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Stored mRNA in Sporangiospores of the Fungus *Mucor racemosus*

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When introduced into nutrient medium under air, the asexual sporangiospores of *Mucor racemosus* germinated within 5 to 8 h, culminating with the emergence of germ tubes. We found that sporangiospores increased 20% in dry weight during the first 60 min of germination, indicating a high degree of synthetic activity. Sucrose density gradient analysis of spore extracts revealed that the percentage of ribosomes associated with mRNA increased from 22.5% in dormant spores to 85% within 10 min after the addition of medium and remained at this level for at least 3 h. L-[^{14}C]leucine was immediately incorporated at a rapid rate into protein of a leucine auxotroph, whereas [^{3}H]uracil or [^{32}P]phosphate was incorporated into RNA at a significant rate only 20 min after the addition of medium. This newly synthesized RNA occurred in polysomes only after 30 min had passed. Pool equilibration of the radioactive precursors was not limiting to these measurements. Polyadenylated RNA was isolated from dormant spores by oligodeoxythymidylic acid-cellulose chromatography and was found to comprise 3.3% of the total cellular RNA. Sucrose density gradient centrifugation revealed the polyadenylated RNA to be heterodisperse in size, ranging from 6S to 20S. It was concluded that *M. racemosus* sporangiospores contain preformed mRNA which is translated commencing immediately upon the addition of nutrient medium.

Mucor racemosus is a zygomycete capable of growing as a yeast or a hypha depending upon the environmental conditions (22). On solid medium the hyphal form of the organism produces asexual sporangiospores which can develop new hyphal germ tubes within 5 to 8 h after reintroduction into a nutrient medium under air. During germination the sporangiospores swell to several times their original size. In addition, there is an exponential accumulation of cellular RNA and protein (16). The mechanism by which *M. racemosus* is able to trigger this biosynthetic activity in dormant spores is unknown.

Several other fungal species, including *Allomyces macrogynus* (20), *Botryodiplodia theobromae* (6), *Blastocladiella emersonii* (5), and *Rhizopus stolonifer* (4, 23), produce dormant spores containing stored mRNA which is translated upon germination. The presence of stored mRNA in the spores suggests that the triggering mechanism for germination in these organisms may be at the level of translation. For example, *B. emersonii* zoospores store polyadenylated RNA [poly(A)$^+$ RNA] in a cellular structure called the nuclear cap (5). This poly(A)$^+$ RNA is associated with single ribosomes in such a manner that the mRNA is stable but not translated until germination commences. A low-molecular-weight inhibitor (possibly a nucleotide), which reversibly binds ribosomes, has been implicated in this control (1).

M. racemosus can reportedly regulate the velocity of translation and the percentage of ribosomes active in protein synthesis as a function of yeast-to-hypha morphogenesis (17) or changes in the growth rate (13). Such observations suggest the possibility of differential gene expression regulated at the translational level. The experiments in this study were designed to determine whether dormant *Mucor* sporangiospores contain stored mRNA, which is translated during germination. Such findings would imply the participation of translational control in *Mucor* gene expression.

MATERIALS AND METHODS

Organism, spore production, and germination. *M. racemosus* (*M. lusitanicus*) ATCC 1216B or a leucine auxotroph (*leu2A*) derived from this strain were used in these experiments. The growth medium (YPG) contained 0.3% yeast extract, 1.0% peptone (Difco Laboratories, Detroit, Mich.), and 2% glucose (pH 4.5). Sporangiospores were produced on YPG agar plates kept at room temperature (22°C) for 7 to 10 days. Spores were harvested by scraping them into TMK buffer (see below) or directly into YPG medium and were incubated at 200 rpm on a shaker under air at room temperature for the desired length of time. All hyphae remained attached to the agar and never

contaminated the spore suspension. Spore concentrations varied over the range of 3.3×10^7 to 1×10^8 spores per ml.

