1983

The Regulation of Gene Expression During the Aerobic Germination of Mucor Racemosus Sporangiospores.

John Edgar Linz
Louisiana State University and Agricultural & Mechanical College

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THE REGULATION OF GENE EXPRESSION DURING THE AEROBIC GERMINATION OF MUCOR RACEMOSUS SPORANGIOSPORES

A Dissertation

Submitted to the Graduate Faculty of the Louisiana State University and Agricultural and Mechanical College in partial fulfillment of the requirements for the degree of Doctor of Philosophy in

The Department of Microbiology

by

John E. Linz
B.A., Albion College, 1976
M.S., University of Illinois, 1979
December 1983
Dedication

I would like to dedicate this work to my parents, Henry and Beatrice Linz, who provided me with support and encouragement in my pursuit of a career in academics.
Acknowledgement

I would like to thank Dr. Michael Orlowski for all of the advice, support, and friendship he has extended to me over the past few years. He has helped to increase my understanding of Microbiology through our conversations and has been a great inspiration and source of motivation in the laboratory.

I would also like to thank the members of my graduate committee: Dr. A. D. Larson, Dr. H. D. Braymer, Dr. D. F. Day, Dr. S. Chang, and Dr. V. R. Srinivasan for their timely advice and constructive criticisms of my work.

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Finally, I would like to thank my wife, Susan, and daughter, Sarah, for their love, understanding and support in my work.
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Abstract

This study was undertaken to investigate the regulation of gene expression during the aerobic germination of *Mucor racemosus* sporangiospores. Sucrose density gradient analysis of spore extracts revealed that the percentage of ribosomes associated with mRNA increased from 23% in dormant spores to 85% after 10 min of germination. L-[¹⁴C]leucine was immediately incorporated at a rapid rate into protein of a leucine auxotroph, whereas [³²P]-phosphate were not incorporated into RNA at a significant rate until 20 min after the addition of medium. Newly synthesized RNA did not appear in polysomes until at least 30 min.

Polyadenylated RNA, comprising 3.3% of the cellular RNA, was isolated from dormant spores by oligodeoxythymidylic acid-cellulose chromatography. Dormant spore RNA was translated in a rabbit reticulocyte cell free system with an efficiency similar to rabbit globin mRNA. It was concluded that sporangiospores store functional mRNA which is translated immediately upon the addition of nutrient medium.

In vivo translation products of the stored mRNA, pulse labeled with L-[³⁵S]methionine, were compared to proteins continuously labeled with L-[³⁵S]methionine during formation of the spore by analysis on 1- and 2-dimensional polyacrylamide gels. Autoradiography of dried gels revealed several proteins which were synthesized during spore
formation but not during early germination. Conversely, other pro-
teins were synthesized during the first 30 min of germination but
not during spore formation, even though the mRNA for these proteins
must have been synthesized at this time. Spores appear to possess a
post-transcriptional regulatory mechanism directing selective trans-
lation in the developing spore.

The relative rates of synthesis of many proteins were observed
to change during the first hour of germination. Many of these changes
were accompanied by corresponding changes in the intracellular levels
of their respective mRNAs as determined by an in vitro translation
assay. Other proteins were identified whose translatable mRNA levels
remained constant while in vivo translation rates decreased. These
data indicate that sporangiospores can effect qualitative and quan-
titative changes in gene expression by regulation at both the trans-
scriptional and post-transcriptional levels.
Introduction

*Mucor racemosus* is a fungus belonging to the class Zygomycetes. Members of the genus *Mucor* play an important role in industry, being utilized in ethanol production and causing significant damage as food spoilage agents. Some species are opportunistic pathogens which cause a rare disease, mucormycosis, which ultimately attacks the central nervous system and is often fatal. *Mucor* also provides an excellent system for the study of cell differentiation in a lower eukaryote due to its ability to alter cell morphology as a function of its environment.

In *Mucor*, the morphogenesis from yeast to hypha is the most widely studied system, but an equally useful morphogenetic system is the germination of sporangiospores to hyphae under aerobic conditions. Several aspects of sporangiospore germination have been studied previously including the effect of cyclic adenosine 3',5'-monophosphate (cAMP) on germination (Wertman and Paznokas, 1981; Orlowski, 1980), the changing patterns of cAMP-binding proteins during germination (Orlowski, 1979), the effect of various growth factors on the initiation of germination (Tripp and Paznokas, 1981; 1982A and B) and the regulation of macromolecular synthesis throughout the germination process (Orlowski and Sypherd, 1978B). This last study determined that the incorporation of radioactive precursors into RNA and protein began immediately
upon the initiation of germination by the addition of nutrient medium
to dormant spores. Sucrose density gradient analysis of dormant spore
extracts revealed that all of the ribosomes were present as inactive
40S and 60S subunits, suggesting that protein synthesis proceeds at a
very low rate, if at all, in dormant spores. The present study was
carried out to determine the triggering mechanism for the burst of
synthetic activity seen in early germination and to determine the
level of regulation of gene expression during this process.

Several fungal species including *Allomyces macrogynus* (Smith and
Burke, 1979), *Botryodiplodia theobromae* (Knight and Van Etten, 1976A),
*Blastocladia emersonii* (Johnson, Lovett, and Wilt, 1977) and
*Rhizopus stolonifer* (Freer and Van Etten, 1978; Van Etten and Freer,
1978) produce dormant spores containing stored mRNA which is trans­
lated upon germination. The presence of stored mRNA in spores implies
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 (Johnson et al., 1977). 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 (Adelman and

*M. racemosus* can reportedly regulate the velocity of transla­
tion and the percentage of ribosomes active in protein synthesis as
a function of yeast-to-hypha morphogenesis (Orlowski and Sypherd, 1978C) or changes in growth rate (Orlowski, 1981; Ross and Orlowski, 1982A and B). Differential synthesis of polypeptides was found during yeast-to-hypha morphogenesis (Hiatt, Inderlied, and Sypherd, 1980), indicating that differential gene expression plays a role in the change in cell morphology.

The data presented in this study reveal that dormant sporangiospores of Mucor racemosus contain stored mRNA which is translated immediately upon initiation of germination. Differential gene expression is important in early germination. The evidence suggests that the regulation of differential gene expression may occur at the transcriptional and post-transcriptional levels. Mucor also appears to have the ability to selectively translate certain mRNAs during spore maturation, storing other transcripts stably until they are translated early in germination.
Literature Review

Introduction

In *Mucor*, the addition of nutrient medium to dormant sporangiospores triggers a series of events called germination. The spores swell to several times their original size and within 5 to 8 hours germ tubes emerge. In *Mucor racemosus*, there is an exponential accumulation of cellular RNA and protein as soon as germination is initiated (Orlowski and Sypherd, 1978C). Cell wall material in *Mucor rouxii* accumulates exponentially throughout germination and is deposited in a uniform and nonpolar fashion during the swelling stage of germination. Just prior to germ tube emergence, cell wall synthesis becomes polarized in the area where the germ tube will emerge (Bartnicki-Garcia and Lippman, 1977). The once metabolically dormant cell is now actively growing in a very ordered manner.

Several questions about germination specifically and about cell differentiation in general arise as a result of these preliminary studies:

i) How is a change in cell environment translated into a change in metabolic activity, macromolecular synthesis, and cell morphology?

ii) In what form is information transferred from the external environment to the cell nucleus?
iii) What specific chain of events takes place to alter gene expression?

iv) At what functional level(s) is gene expression regulated?

It is the purpose of this section to present information from the literature pertaining to the above questions in order to put the present study on Mucor spore germination into perspective.

Fungal Spore Germination

The germination of fungal spores is one example of how a change in cell environment elicits a change in metabolism, macromolecular synthesis and cell morphology (for reviews, see: Brambl, Dunkle, and Van Etten, 1978; Lovett, 1975).

Dormancy in fungal spores can be defined as a rest period or irreversible interruption of the phenotypic development of the organism (Sussman, 1966). Fungi have evolved a variety of mechanisms for maintaining the dormant state in spores including nutritional barriers, metabolic blocks, or self inhibitors (Macko et al., 1976). Breaking of spore dormancy can result from hydration of the spores. For example, in Rhizopus stolonifer, addition of water to dormant sporangiospores causes metabolic activity to commence and initiates the formation of polyribosomes (Nickerson, Freer, and Van Etten, 1981). In Mucor racemosus, a wide variety of compounds including glucose, mannose, glutamate, and xylose are reported to initiate sporangiospore germination depending on the conditions of spore production (Tripp and Paznokas, 1981; 1982A and B). The actual mechanism by which initiation takes place is not known.
The majority of fungal spores studied seem to be blocked from germination at some specific point in a metabolic pathway or macromolecular synthesis. Early studies toward discovering this block centered on the DNA of dormant and germinating spores. A base composition comparison (Minagawa, Wagner, and Strauss, 1959) and DNA: DNA hybridization experiments (Dutta and Chaudhuri, 1975) on Neurospora crassa conidal and mycelial DNA revealed no alteration of DNA during germination. The buoyant density in CsCl gradients and the thermal denaturation profile of dormant spore DNA were identical to those of germinated spore DNA in Botryodiplodia theobromae and Rhizopus stolonifer (Dunkle and Van Etten, 1972). In the fungi studied, there was no alteration, loss, or amplification of DNA during vegetative development.

It is assumed that protein synthesis in dormant spores takes place at a very low rate, if at all. One of the earliest events during germination of many fungal spores is a rapid increase in protein synthesis, which seems to be necessary for germ tube emergence (Brambl et al., 1978). A search was conducted for functional components of the translation apparatus in dormant spores.

Early evidence for the presence of mRNA in dormant spores came from the discovery of polysomes in uredospores of Uromyces phaeoeli (Staples, Bedigan, and Williams, 1968), conidia of Botryodiplodia theobromae (Brambl and Van Etten, 1970; Brambl, 1975), and conidia of Neurospora crassa (Mirkes, 1974). Later, poly(A)⁺ RNA was purified from dormant spores of Allomyces macrogynus (Smith and Burke,
1979), *Botryodiplodia theobromae* (Knight and Van Etten, 1976A), *Blastocladiella emersonii* (Johnson et al., 1977), *Rhizopus stolonifer* (Freer and Van Etten, 1978; Van Etten and Freer, 1978), and *Dictyostelium discoideum* (Giri and Ennis, 1978). The poly (A)$^+$ RNA from *Dictyostelium discoideum* and *Rhizopus stolonifer* spores was translated in a wheat germ cell-free protein synthesizing system, yielding significant incorporation of labeled amino acids into protein. This implies that at least part of the poly (A)$^+$ RNA is functional mRNA.

