identically. Strains *25Cts* and *34Cts* did not complement with each other in heterokaryons, though each complemented with other mutant strains. Hence, the two mutant isolates are believed to represent a single recessive mutation, or two very close alleles at a single locus; further studies were carried out mainly on strain *34Cts*.

The functional test of allelism was confirmed by a genetic test; there were no wild-type recombinants in over 2,000 progeny of a cross between strains *25Cts* and *34Cts*. A cross between strain *34Cts* and the *Alcoy* tester strain (FGSC #997) indicated that the mutation was on linkage group I or IV. Crosses with strains bearing nutritional markers on each of those linkage groups localized the ts mutation to linkage group IV, about 19 map units from *pyr-1*. Tetrad analysis of a cross between strains *pyr-1* and *34Cts* showed that the ts defect segregates as a single gene defect, and that the *34Cts* lesion is proximal to *pyr-1* lesion. In fact, *34Cts* appears to be the most proximal marker reported on the right arm of linkage group IV in *N. crassa*. (Details of these crosses will be presented by Stalder, Towe, and Loo; manuscript in preparation.)

General growth properties. Conidia of *Neurospora* germinate asynchronously at a rate determined by temperature, nutrients, and unknown factors particular to individual cultures. At 20 C in liquid inositol medium, conidia from

strain *34Cts* or its *inos a* parent strain began forming germ tubes at about 2 h, and 80% of the spores were germinated within 8 h after the start of incubation (Fig. 1). At 35 C, *inos a* conidia germinated within 1 to 4 h, whereas *34Cts* conidia only began to germinate after about 11 h. Even when *34Cts* conidia were preincubated at 20 C for a few hours and then shifted to 35 C, conidia failed to germinate (Fig. 2). After a 3-h preincubation and shift to 35 C, conidia that had not germinated at the time of transfer remained ungerminated, and germinated conidia showed very little germ tube elongation. That is, the ts function in strain *34Cts* is required very late in the pregermination period, and may be required throughout the pregermination period.

These microscope observations were supported by measures of dry weight (Fig. 3). Like germination, growth of mutant *34Cts* was similar to that of the parent strain at early times at 20 C. Conidia from strains *inos a* and *34Cts* doubled in dry weight after 5 to 7 h in liquid cultures at 20 C, though subsequent doubling times were longer for strain *34Cts* than for strain *inos a*. At 35 C, *inos a* conidia doubled in dry weight in about 4 h, whereas *34Cts* conidia required about 12 h for their first doubling. If conidia were preincubated at 20 C and shifted to 35 C, *inos a* conidia showed an increased rate of weight gain, whereas *34Cts* conidia showed very little weight gain.

The mutant was also grown in race tubes at 20 C, to allow germination and some hyphal elongation. Upon transfer to 35 C, hyphal elongation was greatly reduced, and stopped within 3 h. Thus, the ts function is also required for vegetative growth after germination.

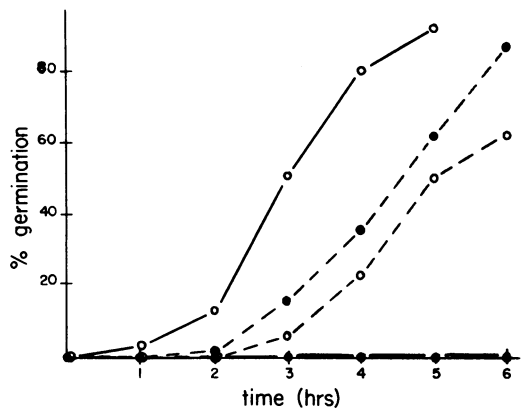

FIG. 1. *Germination of strains inos a* (○) *and 34Cts* (●) *as a function of time at 20 C* (- - -) *and 35 C* (——).

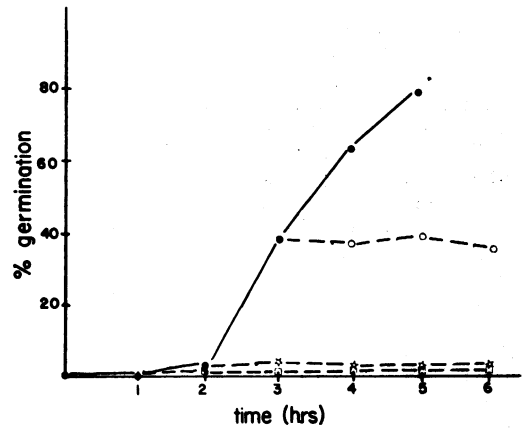

FIG. 2. *Germination of strain 34Cts as a function of time at 20 C* (○), *and at 35 C after preincubation at 20 C for 1* (□), *2* (✲), *and 3* (●) *h.*

