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Evidence for the Presence of Messenger Ribonucleic Acid in *Allomyces macrogynus* Mitospores

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Sucrose density gradient analysis was used to show that polysomes were present in the mitospores of *Allomyces macrogynus*. Fifty percent of the spore monosomes were shown to be resistant to dissociation by 0.8 M KCl, indicating that messenger ribonucleic acid (mRNA) was bound to them. These polysomes and all the spore ribosomes were contained in the nuclear cap. Only 4S RNA could be demonstrated in the extra-cap fraction. Hybridization studies using ^{3}H-labeled polydeoxythymidylic acid indicated that polyadenylate was present to the extent of 0.08% of the total spore RNA. Sixty-eight percent of the polyadenylic acid is found in the nuclear cap, and 32% is found in the extra-cap fraction. It was demonstrated that [^{3}H]uridine was taken up by the spores and converted to uridine triphosphate. Lack of incorporation of ^{3}H into RNA indicated that the spores do not synthesize RNA. Thus, the mRNA found in spores is synthesized prior to spore formation.

The spores of many fungi appear to contain mRNA which is stored and then translated during germination. Indirect evidence for this includes the following observations. Mitospores of *Allomyces* germinate in the presence of the RNA synthesis inhibitor actinomycin D (4), protein synthesis occurs in *Botryodiplodia theobromae* conidiospores in the absence of detectable RNA synthesis (3), and protein synthesis occurs in germinating conidia of *Peronospora tabacina* in the presence of the RNA synthesis inhibitors fluorouracil and ethidium bromide (7, 8).

That fungal spores do contain mRNA has been directly demonstrated by two lines of study. RNA containing 3'-polyadenylate [poly(A)] sequences has been found in spores of *Blastocladiella emersonii* (9, 10), *B. theobromae* (12), and *Rhizopus stolonifer* (22). Secondly, polysomes have been found in ungerminated conidiospores of *Botryodiplodia* (2, 3), uredospores of *Uromyces phaseoli* (25), conidia of *Neurospora crassa* (19, 20), and zoospores of *B. emersonii* (9, 16). The presence of either poly(A) or polysomes indicates that mRNA is present.

When grown on agar, *Allomyces macrogynus* produces sporangia at the tips of the hyphae. Sporangia are completely isolated from the tips of the hyphae by a membrane. The sporangium is a multinucleate, coenocytic mass. When agar-grown plants are flooded with a dilute salt solution (4) at room temperature, membrane vesicles appear, fuse, and cleave out individual, uninucleate spores. The spores are released from the sporangium.

Previous work in our laboratory using actinomycin D suggested that spores of the phycomycete *A. macrogynus* contain stored mRNA which is translated during germination. *A. macrogynus* is a particularly interesting organism in which to study the activation and translation of stored mRNA because of the presence of the "nuclear cap" in the spores. This horseshoe-shaped organelle is membrane bound and surrounds, but is distinct from, the cell nucleus. Electron microscope studies indicate that all of the spore ribosomes appear to be in the cap. The cap membrane breaks down during germination, and the ribosomes are dispersed throughout the cytoplasm of the cell (6).

This paper reports the results of studies examining spores for the presence of mRNA and the distribution of poly(A) in the cap and extra-cap fractions. We also determined whether spores would incorporate RNA precursor and whether they synthesized RNA.

MATERIALS AND METHODS

Culture conditions. All conditions for the growth of *A. macrogynus* and preparation of mitospores have been described previously (4). Released spores were collected by centrifugation at 1,500 rpm for 2.5 min in a Sorvall model GLC-1 centrifuge (Ivan Sorvall, Inc., Norwalk, Conn.).

Isolation and sucrose density gradient analysis of polysomes. Polysome isolations and gradient analysis were performed using the procedure of Leaver and Lovett (13). Rat liver RNase inhibitor (3%, wt/

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vol) prepared by the method of Avadhani and Buetow (1) and heparin at 100 μg/ml were added to the polysome buffer (0.25 M NaCl–0.01 M $MgCl_2$–0.25 M sucrose–0.05 M Tris [pH 8.5]). The ribosome pellets were resuspended in TKM buffer (0.06 M KCl–0.006 M $MgCl_2$–0.05 M Tris-hydrochloride [pH 7.6]) before density gradient analysis.

