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Protein and Ribonucleic Acid Synthesis During the Diploid Life Cycle of *Allomyces arbuscula*

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The diploid life cycle of *Allomyces arbuscula* may be divided into four parts: spore induction, germination, vegetative growth, and mitosporangium formation. Spore induction, germination, and mitosporangium formation are insensitive to inhibition of actinomycin D, probably indicating that stable, pre-existing messenger ribonucleic acid (RNA) is responsible for these developmental events. Protein synthesis is necessary during the entire life cycle except for cyst formation. A system for obtaining synchronous germination of mitospores is described. During germination there is a characteristic increase in the rate of synthesis of RNA and protein although none of the other morphogenetic changes occurring during the life cycle are necessarily accompanied by an appreciable change in the rate of macromolecular synthesis.

Several investigators have emphasized the potential usefulness of aquatic fungi for studies of the mechanisms of cellular differentiation and morphogenesis (6, 14). Among the phycomycetes, *Allomyces arbuscula* is an especially appropriate model system for the correlation of cytological and biochemical changes occurring during differentiation. *Allomyces* possesses stable alternation of haploid and diploid life cycles (Fig. 1), a property common to members of the family *Blastocladiaceae* (5). The haploid and diploid stages seem to be identical in nutritional requirements and physiology; they apparently differ only morphologically, the haploid producing paired gametangia, and the diploid producing mito- and meiosporangia (20). Both the diploid and the haploid stages produce several differentiated cell types: mitospores (2N) and meiospores (1N) in the diploid, male and female gametes in the haploid, and an encysted single cell stage, rhizoidal growth, hyphal growth, and reproductive structures in both. The life cycle can be conveniently reproduced on defined medium, and large amounts of the various cell types can be obtained after proper manipulation of growth conditions (8, 20, 21). Furthermore, the numerous cytological and physiological studies already published provide an invaluable background for the present biochemical studies (2, 5, 9, 12, 19, 21, 25, 28, 30).

Our initial studies with this organism were designed to determine the characteristics of macromolecular synthesis associated with development and differentiation and to begin to identify those points during the life cycle which are the most critical for the initiation of these processes.

The studies to be reported were concerned with the temporal variation of the rate of ribonucleic acid (RNA) and protein synthesis during the diploid stage. In particular, this communication examines the point in the life cycle at which the genome is activated or at least genome activity is dramatically increased and the several times during the life cycle when it is probable that development is controlled by pre-existing stable messenger RNA (mRNA). In many of these instances differentiation occurs without profound changes in the rate of synthesis of RNA or protein.

MATERIALS AND METHODS

Organisms and media. All experiments were performed with a stock of *Allomyces arbuscula* strain Brazil 2 G derived from a single meiospore (1N) isolate of the original Brazil 2 G strain that was kindly provided by Ralph Emerson. The gametophyte plant developed from this meiospore was induced to release gametes at maturity, and the zygotes obtained from this were used as the initial source of diploid material. This strain has been designated B2G-15-D. The preparation of YpSs me-

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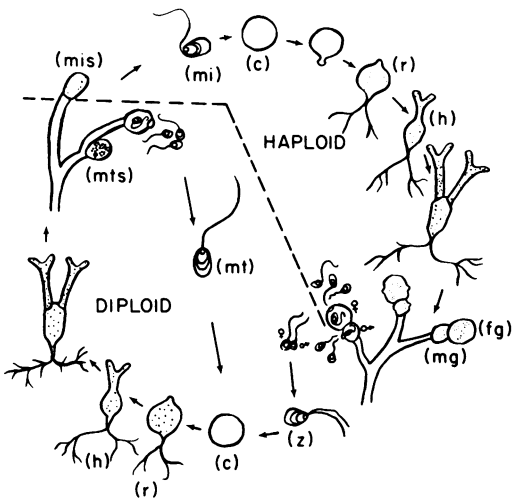

FIG. 1. *Life cycle of Allomyces arbuscula. (mt) Mitospores, (c) cyst, (r) rhizoid growth, (h) hyphae growth, (mts) mitosporangium, (mis) meiosporangeum, (mi) meiospore, (mg) male gametangium, (fg) female gametangium, and (z) zygote.*

dium, Machlis B medium (MB), and dilute salt solution (DS) has been described elsewhere (7, 17). MB medium was modified by the addition of 15 g of soluble starch per liter in place of glucose, 400 μg of L-leucine/ml, and 400 μg of L-lysine/ml. In some cases, 1.0 to 4.0 mg of an equal mixture of Casamino Acids (Difco) and tryptone (Difco) was added per ml to the MB medium.