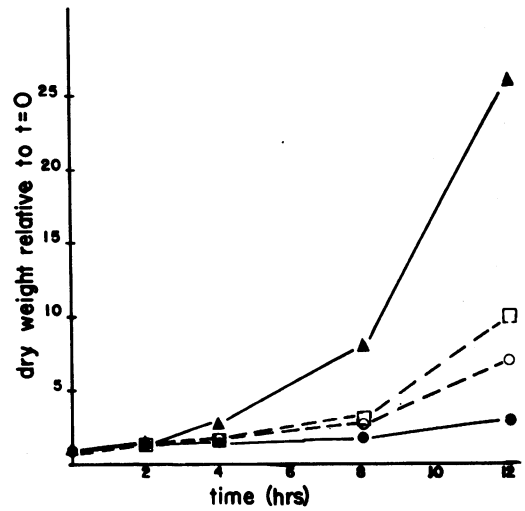

FIG. 3. *Dry weight relative to that at t = 0 in inos a after incubation at 35 C* (▲) *and 20 C* (□), *and in 34Cts after incubation at 35 C* (●) *and 20 C* (○). *Dry weights at t = 0 were 2.0 mg/10⁸ spores for inos a and 2.2 mg/10⁸ spores for 34Cts.*

The ts defect cannot be corrected by nutritional supplementation. Conidial suspensions, plated on complete medium at 35 C, did not give rise to colonies, even after several days of incubation. Mutant conidia were also tested on plates supplemented with inositol and arginine, or inositol and histidine at 35 C, since the uptake of these amino acids may be inhibited by the presence of other amino acids in complete medium. Again, no colonies appeared. Therefore, a simple ts auxotrophic mutation has been ruled out as the source of growth restriction.

There was no loss of viability in *34Cts* conidia incubated in liquid inos medium at 20 C or *inos a* conidia incubated at 20 or 35 C. But when *34Cts* conidia were preincubated for 12 h at 35 C in liquid inos medium, only 70% of the spores formed colonies after plating on inos sorbose at 20 C. After 24 h at 35 C, the viability of *34Cts* conidia had dropped to about 20%.

Continuous labeling studies. To define the ts defect more precisely, conidia were incubated in inos medium with [^{14}C]uracil and [^{3}H]leucine to monitor macromolecular synthesis. In temperature shift experiments, conidia were allowed to grow for 4 h at 20 C. Then half of the culture was shifted to 35 C, while the other half was kept at 20 C, as a control. Under these conditions, *inos a* cultures accumulated about 3.5 times as much labeled DNA, RNA, and protein at 35 C than they did at 20 C, in the 4 h after the 20 C preincubation (Fig. 4). On the other hand, cultures of *34Cts* conidia accumulated less labeled material at 35 than at 20 C, after preincubation (Fig. 4). Although it is difficult to discern from this figure, the drop in the accumulation of label was seen first in protein, with the rate falling below the 20 C rate in the first 30 min. Nearly equal amounts of DNA and RNA were labeled in that time at 20 and 35 C. Subsequently, the accumulation of all three labeled macromolecules declined; the ratios of 35 C labeled material to 20 C labeled material at 4 h after the 20 C preincubation were about 0.25 for DNA, RNA, and protein.

In other continuous labeling studies, conidia were incubated directly at 20 and at 35 C to see whether the ts gene product played the same role in ungerminated conidia as in germinating conidia. Again, conidia from *inos a* accumulated more labeled macromolecules at 35 than at 20 C. After 1 h of incubation, about twice as much labeled RNA and protein accumulated at 35 than at 20 C. DNA synthesis had not begun in either culture during the first hour. Mutant conidia also accumulated more labeled RNA in the first hour at 35 than at 20 C. However, their accumulation of labeled protein was only equivalent at 20 and 35 C for 15 to 30 min, and then lower at 35 C.

Thus, temperature shift and direct incubation studies indicated that the ts defect initially inhibited protein synthesis, and that its effect was slightly less inhibitory during the first 15 min of conidial growth. The latter observation may be due to a different role of the gene product early in germination, or to a packaged gene product that was made under permissive conditions during conidiation, or to differences in equilibration between the endogenous amino acid pools and the exogenous label.