In some cases, the ribosomes were treated before gradient analysis as follows. RNase treatment consisted of a digestion with RNase A (2 μg/ml) incubated at 4°C for 10 min. Treatment with KCl was at a concentration of 0.8 M for 30 min at 4°C. Sucrose gradients also contained 0.8 M KCl. EDTA was added at a concentration of 10 mM for 20 min at 4°C, and puromycin was added at 1 mM in the presence of 0.8 M KCl. The samples were incubated with puromycin for 20 min at 37°C.

The optical density traces of the gradients were monitored by use of an Instrument Specialties Co. (ISCO) model 224, type 4, dual-beam UV flow analyzer with a 5-mm flow cell and recorded using an ISCO model UA-2 UV analyzer (Instrument Specialties Co., Lincoln, Neb.).

Optical density traces of polysome sucrose density gradients were enlarged, traced, and cut out. Areas under the subunit, monosome, and polysome peaks were weighed, and the percentage of each was calculated as the ratio compared to the combined weights of all three areas of the gradient.

Isolation of nuclear caps. Nuclear caps were isolated by modification of the method of Lovett (15). The nuclear cap pellet was suspended in 5 ml of cap buffer (0.075 M sucrose–10% glycerol–0.003 M $MgCl_2$ [pH 6.2]) containing 100 μg of heparin per ml and 3% (vol/vol) rat liver RNase inhibitor. Caps were resuspended in 5 ml of cap buffer and pelleted. The pellets were then suspended in 5 ml of an appropriate buffer and examined under the phase-contrast microscope. The cap preparations were almost entirely intact horseshoe-shaped nuclear caps, were free of any whole cells, and contained a minimal amount of cell debris. The supernatants from the cap-pelleting steps were pooled, and heparin (100 μg/ml) and rat liver RNase inhibitor (3% vol/vol) were added. This is the extra-cap fraction.

When RNA was to be extracted, the caps were suspended in sodium acetate buffer. When polysomes were isolated, the cap pellet was suspended in polysome buffer. The extra-cap fraction was adjusted to the final concentration using 10-times-concentrated stocks of the appropriate buffer.

Isolation of RNA. The spores, nuclear caps, or extra-cap fraction were suspended in 0.01 M sodium acetate–0.10 M NaCl–and 0.01 M EDTA (pH 5.2). Sodium dodecyl sulfate was added to a final concentration of 2%. An equal volume of phenol–chloroform–isoamyl alcohol (64:32:4), presaturated with acetate buffer, was added, and the suspension was well mixed. The aqueous and organic phases were separated by centrifugation, and the aqueous phase was then dialyzed against TKM-2 buffer (0.01 M Tris–0.01 M KCl–0.01 M $MgCl_2$ [pH 7.6]). After dialysis, the RNA samples were treated with DNase (20 μg/ml) for 30 min on ice and then extracted with the phenol mixture. After centrifugation to separate the phases, the aqueous phase was made 2% in potassium acetate. RNA was precipitated at −20°C by the addition of two volumes of cold ethanol. Two subsequent precipitation steps were performed, and the final RNA pellet was suspended in 0.1× concentration of standard saline citrate (0.015 M NaCl and 0.0015 M sodium citrate). RNA concentration was determined by the Orcinol method (24).

Sodium dodecyl sulfate-polyacrylamide gels. All gel procedures used were the methods of Loening (14). After electrophoresis, the gels were transferred to quartz tubes and scanned by transmitted UV light (254 nm) in a model 224 UV flow analyzer, type 4 (ISCO). Radioactive gels were fractionated into 0.1-mm slices using a Gilson Aliquogel Fractionator (Gilson Medical Electronics, Inc., Middleton, Wis.). The fractions were solubilized in 30% H_2O_2 overnight at 35°C and counted in Triton/toluene scintillation fluid. They were counted on an Isocap 300 liquid scintillation counter (Nuclear Chicago, Des Plaines, Ill.).

Measurement of uridine and UTP pool radioactivity. Intracellular [^{3}H]uridine and [^{3}H]UTP were detected using the procedures of Randerath and Randerath (21). Unlabeled standards were mixed with the spore extracts. Extracts were analyzed by one-dimensional chromatography on polyethyleneamine cellulose plates. Chromatography was done using both a LiCl and an NH_4HCO_3 solvent system.