Whole ribosomes, functional in cell free protein synthesis, have been isolated from the dormant spores of *Aspergillus oryzae* (Horikoshi and Ikeda, 1968), *Botryodiplodia theobromae* (Van Etten, 1968) and several other fungi. These ribosomes generally had lower activity than did ribosomes isolated from germinating spores. This may be in part due to artifacts of isolation. The ribosomal RNAs from dormant spores of *Neurospora crassa* (Henney and Storck, 1963), *Aspergillus oryzae* (Horikoshi, Ohtaka, and Ikeda, 1965) and *Botryodiplodia theobromae* (Knight and Van Etten, 1976A) were identical to the ribosomal RNAs from germinated spores, suggesting any difference in ribosome activity is due to the ribosomal proteins or associated factors.

Active elongation factors have been purified from dormant spores of *Botryodiplodia theobromae* (Van Etten and Brambl, 1968) and *Aspergillus oryzae* (Horikoshi and Ikeda, 1968) among others. In
addition, active aminoacyl tRNA synthetases were extracted from some of these same organisms (Merlo et al., 1972, Horikoshi et al., 1969).

Transfer RNAs (tRNA) from dormant spores of several species also appear to be identical to their counterpart in germinated spores (Henney and Storck, 1963; Tanaka et al., 1966; Van Etten, Koski, and El-Olemy, 1969). However, in Aspergillus oryzae, differences were detected in the number of isoaccepting species of leucyl and methionyl tRNA, although the significance of this is not apparent (Horikoshi et al., 1969).

The evidence suggests that the initiation of protein synthesis in germinating spores is not limited by the absence of a component of the translation machinery, but by the activation or modification of some molecule already present.

Self-inhibitors of germination in some fungal spores function by preventing protein synthesis. The self-inhibitor in zoospores of Blastocladiiella emersonii mentioned earlier is just one example (Adelman and Lovett, 1974). A similar inhibitor of protein synthesis has been found in Dictyostelium discoideum spores (Bacon, Sussman, and Paul, 1973). In these organisms, hydration of spores initiates germination by diluting out the self-inhibitor.

Another possible mechanism for blocking or regulating protein synthesis in dormant spores is at the level of mRNA storage and processing. In animal cells, functional mRNA is derived post-transcriptionally from large precursor RNAs, collectively termed
heterogeneous nuclear RNA (HnRNA) (Darnell, 1968; Darnell et al., 1973). Processing of the HnRNA includes the addition of a polyadenylic acid [poly(A)] "tail" (approximately 150-200 nucleotides in length) to the 3' end, addition of a specially methylated guanine "cap" to the 5' end, excision of excess nucleotides from the middle (introns) and ends of the molecule and the methylation of several internal adenine residues. Ninety percent of the HnRNA remains in the nucleus while 10% moves to the cytoplasm as functional mRNA after having been complexed with specific proteins to form so-called ribonucleoprotein particles (RNP's). Selective processing and transport of HnRNA could prove to be an important regulatory mechanism.

Attempts to detect the HnRNA in Phycomyces blakesleeanus (Gamow and Prescott, 1972), Rhizopus stolonifer (Roheim, Knight, and Van Etten, 1974), and Botryodiplodia theobromae (Knight and Van Etten, 1976A) were initially unsuccessful. However, a more recent study in Rhizopus stolonifer demonstrated RNA processing in poly(A)+ RNA synthesized during the first 15 min of germination (Freer, Mayama, and Van Etten, 1977). Newly synthesized mRNA was about 1200 nucleotides in length while mRNA from polysomes was on the average only 860 nucleotides. About 50 adenine residues were removed from the 3' end and 300 residues were removed from the 5' end of the mRNA. HnRNA has also been purified from dormant spores of Dictyostelium discoideum. This HnRNA was only 20% larger than functional mRNA and, on the average, smaller than HnRNA from animal cells (Firtel and Lodish, 1973).
Alterations of ribosomal proteins or associated protein factors (including initiation, elongation, and termination factors) by covalent modifications such as methylation or phosphorylation present a potential mechanism of regulation in germinating spores. The intracellular concentration of cAMP could be a mediator in this type of protein modification. High levels of cAMP added to germination medium stimulated the rate of germination of *Mucor racemosus* sporangiospores (Wertman and Paznokas, 1981) although normal germ tube development was inhibited (Orlowski, 1980). Cyclic AMP pools remained at high levels until germ tubes emerged, and then decreased sharply. Cyclic AMP-binding protein activity was shown to be correlated to cAMP pool levels during germination (Orlowski, 1979). Some of the cAMP-binding proteins are likely to be cAMP-dependent protein kinases, which could be involved in phosphorylation and activation of proteins in dormant spores. One possible candidate for this type of activation is a protein from the small ribosomal subunit (S-6) of *Mucor racemosus*. This protein was found to be phosphorylated to varying degrees in yeast and hyphal cells but was unphosphorylated in dormant spores (Larsen and Sypherd, 1980). Similarly, the elongation factor EF-1α in this same organism is methylated at several lysine residues in vegetative cells, but is unmethylated in dormant spores (Hiatt et al., 1982).

Since many fungal spores contain stored mRNA, RNA transcription probably does not play a role in the breaking of spore dormancy. The regulation of RNA transcription is, however, an indirect means of regulating protein synthesis during germination. Increasing the
transcription of ribosomal RNA and mRNA could result in increased rates of protein synthesis by providing part of the raw materials necessary in translation. Transcription is, in turn, a function of the relative concentration and specific activity of RNA polymerase I, II, and III inside the cell. The transcriptional specificity of these enzymes in fungi is generally thought to be the same as in animal cells. That is, RNA polymerase I is responsible for synthesis of rRNA, RNA polymerase II for synthesis of mRNA, and RNA polymerase III for synthesis of small RNA's. In *Rhizopus stolonifer*, these enzymes were purified from dormant and germinated spores (Gong and Van Etten, 1972). Dormant spores contained active RNA polymerase I and III while germinated spores contained all three enzymes. RNA polymerase II activity was not detected until three hours of germination, but mRNA was synthesized within the first 15 min. A similar study in *Blastocladiella emersonii* revealed no differences in the RNA polymerases purified from dormant and germinated spores (Horgen, 1971). It is still unclear as to the exact relationship between RNA polymerase activity and regulation of transcription *in vivo*. The presence of polyamines such as putrescine and spermidine may have a direct bearing on this issue. The affinity of these highly charged molecules for DNA could regulate transcription by affecting RNA polymerase binding. A preliminary study on polyamine levels during germination of *Rhizopus stolonifer* spores did show a direct correlation between polyamine concentration and the rate of RNA synthesis (Nickerson et al., 1977).
There is a general lack of information as to the nature of proteins synthesized early in spore germination. An SDS/polyacrylamide gel analysis of proteins synthesized early in *Rhizopus stolonifer* spore germination detected changes in the rates of synthesis of several proteins, although the functions of these proteins were not determined (Freer and Van Etten, 1978). These changes were attributed to regulation at the level of transcription, because similar changes were noted in mRNA levels when assayed in a cell-free protein synthesizing system. In their review of fungal spore germination, Brambl, Dunkle and Van Etten (1978) speculated that the kinds of proteins most likely to be synthesized early in germination would be those involved in cell wall assembly, membrane synthesis, respiration, or proteins such as kinases or methylases which modify existing macromolecules.

**Yeast-to-Hypha Morphogenesis in Mucor**

In order to better understand the morphogenetic events associated with aerobic sporangiospore germination in *Mucor*, it is useful to examine the information available on the regulation of macromolecular synthesis during the yeast-to-hypha morphogenesis. In both cases, spherical growth by diffuse intercalation of cell wall precursors gives way to polarized linear growth. Several environmental factors directly affect the morphology of *Mucor* cells (Sypherd et al., 1978), but a change in atmosphere is the easiest to manipulate and the most widely studied system. The cells grow as budding yeasts under CO$_2$ in the presence of a fermentable hexose (Bartnicki-Garcia
and Nickerson, 1962). Changing the atmosphere to air results in the emergence of germ tubes followed by hyphal growth. Analysis of macromolecular synthesis following the atmospheric shift determined that the specific rate of protein synthesis (Orlowski and Sypherd, 1977), RNA synthesis (Orlowski and Sypherd, 1978A), and lipid synthesis (Ito, Cihlar, and Inderlied, 1982) increased significantly. At least part of the increase in the rate of protein synthesis was shown to be the result of an increase in the velocity of ribosome movement along mRNA (Orlowski and Sypherd, 1978C). Analysis of proteins synthesized before and after the atmospheric shift by means of 2-dimensional polyacrylamide gel electrophoresis (O'Farrell, 1975) demonstrated that differential polypeptide synthesis and differential gene expression occurred during the morphogenesis (Hiatt et al., 1980).

More recent studies on the regulation of protein synthesis during morphogenesis revealed that the increase in the specific rate of protein synthesis was due to an increase in growth rate accompanying the atmospheric shift and not due to morphogenesis per se (Ross and Orlowski, 1982A). Growth conditions were determined which would maintain cell morphology yet allow variation in growth rate (Orlowski, 1981; Ross and Orlowski, 1982B). Under alternative conditions, morphogenesis could occur without a significant change in growth rate (Ross and Orlowski, 1982A). Yeast cells at high growth rates contained more ribosomes, had a higher percentage of active ribosomes, and had a faster polypeptide chain elongation rate than did slower growing cells. Hyphal cells at faster growth rates also contained
more ribosomes, and had a faster polypeptide chain elongation rate than did slower growing cells. However, the percentage of active ribosomes was significantly lower in fast growing hyphae than in slow growing hyphae. In a morphogenetic shift without a change in growth rate, these three parameters remained essentially constant. Mucor cells can regulate the rate of protein synthesis by at least 3 mechanisms during a change in growth rate.

Internal cAMP pools may play a role in determining the morphology of Mucor cells. High cAMP levels were found in yeast cells and low cAMP concentrations were found in hyphal cells (Larsen and Sypherd, 1974) during a CO₂-to-air morphogenetic shift. It was later established that internal cAMP levels are a function of morphology and not the rate of growth or protein synthesis (Orlowski and Ross, 1981). Polyamines, when added to the growth medium, influenced the size of cAMP pools (Orlowski, unpublished data). High levels of polyamines, such as spermidine, lowered the internal levels of cAMP. During morphogenesis from yeasts to hyphae, the activity of ornithine decarboxylase, one important enzyme in polyamine synthesis, increased 30 to 50 fold (Inderlied, Cihlar, and Sypherd, 1980). The level of polyamines may indirectly regulate the morphology of Mucor cells by controlling the size of cAMP pools in vivo. Polyamines may also play a direct role in the regulation of protein synthesis. These polycations have been reported to significantly affect the rate of protein synthesis (Tabor and Tabor, 1976) and are routinely added to cell-free protein synthesizing systems to stimulate activity. A
direct correlation between ornithine decarboxylase activity and an increase in the rate of protein synthesis has been established (Inderlied et al., 1980).