Density gradient analysis of ribosomes. Protein synthesis was stopped by the addition of cycloheximide (200 μg/ml) directly into the culture medium followed by an additional 2 min of shaking. A time zero (dormant spores) was obtained by harvesting spores directly into TMK buffer (50 mM Tris-hydrochloride [pH 7.25], 10 mM magnesium acetate, 500 mM KCl) containing cycloheximide (200 μg/ml) or cycloheximide plus verrucarin (25 μg/ml). The cells were collected and washed with cold TMK buffer plus antibiotics on a membrane filter (Millipore Corp., Boston, Mass.; type AA, pore size, 8 μm). The cells were broken by grinding in a mortar under liquid N_2. Spore extracts (15,000 × *g*, supernatant fractions) were prepared in TMK buffer plus antibiotics, and the ribosomes were fractionated by centrifugation on 10 to 40% linear sucrose density gradients as described by Orlowski and Sypherd (17). Gradients were scanned at 254 nm with an ISCO model 640 density gradient fractionator equipped with a chart recorder. The percentage of ribosomes in polysomes and active monosomes versus inactive subunits was calculated from the absorbance profiles by the procedure of Martin (10).

For measuring [^{32}P]phosphate incorporation into ribosome fractions, spores were harvested in YPG medium containing 8.5 μCi of [^{32}P]phosphoric acid per ml, and the above procedure was carried out. Fractions (6 drops) were collected into cold 10% trichloroacetic acid and kept on ice for 30 min. The precipitate in each fraction was collected on a glass fiber filter (Reeve Angel, Clifton, N.J.), washed four times with 1-ml volumes of 10% trichloroacetic acid, dried under a heat lamp, placed into liquid scintillation counting fluid, and assayed for radioactivity with a Beckman LS-200 liquid scintillation spectrometer.

Measurement of RNA and protein synthesis with radioactive precursors. For measurement of RNA synthesis, sporangiospores were harvested into YPG medium containing [^{3}H]uracil (10 μCi/ml) or [^{32}P]phosphoric acid (20 μCi/ml). Samples (100 μl) of culture were withdrawn at 2-min intervals and placed into 1 ml of ice-cold 10% trichloroacetic acid. After 30 min on ice, the samples were filtered, washed, dried, and assayed for radioactivity as described above. The same procedure was followed for measuring protein synthesis except that spores of a leucine auxotroph (*leu2A*) were harvested into YPG containing L-[^{14}C]leucine (1 μCi/ml). Samples (100 μl) of culture were placed into 1 ml of 10% trichloroacetic acid, heated at 90°C for 30 min, cooled on ice for 30 min, filtered, washed, dried, and assayed for radioactivity as above.

RNA purification. RNA was labeled by harvesting spores in YPG containing [^{32}P]phosphoric acid (8.5 μCi/ml) and incubating for the appropriate time. The spores were collected, washed, and broken, and the RNA was purified by phenol extraction and ethanol precipitation as described previously (15).

Density gradient analysis of RNA. The purified RNA was redissolved in sterile ANE buffer (10 mM sodium acetate, 100 mM NaCl, 1 mM EDTA, pH 5.3) containing 50% formamide, heated to 55°C for 10 min, and fractionated on 5 to 20% linear sucrose density gradients as described previously (15). The gradients were scanned at 254 nm, and 6-drop fractions were collected into cold 10% trichloroacetic acid. Each fraction was filtered, washed, dried, and assayed for radioactivity as described above.

Oligo(dT)-cellulose fractionation of RNA. RNA for oligodeoxythymidylic acid [oligo(dT)]-cellulose fractionation was purified as described previously (15) and redissolved in TK buffer (10 mM Tris-hydrochloride [pH 7.5] and 500 mM KCl). A volume of solution containing 135 μg of RNA was loaded onto an oligo(dT)-cellulose column (0.5 g [dry weight], 1-ml bed volume) which had been prewashed with 0.1 N KOH followed by TK buffer. Non-poly(A)$^+$ RNA ("bulk" RNA) was eluted from the column with approximately 15 ml of TK buffer collected in 8-drop fractions. Poly(A)$^+$ RNA (putative mRNA) was eluted from the column with approximately 10 ml of TO buffer (10 mM Tris-hydrochloride, pH 7.5) collected in 8-drop fractions. The fractions were collected directly into liquid scintillation fluid, and radioactivity was assayed as above.

