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INEFFICIENCY OF THE STRINGENT RESPONSE IN THE FUNGUS MUCOR

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SUMMARY

Removal of a required amino acid from the growth medium or addition of cycloheximide caused an immediate stoppage of growth and protein synthesis in the fungus Mucor racemosus. However, RNA synthesis persisted for several hours at rates that only gradually decreased under the same circumstances. An analysis of the major classes of RNA synthesized during the first hour of treatment showed that cycloheximide preferentially inhibited rRNA synthesis, whereas amino acid starvation slowed synthesis of all RNA species uniformly. Neither treatment affected the percentage of mRNA synthesized. The partial and delayed effects of amino acid starvation and cycloheximide treatment on RNA synthesis reported here suggest the absence of or the gross inefficiency of a classical stringent response in M. racemosus.

The stringent response has been well characterized in bacteria. It involves the immediate cessation of most RNA synthesis in response to amino acid starvation (1,2). Stoppage of protein synthesis per se, as by means of chloramphenicol, is not sufficient to cause the stringent response (1,2). Rather, the accumulation of uncharged tRNA's in the ribosomal acceptor sites stimulates the generation of ppGpp which inhibits transcription by interferring with the interaction of RNA polymerase and promoter regions on the DNA (3-5). Complete and immediate inhibition of rRNA synthesis is the most pronounced effect of ppGpp on transcription. Additional effects of this nucleotide include blocking the synthesis of most tRNA's and the mRNA's specific for ribosomal and numerous other proteins while stimulating the synthesis of mRNA's specific for amino acid biosynthetic enzymes (1,2). An effect analogous to the stringent response in bacteria has been described in the ascomycetous fungus Saccharomyces cerevisiae (6-9). The effect is different, however, in that the stoppage of protein synthesis by any of several means is invariably accompanied by a rapid cessation of RNA synthesis. Starvation of amino acid auxotrophs

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(6-9), cycloheximide treatment (10) and the use of temperature-sensitive mutants (10) all mimick stringency. Lowering of charged tRNA levels is therefore not a sine qua non of eukaryotic stringency. The molecular mechanism of this stringency must also differ from the prokaryotic paradigm in that ppGpp is absent from eukaryotic cells (11). Several observations are made in the present paper which suggest that the stringent response, if indeed there is one, may be regulated in yet another, less efficient, way by the phycomycetous fungus Mucor racemosus.

MATERIALS AND METHODS

Mucor racemosus (ATCC 1216B) was the parental strain for all auxotrophs. Leucine auxotrophs Leu-1A, Leu-2A and Leu-2B were provided by J. Peters (12). Sporangiospores were produced on YPG agar plates as previously described (13). The defined medium of Larsen and Sypherd (14) was supplemented with 1 μmol/ml of L-leucine. All cultures were inoculated with 10^6 spores/ml, sparged with water-saturated air, and shaken (100 rpm) at 22°C. Approximately 10-12 hours later, when the spores had germinated into short hyphae, protein synthesis was stopped by adding cycloheximide to the medium (200 μg/ml final concn) or collecting the cells on a filter (Millepore, type AA, 0.8 μm pore size), washing them with and resuspending them in fresh growth medium minus leucine. As a control some cells were filtered, washed and resuspended in fresh medium still containing leucine.

The procedures of Orlowski and Sypherd (15,16) were used to pulse-label cells with L-[U-^{14}C]proline (260 mCi/mmol; final concn, 10 μCi/ml) to measure the kinetics of protein synthesis or [5,6-^{3}H]uracil (32.5 Ci/mmol; final concn, 20 μCi/ml) to measure the kinetics of RNA synthesis. An analysis was made of the RNA synthesized under the various experimental conditions described above. The cells were exposed to [^{3}H]uracil (final concn, 4 μCi/ml) for one hour following the appropriate experimental manipulation. RNA was extracted from the cells and fractionated on sucrose density gradients or oligo-dT cellulose columns as described by Orlowski and Sypherd (16). Intracellular amino acid pools were measured as previously described (15).

RESULTS AND DISCUSSION

Addition of cycloheximide or removal of a required amino acid from the growth medium resulted in the immediate cessation of growth of M. racemosus (Fig. 1). Both experimental manipulations also caused a rapid stoppage of