The nucleotides were visualized under UV light using the Transilluminator of Ultra-Violet Products, Inc. (San Gabriel, Calif.), and the spots were marked on the back of the plate with a pencil. The spots were cut out and counted. Counting was done on a Nuclear Chicago Isocap 300 liquid scintillation counter. Corrections were made for the reduced efficiency of counting on the polyethyleneamine plates.

Assay for the presence of poly(A) present in RNA preparations was measured by the method of Kaufman and Gross (11). [^{3}H]polydeoxythymidylic acid [poly-(dT)] was kindly provided by J. Milner.

27S RNA isolated from *Allomyces* was used as a control to check the specificity of hybridization of [^{3}H]poly(dT).

Materials. DNase, RNase-free, and RNase A were secured from Worthington Biochemical Corp., Freehold, N.J. [^{32}P]phosphoric acid and [^{3}H]uridine were secured from New England Nuclear Corp., Boston, Mass. Heparin was purchased from Sigma Chemical Co., St. Louis, Mo. PEI-cellulose plates were obtained from Brinkman Instruments, Inc., Westbury, N.Y.

RESULTS

Presence of polysomes in ungerminated spores. The ribosome fraction of spores was examined for the presence of mRNA in the spores. Ribosomes were isolated from ungerminated spores, and the ribosomal fraction was characterized by means of sucrose density gradient analysis. A polysome fraction was readily evident (Fig. 1a). Peaks containing up to seven ribosomes could be distinguished. Ribosomes isolated from actively growing 16-h cultures are shown for comparison (Fig. 1b). The percentage of ribosomes in polysomes present in ungerminated spores varied from approximately 25 to

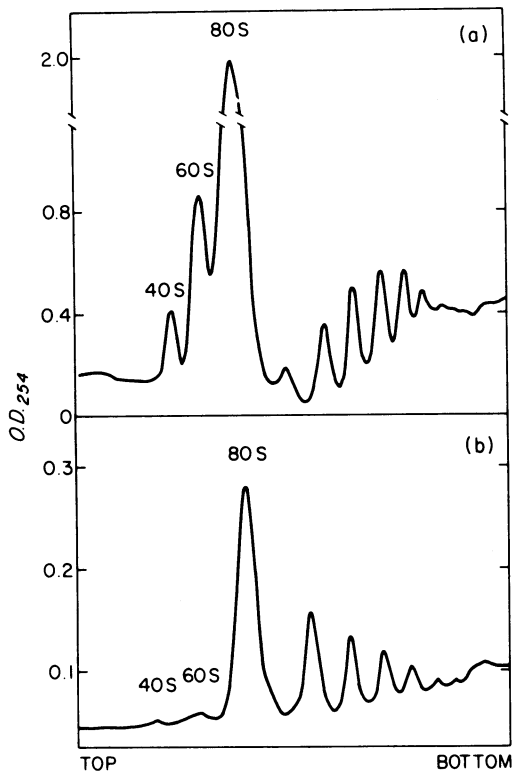

FIG. 1. *Polysome content of A. macrogynus. (a) Polysomes from ungerminated spores. (b) Polysomes from 16-h plants. Spores and plants were obtained as described in the text. Preparation and analysis of polysomes are also described in the text. O.D.*$_{254}$*, Optical density at 254 nm.*

35%, depending on the preparation, whereas those found in 16-h cultures ranged from 55 to 70%, double the fraction present in spores. Spore ribosome preparations also exhibited a considerably greater percentage of ribosomal subunits than found in the 16-h culture. The lesser amount of polysomes and greater amount of subunits seem reasonable since spores do not synthesize protein, whereas 16-h cultures are actively engaged in protein synthesis. The absorbance increases at the bottom of the gradient (Fig. 1a). This increase appeared even after treatment of the polysomes with various chemicals (KCl, EDTA) (see Fig. 3). Preliminary characterization of this rapidly sedimenting material showed it to be almost entirely hexose, and we believe it is a polysaccharide. The percentage of polysomes estimated for ungerminated spores is corrected for the presence of this material.

