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Dependence of Ribonucleic Acid Synthesis on Continuous Protein Synthesis in Yeast

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Using an auxotrophic strain of *Saccharomyces cerevisiae*, we examined the kinetics of ribonucleic acid (RNA) synthesis following inhibition of protein synthesis caused by amino acid starvation or cycloheximide. Removal of a required amino acid immediately stopped net protein synthesis. After a brief lag, RNA synthesis also ceased. Cycloheximide, a ribosome-inhibiting drug, also immediately halted net protein synthesis. Again RNA synthesis stopped after a brief lag. Although cycloheximide and amino acid starvation affect different steps in protein biosynthesis, both inhibited RNA synthesis in identical fashion. This indicates that amino acids do not play a unique role in the control of RNA production in rapidly growing yeast; rather, it suggests that RNA synthesis is responsive to the overall rate of protein synthesis itself.

Microorganisms, including yeast, can rapidly adjust their rate of ribonucleic acid (RNA) synthesis in response to changes in growth conditions (15, 16, 25). The mechanisms microbial cells use to regulate RNA production have been investigated most thoroughly in bacteria (2, 6, 8). Early studies with *Escherichia coli* indicated that protein synthesis somehow regulated the rate of RNA production (15, 21). The mechanism by which protein synthesis exerted control was clarified with the observations that amino acid starvation immediately halted RNA synthesis, whereas interference of protein synthesis with chloramphenicol, or other ribosome-inhibiting drugs, permitted RNA synthesis to continue (1, 6, 14). Thus it was shown that RNA synthesis was governed by the amino acid supply rather than by the overall rate of protein synthesis itself (1, 14). The unique role played by amino acids was further supported by the discovery of the "ribonucleic acid control" (RC) locus (24). The normal allele of the RC locus (RC^{str}-stringent control) allows the typical rapid inhibition of RNA synthesis following amino acid deprivation. In cells possessing the so-called relaxed control allele (RC^{rel}), RNA synthesis continues in the absence of amino acids. It is now known that amino acids regulate RNA synthesis indirectly through combination with their respective transfer RNA species (20).

In *Saccharomyces cerevisiae*, a relationship also exists between the synthesis of RNA and protein. However, in contrast to the situation in *E. coli*, RNA production is affected when protein synthesis is inhibited by a variety of independent methods (5, 11, 12, 17, 25), some of which have no apparent relationship to changes in amino acid levels. It was the purpose of this investigation to further characterize the interdependence of RNA and protein synthesis in yeast. Specifically, we wished to determine whether RNA synthesis exhibited differential responses to inhibitions of protein synthesis caused by amino acid starvation and cycloheximide. Cycloheximide (13) is a ribosome-inhibiting drug active in eukaryotes; it is similar in its mode of action to chloramphenicol which is inactive on eukaryotic ribosomes (22, 23, 26).

Although cycloheximide and amino acid starvation block protein biosynthesis at distinctly different steps, both inhibited RNA synthesis in an identical manner. This finding indicates that amino acids, per se, do not play a unique role in control of RNA synthesis during steady-state growth. In conjunction with other data, it suggests that continuous protein synthesis itself is involved in the regulation of RNA production in yeast.

MATERIALS AND METHODS

Organism and growth conditions. *S. cerevisiae* 2439-6C, a nonclumpy haploid strain, was provided by D. Hawthorne. Its genotype was *a, ade-1, ade-2, ade-5,7, ura-1, ura-4, lys-2, trp-1, tyr-1, tyr-6*; the symbols refer to mating type and nutritional requirements for adenine, uracil, lysine, tryptophan, and

tyrosine, respectively; in addition, the block imposed by *tyr-6* caused a requirement for phenylalanine. Experiments were performed with cells grown in synthetic complete (SC) medium containing, in grams per liter: yeast nitrogen base (Difco), 6.7; glucose, 10; adenine, 0.02; uracil, 0.02; L-arginine, 0.02; L-leucine, 0.03; L-lysine, 0.04; L-histidine, 0.02; L-methionine, 0.02; L-isoleucine, 0.02; L-valine, 0.15; L-tryptophan, 0.02; L-tyrosine, 0.02; L-phenylalanine, 0.05; L-threonine, 0.1; L-aspartic acid, 0.1; and L-glutamic acid, 0.1.