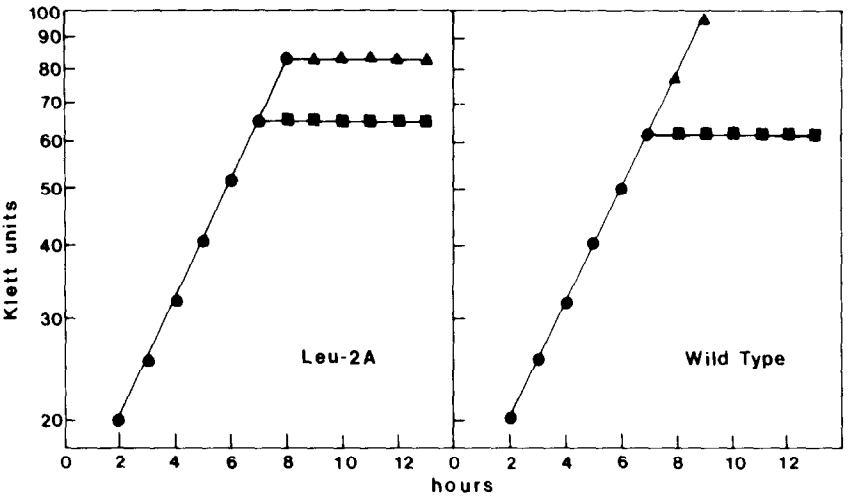

Figure 1. Effect of leucine starvation and cycloheximide treatment on growth of M. racemosus. Growth was monitored with a Klett-Summerson colorimeter (no. 66 filter). The response of leucine auxotroph Leu-2A is compared to that of the wild type. All other leucine auxotrophs behaved similarly. Symbols: (●), growth prior to treatment; (■), growth following addition of cycloheximide to the culture; (▲), growth following removal of leucine from the culture.

protein synthesis (Figs. 2 and 3). An examination of the intracellular leucine pools in strain Leu-2A indicated that they were depleted within the first 30 minutes of leucine starvation (data not shown). However, RNA synthesis continued for at least three hours (Fig. 2) with little reduction in rate over the first hour (Fig. 3). The rate of RNA synthesis was noticeably reduced after that time, but to a greater degree by cycloheximide than by amino acid starvation (Fig. 3). Minor variations in the rapidity of this response were noted among the several auxotrophic strains (Fig. 2). The significance of this variability is presently unclear.

The nature of the RNA synthesized during the first hour of cycloheximide treatment or amino acid starvation was examined. Cells under the appropriate conditions were given a one-hour pulse of [^{3}H]uracil. The labelled RNA was extracted and separated into 4S tRNA, 18S and 25S rRNA and 32S precursor-rRNA on sucrose density gradients, and into polyA-containing mRNA and "bulk" RNA (non-mRNA) on oligo-dT cellulose columns. The sucrose gradients were scanned at 254 nm in an ISCO density gradient fractionator (model 640) and radioactivity