Pulse-labeling studies. The failure to accumulate labeled macromolecules might be due to a decrease in synthesis, or an increase in degradation. Pulse-labeling experiments were carried out to distinguish between these two possibilities by minimizing the role of degradation. They

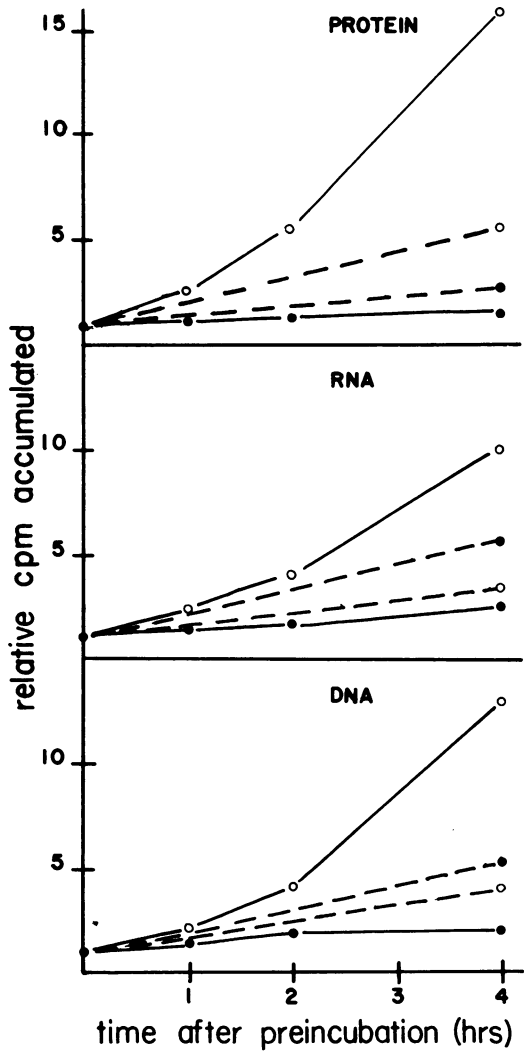

FIG. 4. *Macromolecular synthesis in strain inos a* (○) *and 34Cts* (●) *at 20 C* (- - -) *and 35 C* (——), *after 4 h of preincubation at 20 C. Cultures were labeled from the start of preincubation with* [^{3}H]*leucine* (*25 μCi/μmol, 3 nM/ml*) *and* [^{14}C]*uracil* (*0.4 μCi/μmol, 7 nM/ml*). *Label accumulation is normalized to counts per minute accumulated in the preincubation period. Under the stated conditions, samples of* 10^{7} *cells accumulated about 2,000 counts/min in RNA and protein, and about 50 counts/min in DNA.*

also dramatized the temporal differences between the inhibition of RNA and protein synthesis.

The temperature shift format was the same as in continuous labeling studies. Samples of cultures at 20 and 35 C were pulse-labeled for 10 or 15 min at various times after the preincubation period. The rate of protein synthesis in *inos a* cultures increased immediately at 35 C, whereas the rate of RNA synthesis increased less steadily (Fig. 5). In cultures of strain *34Cts*, the rate of protein synthesis at 35 C was lower than that at 20 C even in the first pulse-labeling period (0 to 15 min after the shift). The rate of RNA synthesis at 35 C exceeded that at 20 C for about 45 min, but fell below the 20 C rate after about 1 h (Fig. 5).

When conidial cultures were incubated directly at 20 and 35 C for pulse-labeling, the results were again consistent with continuous labeling data. That is, the rates of RNA and protein synthesis were greater at 35 than at 20 C in *inos a* cultures, whereas in mutant cultures the rates of protein synthesis were equivalent at the two temperatures for about 15 min and subsequently lower at 35 C. The rate of RNA synthesis was greater at 35 than at 20 C for at least 1 h.

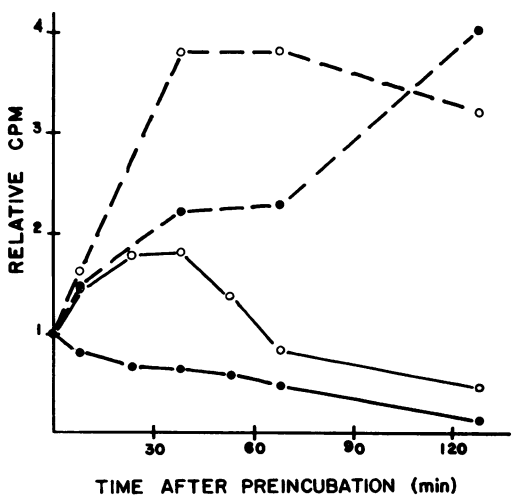

FIG. 5. *Rates of RNA synthesis* (O) *and protein synthesis* (●) *in inos a* (- - -) *and 34Cts* (——) *at 35 C after a 4-h preincubation period at 20 C. Label incorporated in a 15-min pulse at 35 C is normalized to incorporated at 20 C just before the temperature shift. Details of pulse-labeling are given in Materials and Methods. Absolute values for the 20 C rates were about 2,000 counts/min in [^{3}H]leucine and 1,000 counts/min in [^{14}C]uracil incorporated by a sample of 10^7 cells.*