Growth conditions. Mitospores were obtained in the following manner. YpSs agar plates of 5- to 10-day-old plants were flooded with 10 to 20 ml of sterile DS. Motile mitospores were released within 2 hr. For germination and growth experiments, mitospores in DS were inoculated to a final concentration of between 5×10^4 to 2×10^5 spores/ml into YpSs broth or MB broth plus additions and incubated at 28 C. The inoculum was never more than 10% of the final volume. The growth vessel was either a 125-ml Erlenmeyer flask with a stirring bar for agitation or a 500-ml Bellco spinner flask.

Spore formation was also studied by growing plants (this refers to either the rhizoidal or hyphal growth stage) (Fig. 1) for various lengths of time in liquid YpSs or MB broth. The mycelium was harvested by centrifugation and resuspended in a volume of DS equal to that of the original culture and incubated until mitosporangia were formed (20).

Labeling. Mitospores were labeled by the addition of ^{3}H-adenine and ^{14}C-Phenylalanine along with the DS when plates were flooded. Germinated spores and the plants were labeled in two ways. (i) Total uptake into trichloroacetic acid-precipitable material, was determined by adding a labeled base or amino acid, or both, to the medium and removing samples at various times. (ii) Determinations of the rate of macromolecule synthesis were made by removing samples from a spinner flask culture and incubating them with label for either 5 or 10 min. Samples were precipitated with cold 10% trichloroacetic acid containing 200 μg of base or amino acids, or both, per ml as carrier, filtered on Whatman GFC filters, and counted in a Packard Tri-Carb scintillation counter.

Staining. Mitospores and rhizoid stage plants were stained by mixing a volume of culture with an equal volume of 2% toluidine blue in water.

Chemicals. Actinomycin D and actidione (cycloheximide) were purchased from Calbiochem, Los Angeles, Calif. ^{3}H-adenine, ^{14}C-phenylalanine, ^{3}H-phenylalanine, and ^{14}C-glutamic acid were purchased from New England Nuclear Corp., Boston, Mass. The specific activities of the isotopes are given with each experiment.

RESULTS

Induction of mitospores. The diploid life cycle can be conveniently divided into four parts: induction of the mitospore, spore encystment and germination, rhizoidal and hyphal growth (vegetative), and the formation of mitosporangia. On flooding of an agar plate culture with DS, the multinucleate cytoplasm in the sporangium cleaves into individual uninucleate motile spores, and within 2 hr the motile spores are released through discharge papillae. The uninucleate nature of the spores was confirmed by microscopic examination of stained material.

To determine whether the induction is merely a rearrangement of existing elements or represents new synthetic events, the uptake of RNA and protein precursors into acid-precipitable material was measured in the presence and absence of inhibitors (Table 1). RNA synthesis appeared to be unnecessary for induction: the number of spores induced in the presence of actinomycin D was the same as that induced in its absence. The RNA synthesized during induction in the absence of actinomycin D might result from some residual vegetative synthesis of some RNA species which are subsequently incorporated into the spore during cleavage. In any case, it is not necessary for further growth since spores induced in the presence of actinomycin D developed normally when compared to spores induced in the absence of actinomycin D.