The strain was maintained on YEPD (19) agar at 5 C. For each experiment, cells were transferred from an actively growing culture on YEPD into 100 ml of prewarmed SC medium (in a 500-ml flask) and incubated with vigorous shaking (350 rev/min) in a New Brunswick G-25 rotary incubator shaker held at 30 C. The liquid cultures were maintained in continuous early log-phase growth for 36 to 48 hr prior to the start of each experiment by subculturing at intervals into fresh medium. Growth was monitored by the increase in absorbance at 600 nm (in a Beckman DU spectrophotometer); where indicated, cell counts were performed with a hemacytometer. During continuous log-phase growth, there was a constant relationship between absorbance and cell concentration with 1.0 absorbance unit corresponding to 6×10^6 cells per ml.

Experimental methods. Our aim was to compare the effects of cycloheximide and amino acid starvation on the kinetics of protein and RNA synthesis during steady-state growth. Cultures received identical treatment prior to cycloheximide addition or amino acid starvation. A rapid harvesting and washing procedure was used both to affect amino acid starvation and as a prelude to cycloheximide treatments. When growing cultures reached an absorbance of about 0.5, they were collected on sterile membrane filters (47 mm, 0.45 μm pore size) and washed with prewarmed, aerated, SC medium. The cells were then dislodged from the filters by agitation in fresh medium. Fifty-milliliter portions of this suspension were dispensed to individual flasks and incubated in a New Brunswick G-77 gyratory waterbath shaker held at 30 C. The experiments were designed so that the control cultures reached an absorbance of 1.0 at the termination of the experiment.

Labeling procedures. Macromolecular synthesis was examined by standard labeling procedures fully detailed by Hartwell (10) and Wehr and Parks (25). For continuous labeling, the log-phase cultures were grown in the presence of the appropriate radioactive precursor for the last 15 to 18 hr prior to the start of an experiment. Short-term labeling was accomplished by adding a small amount of high specific activity precursor to the cultures at the start of an experiment (t = 0). In continuous labeling experiments, RNA and protein synthesis were measured in separate cultures run simultaneously. For short-term labeling, a single culture was divided at the start of an experiment, with RNA and protein synthesis being examined in separate flasks. Cycloheximide was a gift from the Upjohn Co. Radioactive compounds were purchased for the New England Nuclear Corp. The uracil-2-^{14}C had a specific activity of 2 mCi/mmole, the uracil-6-^3H was 5 mCi/μmole and the lysine-G-^3H was 5.3 mCi/μmole.

RESULTS

RNA and protein syntheses were followed by the incorporation of radioactive precursors into the macromolecular fraction. In Fig. 1, the net synthesis of RNA and protein, measured with ^{3}H-uracil and ^{3}H-lysine, respectively, was examined during growth. The mass and cell number doubling time, as well as the doubling time for protein and RNA content, was 100 min. These conditions indicated steady-state logarithmic growth so all subsequent experiments were performed using cultures harvested during this portion of the growth curve (i.e., between 5×10^5 and 5×10^6 cells per ml).

Effects of cycloheximide on protein and RNA synthesis. The effects of three concentrations of cycloheximide (1.0, 10, and 100 μg per ml) on growth and on the accumulation of protein and RNA are shown in Fig. 2. Addition of the drug immediately reduced the rate of protein synthesis. Significant inhibition was