These studies were repeated with pulse periods as short as 4 min to further reduce the effects of degradation, and the same results were obtained. Therefore, the simplest interpretation is that the mutant is ts for some process required for protein synthesis. However, a rigorous interpretation of any labeling experiment requires the knowledge that exogenous label is entering the cells and equilibrating with internal precursor pools. Although the specific activities of precursor pools have not been measured, label uptake was monitored by filtering pulse-labeled samples and washing them with ice-cold water. This should preserve some of the radioactive molecules that were taken into the cell but not incorporated into macromolecules. Mutant *34Cts* took up greater amounts of both labels at 35 than at 20 C, even though it incorporated less of them into macromolecules at 35 C.

Polysome profiles. The inhibition of protein synthesis could arise from blocks in peptide chain initiation, elongation, or termination. It has been shown in mammalian cells (3), in yeast (7), and in *Neurospora* (E. Crane, Master's thesis, University of Washington, Seattle, 1972) that the inhibition of chain elongation and termination by cycloheximide causes a stabilization of the ribosomes in polysomes. On the other hand, inhibition of initiation causes a rapid breakdown of polysomes into monosomes (7).

Extracts of the mutant and parent strain were prepared before a shift to 35 C and at various times thereafter. The *inos a* parent strain showed a slight increase in polysomes after the shift from 20 to 35 C. However, the polysome content in mutant extracts decreased from 85% before the shift to 75 and 53% at 10 and 20 min after the shift, respectively. The rate of polysome decay was somewhat variable, in that more drastic decreases could be observed. Figure 6 shows the spectrophotometric tracings of polysomes in strain *34Cts* before a shift and 20 min after the shift; there is a 3.6-fold reduction in the percentage of ribosomes in polysomes. The consistent result is, however, that there is a gradual but incomplete decrease in polysome content.

This decrease is consistent with a ts effect upon peptide chain initiation. Not only does the polysome content decrease, but so does the average size of the remaining polysomes. If the amount of A_{260} representing polysomes containing seven or more ribosomes is compared to that representing two to six ribosomes, the ratio decreases upon incubation at 35 C (Table 1). The average polysome size decreased by about

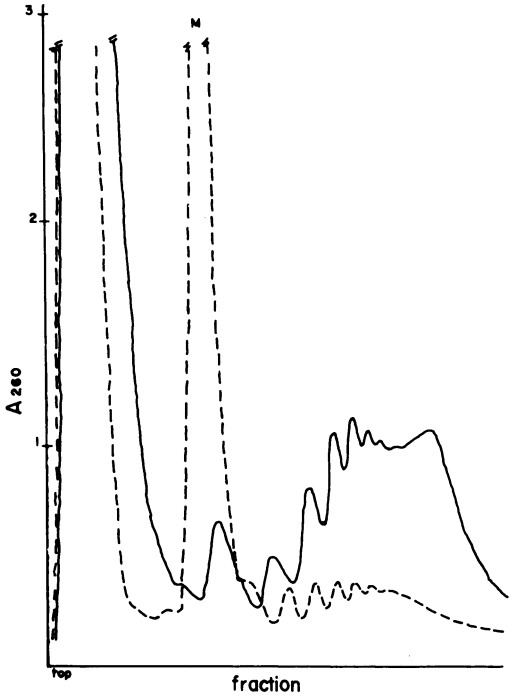

FIG. 6. *Polysome profiles of strain 34Cts after 4 h at 20 C (——), and 20 min after a shift to 35 C (- - -). The percentage of ribosomes in polysomes is 92% at 20 C and 25% at 35 C. The monosome peak is labeled "M" for reference.*

45% after 20 min at 35 C, and this size decrease could account for most of the decrease in protein synthesis observed in labeling studies. Since it is difficult to resolve the larger polysomes, and they contribute heavily to the average size estimate, a strict correlation between average polysome size and rate of protein synthesis cannot be drawn from these data. In general, the polysome profiles support the theory that peptide chain initiation, rather than elongation, is defective in the mutants.

Inhibition of energy metabolism. Hartwell and McLaughlin (8) have pointed out that various defects can interfere with the initiation of translation. These include defects in energy metabolism, messenger synthesis or processing, and the translational machinery per se. The first possibility may be ruled out by comparing macromolecular synthesis in the mutant at 35 C, and in the parent strain treated with specific inhibitors.