Dormant sporangiospores (time zero) were harvested directly into TMK buffer containing cycloheximide (200 μg/ml) and the energy poisons NaN_3 (10 mM) and NaF (10 mM), which were shown to completely prevent any RNA synthesis. The RNA was purified as described above and labeled by methylation with [^{3}H]dimethyl sulfate in a procedure described by Knight and Van Etten (7). This RNA was fractionated on an oligo(dT)-cellulose column as above. These fractions were filtered, washed, dried, and assayed for radioactivity as shown above.

Measurements of free intracellular amino acids and nucleotide pools. Free intracellular amino acids were extracted and quantitated as previously described (14) except that 10% formic acid was used instead of acetic acid.

Nucleotide pools were labeled by harvesting spores in YPG medium containing [^{32}P]phosphoric acid (200 μCi/ml). At various times 2-ml samples were withdrawn, and the pools were extracted into 10% formic acid as above. Individual nucleotides were separated by DEAE-cellulose thin-layer chromatography (19). After development and drying, the thin layers were exposed to Kodak X-Omat X-ray film for 24 h at −40°C. The films were developed with Kodak X-ray film developer at 22°C and fixed with Kodak rapid fixer. The resultant spots were identified by comparing the R_f values with those of standards run at the same time. Duplicate spots for each ribose nucleoside triphosphate were cut out from the DEAE-cellulose thin layer. One was placed in liquid scintillation counting solution and assayed for radioactivity as described above, whereas the other spot was eluted with 2 M NaCl, and the nucleotide concentration was calculated from the absorbance at 260 nm. ATP pools were also measured by the luciferin-luciferase assay system of Stanley and Williams (21).

Radioisotopes. [^{3}H]uracil (32.5 Ci/mmol) and [^{32}P]phosphoric acid (285 Ci/mg) were purchased from ICN Pharmaceuticals, Irvine, Calif. L-[^{14}C]leucine (289 mCi/mmol) and [^{3}H]dimethyl sulfate (4.7 Ci/mmol) were obtained from New England Nuclear Corp., Boston, Mass.

Biochemicals. Cycloheximide, verrucarin, oligo(dT)-cellulose, nucleotide standards, and firefly lantern

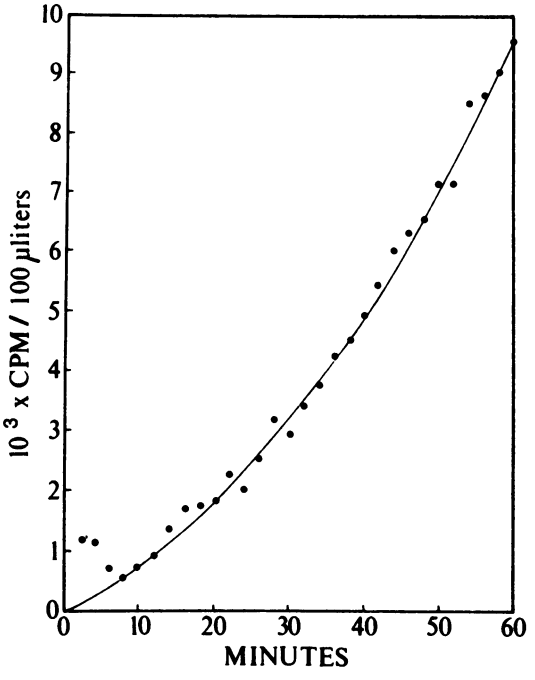

FIG. 1. Incorporation of L-[^{14}C]leucine into hot trichloroacetic acid-insoluble material in germinating spores of a leucine auxotroph (*leu2A*) derived from *M. racemosus* 1216B. Spores were harvested directly into YPG medium containing L-[^{14}C]leucine and incubated with shaking at room temperature under air. Samples (100 μl) were withdrawn from the culture every 2 min and treated as described in the text. Ordinate, Radioactivity incorporated; abscissa, time after exposure of spores to medium.

extract were obtained from Sigma Chemical Co., St. Louis, Mo. DEAE-cellulose thin layers were purchased from Brinkmann Instruments Inc., Westbury, N.Y.