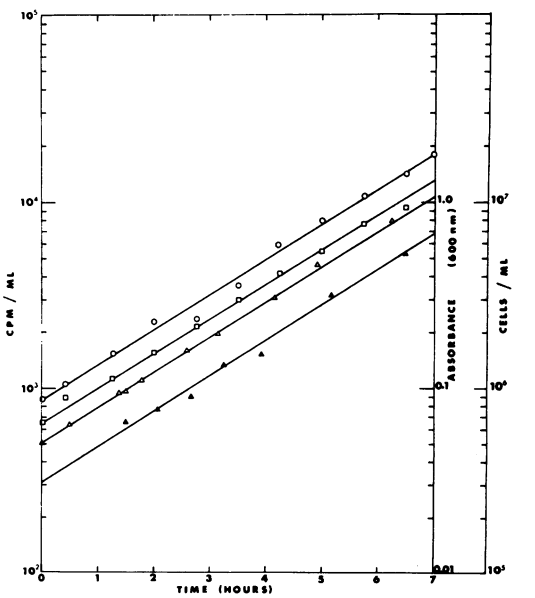

FIG. 1. *RNA and protein accumulation during growth of strain 2439-6C. At zero time, two separate cultures grown for 16 hr in medium containing either ^{3}H-uracil (0.25 μCi/ml) or ^{3}H-lysine (0.25 μCi/ml) were subcultured into fresh labeled medium. At intervals over the next 7 hr, samples were removed from each culture for the determination of cell concentration (▲) and absorbance at 600 nm (△). In one culture, net protein content (○) was determined by the incorporation of ^{3}H-lysine. Net RNA content was determined in the other culture (□) by the incorporation of ^{3}H-uracil.*

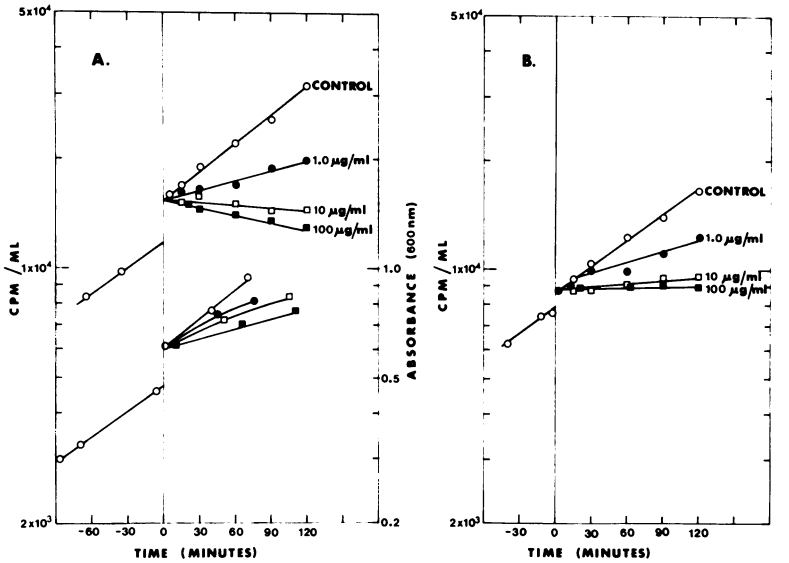

FIG. 2. *Effects of cycloheximide on growth and on the accumulation of protein and RNA. At zero time, two separate cultures (A and B) growing in the presence of either ^{3}H-lysine (0.25 μCi/ml) or ^{3}H-uracil (0.25 μCi/ml) were harvested and washed, and each was resuspended in fresh medium. Each suspension was quickly divided into four samples; one sample (control) remained untreated; sufficient cycloheximide was added to each of the other samples to yield final drug concentrations of 1.0, 10, and 100 μg per ml. The ^{3}H-lysine and ^{3}H-uracil concentrations were maintained constant throughout these manipulations. (A) Protein accumulation was monitored by the incorporation of ^{3}H-lysine (upper series of curves), and growth was followed by increased absorbance at 600 nm (lower series of curves). (B) RNA accumulation was monitored with ^{3}H-uracil. In both A and B, the control points prior to zero time were obtained before the cultures were harvested. The offset in the curves at t = 0 resulted when the cells were resuspended at slightly higher density after harvesting and washing.*

observed at 1.0 μg of drug per ml; at 10 μg/ml and above, net accumulation of protein ceased. At the higher drug concentrations, the cellular protein content actually declined slightly during the course of the experiment. Short-term labeling showed that protein synthesis occurred at the higher drug concentrations, but synthesis was exceeded by a more rapid rate of protein turnover.