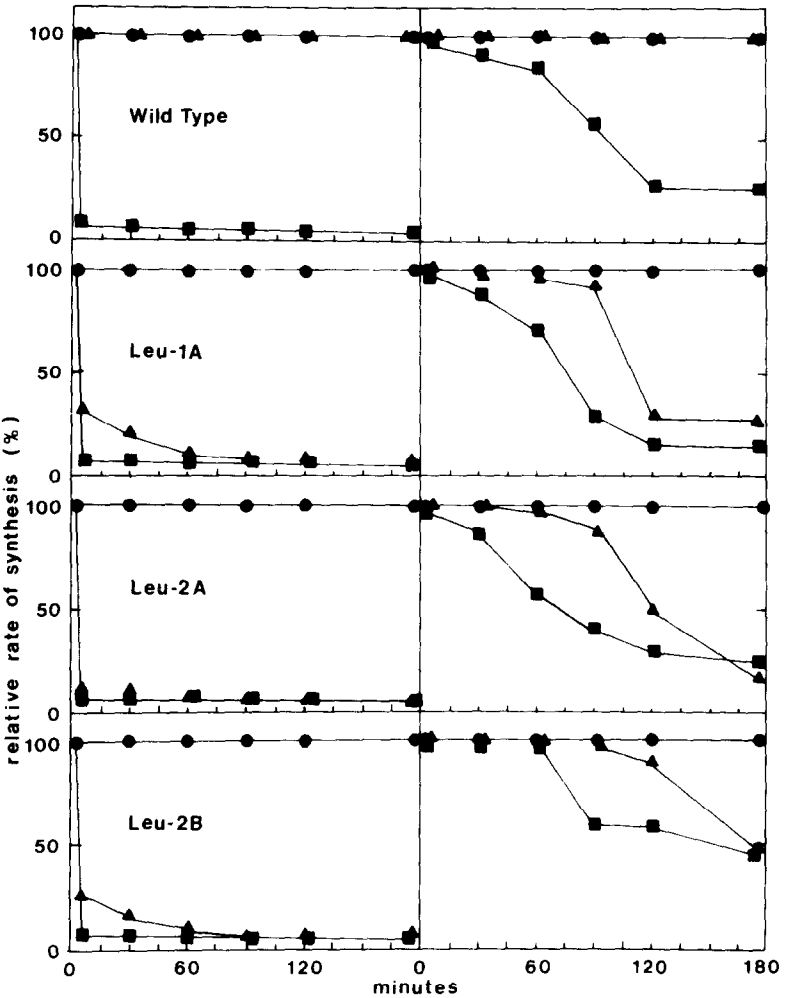

Figure 2. Effect of leucine starvation and cycloheximide treatment on rates of protein and RNA synthesis in M. racemosus. The responses of leucine auxotrophs Leu-1A, Leu-2A and Leu-2B are compared with that of the wild type. The cells were pulsed with L[^{14}C]proline or [^{3}H]uracil at the indicated times to measure the kinetics of protein or RNA synthesis (15,16). All rates are expressed as a percentage of the rates measured in untreated control cultures. Left hand panels: rates of protein synthesis. Right hand panels: rates of RNA synthesis. Symbols: (●), untreated control culture; (■), cycloheximide-treated culture; (▲), leucine-starved culture.

under the peaks was quantitated in a liquid scintillation spectrometer following precipitation in cold trichloroacetic acid. Hence the relative and absolute rates of synthesis for each class of RNA could be determined.

Starvation for leucine did not cause any significant changes in the relative rates of synthesis of any of the major classes of RNA in strains Leu-2A

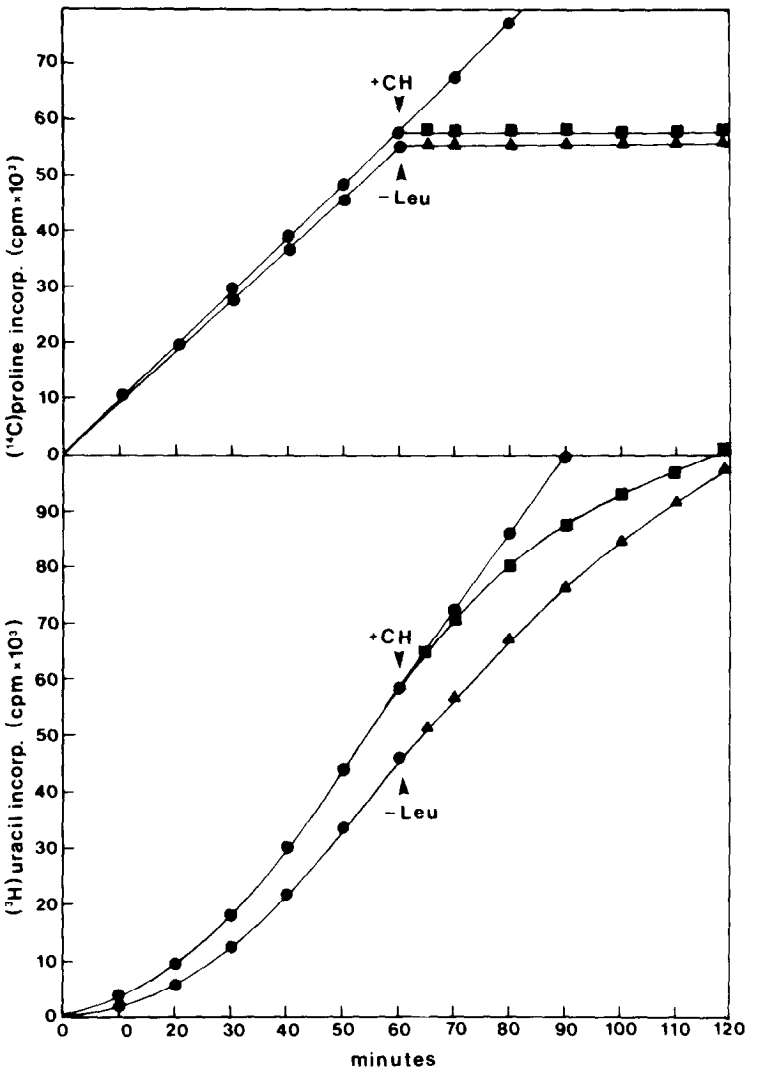

Figure 3. Kinetics of protein and RNA synthesis in M. racemosus, strain Leu-2A, immediately following leucine starvation and cycloheximide treatment. L[^{14}C]proline and [^{3}H]uracil incorporation was measured (15,16) at 10-minute intervals to ascertain constant rates of protein and RNA synthesis. After 60 minutes, cycloheximide was added or leucine was removed from the culture (note arrows) and the measurement of radioisotope incorporation kinetics was continued. Top panel: kinetics of protein synthesis. Bottom panel: kinetics of RNA synthesis. Symbols: (●), untreated control culture; (■), cycloheximide-treated culture; (▲), leucine-starved culture.