RESULTS

Incorporation of L-[^{14}C]leucine into protein during sporangiospore germination. Spores of *M. racemosus* increased 20% in dry weight during the first 60 min of germination (data not shown). To identify the nature of this newly synthesized material, sporangiospores of a leucine auxotroph (*leu2A*) were harvested in YPG medium containing L-[^{14}C]leucine (see above). Radioactivity was incorporated into protein as soon as the spores were exposed to the medium. The kinetics of its accumulation were exponential for the duration (60 min) of the experiment (Fig. 1). The presence of cycloheximide in the medium completely prevented any protein synthesis (data not shown). Previously performed analyses of exogenous L-[^{14}C]leucine entry into the intracellular pools of several morphological forms of *M. racemosus* showed that equilibration was extremely rapid and not limiting to the apparent rate of leucine incorporation into protein (14). The present amino acid pool analysis of the *leu2A* strain revealed that the sporangiospores contained no intracellular leucine. Leucine accumulation commenced as soon as the spores were exposed to an exogenous source of the amino acid (YPG medium).

Ribosome analysis. The percentage of ribosomes associated with mRNA (polysomes plus 80S monosomes) increased from 35% in dormant spores to 85% within 10 min after introduction of the spores to nutrient medium and remained at this value for at least 3 h (Fig. 2). When verrucarin was added to the system (see above), 22.5% of the ribosomes were bound to mRNA in the dormant spore. Verrucarin is a protein synthesis initiation inhibitor which prevents ribosomes from binding to mRNA (3). Cycloheximide is an elongation inhibitor (18), and when present alone in the system, possibly allowed a low level of ribosomal subunit binding to mRNA during harvesting. It should not be assumed that

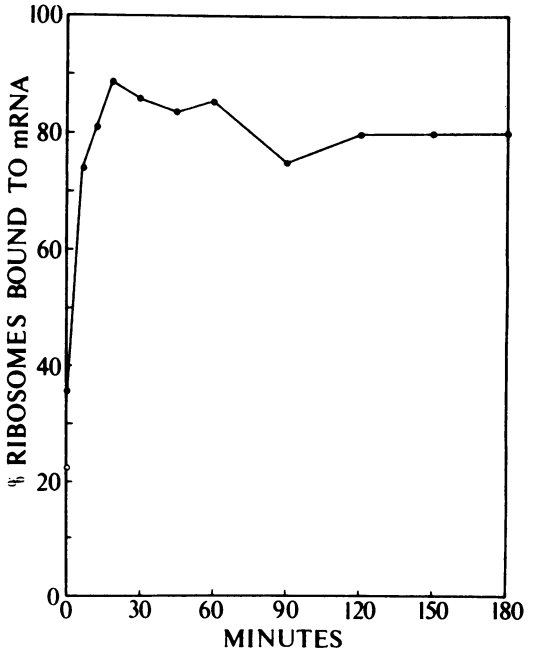

FIG. 2. Percentage of ribosomes associated with mRNA (polysomes plus 80S monosomes) in germinating sporangiospores of *M. racemosus*. Spore extracts were fractionated on sucrose density gradients as described in the text. Ordinate, Percent mRNA-bound ribosomes; abscissa, time after exposure of spores to medium. Symbols: O, spores treated with cycloheximide plus verrucarin; ●, spores treated with cycloheximide alone.