Two instances of potential control involving post-translational modification of gene products have been studied as a function of Mucor morphogenesis. These studies examined the phosphorylation of ribosomal protein S-6 and the methylation of elongation factor EF-1α.

Ribosomal protein S-6 was shown to exist in four alternative forms containing from zero-to-three covalently bound phosphate groups (Larsen and Sypherd, 1979). The degree of phosphorylation was shown to be a correlate of both the morphology and physiology of the cell. The protein was completely unphosphorylated in dormant sporangiospores and maximally phosphorylated in rapidly growing hyphae. Yeasts and slow-growing hyphae displayed intermediate levels of phosphorylation (Larsen and Sypherd, 1980). The extent of phosphorylation in protein S-6 may play a role in determining the qualitative or quantitative activity of the ribosome.

In a similar study, elongation factor EF-1α was found to exist in various states of methylation in vivo (Hiatt et al., 1982). The protein was found to be highly methylated in hyphal cells but unmethylated in dormant spores. Considerably larger quantities of the methylated protein were present in hyphae than in yeasts. Intracellular pools of the methyl donor S-adenosylmethionine expanded significantly following an atmospheric shift from CO₂ to air concomitant with an increase in the methylation of total cellular protein.
The observed correlations suggest a possible role for protein methylations in the qualitative or quantitative regulation of protein synthesis during Mucor morphogenesis. However, that potential role has yet to be defined.

The increase in the specific rate of RNA synthesis in Mucor yeasts following a morphogenetic shift, may be in part explained by a shift in RNA polymerase activity. In Mucor rouxii, all three RNA polymerases common to higher eukaryotes have been purified and characterized (Young and Whitely, 1975A). The specific activity and relative concentrations of these enzymes were assayed as a function of a CO₂-to-air atmospheric shift resulting in a yeast-to-hypha transition (Young and Whitely, 1975B). The specific activities of RNA polymerases I and II increased after the shift with the relative concentration of RNA polymerase I nearly doubling. RNA polymerase III activity remained unchanged.

Other Fungal Cell Differentiation Systems

In Dictyostelium discoideum, fruiting body formation can be initiated by amino acid starvation. Changes in the relative rates of synthesis of 100 proteins were detected during cell differentiation (Alton and Lodish, 1977A). Regulation of protein synthesis was primarily at the level of transcription. In subsequent studies concentrating on the first 60 min after initiation of cell differentiation (Alton and Lodish, 1981), both transcriptional and translational regulation of protein synthesis were detected. One important conclusion
reached in these studies was that ribosome heterogeneity is correlated to the cell differentiation.

Sporangium formation in the oomycete Achlya ambisexualis can be initiated by nutrient starvation. The studies on this organism suggest that gene expression during sporangium formation is primarily regulated at a pretranslational level (Gwynne and Brandhorst, 1982).

Starvation for nutrients induces ascus and ascospore formation in Saccharomyces. The level of poly(A)$^+$ RNA was found to remain constant during cell differentiation, however the incorporation of amino acids in a cell free protein synthesizing system decreased 2 fold (Kraig and Haber, 1980). The percent of active ribosomes decreased 3 fold during this time period. The decrease in activity of the ribosomes was due to a decrease in the formation of initiation complexes and was found to be a function of starvation rather than cell differentiation per se. A set of highly phosphorylated nucleotides (HPN) has been purified from starvation induced yeast cells (Rhaese et al., 1979). The presence of these HPNs was demonstrated to cause changes in transcription leading to ascus formation. Analysis of proteins synthesized after ascus induction revealed the synthesis of 11 new proteins and the inhibition of synthesis of nine others (Trew, Friesen, and Moens, 1979).

These examples imply that lower eukaryotes have the ability to alter gene expression through regulation at many possible levels, often simultaneously. More information is needed on the exact mechanisms of regulation and on the transcellular information relay
systems which may activate or inactivate these mechanisms in response to the cellular environment.
Materials and Methods

Organism and Cultivation

*Mucor racemosus* (*M. lusitanicus*) ATCC 1216B or a leucine auxotroph (leu 2A) derived from the parent strain (Peters and Sypherd, 1978) were used in all experiments. Stock cultures, originally obtained from Paul Sypherd (University of California, Irvine), were routinely maintained on a solid growth medium (YPG agar) composed of 2% (wt./vol.) glucose, 1% (wt./vol.) Bacto-Peptone, 0.3% (wt./vol.) Bacto-Yeast Extract, and 3% Bacto-Agar (Difco Laboratories, Detroit, Michigan). The medium was adjusted to pH 4.5 with $\text{H}_2\text{SO}_4$. A small amount of aerial hyphae containing sporangiospores from a previous stock culture was inoculated in the center of YPG agar plates (100 mm) which were incubated at room temperature (22° C) for two weeks, at which time stock cultures were transferred to fresh medium. Liquid growth medium (YPG) was the same as indicated above minus the agar. Glucose was always autoclaved separately from the other components of the medium.

Spore Production

Sporangiospores for germination experiments were produced in sterile pyrex baking dishes (8 in. dia.) containing 250 ml of YPG agar or 100 mm petri plates containing 20 ml of YPG agar. An inoculum was prepared by adding 10 ml of liquid YPG to a 7 to 14 day stock culture of *M. racemosus*. Scraping the submerged mycelial surface with
a sterile glass rod released the sporangiospores from the sporangia and suspended them in the liquid medium, leaving the hyphae and sporangiophores attached to the agar surface. A small quantity of this pure spore suspension (50 μl per pyrex baking dish, 20 μl per petri plate) was evenly distributed over the agar surface using a sterile glass rod. The cultures were incubated at room temperature for a period of 7 to 10 days, at which time the agar surface was completely covered with aerial hyphae bearing the sporangia containing the grey-black sporangiospores to be used in germination experiments.

Germination

After 7 to 10 days of incubation, sporangiospores were harvested by adding liquid YPG medium to the cultures and scraping the mycelial surface with a sterile glass rod. This resulted in pure spore suspensions containing from $3.3 \times 10^7$ to $1.2 \times 10^8$ spores/ml. Purity of spore suspensions and spore concentrations were routinely checked under a light microscope by means of a hemacytometer. Spore suspensions were incubated with shaking (200 RPM) on a rotatory shaker at room temperature while bubbling water-saturated sterile air into the medium. These conditions resulted in the emergence of hyphal germ tubes from the sporangiospores within 5 to 8 hours. Some spore suspensions ($2 \times 10^5$ spores/ml) were incubated with shaking (200 RPM) at room temperature while bubbling CO$_2$ gas through the culture at a flow rate of 0.5 volume of gas per volume of culture fluid per min. All sporangiospores produced budding yeast cells under these conditions.
Preparation of L-[\textsuperscript{35}S]Methionine from [\textsuperscript{35}S]Sulfate

L-[\textsuperscript{35}S]Methionine was prepared from H\textsubscript{2}\textsuperscript{35}SO\textsubscript{4} (New England Nuclear Corp., Boston, Mass.; specific activity 1490 Ci/mg atom) by the procedure of Crawford and Gesteland (1973). Escherichia coli B (obtained from Nova Scientific, Burlington, N.C.) was inoculated into 100 ml of medium (S medium, Roberts et al., 1937) containing NH\textsubscript{4}Cl (2.0 g/liter), Na\textsubscript{2}HPO\textsubscript{4} (6.0 g/liter), KH\textsubscript{2}PO\textsubscript{4} (3.0 g/liter), NaCl (3.0 g/liter), MgCl\textsubscript{2} (0.010 g/liter), and glucose (10 g/liter, autoclaved separately). The culture was incubated with shaking (200 RPM) at 37°C while measuring cell growth spectrophotometrically at 550 nm.

When the growth rate leveled off (A\textsubscript{550} = 0.2) due to sulfur starvation, 3 ml of cells were added directly to a vial containing 25 mCi of H\textsubscript{2}\textsuperscript{35}SO\textsubscript{4} which was capped tightly, and allowed to sit for 6 hours at room temperature. The cells were pelleted by centrifugation at 15,000 x g for 10 min, the supernatant fluid removed, and the cell pellet resuspended in 20 µl of 0.05 M dithiothreitol and 180 µl of 6N HCl. This solution was added to a glass tube which was evacuated, sealed, and heated at 105°C for 24 hours. The resulting hydrolysate was dried in a Speed Vac Concentrator (Savant Instruments, Hicksville, N.Y.) and resuspended in 0.2 ml of sterile water. This solution was spotted in a 15 cm wide strip on Whatman 3MM chromatography paper.

The methionine was purified by ascending chromatography using a butanol/acetic acid/water (4:1:5) solvent system in a pre-equilibrated sealed chamber. After five hours, the paper was dried, the L-[\textsuperscript{35}S]-methionine located by autoradiography (Kodak XAR-1 Film, 60 sec
exposure), and the strip containing the methionine eluted into 10 mM dithiothreitol. The final yield of L-[\textsuperscript{35}S]methionine (5.36 mCi) represented about 20% of the initial H\textsubscript{2}\textsuperscript{35}SO\textsubscript{4}. The specific activity was approximately 300 Ci/mmol.

Determination of the Kinetics of RNA and Protein Synthesis

For measurement of the kinetics of RNA synthesis during germination, sporangiospores were harvested into YPG medium containing \[^{3}H\]uracil (32.5 Ci/mmol, 10 μCi/ml final concentration) or \[^{32}P\]-phosphoric acid (carrier free, 20 μCi/ml final concentration), both purchased from ICN Pharmaceuticals, Irvine, Calif. Samples (100 μl) of culture were withdrawn at 2- or 5-min intervals, placed into 1 ml of ice-cold 10% trichloroacetic acid, and kept on ice for 30 min. The samples were filtered onto glass fiber filters (Reeve Angel, Clifton, N.J.), washed 4 times with 1-ml volumes of cold 10% trichloroacetic acid, dried under a heat lamp, placed in 5 ml of liquid scintillation counting fluid, (0.4% 2.5-diphenyloxazole and 0.01% [2,2'-p-phenylenebis (5 phenyloxazole)] in toluene) (Orlowski and Sypherd, 1977) and assayed for radioactivity with a Beckman LS-200 liquid scintillation spectrometer. To determine the effect of antibiotics and growth inhibitors on the kinetics of RNA synthesis, the following combinations of compounds were added to the growth medium in the indicated concentrations: actinomycin D (200 μg/ml) plus daunomycin (200 μg/ml) or sodium azide (10 mM) plus sodium fluoride (10 mM).
L-[35S]Methionine (prepared from H2SO4 as described above; 300 Ci/mmol, 4μCi/ml final concentration) or L-[14C]leucine (New England Nuclear Corp., Boston, Mass.; 289 mCi/mmol, 1μCi/ml final concentration) was added to YPG medium for measurement of the kinetics of protein synthesis during germination. The procedure was the same as that outlined for RNA synthesis except that a leucine auxotroph (leu2A) was employed for incorporation of radioactive leucine. Samples 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 described above. In some experiments cycloheximide (200 μg/ml) was added to the growth medium to inhibit incorporation of radioactive precursors into protein.