RNA synthesis (Fig. 2) was also inhibited by cycloheximide. In the presence of 1.0 μg of drug per ml, net RNA synthesis continued for 15 to 20 min, at, or near, the normal rate and then fell to a slower rate for the remainder of the experiment. However, with drug concentrations of 10 and 100 μg per ml, which caused severe inhibition of protein synthesis, net RNA synthesis quickly fell to very low rates with the inhibition being more severe at the higher concentration. As expected, the drug inhibited growth (Fig. 2). Next, short-term labeling was used to more closely examine the effects of cycloheximide on RNA synthesis (Fig. 3). The ^{3}H-uracil was added together with the drug, and also 60 min after the drug (Fig. 3). Considering first the effects of 10 and 100 μg per ml, we found that when drug and label were added simultaneously the incorporation pattern indicated that RNA synthesis continued at an appreciable rate for about 30 min and then rapidly declined to a lower rate. Thus, a lag existed before cycloheximide had its final or complete effect on RNA synthesis. This lag was more clearly shown when the label was first added 60 min after the drug. Here the ^{3}H-uracil incorporation only detected the slow rate of synthesis characteristic of the period after cycloheximide has had its final effect of RNA synthesis. Like protein synthesis, RNA synthesis continued at an appreciable rate in the presence of 1.0 μg of cycloheximide per ml.

Effects of amino acid starvation on protein and RNA synthesis. Rapid amino acid removal was accomplished by collecting cultures on membrane filters and washing them with SC medium lacking a single amino acid. Tyrosine starvation, like cycloheximide, immediately halted the net synthesis of protein (Fig. 4). RNA synthesis was also rapidly affected by starvation; net synthesis continued for 15 to 20 min, at, or near, the control rate and then quickly fell to a very low rate for the re-

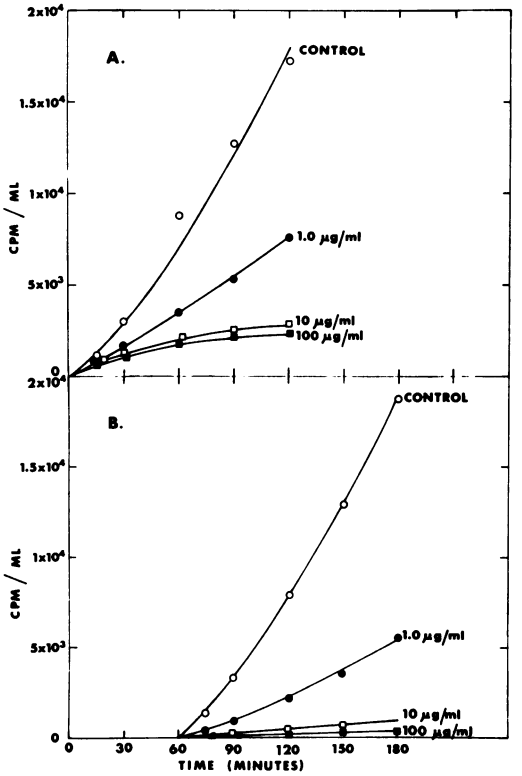

FIG. 3. *Effects of cycloheximide on RNA synthesis. At zero time, a culture was harvested, washed, and resuspended in fresh medium. The suspension was divided into two sets (A and B) of four samples each. One sample of each set (control) was left untreated; the remaining samples in each set had sufficient cycloheximide added to give final drug concentrations of 1.0, 10, and 100 µg/ml. At zero time, ^{3}H-uracil (to a final concentration of 0.5 µCi/ml) was added to all the samples in A. (B) The ^{3}H-uracil (0.5 µCi/ml) was added to all samples at 60 min. RNA synthesis was monitored by the incorporation of the added ^{3}H-uracil.*