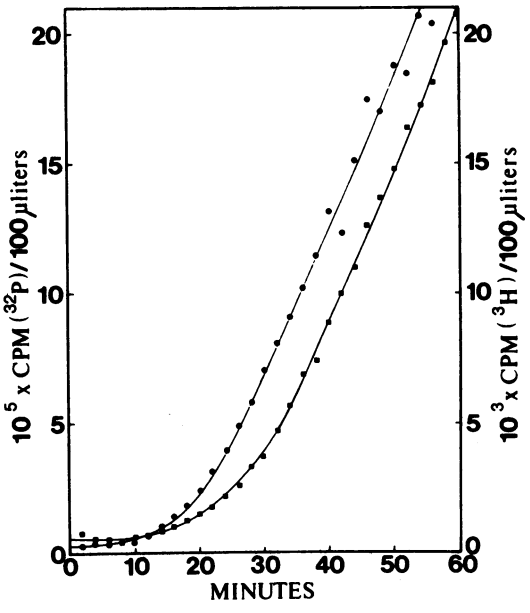

FIG. 3. Incorporation of [^{3}H]uracil and [^{32}P]phosphate into cold trichloroacetic acid-insoluble material in germinating sporangiospores of *M. racemosus*. Spores were harvested directly into YPG medium containing [^{3}H]uracil or [^{32}P]phosphoric acid and incubated with shaking at room temperature under air. Samples (100 μl) were withdrawn from the culture every 2 min and treated as described in the text. Ordinate, Radioactivity incorporated; abscissa, time after exposure of spores to medium. Symbols: ●, [^{3}H]uracil label; ■, [^{32}P]phosphate label.

the mRNA-bound ribosomes in the dormant spore are actively translating the message, since the spores of *leu2A*, which contain no free leucine, also possess approximately 35% of their ribosomes in association with mRNA. Unfortunately, dormant spores cannot be assayed for ongoing protein synthesis in a radioisotope incorporation experiment, because mere wetting of the spores probably breaks dormancy (see below).

Incorporation of [^{3}H]uracil and [^{32}P]phosphate into RNA during sporangiospore germination. There was a lag period of at least 10 min before labeled precursors were incorporated into RNA (Fig. 3). From 10 to 20 min, incorporation took place at a relatively low rate and did not reach a maximum until after 20 min. Measurements of uptake of [^{3}H]uracil indicated that this compound equilibrated with the free nucleotide pools in *M. racemosus* at rates nonlimiting to the measurement of RNA synthesis (15). Thin-layer chromatography of ^{32}P-labeled ribose nucleoside triphosphates showed that high levels of radioactivity had already accumulated in the pools within 5 min (data not shown), yet labeling of RNA did not begin until after 10 min. Uptake of [^{32}P]phosphate into pools was not the limiting step for the lag seen in RNA labeling. ATP pools per se were also measured at frequent intervals by the luciferin-luciferase assay (21). The level of intracellular ATP was halved between 2 and 10 min and then expanded to near its original value by 40 min (200 ± 10 ng of ATP per mg [dry weight] of cells).

Analyses of RNA synthesized during sporangiospore germination. ^{32}P-labeled RNA was purified from sporangiospores as described above. The RNA was fractionated on 5 to 20% sucrose density gradients which separated the RNA into three peaks: 4 to 6S, 18S, and 25S, representing tRNA and the four species of rRNA. [^{32}P]phosphate was incorporated at low levels into the 4 to 6S peak by 10 min, but not into the 18S and 25S RNA species until 15 min of germination. The labeling in the 4 to 6S peak occurred at a greater rate than in the other RNA species until 20 min, after which the incorporation of radioactivity into 18S and 25S increased rapidly to a rate representative of whole cell labeling (data not shown).

Purified RNA, labeled for various lengths of time, was also fractionated by oligo(dT)-cellulose chromatography. An example of such a fractionation, using RNA methylated with [^{3}H]dimethyl sulfate, is shown in Fig. 4. The RNA peak eluted from the column in high-salt buffer (peak A) was composed of tRNA and rRNA. The second peak (peak B), eluted from the column in low-ionic-strength buffer, was a poly(A)$^+$ form of RNA and represented presumptive mRNA. When *Mucor* RNA from peak A was passed through the column a second time in high-ionic-strength buffer, it did not bind. All RNA from peak B bound the column in a second passage and was eluted in low-ionic-strength buffer. None of the labeled RNA bound to a cellulose column lacking the oligo(dT) ligands. The radioactivity in peaks A and B was added at each time point. [^{32}P]phosphate incorporation into poly(A)$^+$ RNA did not begin until after 10 min of germination and did not attain a significant rate until 20 min had passed (Fig. 5). ^{32}P-labeled poly(A)$^+$ RNA from spores 40 min into germination was further fractionated on 5 to 20% sucrose density gradients together with unlabeled *Mucor* marker RNA. The poly(A)$^+$ RNA was heterodisperse in size, ranging from 6S to 20S (data not shown).