**Measurement of Free Intracellular Amino Acid and Nucleotide Pools**

Free intracellular amino acids were extracted and quantitated as described by Orlowski and Sypherd (1977), except that 10% formic acid was used instead of acetic acid. Samples (2 ml) of sporangiospores were withdrawn at various times during germination, filtered rapidly on a membrane filter (Millipore Corp., Boston, Mass.; type HA, pore size 0.45 μm), washed quickly with distilled water, immersed in 2 ml of 10% formic acid, and incubated on ice for 60 min. The samples were vortexed, the filters removed, and the spores pelleted by centrifugation at 15,000 x g for 10 min. Portions (1.5 ml) of the supernatant fraction were dried in a Speed Vac Concentrator, resuspended in 300 μl of sodium citrate buffer (Beckman), and the amino acid concentrations determined on a Beckman Model 139 C.
amino acid analyzer. The remaining spore pellet was transferred to a tared aluminum weighing pan, heated at 100°C for 24 hours, and the dry weight determined using an analytical balance.

Nucleotide pools were radioactively labeled by harvesting spores into YPG medium containing $^{32}$P phosphate (carrier free, 200 μCi/ml). At various times samples (2 ml) were withdrawn and the pools extracted into 10% formic acid as above. Individual nucleotides were separated on DEAE cellulose thin layers (Brinkman Instruments Inc., Westbury, N.Y.) by the procedure of Randerath and Randerath (1967). Thin layers were developed for 100 min in a solvent system consisting of 0.04 N HCl. After development and drying, the thin layers were exposed to Kodak X-Omat X-ray film for 24 hours at -40°C. The films were developed with Kodak GBX X-ray developer at 22°C and fixed with Kodak rapid fixer. The resulting spots were identified by comparing the $R_f$ values with those of standards run at the same time. Spots for each ribose nucleoside triphosphate were cut out from the thin layer, placed into 5 ml of liquid scintillation counting fluid, and assayed for radioactivity as described above. ATP pools were also measured by the luciferin-luciferase assay system of Stanley and Williams (1969). The reaction mixture was prepared in a glass scintillation vial and contained 2.0 ml of 10 mM magnesium sulfate and 20 mM sodium arsenate (pH 7.4), and 20 μl of spore extract brought up to 100 μl in 30 mM sodium arsenate (pH 7.4). Forty μl of firefly lantern extract (Sigma) was added to start the reaction.
The scintillation vial was immediately placed in the counting chamber of a Beckman LS-200 liquid scintillation spectrometer set on the sample repeat mode for 0.1 min counts in the $^3$H channel. ATP standards were used to prepare an ATP standard curve from which sample values were calculated and normalized to dry weight of spores.

**Density Gradient Analysis of Ribosomes**

Protein synthesis in germinating sporangiospores 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 on a membrane filter (Millipore Corp., Boston, Mass.; type AA, pore size 8 μm), washed with cold sterile TMK buffer plus antibiotics, and broken by grinding for 5 to 10 min in a sterile mortar (baked at 100°C for 48 hours) under liquid N$_2$. The frozen material was resuspended in sterile TMK buffer plus antibiotics and centrifuged at 15,000 x g for 15 min at 4°C to pellet the cell debris.

The absorbance at 260 nm of the supernatant fraction was determined in a Beckman Model 35 spectrophotometer. Some samples were incubated on ice for 10 min with 10 μg/ml RNase A (Sigma). Aliquots of supernatant fluid containing 8 A$_{260}$ (RNase-treated or RNase-free) were carefully layered on top of 11-ml linear 10-to-40% (wt./vol.)
sucrose density gradients (buffered with TMK) resting on 0.8-ml 2M sucrose cushions. The gradients were centrifuged at 150,000 x g for 100 to 110 min at 4°C in a Beckman Model L2-65B ultracentrifuge using an SW-41Ti rotor. Gradients were fractionated and 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 (percent of ribosomes associated with mRNA) versus inactive subunits was calculated from the absorbance profiles (by weight) by the procedure of Martin (1973).

For measuring $^{32}$P phosphate incorporation into ribosome fractions, spores were harvested into YPG medium containing $^{32}$P phosphate (carrier free, 8.5 µCi/ml). Spore extracts were prepared and fractionated on 10-to-40% linear sucrose density gradients. Fractions (6 drops) were collected into 1 ml of cold 10% trichloroacetic acid, kept on ice 30 min, filtered, washed, dried, and assayed for radioactivity as described above.

Some cell extracts were prepared for density gradient analysis from dormant spores which had been harvested into nonaqueous solvents such as freon, heptane, and mineral oil. The same procedures as described above were followed except that the spores were filtered from the solvent and broken with grinding under liquid $N_2$ without washing them in TMK buffer. This was done to prevent any wetting which would occur during even a brief buffer wash.
RNA Purification

RNA for Oligo(dT)-cellulose and sucrose density gradient analysis was purified by the procedure of Orlowski and Sypherd (1978A). Spores were collected at the appropriate time during germination by filtration, washed quickly with cold sterile TMK buffer, and broken with grinding for 5 to 10 min in a sterile mortar under liquid N₂. Spores to be assayed for polyadenylated RNA were incubated in the presence of cycloheximide (200 μg/ml) for 5 min and washed with cold sterile TNE buffer (100 mM Tris-HCl, pH 9.0; 100 mM NaCl; and 1 mM EDTA) containing cycloheximide (200 μg/ml) prior to grinding. Frozen cell material was resuspended in either cold sterile TMK buffer with cycloheximide (200 μg/ml) for sucrose density gradient analysis or cold sterile TNE buffer with cycloheximide (200 μg/ml) for preparation of polyadenylated RNA. An amount of sodium dodecyl sulfate (SDS) was added to bring the solution to 1% (wt./vol.). The cell suspension was extracted 3 times with 2 volumes of cold water-saturated (or TNE-saturated) redistilled phenol containing 0.1% (wt./vol.) 8-hydroxyquinoline. Ammonium formate was added to the aqueous phase (final concentration of 0.3 M) followed by 2 volumes of absolute ethanol and refrigerated at -20°C overnight. The precipitated RNA was collected by centrifugation at 30,000 x g for 20 min at 4°C, lyophilized, dissolved in a small volume of ice-cold buffer (50 mM Tris-HCl, pH 7.25, and 10 mM MgCl₂), and treated with DNase (10 μg/ml, Sigma) on ice for 10 min. The solution was re-extracted with 2 volumes of
water-saturated (or TNE-saturated) phenol and precipitated with ammonium formate and ethanol. The RNA was pelleted by centrifugation, lyophilized and then dissolved in ANE buffer (10 mM sodium acetate, 100 mM NaCl, and 1 mM EDTA, pH 5.3) for sucrose density gradient analysis or TK buffer (10 mM Tris-HCl, pH 7.5; 500 mM KCl) for oligo (dT)-cellulose fractionation.

Density Gradient Analysis of RNA

RNA in germinating sporangiospores was radioactively labeled by harvesting spores into YPG medium containing [\(^{32}\)P]phosphate (carrier free, 8.5 μCi/ml). Spore samples were collected by filtration at various times during germination. The RNA was purified by the procedure outlined above and dissolved in ANE buffer. Aliquots of the RNA preparation containing 4 A\(_{260}\) were carefully layered on top of 11-ml 5-to-20% linear sucrose density gradients (in ANE buffer) resting on 0.8-ml 2M sucrose cushions. The gradients were centrifuged at 77,000 x g for 20 hours at 4°C in an SW-41 Ti rotor. The gradients were fractionated and scanned at 254 nm on an ISCO density gradient fractionator. Fractions (6 drops) were collected into 1 ml of cold 10% trichloroacetic acid, kept on ice 30 min, filtered, washed, dried, and assayed for radioactivity as described above.

Oligo (dT)-Cellulose Fractionation of RNA

RNA in germinating sporangiospores was radioactively labeled by harvesting spores in YPG containing [\(^{32}\)P]phosphate (carrier free,
8.5 μCi/ml. Spore samples were collected, the RNA purified, and resuspended in TK buffer as described above. A volume of solution containing 135 μg of RNA (1 A260 = 45 μg RNA/ml) 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. Nonpolyadenylated RNA ("bulk" RNA) was eluted from the column with approximately 15 ml of TK buffer and collected in 8 drop fractions. Polyadenylated RNA (putative mRNA) was eluted from the column with approximately 10 ml of TO buffer (10 mM Tris-HCl, pH 7.5) collected in 8 drop fractions. The fractions were collected directly into an aqueous scintillation counting fluid (Miller and Sypherd, 1973) and assayed for radioactivity as shown above.

One culture of dormant sporangiospores was harvested directly into TMK buffer containing cycloheximide (200 μg/ml) and the energy poisons sodium azide (10 mM) and sodium fluoride (10 mM), which were shown to completely prevent any RNA synthesis. The RNA was purified as described above and radioactively labeled by methylation with [3H]dimethyl sulfate in a procedure described by Knight and Van Etten (1976B) and originally outlined by Gaubatz and Cutler (1975). The purified RNA was resuspended in 0.2 M sodium phosphate buffer (pH 7.8) to a final concentration of approximately 100 A260/ml. A portion (0.2 ml) of this solution was added to an ampoule containing 5 mCi of [3H]dimethyl sulfate (New England Nuclear Corp., Boston, Mass.; 4.7 Ci/mmol) and incubated at 25°C for 100 min. One ml of 0.2 M
sodium phosphate buffer (pH 7.8) was added to stop the reaction. The RNA sample was dialyzed overnight against 4 changes of 200 volumes 0.1X SSC (0.15 M NaCl, 0.015 M sodium citrate, pH 7.0), and precipitated by the addition of 2 volumes of absolute ethanol (−20°C overnight). The RNA was pelleted by centrifugation at 20,000 x g for 20 min at 4°C and resuspended in 0.2 ml TK buffer. The concentration of the RNA solution was determined by the absorbance at 260 nm. One hundred thirty-five µg of the RNA were fractionated on an oligo (dT)-cellulose column. Fractions (8 drops) were collected into 2 ml of 10% trichloroacetic acid, kept on ice for 30 min, filtered, washed, dried and assayed for radioactivity as described above. An identical 135-µg sample of ^3H-labeled RNA was fractionated on an oligo (dT)-cellulose column and the fractions containing polyadenylated RNA were pooled. The pooled fractions were mixed with 4 A_{260} of unlabeled Mucor RNA and precipitated with ammonium formate (0.3 M final concentration) and 2 volumes of absolute ethanol (−20°C overnight). The RNA was pelleted by centrifugation at 20,000 x g for 20 min at 4°C and resuspended in ANE buffer. This solution was brought to 50% (vol./vol.) formamide, heated to 55°C for 10 min and fractionated on a linear 5-to-20% sucrose density gradient. Fractions (6 drops) were collected into 1 ml of 10% trichloroacetic acid, kept on ice 30 min, filtered, washed, and assayed for radioactivity as described above.
Prelabeling/Pulse Labeling of Sporangiospore Proteins