Further analysis demonstrated that, in addition to the polysomes, approximately 50% of the monosomes found in the spore had mRNA bound to them. This was determined by examining the behavior of spore ribosomes treated with 0.8 M KCl, followed by sucrose gradient analysis in the presence of 0.8 M KCl. In 0.8 M KCl, eucaryotic monosomes are dissociated into 40S and 60S subunits if they are not associated with mRNA (17, 18). Figure 2 illustrates the behavior of spore ribosomes on gradients with (Fig. 2a) and without (Fig. 2b) high KCl. Although there was a loss of resolution in the high KCl gradients, analysis showed that there was approximately the same percentage of ribosomes in the polysome region in the high KCl gradient as in the control. In the high KCl gradient, only 50% of the monosome region relative to the control shifted into the 60S and 40S subunit region. Since all the ribosomes in the polysome region contain bound mRNA, one would expect them to be resistant to dissociation, as is the case. By the same token, since approximately

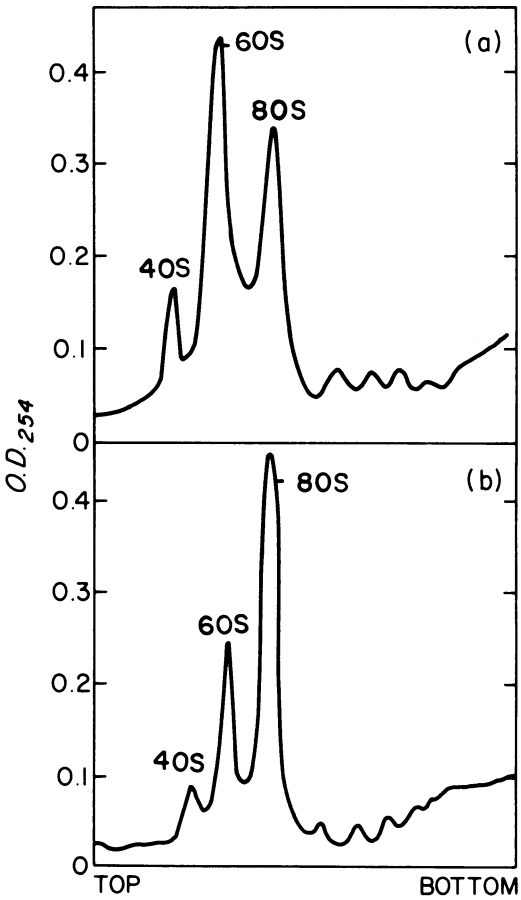

FIG. 2. *Effect of KCl on spore polysomes. (a) Polysomes treated with 0.8 M KCl for 30 min at 4°C. Sucrose gradient also contained 0.8 M KCl. Polysomes were prepared and analyzed as detailed in the text. (b) Profile of untreated polysomes from spores. O.D.*$_{254}$*, Optical density at 254 nm.*

50% of the monosomes are resistant to dissociation by high KCl, they also appear to have bound mRNA.

That the apparent polysomes were not simply aggregates of ribosomes but actually contained mRNA was checked by several controls. Mild RNase treatment (Fig. 3a) results in a shift of ribosomes from the polysome to the monosome region of the gradient but no increase in subunits, as would be expected from the partial degradation of mRNA. When spore ribosomes were RNase treated and then subjected to high KCl treatment (not shown), the shift from the monosome to subunit region was the same as in the non-RNase-treated sample. Thus, the RNase treatment apparently does not digest all of the RNA bound to the monosome, but clips between the monosomes. Figure 3b shows the effect of EDTA on the polysomes. EDTA has been shown to dissociate polysomes into subunits and does so in our system.

The second set of controls (not shown) demonstrated that the spore monosomes which did not dissociate in high KCl had bound mRNA and were not artifacts of cell breakage. Polysomes were prepared from an actively growing 16-h vegetative culture and from a 16-h culture which was "shifted-down" nutritionally. In this latter case there is a loss of polysomes and a concomitant increase in monosomes relative to the actively growing culture.

A significantly greater percentage of the monosomes from the shifted-down culture were dissociated in high KCl than from the actively growing culture. Since one presumes that a higher percentage of the monosomes in the shifted-down culture did not contain bound mRNA, this greater degree of dissociation is what one would predict. Also, a larger percentage of the monosomes from the actively growing culture were dissociated in high KCl than of the spore monosomes. Since the percentage of polysomes in actively growing plants is higher than in spores, and since the breakage conditions for obtaining plant polysomes are more vigorous than for obtaining spore polysomes, this result makes it unlikely that spore monosomes with bound mRNA are the product of polysome breakage.