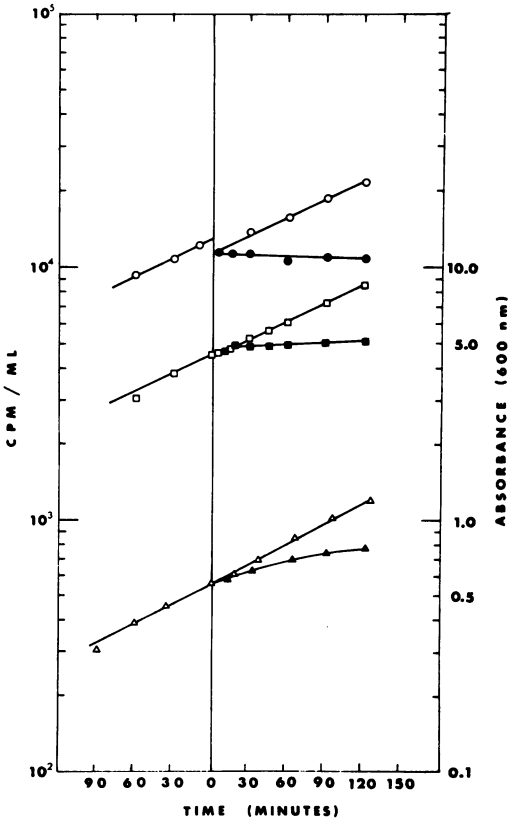

FIG. 4. *Effects of tyrosine starvation on growth and on the accumulation of protein and RNA. At zero time, two separate cultures growing in the presence of either ^{3}H-lysine (0.25 µCi/ml) or ^{14}C-uracil (0.05 µCi/ml) were harvested, washed, and resuspended in tyrosine-free medium. Each suspension was quickly divided into two samples; one sample remained in the tyrosine-free medium; sufficient tyrosine was added to the other sample (control) to yield the standard concentration (20 µg/ml). Throughout these manipulations, the concentration of ^{3}H-lysine and ^{14}C-uracil was maintained constant. The upper set of curves show protein accumulation, as ^{3}H-lysine incorporation, in the tyrosine-starved samples (●) and in the control sample (○) containing tyrosine. The middle set of curves show RNA accumulation, as ^{14}C-uracil incorporation, in the tyrosine-starved (■) and control (□) samples. The bottom curves show growth, measured by absorbance at 600 nm, in the starved (▲) and control (△) samples. Control points prior to zero time were obtained before the cultures were harvested, washed, etc.*

mainder of the experiment. Short-term labeling was used to examine more closely the effects of tyrosine starvation on the kinetics of RNA and protein synthesis (Fig. 5). This experiment confirmed the immediate effects of amino acid removal on protein synthesis and verified that RNA synthesis continued for a brief period before becoming fully inhibited. Tryptophan starvation of strain 2439-6C had exactly the same effects on protein and RNA synthesis as tyrosine removal. In other auxotrophic strains, we have observed the characteristic effects of amino acid starvation after removal of arginine, leucine, or histidine.

The results presented above indicate that amino acid deprivation and cycloheximide inhibit RNA synthesis in a similar, if not identical, fashion. This similarity was specifically reexamined in another experiment (Fig. 6). A single batch of cells was collected, washed, and resuspended in either tyrosine-free medium or in complete medium containing 20 µg of cyclo-

heximide per ml. The kinetics of inhibition of RNA synthesis were indeed identical in both cases.

Resumption of protein and RNA synthesis. We next examined the resumption of protein and RNA synthesis after cycloheximide removal and upon readdition of a previously removed amino acid. Cells were exposed to cycloheximide (20 μg/ml) for 2 hr and then rapidly washed to remove the drug (Fig. 7). RNA and protein syntheses were then followed for 2 hr, starting immediately after the cells were placed in drug-free medium. Removal of the drug allowed an immediate resumption of protein synthesis. RNA synthesis began at the control rate about 20 min later. Next, tyrosine was readded to a culture which had been starved for 2 hr (Fig. 7). Again, protein synthesis resumed immediately, rapidly followed by the resumption of RNA synthesis. These experiments provided some evidence