The proteins in dormant sporangiospores were prelabeled by allowing sporulation of a culture at room temperature on YPG agar containing either \( \text{H}_2\text{SO}_4 \) (1490 Ci/mg atom, 250 μCi/ml final concentration) or L-[\(^{35}\)S]methionine (New England Nuclear Corp., Boston, Mass.; 1194 Ci/mmole, 100 μCi/ml final concentration). After 7 days of incubation, the sporangiospores were harvested directly into sterile water containing cycloheximide (500 μg/ml) to prevent further incorporation of the labeled amino acid into protein. The proteins of germinating sporangiospores were pulse labeled by harvesting the spores into YPG medium. At the appropriate time during germination, the spores were exposed to L-[\(^{35}\)S]methionine (approximately 300 Ci/mmol, prepared from \( \text{H}_2\text{SO}_4 \); 160 μCi/ml final concentration) for 15 min. Cycloheximide (500 μg/ml) was added at the end of pulse periods to prevent further incorporation of label into protein. Four consecutive 15-min pulse periods were used during the first 60 min of germination. To determine the specific activity of L-[\(^{35}\)S]methionine in free intracellular methionine pools during pulse periods, two identical spore samples (2 ml) were rapidly filtered, washed, and the amino acid pools extracted into 10% formic acid as outlined above.

The concentration of amino acids in one sample was determined on a Beckman Model 139 C amino acid analyzer. The methionine from the other spore extract was purified by paper chromatography (Whatman 3MM paper) using the butanol/acetic acid/water solvent system described above. Spots were located by autoradiography on Kodak XAR-5 X-Ray
film (exposure time 16 hours at -70°C), cut out, immersed in 5 ml of liquid scintillation counting fluid, and the radioactivity assayed in a Beckman LS-200 liquid scintillation spectrometer. The dry weight of each spore sample was determined as described above.

Measurement of Protein Turnover

Dormant sporangiospores, whose proteins were radioactively pre-labeled with L-[35S]methionine, were harvested into YPG containing a large excess of unlabeled L-methionine (2 mM). The spores were rapidly filtered, washed with YPG containing unlabeled L-methionine (2 mM), resuspended in this medium, and incubated at room temperature under air. Samples (100 μl) were withdrawn every 5 min, pipetted into 1 ml of 10% trichloroacetic acid, heated at 90°C for 30 min, cooled on ice for 30 min, filtered, washed, and assayed for radioactivity as described above.

Cell Free Protein Synthesizing System

Total cellular RNA for translation in vitro was purified from M. racemosus sporangiospores by a modification of the procedure of Alton and Lodish (1977A). Sodium azide (10 mM) and sodium fluoride (10 mM) were added to separate cultures of germinating sporangiospores at various times to totally inhibit further RNA synthesis. Some sporangiospores were harvested directly into sterile water containing sodium azide (10 mM) and sodium fluoride (10 mM) to obtain a time zero (dormant spores). Spore suspensions were filtered rapidly on membrane filters (Millipore Corp., Boston, Mass.; type AA, pore
size 8 μm) and broken by grinding for 5 to 10 min in a sterile mortar under liquid N₂. The frozen cell material was added to 4 ml of sterile buffer (HMK) containing 50 mM HEPES (pH 7.5), 40 mM magnesium acetate and 20 mM KCl. Sodium dodecyl sulfate was added to the solution (0.2% final concentration) which was agitated gently. This solution was thoroughly mixed with an equal volume of water-saturated redistilled phenol containing 0.01% 8-hydroxyquinoline, followed by the addition of 0.2 ml of 4 M sodium acetate (pH 7.5) and further agitation. Following the addition of 4 ml of chloroform, the mixture was agitated and the phases separated by centrifugation at 15,000 x g for 10 min at 2°C. The phenol-chloroform phase was removed to a new sterile tube and re-extracted with 2 ml of HMK. The aqueous phase and interphase were extracted with phenol-chloroform and the phases separated by centrifugation. The two aqueous layers were combined and re-extracted with phenol-chloroform once (6 mls of each) and chloroform alone 2 times. Two volumes of absolute ethanol were added and the RNA was precipitated at -20°C overnight. The RNA was pelleted by centrifugation at 15,000 x g for 45 min at 0°C, dissolved in 5 ml of 0.2 M potassium acetate (pH 7.5) and reprecipitated with 2 volumes of absolute ethanol at -20°C overnight. The RNA was collected by centrifugation at 15,000 x g for 45 min at 0°C, dissolved in a small volume of sterile distilled water and lyophilized to dryness to remove traces of ethanol. The dried RNA was again dissolved in a small volume of water and the RNA concentration determined by the absorbance at 260 nm. All RNA samples were stored frozen at -70°C.
Translation of *Mucor* RNA *in vitro* was carried out in a commercial rabbit reticulocyte cell-free protein synthesizing system (Bethesda Research Laboratories, Gaithersburg, Md.). This kit consisted of: i) a rabbit reticulocyte lysate (3 x concentrated) containing 3.5 mM MgCl₂, 0.05 mM EDTA, 25 mM KCl, 0.5 mM dithiothreitol, 25 μM hemin, 50 μg/ml creatine kinase, 1 mM CaCl₂, 2 mM EGTA, and 70 mM NaCl; ii) a protein biosynthesis reaction mixture (10 x concentrated) containing 250 mM HEPES, 400 mM KCl, 100 mM creatine phosphate, and 500 μM of each of the 19 amino acids (minus L-methionine); iii) rabbit globin mRNA (5 μg/0.1 ml) in sterile water; iv) 2 M potassium acetate, pH 7.2; and v) 20 mM magnesium acetate, pH 7.2. All stocks were stored frozen at -70°C.

Each of the stock reagents was added to an acid-washed sterile 1.5-ml microcentrifuge tube (West Coast Scientific, Berkeley, Calif.) on ice in the following amounts and in the order given to make 20 μl of master mix per cell free reaction:

1. potassium acetate 1.6 μl (168 mM final concentration)**
2. magnesium acetate 1.3 μl (1.2 mM final concentration)
3. L-[³⁵S]methionine* 4.1 μl (≈ 50 μCi)
4. reaction mixture 3.0 μl
5. reticulocyte lysate 10.0 μl


**This concentration of potassium acetate was optimum for synthesis of high molecular weight proteins.
Ten μl of solution containing RNA (0-45 μg) was added to the master mix followed by gentle mixing (30 μl total volume). The reaction mixture was incubated at 30°C for 60 min. Five μl of RNase A (Sigma, 10 mg/ml stock) was added to the solution and it was incubated for 15 min at 37°C. The reaction was stopped by lowering the temperature to 0°C in an ice bath. To measure the incorporation of L-[35S]-methionine into protein, 5 μl of cell-free reaction mixture were added to 2 ml of 10% trichloroacetic acid containing 200 μg of bovine serum albumin as carrier, boiled 15 min, and cooled on ice for 30 min. The resultant precipitate was collected by filtration, washed, dried, and assayed for radioactivity as described above. The remainder of the reaction mix (30 μl) was precipitated with 10 volumes of 80% (vol./vol.) acetone and cooled on ice for 10 min. The precipitate was collected by centrifugation for 20-30 sec in an Eppendorf Model 5412 microcentrifuge and resuspended in 30 μl of buffer (SDS sample buffer) containing 50 mM Tris-HCl (pH 7.0), 2% (wt./vol.) sodium dodecyl sulfate, 5% (vol./vol.) β-mercaptoethanol and 20% sucrose. The samples were stored frozen at -20°C.

Polyacrylamide Gel Electrophoresis of Proteins

Proteins were extracted from M. racemosus cells for 2-dimensional isoelectric focusing/SDS polyacrylamide gel electrophoresis using the modifications of Hiatt, Inderlied, and Sypherd (1980) of the original procedure of O'Farrell (1975), except that SDS was not included in the extraction buffers. Sporangiospores were collected on membrane filters (Millipore Corp., Boston, Mass.; type AA,
pore size 8 μm) at various times during germination, washed with cold sonication buffer (Solution B of O'Farrell, 1975) containing 0.01 M Tris-HCl (pH 7.4), 5 mM MgCl₂, and 50 μg/ml of pancreatic RNase (Sigma) with cycloheximide (500 μg/ml), and broken by grinding them for 5 to 10 min in a sterile mortar under liquid N₂. The frozen cell material was suspended in 0.5 ml of sonication buffer containing 1 mM phenylmethylsulfonyl fluoride (PMSF). DNase was added (50 μg/ml final concentration) to the suspension and it was incubated on ice for 15 min. The solution was lyophilized, and the dry cell material extracted twice at 25°C for 15 min with 250 μl of lysis buffer (Solution A of O'Farrell, 1975) which contains 9.5 M urea (Sigma), 2% (wt./vol.) nonidet-P40 (Sigma), 1.6% ampholines (pH 5-to-8) (Sigma), 0.4% ampholines (pH 3-to-10) (Sigma), and 5% (vol./vol.) β-mercaptoethanol. Nonsolubilized cell debris was pelleted by centrifugation for 5 min in an Eppendorf Model 5412 microcentrifuge. The supernatant fraction was removed and stored frozen at -20°C. Small aliquots of the protein samples in lysis buffer were prepared for SDS/polyacrylamide gel electrophoresis. The protein was precipitated by the addition of 10 volumes of 10% trichloroacetic acid and kept on ice for 30 min. The precipitate was collected by centrifugation and solubilized in 50 μl of SDS sample buffer by boiling for 3 min. These samples were loaded directly onto slots of an SDS polyacrylamide gel and electrophoresed as described below.

Samples of proteins synthesized in vitro, prepared as described above, could either be analyzed directly by SDS/polyacrylamide slab
gel electrophoresis or prepared for 2-D polyacrylamide gel electrophoresis (O'Farrell, 1975) by the procedure of Alton and Lodish (1977A). Protein samples in SDS sample buffer were thawed by boiling for 1 min and 10 μl were mixed with 5 mg of urea. The urea was completely dissolved and the sample buffer was diluted with 50 μl of lysis buffer (Solution A of O'Farrell, 1975). These samples were stored frozen at -20°C.