Protein synthesis does, however, seem to be necessary for spore induction. Cycloheximide at concentrations as low as 0.1 μg/ml inhibited mitospore induction to a level of 0.01% or less than that of control cultures. The protein synthesis taking place appeared to be directed by stable mRNA since the amount of synthesis was the same in the presence or absence of actinomycin D. The time at which this synthesis

TABLE 1. *Effect of inhibitors on mitospore induction*[a]

Additions	No. of spores/ml	Incorporation (pmoles/ml)	
		Adenine	Phenyl-alanine
None	10^5	19	20
Actinomycin D, 20 μg/ml	10^5	<0.3	16
Cycloheximide, 20 μg/ml	10^3	<0.3	<0.2

[a] Ten milliliters of DS solution containing 20 μCi of ^{3}H-adenine (505 μCi/μmole) and 1 μCi of ^{14}C-phenylalanine (16.5 μCi/μmole) was added to each plate and incubation was carried out for 120 min before measurement of isotope incorporation.

occurred was determined by flooding the plates and then adding cycloheximide (20 μg/ml) after different times of incubation. Addition during the first 15 min completely inhibited induction, addition from 15 to 30 min resulted in an increased percentage of spores relative to a control incubated for 2 hr without cycloheximide, and addition after 30 min was without effect. Addition at 30 min resulted in about 10% binucleate, double-sized spores. This suggests that the processes of cleavage of individual spores and that of the other events of induction may be separate.

Germination and outgrowth. The next stages of the life cycle are encystment, germination, and the initial outgrowth of the rhizoid from the germling. In order to enumerate individual events occurring during this time, a method for the synchronous germination and outgrowth of the mitospores was developed following the observation of Machlis (18). This was achieved by adding 400 μg of L-leucine and 400 μg of L-lysine per ml to MB medium and incubating at 28 C rather than at room temperature as has previously been the case (Fig. 2). Under these conditions the spores retracted their flagella within 20 min and formed cysts. Germination began by 30 min and was essentially complete by 80 min as shown in Fig. 2. The actual germination time may be slightly less since the kinetics were monitored by the appearance of a germ tube, which may not be the earliest event in germination. The timing of these events was very reproducible and represented a considerable acceleration of germination when compared to incubation at 25 C in unmodified MB medium. Under the latter conditions a mixed population of spores, cysts, and germlings was present for a 7-hr period after inoculation of the medium.

The rate of synthesis of RNA and protein was measured by means of the total uptake into trichloroacetic acid-precipitable material of precursor by germinating spores (Fig. 3a). Zero time was taken as the inoculation of spores into medium capable of supporting growth. Synthesis of RNA and protein commenced at about 20 min, at which time encystment was complete. The rate of incorporation of adenine, as determined in a similar experiment, revealed some interesting characteristics (Fig. 3b): it increased with time to a maximum value, decreased, and then reached a constant value. The rate of protein synthesis followed precisely the same pattern after a 40-min lag, and the nature of both incorporation curves was quite reproducible. Such rate increase coupled with the apparent lack of synthesis of RNA by the spore is suggestive of activation of the genome upon germination. It will be necessary to examine the type and amount of RNA made during germination to be able to rule out mechanisms other than genome activation to explain the results obtained. Further analysis is necessary also to determine the significance of the decrease in rate of synthesis that seems to take place.

The effects of actinomycin D and cycloheximide on germination and outgrowth were also examined (Table 2). Growth proceeded in actinomycin D to the rhizoid stage, whereas cycloheximide stopped growth after encystment (see Fig. 1). Thus, it appears that protein synthesis, but not RNA synthesis, is necessary to

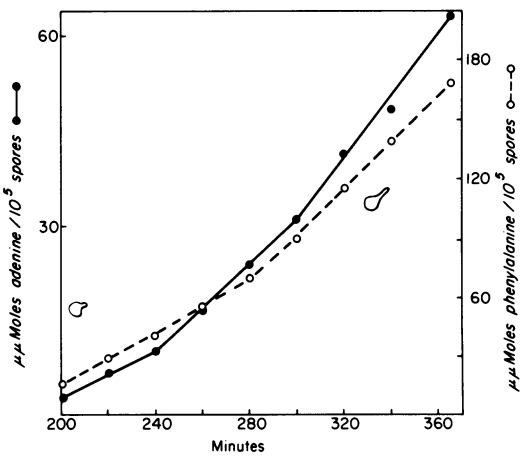

FIG. 2. *Synchronous germination of mitospores. Spores at a final concentration of 10^5/ml were inoculated into modified MB medium (see Materials and Methods) and incubated at 28 C.*