In *Neurospora*, chitin is synthesized by the energy-dependent addition of *n*-acetyl glucosamine to preexisting chitin. Synthesis may be monitored by the incorporation of [^{14}C]glucose into the cell wall. Although [^{14}C]glucose may be converted into precursors for other macromolecules, it is preferentially incorporated into material that remains with the cell wall when ghosts are made by dissolving the cytoplasm with ethanol and NaOH (5). In control experiments, the counts remaining in cell wall ghosts were equivalent to those precipitated by 5% trichloroacetic acid in short pulses, and nearly so in longer labeling periods.

When protein synthesis in *inos a* was inhibited by 10 μg of cycloheximide per ml, the incorporation of labeled glucose into cell wall continued at a somewhat depressed rate (Fig. 7A). However, when energy metabolism in *inos a* was inhibited by 0.05 M arsenate, both protein and cell wall synthesis were quickly inhibited. When *34Cts* was shifted to 35 C in the presence of [^{3}H]leucine and [^{14}C]glucose, protein synthesis declined, but cell wall synthesis increased transiently (Fig. 7B). Furthermore, after treatment of the parent strain with arsenate, protein and RNA synthesis declined at the same time. This was not seen in the mutant after a shift to 35 C (Table 2). These data indicate that the decrease in polysome content and protein synthesis in the mutant does not result from a defect in energy metabolism.

Characterization of RNA made after a temperature shift. A ts defect in messenger RNA (mRNA) synthesis seemed unlikely, since

TABLE 1. *Polysomes in strain inos a and 34Cts after incubation at 20 and 35 C*

Strain	Growth condition	Polysomes of >7 ribosomes/ polysomes of 2–6 ribosomes	Ribosomes in polysomes (%)
inos a	3 h, 20 C	0.99	93
	3 h, 35 C	1.76	87
	3.5 h, 20 C	1.0	74
	3.5 h, 20 C, followed by 15 min, 35 C	1.1	91
34Cts	4 h, 20 C	0.85	95
	4 h, 35 C	0.25	83
	4 h, 20 C	1.03	85
	4 h, 20 C, followed by 10 min, 35 C	0.74	75
	4 h, 20 C, followed by 20 min, 35 C	0.6	53
	4 h, 20 C	1.36	92
	4 h, 20 C, followed by 20 min, 35 C	0.7	25

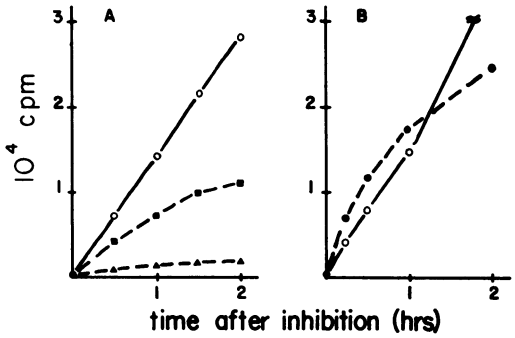

FIG. 7. *Incorporation of [^{14}C]glucose by treated cultures of strains inos a (A) and 34Cts (B). Cultures were preincubated for 4 h at 20 C, and [^{14}C]glucose (8 mCi/mmol, 20 nM/ml) was added at the onset of treatment. (A) Incorporation by inos a at 20 C in the presence of 10 μg of cycloheximide per ml (■), 0.05 M arsenate (▲), and no inhibitor (○). (B) Incorporation by 34Cts at 20 C (○) and 35 C (●).*

TABLE 2. *Inhibition of RNA and protein synthesis in 4-min pulse label of inos a treated with arsenate (ARS) (0.05 M) at 20 C, and of 34Cts shifted to 35 C*

Time (min) of pulse label after treatment	*inos a*, 20 C (+ARS/-ARS)		*34Cts* (35 C/20 C)	
	RNA	Protein	RNA	Protein
0–4	0.77	0.83	1.57	0.81
5–9	0.59	0.55	1.64	0.69
10–14	0.48	0.55	1.30	0.45
15–19	0.42	0.59	1.34	0.48
20–24	0.55	0.41	1.19	0.45

protein synthesis was inhibited before RNA synthesis at 35 C. However, it was possible that a decline in mRNA transcription was masked by an increase in ribosomal RNA (rRNA) synthesis, or that messengers were transcribed but rendered untranslatable by a ts defect in processing. If this were so, mRNA in polysomes at 35 C could only be that transcribed before the shift to 35 C.

To investigate this possibility, labeled RNA precursors were added to a *34Cts* culture at the time of a shift to 35 C, to monitor RNA made at 35 C. Incubation was continued for some time, before RNA was extracted and characterized. An unshifted control culture was treated in the same way. The optical density profile of unlabeled carrier RNA was used to assign sedimentation values to different regions of sucrose gradients.