The concentration of protein in samples was determined by a modification of the method of Lowry et al. (1951). Proteins were precipitated from solution by the addition of 2 ml of 10% trichloroacetic acid. After incubating the mixtures on ice for 30 min, the precipitates were collected on glass fiber filters (Reeve Angel, Clifton, N.J.) and resuspended by heating at 90°C for 30 min in 1 N NaOH. Following the addition of the appropriate reagents, the absorbance at 750 nm was determined. Bovine serum albumin (Sigma) was used to construct a standard curve from which unknown protein concentrations were calculated. The amount of L-[\text{35S}]methionine incorporated into proteins in vivo was determined in a similar manner. Aliquots of protein in lysis buffer were dispersed into 2 ml of 10% trichloroacetic acid, heated at 90°C for 30 min, and kept on ice 30 min, filtered, washed, dried, and assayed for radioactivity as described above. The specific activity of protein samples was then calculated by the following formula:

\[
\frac{\text{CPM of L[35S]methionine incorporated}}{\mu g \text{ protein}}
\]
Isoelectric focusing gels (first dimension) were prepared according to the procedure of O'Farrell (1975). Acid-washed glass tubes (13 cm long; 3 mm, inside diameter) were coated with Sigmacote (Sigma) and sealed at the bottom with Parafilm. Ten ml of gel mixture (to make approximately 7-8 gels, 12.5 cm in length) were made in a 125-ml sidearm flask from the following components present in the indicated amounts: 5.5 g urea, 1.33 ml acrylamide stock (29.2% [wt./vol.] acrylamide [Sigma], 0.8% [wt./vol.] bisacrylamide [Sigma]), 2 ml nonidet P40 (10% [vol./vol.] stock solution), 1.97 ml water, 0.4 ml ampholines (pH range 5-8), and 0.1 ml ampholines (pH range 3-10). The urea was completely dissolved and 10 µl of fresh 10% (wt./vol.) ammonium persulfate (Sigma) were added. The solution was degassed for 1 min. Seven µl of TEMED (Bio Rad Industries, Richmond, Calif.) were added, the solution was mixed gently, and the gel solution dispensed into the sealed tubes (using a syringe fitted with narrow tygon tubing) to within 0.5 cm of the top. The gels were initially overlaid with a small volume of gel overlay solution (8 M urea, Solution H of O'Farrell, 1975) for 2 hours and then replaced with 20 µl of lysis buffer covered with water for an additional 2 hours. The parafilm was removed and the bottom end of the tubes were covered with dialysis membrane held in place by a rubber grommet. Twenty µl of fresh lysis buffer were then added to the top of the gels and the tubes were filled with 0.02 M NaOH (degassed). The gels were then prerun in a Bio Rad Model 155 tube gel apparatus (Bio Rad Industries, Richmond, Calif.), with 0.02 M NaOH (thoroughly degassed) in the top
reservoir and 0.01 M $\text{H}_3\text{PO}_4$ in the bottom reservoir under the following sequence of conditions: 200 volts for 15 min; 300 volts for 30 min; and 400 volts for 30 min. The upper reservoir buffer was removed along with the lysis buffer and NaOH from the surface of the gels. Protein samples in lysis buffer were carefully loaded onto gels which were covered with fresh 0.02 M NaOH. The upper reservoir was refilled with fresh 0.02 M NaOH. The gels were run for 15 hours at 400 volts followed by 1 hour at 800 volts (6,800 v x hours). The pH gradient was determined by cutting a gel into 5 mm sections. Each section was placed in a vial containing 2 ml of degassed water. The vial was capped, shaken 15 to 30 min, and the pH measured on a pH meter. A typical gel had a pH range from pH 4.5 to pH 7.1. All first dimension gels were run immediately in the second dimension as described below.

SDS/polyacrylamide gels (second dimension) were prepared as follows. Acid-washed glass plates (16.5 cm x 14.5 cm; 1.7 cm bevelled notch on front plate) were coated with Sigmacote, and separated by 1.5-mm spacers coated with vaseline. The resolving portion of the second dimension gel was a linear 8-to-15% polyacrylamide gradient (8 cm in length) with the buffering system described by Orlowski (1979) from the original procedure of Laemmli (1970). Eleven ml of 15% acrylamide stock solution (0.375 M Tris-HCl[pH 8.8], 0.45% bisacrylamide, 0.1% sodium dodecyl sulfate) were poured into one chamber of a gradient maker and 11.5 ml of 8% polyacrylamide stock solution (same buffer) were poured into the other
chamber. The two solutions were mixed continuously as the gradient was formed. The gel was overlaid with a 1:10 dilution of the buffer and the gel allowed to polymerize overnight. The overlay buffer was removed and a 4% polyacrylamide stacker (0.1% bisacrylamide, 160 mM Tris-HCl [pH 7.0], 0.007% [wt./vol.] sodium dodecyl sulfate) was poured to the base of the notch in the glass plate. The stacker gel was overlaid with a 1:10 dilution of the stacker buffer for 1 hour. The overlay solution was removed. The first dimension gel was extruded from its tube onto parafilm, and then sealed onto the top of the stacking gel in 2-to-3 ml of melted 1% agarose (Sigma, Type 1) in SDS sample buffer. The gel was electrophoresed in a standard vertical slab gel apparatus for 20 min at 20 mAmps with SDS electrode buffer (25 mM Tris/glycine [pH 8.2] and 0.1% sodium dodecyl sulfate) in the bottom reservoir and High SDS electrode buffer (same buffer but 2.0% sodium dodecyl sulfate) in the top reservoir. The High SDS buffer was removed from the top reservoir and replaced with SDS electrode buffer containing 80 µl of bromphenol blue tracking-dye solution. The gel was electrophoresed at 20 mAmps until the dye front passed through the resolving gel (about 9.5 hours). In some gels, protein molecular weight standards (bovine serum albumin, 66,000 daltons; ovalbumin, 45,000 daltons; trypsinogen [bovine pancreas] 24,000 daltons; and lysozyme, 14,300 daltons [all obtained from Sigma]) were loaded into a slot formed in the agarose seal adjacent to the first dimension gel. These standards were electrophoresed as described above.
One-dimensional SDS/polyacrylamide gel electrophoresis was carried out with the same resolving gel (a linear 8-15% polyacrylamide gradient) and stacker as described above, but a slot former was placed in the stacker during polymerization to form sample wells. Protein samples were boiled in SDS sample buffer for 3 min. Three μl of bromphenol blue tracking dye were added to the samples which were loaded into the sample wells. Protein molecular weight markers (same as above) were electrophoresed in a separate sample well. The electrode buffer in the upper and lower reservoirs was the same as that described above (SDS electrode buffer). Gels were electrophoresed at 15 mAmps until the dye front passed into the resolving gel. The current was increased to 25 mAmps and held constant until the dye front passed through the resolving gel.

Gels were fixed and stained overnight in a solution of 0.5% (wt./vol.) Coomassie Brilliant Blue R-250 (Baker Chemical Co. Phillipsburg, N.J.) in methanol/water/acetic acid (5:4:1). The gels were destained for 24 hours in 2 to 3 changes of methanol/water/acetic acid (2:7:1) and photographed through an orange filter in transmitted light with Polaroid Land Film (Type 55, positive/negative). Gels were transferred to methanol/water/acetic acid (3:6:1) containing 3% (vol./vol.) glycerol, shaken for 24 hours, and dried under vacuum at 65°C onto Whatman 3 MM chromatography paper using an apparatus manufactured by Miles Laboratories (Elkhart, In.).

Dried gels containing proteins labeled with L-[35S]methionine were autoradiographed by exposing them to Kodak XAR5 X-ray film at
-70°C. In the case of 2-D gels, exposure was adjusted such that the product of radioactivity and time was equivalent to $10^6 \text{ CPM} \times 150$ hours (Hiatt et al., 1980). In the case of 1-D gels, image density as a function of exposure time was determined empirically for each gel and the optimum autoradiograph chosen for analysis and presentation. Autoradiographs were developed for 3 to 5 min in Kodak GBX developer and fixed in Kodak Rapid Fixer. The autoradiographs were photographed with Kodak 35 mm Technical Pan Film (ASA 50). Negatives were developed in Kodak HC110 developer (dilution F). All prints were prepared on Kodak Ektamatic SC Paper (F) and processed on an automatic print processor (Ilford Inc., New York, N.Y.) using Ilford activator and stabilizer.
Results

Incorporation of L-\[^{14}\text{C}]\text{Leucine into Protein During Sporangiospore Germination}

Sporangiospores of *M. racemosus* increased 20% in dry weight during the first 60 min of germination in YPG medium (Table 1). To identify the nature of this newly synthesized material, sporangiospores of a leucine auxotroph (leu 2A) were harvested in YPG medium containing L-\[^{14}\text{C}]\text{leucine. 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 (Figure 1). The presence of cycloheximide in the medium completely prevented any protein synthesis (data not shown). Previously performed analysis of exogenous L-\[^{14}\text{C}]\text{leucine entry into the intracellular pools of several morphological forms of *M. racemosus* showed that equilibration was rapid and not limiting to the apparent rate of leucine incorporation into protein (Orlowski and Sypherd, 1977). The present amino acid pool analysis of the leu2A strain revealed that dormant sporangiospores contain no intracellular leucine (Table 1). Leucine accumulation was detected shortly after the spores were exposed to an exogenous source of the amino acid (YPG medium), and followed the same general accumulation pattern as total amino acid pools.
Ribosome Analysis

Sucrose density gradient analysis of spore extracts showed that the percentage of ribosomes associated with mRNA (polysomes plus 80S monosomes) increased from 25-to-30% 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 hours (Figure 2). When verrucarrin was added to the system, 22.5% of the ribosomes were bound to mRNA in the dormant spore. Verrucarrin is a protein synthesis initiation inhibitor which prevents ribosomes from binding to mRNA (Cundlife, Cannon, and Davies, 1974). Cycloheximide is an elongation inhibitor (Pestka, 1971) and when present alone in the system, possibly allowed a low level of ribosomal subunit binding to mRNA during harvesting. In order to preclude the effects of hydration and the consequent breaking of dormancy upon the formation of ribosome-mRNA initiation complexes, some spores were harvested in nonaqueous solvents. The percentage of ribosomes bound to mRNA was found to be 26.5% in mineral oil harvested spores and 20% in heptane harvested spores. Wet- and dry-harvested spores are thus comparable with regard to this parameter (Table 2).

It should not be assumed that ribosomes bound to mRNA in the dormant spore are active in translation, since the spores of leu2A, which contain no detectable free leucine pool, also possess approximately 35% of their ribosomes associated with mRNA. It was noted that the percentage of ribosomes bound to mRNA in dormant spores decreased
to 0 in 21 day old cultures. Older cultures also showed a lower degree of viability than 7 day spores (Table 2). These data may imply the presence of low levels of metabolic activity in dormant spores.