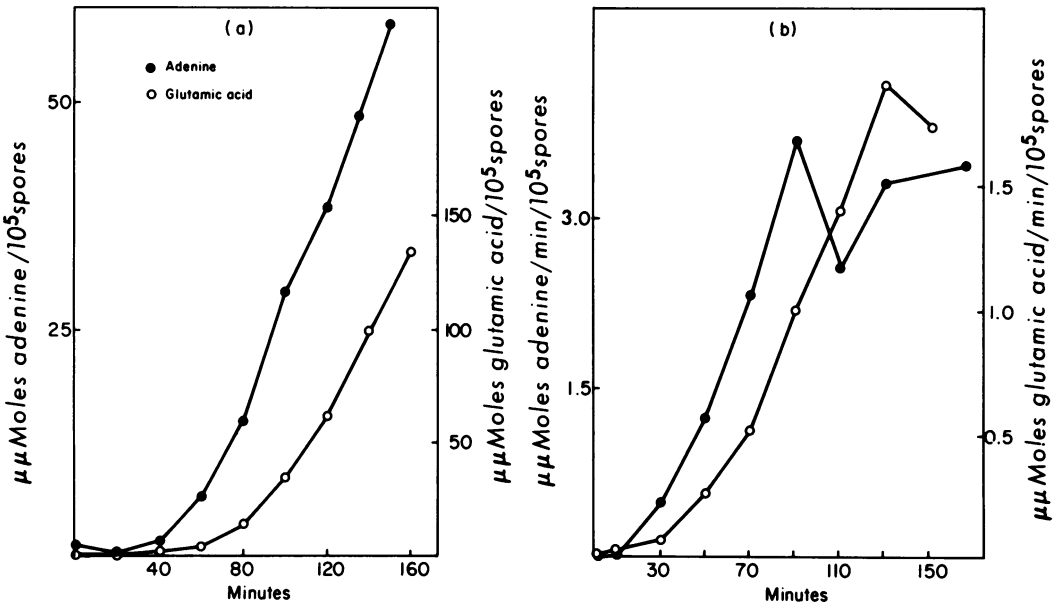

FIG. 3. *Uptake of glutamic acid and adenine by germinating mitospores. Mitospores inoculated into modified MB medium containing 2 μCi of ^{3}H-adenine per ml (135 μCi/μmole) and 0.1 μCi of ^{14}C-glutamic acid per ml (147 μCi/μmole). (a) Total uptake; (b) slopes of total uptake during 20-min intervals.*

support further growth after encystment. Protein and RNA synthesis were measured during germination in the presence of actinomycin D (Fig. 4a and b). Protein as measured by amino acid incorporation into acid-precipitable material appeared to accumulate at 50% of the control rate for the first 80 min of germination and continued at about 25% of the control rate until 120 min before ceasing; RNA synthesis was almost completely inhibited. It appears that some mRNA is made in the absence of actinomycin D because of the higher rate of protein synthesis in the control without actinomycin D, but this RNA is not necessary for germination. Any mRNA necessary for further growth may apparently be synthesized later. When actinomycin D was removed from the medium at 3 hr, the plants developed normally after a lag.

Post germination. Synthesis beyond the initial 3-hr period was investigated by adding precursor to 3-hr cultures and measuring uptake into acid-precipitable material from this point. RNA and protein synthesis continued at a linear rate until 4.5 to 5 hr. At that time the rate of RNA synthesis doubled and, approximately 30 min later, the rate of protein synthesis doubled (Fig. 5). Nuclear staining indicated that this is approximately the time of the first nuclear division. The increase in rate is consistent with the two daughter nuclei synthesizing at the same rate as the single nuclei prior to mitosis (Fig. 5).

TABLE 2. *Effect of actinomycin D and cycloheximide on growth*[a]

Growth stage	Actinomycin D	Cycloheximide
Spore induction	−[b]	+[c]
Encystment	−	−
Germination	−	+
Hyphal growth	+	+
Mitosporangium formation	−	+

[a] Both inhibitors were present at 20 μg/ml.
[b] Indicates no inhibition.
[c] Indicates inhibition.