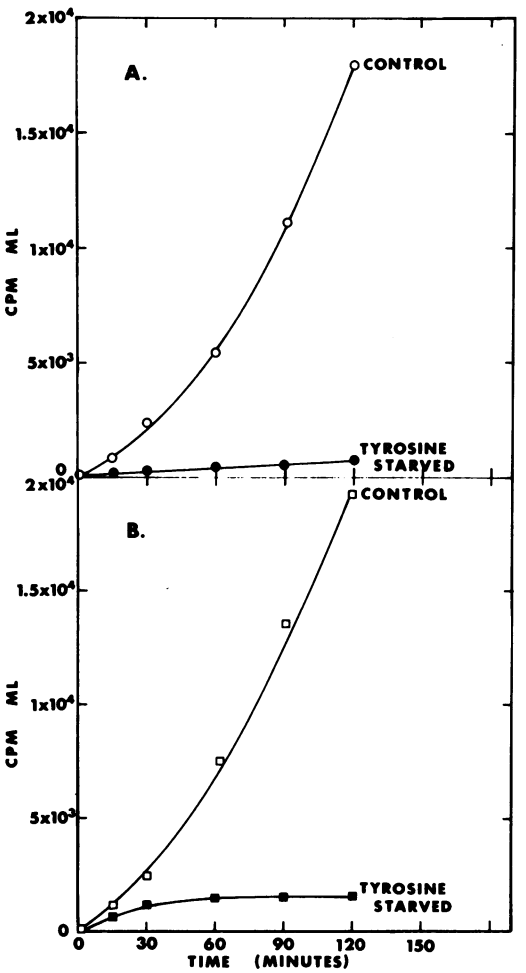

FIG. 5. *Effects of tyrosine starvation on protein and RNA synthesis. At zero time, a culture growing in nonradioactive medium was harvested, washed, and resuspended in tyrosine-free medium. The suspension was divided into two sets (A and B) of two samples; tyrosine was readded to one sample from each set as a control. In set A, ^{3}H-lysine (final concentration of 0.5 μCi/ml) was added at zero time to monitor protein synthesis. In set B, ^{3}H-uracil (final concentration, 0.5 μCi/ml) was added to monitor RNA synthesis.*

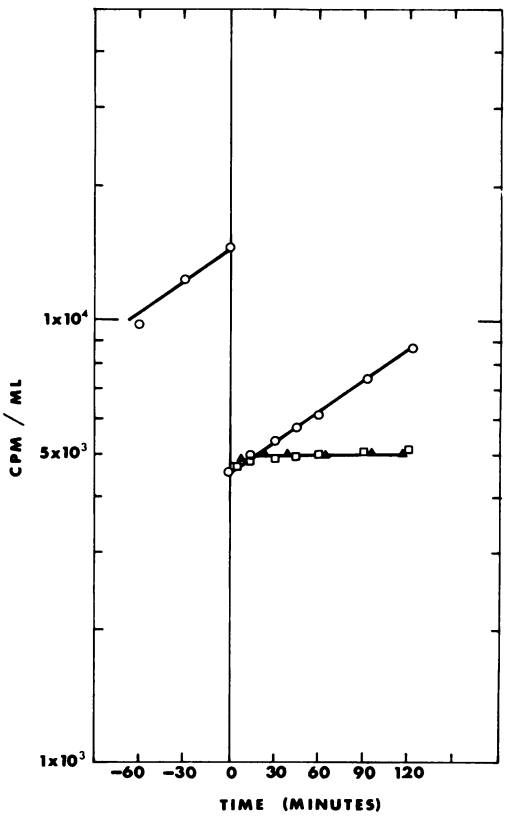

FIG. 6. *Comparison of cycloheximide and tyrosine starvation on the accumulation of RNA. At zero time, a culture growing in the presence of ^{14}C-uracil (0.05 μCi/ml) was harvested, washed, and resuspended in tyrosine-free medium. The suspension was quickly divided into three samples. In the control sample (○), tyrosine was readded to the original concentration. In the second sample (□), tyrosine was readded along with sufficient cycloheximide to give a final drug concentration of 20 μg/ml. The third sample (▲) was maintained in tyrosine-free medium. In each sample, the ^{14}C-uracil concentration was identical to that present during the final period of growth. RNA accumulation was measured by the incorporation of the ^{14}C-uracil. The control points before zero time were obtained prior to harvesting and washing the culture.*