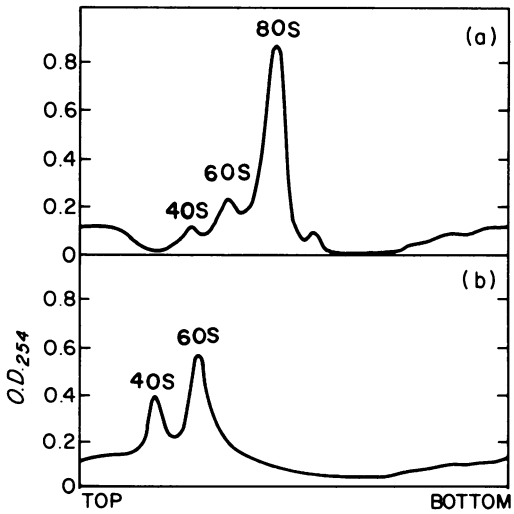

FIG. 3. *Effect of various pretreatments on spore polysomes. (a) Polysomes treated with 2 μg of RNase A per ml for 10 min at 4°C. (b) Polysomes treated with 10 mM EDTA for 20 min at 4°C. Polysomes were prepared and analyzed as detailed in the text. $O.D._{254}$, Optical density at 254 nm.*

Finally, under the conditions employed here in the isolation of spore ribosomes, it is unlikely that the polysomes were obtained from spores which had begun to germinate. The harvested spores were routinely examined by phase-contrast microscopy, and less than 0.5% had encysted, the first step in germination.

The location of the polysomes within the *Allomyces* spore was determined by isolation of the nuclear cap and examination of both the cap and extra-cap fractions. The polysome profile (not shown) of the nuclear cap fraction was identical to that from whole spores, whereas there was no trace of either monosomes or polysomes found in the extra-cap fraction. All of the spore ribosomes are found in the nuclear cap.

Distribution of stable RNA in spores. The distribution of RNA in the spore was further analyzed by polyacrylamide gel electrophoresis. Because of the way the ribosomes are isolated, the previous gradient analysis would not have detected any free mRNA or protein-bound mRNA particles whereas gel analysis of total cell RNA could. The gel analysis is also more likely to detect the presence of a small amount of rRNA in the extra-cap fraction than the gradient analysis.

Spores were obtained from cultures which had been prelabeled with $^{32}PO_4$. This prelabeling increased our ability to detect small amounts of RNA. The RNA was then prepared from the cap and extra-cap fractions (Fig. 4). Figure 4a is the profile from a 2.8% gel of the cap fraction, showing the two large rRNA species. We established that the rRNA's from *Allomyces* are 27S and 19S rather than the more typical 28S and 18S found in many eucaryotes by the use of *methyl*-^{14}C-labeled RNA from purified ribosomes (unpublished data). Figure 4b is the profile of a 10% gel (run with *Escherichia coli* RNA marker) of the cap fraction, showing the presence of both 5S and 4S RNA species.

In contrast to the cap fraction, the extra-cap

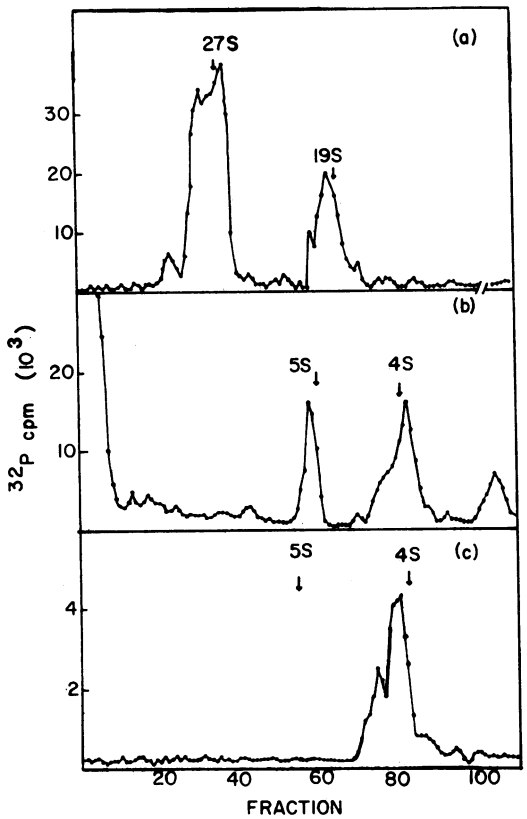

FIG. 4. *SDS polyacrylamide gels of RNA isolated from nuclear cap and extra-cap fractions of ungerminated spores of A. macrogynus. Vegetative cultures were labeled with 20 μCi of $^{32}PO_4$ per ml during growth, and the prelabeled spores were harvested as detailed in the text. Preparation of the cap and extra-cap fractions, RNA isolation procedures, and electrophoretic conditions are given in Materials and Methods. (a) 2.8% acrylamide gel of nuclear cap RNA. (b) 10% acrylamide gel of nuclear cap RNA (E. coli marker RNA included). (c) 10% acrylamide gel of extra-cap RNA (E. coli marker RNA included.*