Whole cell RNA was extracted from cultures of strains *inos a* and *34Cts* labeled for 10 min with [^{14}C]uracil or [^{3}H]uridine at 20 C, or after a shift to 35 C. Extracted RNA was centrifuged in sucrose gradients, and fractions were collected to obtain a profile of the labeled RNA. A fraction of mRNA is expected to sediment in the 6 to 14*S* range, faster than transfer RNA (tRNA) or 5*S* RNA, but slower than rRNA. In the *inos a* cultures, similar percentages of labeled 4 to 5*S* RNA, 6 to 14*S* RNA, and larger RNA were made at both temperatures (Table 3). Similar percentages were also seen in unshifted and shifted cultures of strain *34Cts*, although considerably fewer total counts were recovered in the 35 C extract of strain *34Cts* in one experiment. This may be due to a difference in grinding of the cells, as well as in increase in degradation, suggested by the higher percentage of smaller labeled RNA molecules in the gradient. However, in the second experiment, with a better recovery of labeled RNA from the 35 C extract of the mutant, there was at least as great a percentage of labeled 6 to 14*S* RNA made at 35 C. These experiments suggest that messenger-sized RNA is indeed synthesized by the mutant after a shift to 35 C.

To see whether such RNA was translated in the mutant, RNA was labeled for 1 h after a shift to 35 C and extracted only from polysomes. Duplicate samples of extracted polysomal RNA were run on sucrose gradients. One set of fractions was trichloroacetic acid-precipitated; the other was gravity-filtered on filters containing poly(U) acid. Since many eukaryotic messengers have been shown to contain tails of polyadenylic acid (1, 11), the ability to bind to poly(U) seemed a suitable criterion for

TABLE 3. *Distribution of labeled whole cell RNA made in a 10-min pulse at 20 and 35 C after 4 h of preincubation at 20 C*

Strain	Sedimentation coefficient (*S*)	Counts/min		Total counts/min (%)	
		20 C	35 C	20 C	35 C
inos a[a]	≤5	8,076	10,004	21.8	30.6
	6–14	1,230	878	3.4	2.6
	≥15	27,685	21,800	4.78	66.7
34Cts[a]	≤5	15,671	3,842	23.0	52.6
	6–14	3,096	518	4.5	7.0
	≥15	49,325	2,945	72.5	40.4
34Cts[b]	≤5	7,480	13,781	14.6	34.9
	6–14	2,638	2,379	5.4	6.0
	≥15	40,153	23,532	80.0	59.1

[a] RNA labeled with [^{3}H]uridine (10 μCi/ml, 2 Ci/mmol).

[b] RNA labeled with [^{14}C]uracil (0.4 μCi/ml, 52 Ci/mol).

mRNA. Again, less labeled polysomal RNA was recovered from *34Cts* at 35 C (Table 4). But there was a significant amount of labeled 6 to 14*S* RNA in polysomes at 35 C. Moreover, this RNA showed a five times greater affinity for poly(U) than did the faster-sedimenting RNA. The presence of cold carrier RNA made it impossible to determine the specific activity of each fraction. The affinity of 4 to 5*S* RNA for poly(U) may be due to breakdown of larger mRNA, although nothing has been done to rule out binding by a naturally occurring small RNA.

In a variation of the above experiment, RNA was labeled for 1 h after a shift to 35 C, and extracted polysomal RNA was run through a poly(U) sepharose column. RNA not binding to the column was collected by running buffer through the column. RNA binding to the column was eluted with formamide. Both binding and nonbinding fractions were concentrated by ethanol precipitation, and centrifuged in sucrose gradients. There was sufficient RNA extracted that a carrier was unneccessary. Figure 8A shows the optical density profile and profile of counts which did not bind to the column; Fig. 8B shows profiles of those which did. Over 80% of the counts recovered from the column eluted in the nonbinding fraction. Of the RNA which did bind, however, almost half of it has the properties of mRNA—a size of 6 to 14*S*, a high affinity for poly(U), and a higher specific activity than stable species of RNA. Thus, it seems that translatable mRNA is made in mutant *34Cts* at 35 C.