Extracts from ^{32}P-labeled spores were layered onto 10 to 40% sucrose density gradients for ribosome analysis, as described above. Radioactivity was incorporated into 40S and 60S subunits by 15 min, but did not appear in polysomes until approximately 30 min (Fig. 6). Therefore,

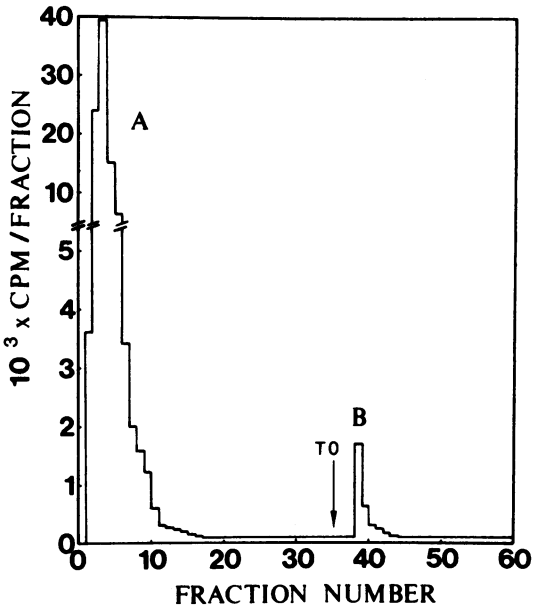

FIG. 4. Fractionation of RNA from dormant sporangiospores of *M. racemosus* on an oligo(dT)-cellulose column. The RNA was purified, methylated with [^{3}H]dimethyl sulfate, and fractionated on an oligo-(dT)-cellulose column as described in the text. The arrow indicates a change in elution buffer from high ionic strength (TK) to low ionic strength (TO). Radioactivity in the eluent fractions was measured and plotted versus fraction number. Peak A represents RNA not retained by the column [non-poly(A)$^+$ RNA]. Peak B represents RNA initially bound by the column and released at low ionic strength [poly(A)$^+$ RNA].

30 min is the earliest possible time at which newly synthesized mRNA could be translated.

Analyses of RNA from dormant sporangiospores. The spores of many fungal species begin active metabolism as soon as they are exposed to an aqueous environment (12), therefore it cannot be assumed that wet-harvested spores are representative of the dormant state. We found that harvesting sporangiospores in buffer containing NaN_3 and NaF (both at 10 mM concentrations) completely inhibited incorporation of [^{32}P]phosphate into RNA (data not shown). (The transcription inhibitors actinomycin D and daunomycin, alone or in combination, failed to stop all RNA synthesis in this system.) RNA purified from spores harvested in this manner was methylated with [^{3}H]dimethyl sulfate and fractionated on an oligo(dT)-cellulose column as described above. Two peaks eluted from the column, one at high ionic strength (bulk RNA) and the second at low ionic strength [poly(A)$^+$ RNA]. The poly(A)$^+$ RNA fraction comprised 3.3% of the total RNA (Fig. 4). Poly(A)$^+$ RNA-containing fractions were pooled. The RNA was precipitated with ethanol, redissolved in ANE buffer with formamide, and fractionated on a 5 to 20% sucrose density gradient together with unlabeled *Mucor* marker RNA. The ^{3}H-labeled poly(A)$^+$ RNA was heterodisperse in size, ranging from 6S to 20S (Fig. 7), a characteristic typical of mRNA.