fraction shows the presence of only 4S RNA (Fig. 4c). No trace of rRNA was evident in the 10% gel or in a 2.8% gel (not shown) from the extra-cap fraction. There was also no evidence of a possible mRNA peak in gels from either fraction. If free mRNA or protein-bound mRNA is present, it exists in quantities too small to be detected by this method or, alternatively, the mRNA is not labeled under these conditions.

RNA synthesis during sporogenesis. To explain the mechanism by which mRNA is stored and then activated for translation during germination, one must determine when the mRNA found in spores is synthesized. We began this investigation by determining whether the mRNA found in spores is synthesized during sporogenesis.

The possible synthesis of any class of RNA in the sporangium during sporogenesis was examined by analysis of spores for the incorporation of either [^{3}H]uridine or $^{32}PO_4$ into polysomes (sucrose gradient), total RNA (acrylamide gels), or mRNA [binding to oligo(dT)]. In no instance was there any indication of incorporation of precursor into any RNA species of the spore. Thus, it does not appear that any RNA found in the spore is synthesized during sporogenesis. It was possible, however, that the apparent lack of synthesis of RNA was due to a lack of incorporation of exogenous precursor into RNA, and we therefore determined whether or not RNA precursor was taken up during sporogenesis.

To induce spores, agar-grown plants were flooded with dilute salt solution containing [^{3}H]uridine. Extracts of the resultant spore harvest were analyzed by means of thin-layer chromatography. There was uptake of the RNA precursor, [^{3}H]uridine, into the spores, and a significant amount of this was converted to UTP (Table 1). Other work done at this time (A. Gee, personal communication) demonstrated that exogenous $^{32}PO_4$ was incorporated into all four nucleotide triphosphates during sporogenesis. Since labeled precursor is found, if RNA was synthesized during this period it could be detected. Thus, RNA, including mRNA, is not synthesized during sporogenesis. Spore mRNA must be synthesized prior to this period and remain stable until germination.

Distribution of poly(A). Although we were unable to demonstrate the presence of mRNA in the extra-cap fraction by either sucrose gradient or gel analysis, we decided to examine this fraction for the presence of poly(A). It has been shown in a wide variety of eucaryotic systems that much of the mRNA population has poly(A) bound to the 3′ end of the message. We used the hybridization of [^{3}H]oligo(dT) to poly(A) for this determination. This is an extremely sensitive probe for the presence of poly(A) (11). RNA isolated from both the nuclear cap and the extra-cap fractions of spores was assayed. Table 2 presents the results of these assays. For comparison, measurements of actively growing 16-h cul-

TABLE 1. *Uptake of [^{3}H]uridine and its conversion to [^{3}H]UTP during sporogenesis*[a]

Chromatography solvent system	[^{3}H]UTP (cpm/10^7 spores)	[^{3}H]uridine (cpm/10^7 spores)
LiCl	3,960	6,450
NH_4HCO_3	3,054	6,600

[a] Cultures were labeled, during sporogenesis, by the inclusion of 2.5 μCi of [^{3}H]uridine (39.4 Ci/mmol) per ml in the spore induction solution (4). Results obtained with two separate solvent systems are shown.

TABLE 2. *Presence of poly(A) in spores and in 16-h cultures*

RNA prepn[a]	Poly(A) present[b] (% of total RNA in fraction)
Whole spore	0.08
Nuclear cap	0.08
Extra-cap	0.20
16-h plants	0.16
27S-rRNA	<0.001

[a] RNA was isolated from each fraction as detailed in the text.

[b] Results for two separate experiments included for each RNA fraction.

tures are included. Data obtained from two separate experiments are given.

In whole-spore RNA preparations, poly(A) is present to the extent of 0.08% of the total RNA. Poly(A) comprises ~0.08% of total nuclear cap RNA and ~0.2% of the extra-cap RNA. From the data, it appears that there is a significant amount of poly(A) present outside the nuclear cap. The division of RNA in the spore is approximately 20% extra-cap:80% cap. Thus, 38% of the total poly(A) is present in the extra-cap fraction, and 62% is present in the nuclear cap fraction.