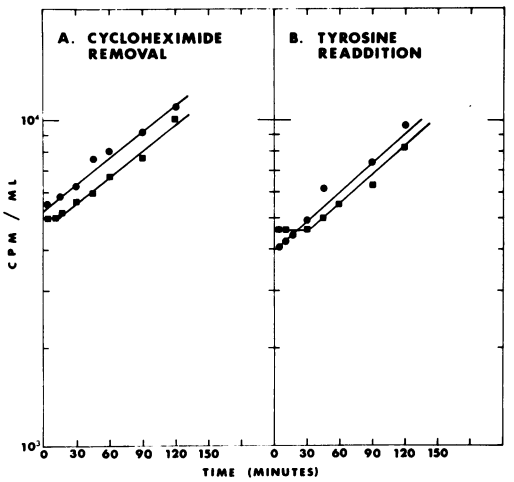

FIG. 7. *Resumption of protein and RNA accumulation upon relief from the inhibition caused by cycloheximide or tyrosine starvation. (A) Removal of cycloheximide. Two hours preceding zero time, a pair of cultures growing in medium containing either ^{3}H-lysine (0.25 μCi/ml) or ^{3}H-uracil (0.25 μCi/ml) were harvested and washed. Each culture was resuspended in complete medium containing 20 μg of cycloheximide per ml. After 2 hr of incubation, the cycloheximide was removed from both cultures by harvesting and washing with drug-free medium. This was considered zero time, and each culture was now resuspended in drug-free medium. Protein accumulation (●) was monitored with ^{3}H-lysine and RNA accumulation (■) with ^{3}H-uracil. The label concentration was held constant throughout the experiment, including the period of incubation with cycloheximide. (B) Readdition of tyrosine. The experiment was conducted in essentially the same manner as described in A, with 2 hr of tyrosine starvation replacing the cycloheximide treatment. At zero time, enough tyrosine was added to each starved culture to give the standard concentration (20 μg/ml). Protein (●) and RNA (■) accumulation were monitored as described in A.*

that the methods we chose to inhibit protein synthesis were specific and did not cause any overall metabolic disturbance.

DISCUSSION

This report demonstrates that RNA production during steady-state growth in yeast is inhibited in identical fashion when protein synthesis is blocked by either amino acid starvation or cycloheximide. Amino acid starvation was accomplished by rapidly removing a required amino acid (tyrosine or tryptophan) from an auxotrophic strain; this treatment immediately stopped net protein synthesis. Amino acid starvation presumably blocks protein synthesis by preventing the formation of a required amino acylated transfer RNA (4, 7, 17, 18). Cycloheximide is a widely used inhibitor of protein synthesis in eukaryotic systems (5, 9, 13). The drug interferes with ribosomal peptide bond formation without affecting amino acylation of transfer RNA (22, 23, 26). Consistent with this interpretation is the observation that cycloheximide-resistant yeasts have altered ribosomes (3, 23). In spite of differences in their mechanism of inhibition of protein synthesis, both amino acid starvation and cycloheximide inhibited RNA synthesis in identical fashion, suggesting a common mode of action. To account for these results, we propose that RNA production in rapidly growing yeast is dependent on continuous protein synthesis. According to this view, amino acids do not play a unique role in the regulation of RNA synthesis; amino acid deprivation simply represents one of a number of possible ways to interfere with protein biosynthesis. Independent support for this proposal is found in the relationship between RNA and protein synthesis in temperature-sensitive mutants (10–12). Of 21 independent mutants blocked at various steps in protein synthesis, all showed reduced RNA synthesis. One mutant (*ts*-187), blocked specifically in initiation, was examined in great detail; at the restrictive temperature (36 C), protein synthesis quickly stopped, and after 20 to 30 min, RNA synthesis also stopped. This is exactly what was found with amino acid starvation and cycloheximide.

In yeast, the apparent absence of a unique amino acid-RNA control system differs sharply from observations made in bacteria. In rapidly growing bacteria (*E. coli*), RNA synthesis quickly responds to a cut off in the amino acid supply but is relatively unaffected by a cessation in protein synthesis caused by chloramphenicol or other ribosome-inhibiting drugs. The rapid response to amino acid starvation, known as "stringent" behavior, is controlled by the RC^{str} gene; the alternative allele of this gene (RC^{rel}) allows the "relaxed" phenotype. In relaxed strains, amino acid starvation, like chloramphenicol, has little effect on RNA synthesis. Although the rapid response of RNA synthesis in yeast to inhibition of protein synthesis bears a formal resemblance to stringent amino acid control, there is no evidence that the same amino acid-sensitive mechanism is involved. In contrast, the results show that during steady-state growth amino acids do not play a unique role in rapid, short-term regulation. This does not rule out the possibility that amino acids perform some other function required for RNA synthesis. For example, DeKloet (5) has shown that amino acids affect RNA synthesis under conditions of limited

growth accompanying a stepdown transition for a rich to a poor medium. Our proposal that RNA synthesis in yeast is controlled by continuous protein synthesis does not identify the actual mechanism(s) involved. As in *E. coli*, the existence of an RC-type system in yeast will require the demonstration of alternative stringent and relaxed states.

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