Incorporation of \(^3\text{H}\)Uracil and \(^{32}\text{P}\)Phosphate into RNA during Sporangiospore Germination

There was a lag of at least 10 min before labeled precursors were incorporated into RNA (Figure 3). From 10 to 20 min incorporation took place at a relatively low rate and did not reach a maximum until after 20 min. The transcription inhibitors actinomycin D and daunomycin, alone or in combination, failed to stop RNA synthesis in this system (Figure 4). However, the energy poisons sodium azide and sodium fluoride completely inhibited incorporation of \(^3\text{H}\)Uracil into RNA (Figure 4). Measurement of the uptake of \(^3\text{H}\)uracil indicated that this compound equilibrated with free nucleotide pools in \textit{M. racemosus} at rates nonlimiting to the measurement of RNA synthesis (Orlowski and Sypherd, 1978A). Thin layer chromatography of \(^{32}\text{P}\)-labeled ribose nucleoside triphosphates (rNTPs) showed that significant levels of radioactivity had accumulated in the pools within 5 min, yet labeling of RNA did not begin until after 10 min. ATP pools were measured at frequent intervals by a luciferin-luciferase assay (Stanley and Williams, 1969). The level of intracellular ATP was halved between 2 and 10 min and then expanded to near its original value by 30 min (Figure 5). When accumulation of \(^{32}\text{P}\) in rNTP pools was normalized to ATP pool size, it was determined
that CPM of $^{32}\text{P}/\mu\text{g}$ ATP increased linearly 14 fold from 4 min to 40 min of germination (Table 3). During this same time span, incorporation of radioactivity into RNA increased exponentially > 200 fold (Table 4) suggesting that uptake of $[^{32}\text{P}]\text{phosphate}$ into pools was not the limiting step for the apparent rate of $^{32}\text{P}$ incorporation into RNA.

### Analyses of RNA Synthesized During Sporangiospore Germination

$^{32}\text{P}$-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. $[^{32}\text{P}]\text{Phosphate}$ 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 RNA species increased rapidly to a rate representative of whole cell labeling (Table 4).

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 $[^{3}\text{H}]\text{dimethyl sulfate}$, is shown in Figure 6. 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 summed for 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 (Figure 7). Poly(A)\(^+ \) RNA comprised approximately 10% of the RNA synthesized between 10 and 60 min of germination. \( {^{32}P} \)-Labeled poly(A)\(^+ \) RNA purified from spores after 40 min of 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 (Figure 8). Therefore, 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 (Nickerson et al., 1981), therefore it cannot be assumed that wet-harvested spores are representative of the dormant state. We found that harvesting sporangiospores in buffer containing \( \text{NaN}_3 \) and \( \text{NaF} \) (both at 10 mM
concentrations) completely inhibited incorporation of \( ^{32}\text{P}\) phosphate into RNA (Figure 4). RNA purified from spores harvested in this manner was methylated with \( ^{3}\text{H}\) 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 \([\text{poly}(A)^{+} \text{RNA}]. \) The \( \text{poly}(A)^{+} \) RNA fraction comprised 3.3% of the total RNA (Figure 8). \( \text{Poly}(A)^{+} \) 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 \text{Mucor} marker RNA. The \( ^{3}\text{H}\)-labeled \( \text{poly}(A)^{+} \) RNA was heterodisperse in size, ranging from 6S to 20S (Figure 9), a characteristic typical of mRNA.

**Analyses of In Vivo Pulse-Labeled Proteins from Germinating Sporangiospores**

\text{Mucor racemosus} sporangiospores were harvested into YPG medium containing \( L-[^{35}\text{S}]\text{methionine} \) and incubated for 60 min to determine the suitability of this amino acid for an in vivo pulse-labeling experiment. Incorporation of radioactivity into protein commenced immediately following exposure of spores to the medium (Figure 10). The kinetics of radioisotope accumulation were exponential for 60 min, nearly identical to results seen for \( L-[^{14}\text{C}]\text{leucine} \) incorporation into a leucine auxotroph (Figure 1). The addition of cycloheximide to the growth medium totally inhibited incorporation of radioactivity into protein.
The proteins of germinating sporangiospores were also pulse-labeled with L-[\textsuperscript{35}S]methionine during 4 consecutive 15-min periods in the first 60 min following the initiation of germination. Determination of the specific activity of proteins purified from sporangiospores after each pulse period revealed that the rate of protein synthesis increased during the first 60 min of germination (Table 5) as might be predicted on the basis of data in Figure 10. Amino acid pools of \textit{M. racemosus} 1216B remained relatively constant over the 60 min period displaying a pattern similar to that observed for pools in the leu 2A auxotroph (Table 6), although concentrations of all amino acids were higher in the wild type than in the mutant. The specific activity of L-[\textsuperscript{35}S]methionine in endogenous pools was found to decrease by 50% during this 60-min period. Therefore the increase in specific activity of proteins must have been due to an increased rate of synthesis rather than an increased availability of the label (Table 7).

Samples of protein extracted from dormant or germinating sporangiospores were analyzed by SDS/polyacrylamide slab gel electrophoresis (Orlowski, 1979) and 2-dimensional isoelectric focusing/SDS polyacrylamide gel electrophoresis (O'Farrell, 1975). The isoelectric focusing gels of the 2-D gels had a pH range of 4.5 to 7.1. Any protein whose isoelectric point falls outside of this range was excluded from the present 2-D gel analysis. SDS/polyacrylamide slab gel electrophoresis theoretically separates proteins which bind SDS
strictly on the basis of size. Therefore changes in major proteins not displayed on the 2-D gels may possibly be seen on the 1-D gels.

Coomassie Blue staining of both 1-D and 2-D gels indicated only minor changes in the total spectrum of sporangiospore proteins during the first 60 min of germination. The total population of proteins isolated from dormant spores (Figure 11, Gel A) and the total population of proteins extracted from spores after 60 min of germination (Gel B) displayed no apparent differences in the constellations of spots visible on the 2-D gels. The same comparison on an SDS/polyacrylamide 1-D slab gel (Figure 12, Gel A; lane 1 and 5) showed only 3 bands (arrows) with different relative concentrations.

In contrast to the previous observation, many changes in composition were noted when a comparison was made between proteins synthesized during sporulation and those actively synthesized during the 4 pulse-labeling periods in the first 60 min of germination. An autoradiograph of an SDS/polyacrylamide 1-D slab gel (Figure 12, Gel B) showed that prelabeled dormant spores (lane 1) displayed 9 bands of protein (designated a, b, c, d, e, f, g, h, and i) which were present in relatively greater amounts than in the pulse-labeled germinating spores (lane 2, 3, 4, and 5). Ten bands of protein synthesized in each of the 4 pulse periods (j, k, l, m, n, o, p, q, r, and s) were present in relatively greater amounts than in the dormant spore. Some of these bands appeared to include new proteins (k, l, o, and p) although the resolution here was limiting. In addition, at
least 4 bands implied changes in the relative rates of protein synthesis during the first 60 min of germination (lane 5, arrows).

Two-dimensional isoelectric focusing/SDS polyacrylamide gel electrophoresis resolved 500-to-600 proteins and showed several striking differences between the population of proteins synthesized during sporulation (Figure 13, Gel A) and those synthesized during the first 60 min of germination (Figure 14, Gel A, B, C, and D).

Many proteins showed increases in relative concentrations in germinating spores (designated 29, 36, 46, 47, 52, and 5-9) while 1 protein showed a decrease (30). At least 8 proteins present in pre-labeled dormant spores were not synthesized during the first 60 min of germination (48, 66, 67, 56, 57, 11, 12 and 2). Four of these proteins (67, 56, 57, and 2) may be specific to spores because they did not appear in Coomassie Blue stained gels of proteins extracted from either the hyphal or yeast forms of the organism (Figure 15). Other proteins (4, 60, 61, and 13) were synthesized during the first 30 min of germination or longer, but were absent in the dormant spore. The mRNA's for these proteins were stored in the dormant spore without being translated until germination was initiated. The appearance of protein 34 may have resulted from the processing of a pre-existing protein (see below). It is interesting to note that protein 60 was found in a Coomassie Blue stained gel of hyphal proteins (Figure 15, Gel A), but not in dormant spores, indicating that it may be important in the development of the hyphal form. Protein 13 was found in hyphae, yeast cells and germinating spores, but not in dormant spores.
Protein 10, which was heavily labeled in the dormant spore, was also synthesized during the first 15 min of germination, but disappeared between 15 and 45 min and then reappeared again later. Differential gene expression was not exclusively an all-or-none phenomenon in this developmental system. Many other proteins were found to have changing relative rates of synthesis during the studied period of germination, including 38, 49, 45, 29, 30, 46, 54, 52, 18-21, and 26-28, which displayed increasing rates of synthesis and 60, 61, 14, 15, 1, 33, 35, 37, 53, and 22-24 which revealed decreasing rates of synthesis.

The apparent changes in composition and rates of synthesis noted above may result from actual changes in rates of protein synthesis or from processing of existing proteins or protein turnover. Prelabeled dormant sporangiospores were washed free of any exogenous label and germinated for 60 min in YPG containing an excess of non-radioactive L-methionine. Samples were collected every 5 min and assayed for radioactivity. The incorporated radioactivity did not decrease over the 60 min period, implying that the proteins synthesized during sporulation were stable (Figure 16). Thus protein turnover was not responsible for the observed changes in protein composition or rates of protein synthesis. An autoradiograph of a 2-dimensional gel of prelabeled proteins extracted at the end of the 60-min germination period showed no apparent changes from proteins extracted immediately from dormant spores (Figure 13, Gel B). Therefore, post-translational processing did not play a major role in the
observed changes in protein composition or rates of protein synthesis. One exception may be protein 34 which appeared after 60 min of germination of prelabeled spores.

Analyses of Functional mRNA Levels in Dormant and Germinating Sporangiospores

A rabbit reticulocyte protein synthesizing system (see Materials and Methods) was used to analyze mRNA populations in dormant and germinating sporangiospores. The cell free system was programmed with increasing amounts of total RNA purified from spores germinated for 60 min in YPG medium. The incorporation of L-[^35]S]methionine into hot trichloroacetic acid-insoluble material was a linear function of input RNA up through 20 µg (Figure 17). At RNA levels higher than 30 µg, the system was markedly inhibited. Twenty µg of RNA were chosen as the amount used in the standard incubation mix because it stimulated a high level of incorporation of radioactivity and still fell on the linear portion of the curve just described. Twenty µg of RNA were added to the standard cell-free reaction mix and incubated for 60 min. Samples (5 µl) were collected at 15-min intervals and assayed for radioactivity. L-[^35]S]methionine was incorporated into protein at a linear rate during the 60-min experiment, indicating that the amount of methionine added (50 µCi) was sufficient to support cell-free synthesis for up to 60 min (Figure 18).