From this result it does appear that there may be mRNA present in the extra-cap fraction. If so, it is not bound to ribosomes. We have not yet, however, ruled out the possibility that this poly(A) material is precursor mRNA from the nucleus or simply tails of poly(A) left from degraded mRNA.

In 16-h cultures, poly(A) is present as 0.16% of total RNA. This is twice the amount found in the ungerminated spores. This twofold difference in poly(A) present in the two cell types may be related to the fact that there is twice the percentage of polysomes present in 16-h cultures as in ungerminated spores, and one would expect a higher level of mRNA in this case.

DISCUSSION

The existence of mRNA in *A. macrogynus* spores was confirmed by demonstrating the presence of polyribosomes. Typically, 25 to 35% of the ribosomes were present as polysomes, and approximately 50% of the monosomes also were shown to have bound mRNA.

A large population of ribosomal subunits is found in ungerminated spores as compared to the small population present in 16-h cultures. This, and the presence of twice the population of polysomes in 16-h cultures, are probably a reflection of the fact that the spores do not synthesize protein whereas 16-h plants are actively engaged in protein synthesis.

There have been conflicting reports as to whether the fungal spores contain polysomes or whether the polysomes form while the spores are being harvested. Evidence has been presented, using spores of *N. crassa* (19) and *R. stolonifer* (26), that polysomes form during the water harvest, whereas this has not been seen using conidiospores of *B. theobromae* (3). We do not have evidence as to whether polysomes are present in the partially differentiated sporangia before flooding with dilute salt solution or whether they are formed during the final differentiation and release of the spore. However, we have shown that RNA synthesis does not occur during the final differentiation and release of the spore, so that if polysomes form during this time, they must do so using mRNA already present in the sporangium.

Other studies have shown that *A. macrogynus* spores do not synthesize protein. This makes it unlikely that the polysomes form during the final induction process since no translation occurs to move the ribosomes along the message. Taken together, then, it appears that *A. macrogynus* spores contain polysomes formed sometime before the spore is formed. The identity of the protein associated with monosomes and the polysomes represents several interesting questions for further study.

By means of hybridization to [^{3}H]poly(dT), it was shown that the spores contain a poly(A) fraction representing 0.08% of the total RNA. Although the majority of the poly(A) (68%) is found in the nuclear cap, a significant amount (32%) is found in the extra-cap fraction.

This extra-cap poly(A) probably does not represent leakage from caps. There was no indication of any ribosomes in this fraction and it does not seem likely, then, that a significant amount of poly(A) could leak without any indication of ribosomes being present. The exact location of this extra-cap poly(A) was not determined. It could be located in the nucleus or the extra-cap cytoplasm. The method of preparation does not allow us to distinguish between these possibilities.

It is interesting to note that the amount and distribution of poly(A) is similar to that found in the spore of the closely related phycomycete *B. emersonii* (10). In *B. emersonii*, poly(A) represents 0.05% of the total RNA, with 80% in the cap and 20% in the extra-cap fraction. The authors also demonstrated that the extra-cap poly(A) had RNA bound to it and was not simply poly(A) tails. It is likely that this is the case in *Allomyces* and that at least a portion of the extra-cap poly(A) represents mRNA. Since we did not directly measure the amount of RNA bound to poly(A), we can only estimate the amount of mRNA in the spores. If we use the

ratio of bound RNA to poly(A) found in *B. emersonii* (~50:1) (10), mRNA would make up 4% of the total RNA in *A. macrogynus* spores. This compares to 2.5% found in spores of *B. emersonii*, 1.8% for *Botryodiplodia* spores (12), and 4 to 5% for *R. stolonifer* spores (22). This estimate may be high, since a ratio of only 10:1 bound RNA to poly(A) has been found for mRNA in several other eucaryotic microorganisms (5, 23).

The amount of mRNA in the spore and the relationship between the cap and extra-cap mRNA are problems yet to be resolved.

These studies have shown that mRNA is present in the ungerminated mitospore of *Allomyces*. They do not show, however, whether this is the mRNA used in the early germination events occurring in the spore. Studies to be reported in a subsequent paper will detail the evidence that at least a portion of this spore mRNA is translated during an early stage of germination of the spore.

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