DISCUSSION

A temperature-sensitive mutant was isolated that failed to germinate or grow at temperatures above 33 C. It did not respond to nutritional supplements. The ts defect mapped as a single gene defect on the right arm of linkage group IV,

TABLE 4. *Distribution of labeled RNA extracted from polysomes of 34Cts*[a]

Region of gradient	Acid-precipitated counts/min		Poly(U) bound counts/min		Fraction (20 C)	Poly(U)/Acid (35 C)
	20 C	35 C	20 C	35 C		
≤5*S*	2,365	2,926	352	322	0.15	0.11
6–14*S*	8,702	5,026	1,315	734	0.15	0.15
≥15*S*	198,871	73,756	2,284	2,105	0.01	0.03
Total	209,938	81,708	3,951	3,161		

[a] Cultures were grown at 20 C for 4 h, before a 1-h pulse-label with [^{3}H]adenine. Acid indicates trichloroacetic acid.

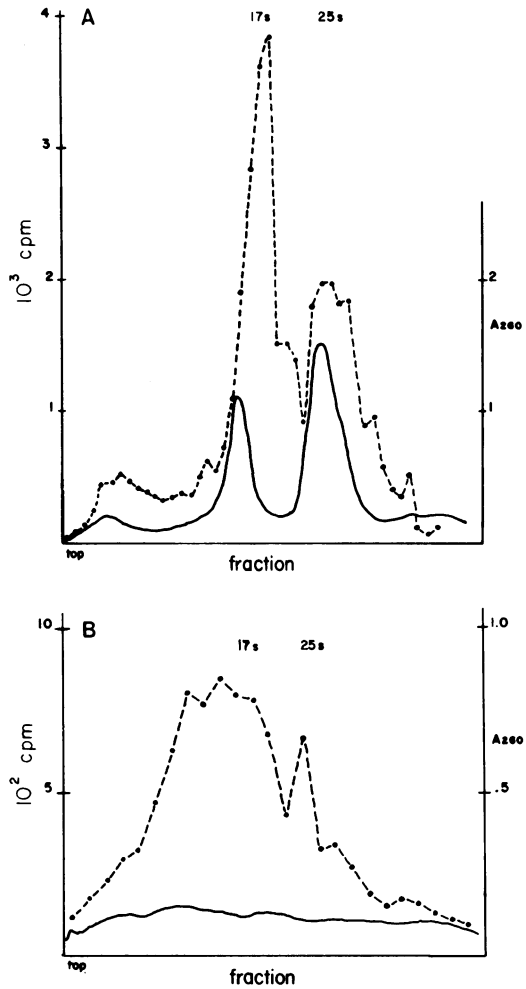

FIG. 8. *Optical density (——) and counts per minute (- - -) profiles of extracted polysomal RNA from strain 34Cts. Cultures were preincubated for 6 h at 20 C. [^{3}H]adenine (17 Ci/mmol, 30 nM/ml) was added at the time of a shift to 35 C and labeling was continued for 1 h. RNA extracted from polysomes centrifuged through 40% sucrose was passed through a poly(U) sepharose column. RNA not binding (A) and RNA binding (B) to the column were concentrated and characterized by sedimentation. Positions of 17S and 25S markers are noted in the figures.*

and was recessive in heterokaryons. It is proposed that the locus defined by this mutant be designated *psi-1* (for protein synthesis initiation).

Continuous-labeling studies showed that the accumulation of labeled macromolecules was normal at 20 C, but decreased at 35 C in the mutant. The accumulation of labeled protein was affected before that of RNA and DNA. This

suggests that protein synthesis was primarily affected by the ts lesion. The subsequent decline of other macromolecular syntheses was not surprising, since studies of bacteria and yeast show a similar coupling between protein and nucleic acid synthesis (14, 16). Moreover, the isolation of this ts mutant required its survival for several days at 33 C, when unbalanced growth might have been fatal.

The interpretation of this ts defect in terms of protein synthesis was supported by pulse-labeling studies. In short labeling periods, with the effects of degradation reduced, less protein was labeled at 35 than at 20 C, even in the first 10 min after a shift to 35 C. RNA synthesis increased briefly after the shift, and then fell after about 45 min. Since the uptake of [^{3}H]leucine and [^{14}C]uracil increased at 35 C, the decline in pulse-labeled molecules probably reflects a real decrease in synthesis.

The hypothesis of a ts defect in protein synthesis is independently supported by polysome profiles of mutant cultures before and after a shift to 35 C. At 20 C, greater than 80% of the ribosomes could be found in polysomes. But the percentage of ribosomes in polysomes gradually decreased to about 40% 20 min after a shift to 35 C. This reduction of polysomes could not be attributed to nonspecific degradation of the mRNA between ribosomes. Such degradation would result in an inverse relationship between polysome size and the number of polysomes of that size. This was clearly not the case in polysomes of the mutant at 35 C. Furthermore, the percentage of polysomes at 35 C could be increased by treating the mutant with a low dose of cycloheximide to impede translocation (unpublished result). This is consistent with the physical interpretation that at 35 C there are fewer ribosomes per mRNA and wider spaces between them, because the ribosomes translocate and "run off" the mRNA at a normal rate, but have difficulty initiating new rounds of translation. The inhibition by cycloheximide would slow down the translocation rate to reestablish a balance between initiation and termination. It is also possible that ribosomes were falling off the mRNA at 35 C, and were stabilized by cycloheximide, but there is little precedent for this interpretation.