When an estimate of the hot water-extractable pool of adenine and glutamic acid was made in material from the synchronous spore germination system, little change in pool sizes was observed over a 4-hr interval beginning from the onset of germination. It should be emphasized that the kinetic picture is influenced by inoculum size and particular growth conditions. Obviously the developmental changes themselves are triggered by processes too subtle to be revealed by the overall kinetic picture.

Formation of mitosporangia. The formation of mitosporangia completes the diploid

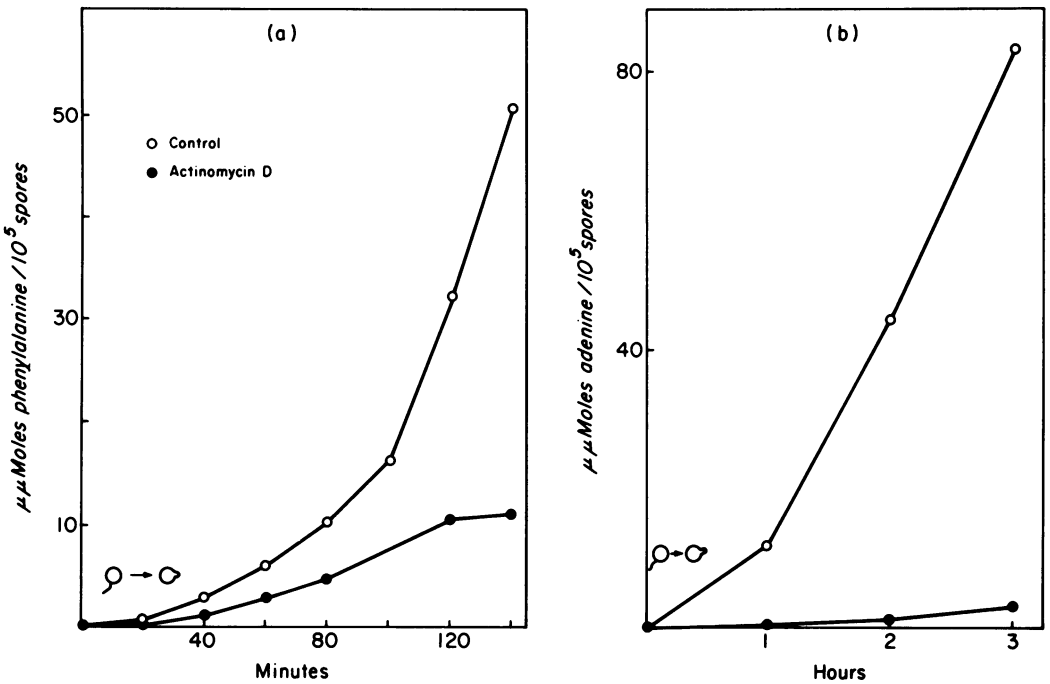

FIG. 4. *Uptake of phenylalanine and adenine during germination in the presence of actinomycin D. Mitospores were inoculated into modified MB medium containing 20 μg of actinomycin D per ml and either phenylalanine or adenine. (a) 1 μCi of ^{3}H-phenylalanine per ml (165 μCi/μmole); (b) 1 μCi of ^{3}H-adenine per ml (135 μCi/μmole).*

life cycle. This process can occur in actively growing plants, since on YpSs plates one of the two branches formed by the growing plant often develops into a mitosporangium while the other hyphal branch continues to grow and divide. However, this does not seem to be the case in liquid medium. Normally, when sporangia do form, they appear at the tips of the hyphae. The process of sporangium formation in liquid medium seems to be initiated by changes in cultural conditions which we have yet to understand. For example, sporangia often appeared in one culture but not in another parallel culture grown simultaneously. Also, the time at which mitosporangia were formed and rate of RNA synthesis of the cultures forming mitosporangia varied greatly. Sometimes sporangia appeared in vigorously growing cultures and at other times in senescent ones. There was a marked variability in the response of these cultures to actinomycin D. In many cases sporangium formation was actually more pronounced in the presence of actinomycin D.