DISCUSSION

The sporangiospores of *M. racemosus* displayed an immediate burst of metabolic activity when exposed to nutrient medium. The dry weight of the spores increased by 20% during the first 60 min of germination, indicating a great deal of synthetic activity. L-[^{14}C]leucine was incorporated into newly synthesized protein at a rapid rate as soon as the spores were exposed to the medium. The percentage of ribosomes associated with mRNA (in polysomes plus 80S monosomes) increased from 22.5% (with verrucarin present) in dormant spores to 85% within

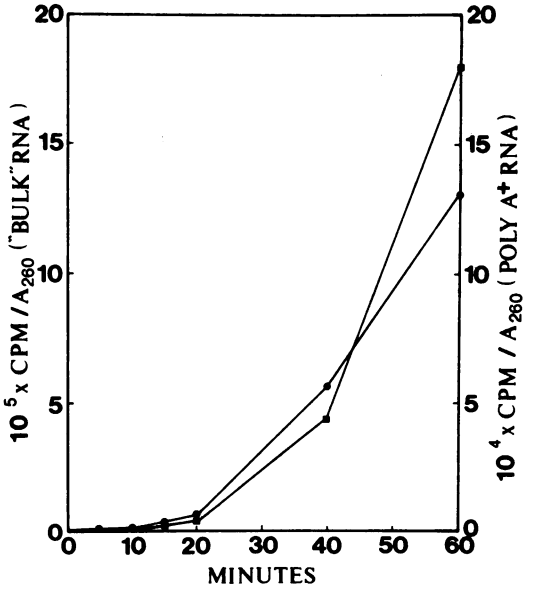

FIG. 5. Time course of poly(A)$^+$ RNA versus bulk RNA synthesis during germination of *M. racemosus* sporangiospores. Spores were harvested directly into YPG medium containing [^{32}P]phosphoric acid and were incubated for appropriate periods of time with shaking at room temperature under air. The RNA was purified and fractionated by oligo(dT)-cellulose chromatography as described in the text. The radioactivity was independently totaled for the bulk RNA fractions and poly(A)$^+$ RNA fractions, was normalized to absorbance at 260 nm (A_{260}), and was plotted versus time of germination. Symbols: ●, bulk RNA; ■, poly(A)$^+$ RNA.

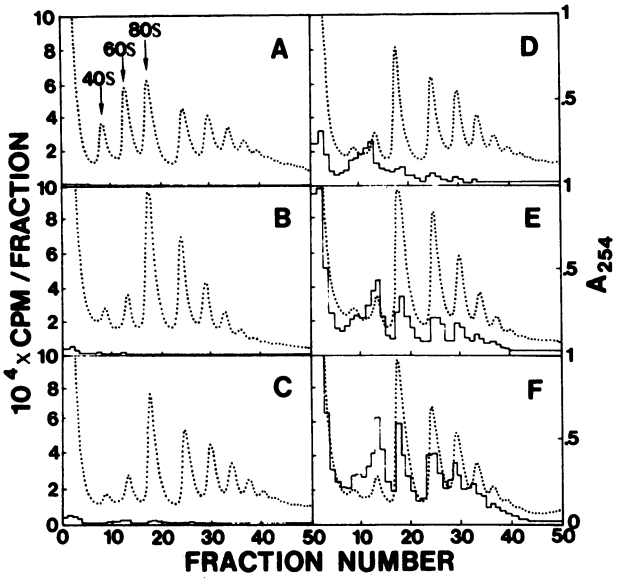

FIG. 6. Newly synthesized RNA: kinetics of appearance in polysomes. Extracts from spores exposed to [^{32}P]phosphoric acid for the indicated times were fractionated on sucrose density gradients as described in the text. Absorbance at 254 nm (.....) and radioactivity (——) were measured in each fraction. The ribosomal subunits and 80S monosomes are identified with arrows. Time after exposure of spores to medium: (A) 5 min; (B) 10 min; (C) 15 min; (D) 30 min; (E) 45 min; (F) 60 min.