(Table 1) or Leu-2B (not shown) but the absolute rates were reduced by 40% for all classes of RNA (Table 1). Cycloheximide, on the other hand, caused a considerable decrease in the absolute and relative rates of synthesis of 18S, 25S and 32S RNA's, while stimulating a significant increase in the relative but

Table 1. Analysis of the Major Classes of RNA Synthesized by M. racemosus, strain Leu-2A, during Leucine Starvation and Cycloheximide Treatment

Culture Conditions	Class of RNA											
	4-6S			18S			25S			32S		
	A	B	C	A	B	C	A	B	C	A	B	C
Control	15.7	18.0	13.5	21.9	25.1	23.5	43.3	49.8	22.9	6.2	7.1	12.1
Leucine Starvation	8.9	17.1	7.6	13.6	26.1	14.5	24.5	47.2	12.9	5.0	9.5	9.7
Cycloheximide Treatment	11.4	71.3	7.8	1.1	6.6	1.5	2.3	14.0	1.2	1.3	8.0	3.1

Leu-2A cells grown in the presence of leucine were exposed to [^{3}H]uracil for 1 hour following transfer to new medium i) still containing leucine (control), ii) lacking leucine or iii) containing cycloheximide. RNA was extracted and fractionated on sucrose gradients as previously described (16). A total of 4.5 A_{260} units of purified RNA was applied to each gradient. Four peaks of the indicated weight classes were recovered. The A_{254} and radioactivity in each class of RNA was quantitated (16). A: Total cpm x 10^4 under peak. B: Percent of total cpm recovered from gradient. C: Cpm x 10^4/ A_{254} of individual peak.

Table 2. Synthesis of mRNA in M. racemosus, strain Leu-2A, during Leucine Starvation and Cycloheximide Treatment

Culture Conditions	"Bulk RNA" (cpm/ A_{254})	mRNA (polyA+RNA) (cpm/ A_{254})	Percent
Control	18.7×10^4	5.6×10^3	3.0
Leucine Starvation	13.7×10^4	4.1×10^3	3.0
Cycloheximide Treatment	5.7×10^4	1.7×10^3	3.5

Labelling conditions were the same as described for Table 1. RNA was extracted and fractionated on oligo-dT cellulose columns as previously described (16). A total of 4.0 A_{260} units of purified RNA was passed through the column and the radioactivity recovered in the "bulk RNA" and mRNA fractions was normalized to this value.

not the absolute rate of tRNA synthesis (Table 1). This notable increase in 4-6S material carrying radioactivity was not a consequence of increased mRNA production. The proportion of labelled RNA containing polyA sequences was not appreciably altered from the control upon starvation for leucine or cycloheximide treatment in strains Leu-2A (Table 2) or Leu-2B (not shown).

If indeed the present data justify the description of a stringent response in Mucor, such response is notably different from that observed either in bacteria (1,2) or in the fungus Saccharomyces (6-10). The response to starvation for a required amino acid is much less efficient in Mucor than in these other organisms. The response to cycloheximide is completely unlike the bacterial response to chloramphenicol (1,2). This response is similar to, though less efficient than, the response of Saccharomyces to cycloheximide in which all rRNA and much tRNA is shut off immediately (10).

Since either leucine starvation or cycloheximide treatment put an immediate end to [^{14}C]proline incorporation, but neither treatment stopped [^{3}H]uracil incorporation in Mucor, the contention made by several authors (6-10) that inhibition of protein synthesis per se elicits a stringent response in fungi cannot be a correct generalization. There is no reason to assume, as other authors have done, that cycloheximide, because it mimicks the effects of amino acid

starvation in Saccharomyces (6-10), affects RNA synthesis via an effect on protein synthesis. It is well established that cycloheximide has many secondary effects on fungal cells, such as an inhibition of transport systems (17) and post-transcriptional processing of RNA's (18). In Mucor, cycloheximide, though it inhibited protein synthesis no more effectively than amino acid starvation, had a much quicker and selective effect on RNA synthesis than did leucine starvation. This may suggest that the drug's effect is mediated on some level other than or in addition to protein synthesis per se.

Lack of a classical stringent response need not be catastrophic during periods of nitrogen starvation in this organism. The wild type of M. racemosus is prototrophic for all required amino acids. Perhaps depletion of some nitrogenous intermediary metabolite other than an amino acid, or an amino acid of general metabolic importance such as glutamate, is the signal to stop all biosynthesis. Inefficiency of the classical stringent response may actually be the rule rather than the exception among eukaryotes. Although S. cerevisiae (6-10) and Neurospora crassa (19) exhibit a classical rapid shutdown of RNA synthesis upon amino acid starvation, Chlamydomonas reinhardi (20), HeLa cells (21), mouse fibroblasts (22) and Chang's liver cells (23) show only a delayed and partial response similar to that described here for M. racemosus.

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