It is possible to study mitosporangium formation by transferring plants from YpSs medium to DS (20). We initially determined that growth in YpSs or MB for as little as 60 min was sufficient to allow plants to form mitosporangia when they were transferred to DS. The effect of inhibitors upon mitosporangium formation was investigated with this regime (Table 3). Young plants in the rhizoid stage did not form mitosporangia when transferred in the presence of actinomycin D or cycloheximide; thus, both RNA synthesis and protein synthesis are necessary. In very young plants, at least, there is some RNA made during growth which is necessary and cannot be replaced after transfer since spores germinated in the presence of actinomycin D will not form mitosporangia when transferred to DS either in the presence or absence of actinomycin D. Plants transferred after they had reached the hyphal growth stage had the ability to form mitosporangia and release spores in actinomycin D but not in cycloheximide. Thus, RNA synthesis is not necessary and the plant presumably contains stable mRNA set aside for the synthesis of mitosporangia.

We were unable to measure any synthesis of either RNA or protein which was specific for mitosporangia formation. This was due to the large amount of turnover of these macromolecules as determined by an initial increase and then decrease in trichloroacetic acid-precipi-

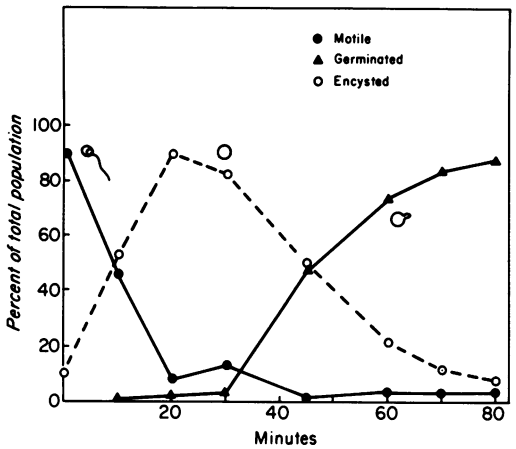

FIG. 5. *Uptake of phenylalanine and adenine during rhizoidal growth. The culture growing in modified MB medium was labeled by the addition of 2 μCi of ^{3}H-adenine per ml (135 μCi/μmole) and 0.1 μCi of ^{14}C-phenylalanine per ml (16.5 μCi/μmole) at 180 min.*

table counts of precursor for either RNA or protein taking place in plants transferred to DS. The kinetics of incorporation were dominated by this turnover rather than by events directly related to mitosporangium formation. The lack of incorporation of adenine in the presence of actinomycin D contrasted to the observed incorporation in its absence indicates that the drug is actually inhibiting RNA synthesis. The results then are consistent with the gradual accumulation of a pool of stable mRNA capable of directing mitosporangium formation which renders the process increasingly refractory to actinomycin D as the plants age.

DISCUSSION

A number of interesting speculations concerning the mechanisms responsible for differentiation during the life cycle can be made from these experiments, the major one being that at particular stages *Allomyces* contains stable mRNA which directs specific developmental stages in a manner analogous to other eukaryotic cells. The stable RNA postulated to direct germination and outgrowth of the mitospore seems to be analogous to stored maternal messenger vital to early development of higher organisms (3, 11, 26, 27, 29, 31). Initial embryonic development in both amphibian and sea urchin embryos has been shown to be under the control of stable mRNA species existing in the egg; *Allomyces* also synthesized mRNA during germination, as shown by the reduction in the rate of protein synthesis effected by actinomycin D. This synthesis of mRNA while development is being directed by other stable messengers is also reminiscent of early embryonic development (11, 26, 27). However, this mRNA synthesized during early germination is either dispensable for further development, or can be replaced after actinomycin D is removed. Studies with *Blastocladiella emersonii*, an organism closely related to *Allomyces*, have also implicated the presence of a stable mRNA that directs protein synthesis during germination, suggesting that this mechanism is common to the aquatic *Phycomycetes* (15). The presence of stable mRNA has also been demonstrated in other fungal systems (13, 23). In the slime mold (23) the mRNA which directs the synthesis of an enzyme necessary for differentiation is made several hours before its expression. In fact, this same mechanism is also to be found in some prokaryotes, namely in spore formation in *Bacillus cereus* (1) and microcyst germination in *Myxococcus xanthus* (24).