10 min. Polysomes have been isolated from ungerminated spores of several other fungal species, including pycnidiospores of *B. theobromae* (2) and conidia of *Neurospora crassa* (11). Polysomes in ungerminated spores can be considered evidence of the presence of stored mRNA. [^{3}H]uracil and [^{32}P]phosphate incorporation into cellular RNA did not begin in *M. racemosus* spores for 10 min and did not reach a significant rate until after 20 min. The spores approximately quadrupled the percentage of ribosomes associated with mRNA and began protein synthesis in the absence of RNA synthesis. Newly synthesized RNA did not appear in polysomes until at least 30 min had passed. These facts suggest that sporangiospores store mRNA, which is immediately translated upon exposure to nutrient medium. Indeed, poly(A)$^+$ RNA, comprising 3.3% of total cellular RNA, was isolated from spores treated with NaN_3 and NaF to totally inhibit new RNA synthesis. The amount of mRNA stored in *Mucor* sporangiospores is comparable to the levels found in spores of other fungi, such as *Blastocladiella* sp., which contained 2.5% of its RNA in the poly(A)$^+$ form (5). The poly(A)$^+$ RNA from *Mucor* spores was shown to be heterodisperse in size, ranging from 6S to 20S, a range similar to that found for stored mRNA from *B. theobromae* pycnidiospores (24).

We propose that the triggering of germination in *M. racemosus* sporangiospores includes regulatory mechanisms affecting translation. (Additional potential mechanisms are not precluded by this assumption.) The spores contain mRNA which is transcribed during sporangiospore maturation, stored in a stable form, and translated immediately upon exposure to nutrient medium. The sites at which translation is blocked could theoretically be at the level of mRNA, tRNAs, the ribosomes, or associated initiation or elongation factors. The high ATP levels in dormant spores suggest that the block is not the absence of available metabolic energy. One intriguing possibility is that the adjustment of ribosome function observed here is controlled by a protein kinase. Protein S-6 of the 40S ribosomal subunit shows a degree of phosphorylation that directly correlates with the rates of cell growth and protein synthesis (9). The S-6 protein is unphosphorylated in dormant sporangiospores of *M. racemosus* but rapidly becomes phosphorylated during germination (8). The significance of the 22 to 35% of ribosomes associated with mRNA in the dormant spore is unclear. One possible explanation is that movement of ribosomes on mRNA is inhibited until germination is triggered. How and why this is are not apparent. The ribosomes may protect the RNA from degradation by intracellular RNases, possibly ex-

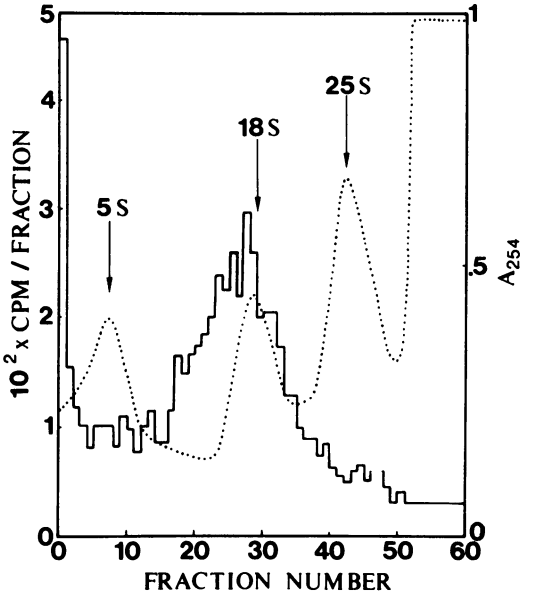

FIG. 7. Size distribution of poly(A)$^+$ RNA from dormant sporangiospores of *M. racemosus*. RNA was purified from spores, methylated with [^{3}H]dimethyl sulfate, and fractionated on an oligo(dT)-cellulose column. The poly(A)$^+$ RNA fractions were pooled, ethanol-precipitated, redissolved in ANE buffer with formamide, and fractionated on a 5 to 20% sucrose density gradient, together with unlabeled *Mucor* marker RNA. Absorbance at 254 nm (A_{254}, dotted line) and radioactivity (solid line) were measured in each fraction. RNA molecules of known size are identified with arrows.

plaining how the mRNA is preserved during spore maturation and dormancy.

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ADDENDUM

We have collected nonhydrated spores by harvesting them, according to procedures described in the text, in a nonaqueous solvent. These spores were broken before exposure to water and were analyzed for ribosome content as described above. The percentage of ribosomes bound to mRNA was found to be 26.5% in mineral oil-harvested spores and 20% in heptane-harvested spores. Thus wet- and dry-harvested spores are comparable with regard to this parameter in *M. racemosus*.

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