Studies with yeast (8) have shown that inhibition of the initiation of protein synthesis may be mediated by defects in energy metabolism, mRNA processing, or translation per se. When a defect in energy metabolism was mimicked by treating the parent strain with arsenate, there was coincident inhibition of protein, RNA, and cell wall synthesis. On the other hand, there was a clear temporal separation of inhibition when the mutant was shifted to 35 C. Thus, the ts defect does not seem to act via the inhibition of energy metabolism.

A ts defect in mRNA processing was also ruled out by characterizing the RNA made in mutant cultures at 35 C. About 5% of the whole cell RNA, labeled in a 10-min pulse at 35 C, sedimented in the 6 to 14*S* region of a sucrose gradient. After longer labeling periods, about 10% of labeled polysomal RNA had the properties of mRNA—a high specific activity, high affinity for poly(U), and a size of 6 to 14*S*.

The major remaining possibility is that some part of the translational machinery is temperature sensitive. In ts yeast mutants, defective in the initiation of protein synthesis, a drastic polysome breakdown occurred in a few minutes at 35 C (8). This immediate response was apparently due to a protein which was temperature sensitive in function. The gradual onset of inhibition in the ts mutant reported here may be explained otherwise. The defective protein may be ts during synthesis, such that only *34Cts* gene products made at the restrictive temperature are dysfunctional. Alternatively, a protein which is ts in function may allow the gradual accumulation of an inhibitor. The former explanation was favored slightly by experiments in which freshly harvested conidia were incubated directly at 35 C. Here, the rate of protein synthesis at 35 C equaled that at 20 C for about 15 min. This would be understandable if the translational machinery at early times were made during conidiation at 20 C and was therefore functional.

If this mutation does result in a protein which is ts during synthesis, it is unlikely to be a ribosomal component. Since the ribosomes are stable cellular units, there would be a large population of functional ribosomes made prior to a temperature shift, and a smaller proportion of dysfunctional ribosomes made after the shift. Ribosomes made before the shift would be expected to function normally in polysomes at 35 C, whereas those made after the shift would be confined to monosomes. However, more ribosomes appeared in the monosome fraction at 35 C than could be accounted for by newly synthesized ribosomes. Moreover, rRNA extracted from polysomes included rRNA that was labeled after the shift to 35 C. Hence, there appeared to be a single population of ribosomes which had difficulty initiating translation, rather than a heterogeneous population of old functional and new dysfunctional ribosomes.

Other likely sites of translational defects are initiation factors (17) and charged tRNAs. As in

bacteria, methionine has been implicated as the N-terminal amino acid in nascent peptides of *Neurospora* (15). If formyl-methionyl tRNA does serve in initiating peptide chains, this ts mutation may interfere with the synthesis or charging of the $tRNA_{f\text{-}met}$, or the formylation of methionine. These possibilities, as well as the possibility of a ts initiation factor, have not been investigated, but they should be amenable to further biochemical analysis.

More exotic possibilities also exist. For example, a mutant of *Escherichia coli* has been described, wherein a ts hydrolase allows the accumulation of mischarged peptidyl tRNA's and thus inhibits the initiation of protein synthesis (12). In addition, Goldstein and Penman (4) have observed a temperature-dependent effect in normal mammalian cells. At 41 C the initiation of protein synthesis was inhibited. This effect was mimicked by low doses of actinomycin at lower temperatures, and was proposed to result from the decay of an RNA which mediates the association of mRNA and ribosomes.

Although the precise molecular defect in this mutant is unknown, its effects on germination are striking. It is not surprising that germination is inhibited in the mutant at 35 C, since de novo activities of amino acid transferases (20), aspartyl transcarbamylase (9), glutamic dehydrogenase (21), and several other enzymes have been association with germination. However, the finding that germination is blocked at 35 C even after several hours of preincubation at 20 C suggests that there is a late critical step that requires ongoing protein synthesis. Moreover, ungerminated ts conidia incubated directly at 35 C do eventually accumulate as much labeled macromolecular material and dry weight as germinating conidia at 20 C. This indicates that germination is not simply the outgrowing of old spore walls. Studies are now in progress to identify possible requirements for germination which may be differentially blocked in this mutant.

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