The involvement of stable mRNA in differentiation is apparent in mitosporangium formation as well as in induction and germination of the mitospore. In each case this mechanism may be important for survival. For example, the mRNA necessary for mitosporangium formation accumulates early in hyphal growth and its translation is induced by starvation conditions. This enables the organism to differentiate rapidly into a motile form which is able to seek out more favorable conditions and reinstate the growth cycle. The ability to completely eliminate hyphal growth, a normal stage in its life cycle, and form mitosporangia is reminiscent of the observation in *Myxococcus xanthus* that fruiting body formation

TABLE 3. *Formation of mitosporangia*[a]

Growth stage	Relative no. of mitosporangia formed (%)	
	Minus actinomycin D	Plus actinomycin D
Rhizoid	10–30	1
Young hyphae	40–50	0–20
First branching	50–60	30–40
Old plants	100	60–100

[a] All percentages are relative to the number of sporangia formed per plant in rhizoid and young hyphae cultures, and per hyphal tip in the other cases. Growth stage refers to the time in the diploid life cycle at which plant material was transferred from YpSs to DS (see Fig. 1).

can be omitted and microcysts formed directly from vegetative organisms.

When conditions are manipulated to eliminate hyphal growth, *Allomyces* greatly resembles *Blastocladiella emersonii*, whose life cycle is somewhat similar. Sporangium formation in *B. emersonii* is also quite resistant to actinomycin D, again indicating the possibility of stable mRNA. This mechanism may not, however, operate in all the *Phycomycetes* since it has been shown that in *Achlya* sporangium formation is inhibited in actinomycin D after induction although it does gradually become refractory to it (10). Further study of other *Phycomycetes* will determine how widespread is the use of stable mRNA to direct sporangial formation.

Several other explanations of these results can be tentatively ruled out. The differentiations which take place in actinomycin D cannot be explained simply by a lack of permeability to actinomycin D because we have demonstrated an inhibition of the uptake of adenine into trichloroacetic acid-precipitable material in the presence of actinomycin D during induction (Table 1), germination (Fig. 4), and formation of the mitosporangia (*unpublished data*). It is possible, however, that only ribosomal RNA (rRNA) synthesis was inhibited and not mRNA. At least in one case mRNA synthesis was shown to be partially inhibited; during germination in actinomycin D the amount of protein synthesis is less than the control, indicating that at least some mRNA synthesis is blocked.

Further, the conclusion that macromolecular synthesis is blocked in the presence of the inhibitors does not seem to be due to a change in the uptake of the precursor. Preliminary estimates of the pool size of the precursors have indicated that there is no significant difference in the pool size of either RNA or protein precursor in the presence of either actinomycin D or cycloheximide as compared to a control culture in which the inhibitors were not present.

Thus, it is most probable that stable mRNA does, in fact, program several aspects of differentiation during the diploid life cycle.

Determination of the rate of RNA and protein synthesis during short intervals through the germination process suggested that rather abrupt changes occur. This may result from activation of parts of the genome resulting in the synthesis of mRNA, rRNA, or both. However, the brief decrease in the rate of synthesis during germination is more difficult to understand. It is probably not a pool phenomenon since we found that the nucleotide pool appears to be equilibrated by this time. It might represent the cessation of synthesis of the mRNA molecules necessary for further growth after the initial direction of translation by the preformed stable mRNA. To help substantiate this hypothesis, it will be necessary to demonstrate that these same molecules are made in the lag period after removal of actinomycin D from spores germinated in its presence. The decrease might also represent some type of damping phenomenon regulating the rate of rRNA synthesis. Clarification of this point awaits good measurements of the relative proportion of rRNA and mRNA present and being synthesized during germination.

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