RNA, purified from dormant spores and from spores sampled at 15-min intervals during the first 60 min of germination, was then translated in the optimized cell-free system for 60 min. Mucor RNA
stimulated the incorporation of radioactive precursor into protein 7.5- to 12.7-fold above the endogenous level of incorporation (Table 8). One μg of pure rabbit globin mRNA stimulated incorporation 14 fold. Since approximately 3.3% of Mucor spore RNA is poly(A)\(^+\) RNA (Figure 8), 20 μg of this RNA should stimulate 66% (9.24-fold over endogenous) as much incorporation of label as 1 μg of globin mRNA. The actual stimulation of incorporation in vitro was at this level or higher. M. racemosus RNA was therefore translated in the reticulocyte system with an efficiency similar to that of a native message (globin mRNA).

RNA from dormant spores was 60-to-70% as efficient in the stimulation of protein synthesis in vitro as RNA purified from spores that had been germinated for 15 min. Poly(A)\(^+\) RNA is not synthesized at a significant level during this time (Figure 7) implying that the increase in translation efficiency was probably due to the processing of stored mRNA. After the initial increase in translation efficiency, the level of translatable mRNA remained relatively constant throughout the first 60 min of germination.

The rabbit reticulocyte cell-free system translated M. racemosus RNA faithfully as determined by electrophoretic separation of in vitro protein synthesis products on 1-D SDS/polyacrylamide slab gels and 2-D isoelectric focusing/SDS polyacrylamide gels. An SDS/polyacrylamide slab gel of spore proteins synthesized in vitro (Figure 19, Gel A) revealed that the majority of protein bands corresponded directly to bands seen on a similar gel of in vivo labeled proteins.
(Figure 12, Gel B). Proteins with molecular weights of up to 200,000 daltons were detected, although large proteins were not quite as numerous as those synthesized in vivo. Four protein bands (arrows) on the 1-D gel changed intensities during the time course of germination suggesting changes in the level of translatable mRNA present in the spore. Levels of mRNA specifying the protein denoted by the top arrow decreased during the first 60 min of development in a pattern identical to that observed for the in vivo synthesis of a protein with the same molecular weight (160,000 daltons) resolved on 1-D (Figure 12, Gel B; top arrow) and 2-D gels (Figure 14; protein 1). The decrease in synthesis of this protein was presumably due to the degradation or some other means of inactivation of its cognate mRNA.

One protein invariably detected on both 1-D (Figure 19, Gel A, Lane 1) and 2-D gels (Figure 19, Gel B, arrow) was identified as an endogenous product of the in vitro protein synthesizing system. This protein had a molecular weight of 47,000 daltons and an isoelectric point of about 6.0. The protein spot has been identified on all gels to avoid possible confusion with Mucor proteins.

Four hundred-to-five hundred of the protein products synthesized in vitro were visible and resolved as individual spots on 2-D isoelectric focusing/SDS polyacrylamide electrophoresis gels (Figure 20). Their molecular weights ranged from 13,000 to 200,000 daltons. A large number of these proteins displayed identity, in terms of
apparent molecular weight and isoelectric point, with proteins labeled during in vivo pulses.

Several proteins, synthesized during sporulation, were not synthesized during early germination (2, 11, 12, 48, 56, 57, 66, and 67). Analysis of mRNA populations by means of in vitro protein synthesis revealed the absence of translatable message for proteins 56 and 57 in dormant spores. The mRNA for protein 48 was present in dormant spores although it was degraded or inactivated early in germination and not translated in vivo or in vitro. Of the 4 proteins synthesized during the first 30 min of germination but not during sporulation (4, 13, 60, and 61), RNA's for proteins 4 and 60 were detected in dormant spores. Translatable mRNA for protein 61 appeared by 15 min and remained at a constant level throughout the first 60 min of germination. Since the level of mRNA synthesis during 0 to 15 min is very low (Figure 7), the mRNA for protein 61 is most likely synthesized as a precursor and stored in the dormant spore. Once germination is initiated the precursor is processed to the final translatable form. Translatable mRNA for protein 13 was not detected in this analysis. The latter observation may imply that this protein is generated by a post-translational event in vivo which cannot be duplicated in the in vitro system. Alternatively, formation of the appropriate translation initiation complex may not take place in vitro due to profound specificities of the components involved.
Levels of translatable mRNA specific for several proteins (5-9, 31, 47, 60, 61) remained constant during the first 60 min of germination. Proteins 5-9, 31 and 47 were also synthesized at a constant rate \textit{in vivo} during this time period. However, proteins 60 and 61 were synthesized at a decreasing rate \textit{in vivo} suggesting a translational level of regulation.

The rates of \textit{in vivo} synthesis of several proteins quantitatively changed during the first 60 min of germination. These changes correlated well with alterations in the levels of specific translatable mRNA's detected by an \textit{in vitro} assay. Proteins 26, 27, 28 and 46 showed increasing rates of synthesis \textit{in vivo} reflecting increased levels of translatable mRNA, whereas proteins 1, 14, 15, 33, 35, 37 and 53 displayed decreasing rates of synthesis \textit{in vivo} reflecting decreases in the concentrations of translatable mRNA's.

Protein 45 is perhaps the most profound example of a gene product subject to translational regulation observed in this study. Protein 45 is synthesized at increasing rates throughout early germination, yet the level of translatable mRNA was found to decrease significantly during this time. This implies that the affinity of the mRNA for ribosomes increases at a rate greater than the rate of mRNA degradation or inactivation. Alternatively, the step time of ribosome movement along mRNA may be independently adjustable for different messages and may vary as a function of developmental stage.

The present study has provided a substantial body of evidence to suggest that protein synthesis is regulated at multiple levels
during the formation and germination of *Mucor* sporangiospores. These levels, at a minimum, encompass differential transcription, post-transcriptional processing and selective translation.
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-[\textsuperscript{14}C]Leucine was incorporated into newly synthesized protein at a rapid rate as soon as the spores were exposed to medium. The percentage of ribosomes associated with mRNA (in polysomes plus 80S monosomes) increased from 22.5% (with verrucarrin present) in dormant spores to 85% within 10 min. Polysomes in ungerminated spores can be considered evidence of the presence of stored mRNA. Spores harvested into nonaqueous solvents also possessed 35% of their ribosomes in association with mRNA, suggesting that the binding of ribosomal subunits to mRNA was not simply a consequence of hydrating the spores. The significance of the 25-to-30% of ribosomes that are associated with mRNA in the dormant spore is unclear.

[\textsuperscript{3}H]Uracil and [\textsuperscript{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 mRNA did not appear in polysomes until at least 30 min had passed.
These data 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 sodium azide and sodium fluoride 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* which contained 2.5% of its RNA in the poly(A)$^+$ form (Knight and Van Etten, 1976A). 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 *Botryodiplodia theobromae* pycnidiospores (Van Etten and Rawn, 1978).

Total cellular RNA purified from dormant sporangiospores stimulated the incorporation of L-$[^{35}S]$methionine into protein in a rabbit reticulocyte *in vitro* system. The efficiency of incorporation was similar to that for a native source of mRNA (rabbit globin message) when corrected for the proportion of total *Mucor* RNA represented by poly(A)$^+$ RNA. This suggests that a large proportion of the stored poly(A)$^+$ RNA is functional mRNA.

The translation products specified by this pool of mRNA stored in the dormant spore and those populations of mRNA present in germinating spores were analyzed by means of polyacrylamide gel electrophoresis. In order to minimize discrepancies or artifacts in the interpretation of polyacrylamide gel electrophoresis, several accepted procedures were followed. Gels to be stained with Coomassie Blue for analysis of the total protein spectrum were loaded with a constant
standard amount of protein so that changes in band or spot intensity reflected real changes in relative protein concentrations. A constant standard amount of isotopically-labeled protein was also loaded onto the first dimension of 2-D gels later to be analyzed by autoradiography. Since the radio-specific activity of proteins varied with the individual sample, the time of X-ray film exposure to the dried gels was adjusted as a linear function of the total radioactivity contained within a given gel to yield the product $10^6 \text{CPM} \times 150$ hours or its equivalent. In this way, changes in spot intensities reflected real changes in relative rates of protein synthesis. Bands or spots on all gels were identified visually by absolute and relative position. Bands or spots migrating to the same position on separate gels were assumed to be the same protein.

When using in vitro protein synthesis as an assay for functional mRNA levels, it is important that incorporation of radioactive precur- sor into protein is a linear function of added RNA. In this study exogenous RNA stimulated incorporation of radioactivity in a relationship with concentration that was linear for up to 20 $\mu$g and at an efficiency similar to that stimulated by a source of native RNA. Endogenous synthetic activity in the cell-free system was low enough that it did not interfere with the analysis of products. The one endogenous protein labelled in the rabbit reticulocyte system was identified in all gels, and did not comigrate with any Mucor proteins of interest. Finally, a large number of in vitro synthesis products migrated to the same position on gels as proteins synthesized
in vivo. The cell-free system described was faithful and efficient, and therefore provided a reliable assay for *M. racemosus* mRNA.

Polyacrylamide gel analysis of in vitro translation products showed that the poly(A)$^+$ RNA in dormant spores is stored in a form which produces proteins identical to those synthesized in vivo during the first 30 min of germination. The ability of spores to store functional mRNA constitutes a form of translational regulation of gene expression.

A recent study performed in this laboratory (Orlowski, Abstr. Annu. Meet. Amer. Assoc. for Microbiol., I-33: 145, 1983) established the presence of ribonucleoprotein particles (RNP's) in dormant spores. These RNP's were found to contain poly(A)$^+$ RNA and an array of approximately 10 major proteins. Sucrose density gradient analysis determined that RNP's in dormant spores sedimented at rates < 80S. After 10 min of germination almost all of the poly(A)$^+$ RNA initially associated with the RNP's sedimented with monosomes and polysomes (> 80S). Nonpolysomal, or free RNP's are presumably the site of stable mRNA storage in dormant spores. RNP particles with properties similar to those of *Mucor* have been detected in *Neurospora crassa* (Mirkes, 1977) and in *Dictyostelium discoideum* (Firtel and Pederson, 1975).

RNA purified from dormant sporangiospores was 30-to-40% less efficient in stimulation of in vitro protein synthesis than was RNA purified from spores undergoing germination, a situation quite comparable to that observed in the related fungus *Rhizopus stolonifer* (Freer and Van Etten, 1978). The rate of RNA synthesis during the
initial 10-to-20 min of germination was so low that the contribution of newly synthesized mRNA to the observed increase in translation efficiency was probably not significant. One realistic alternative explanation for this increase might postulate a significant amount of dormant spore mRNA stored in a form which requires processing before it can be translated. Upon the addition of medium, RNA processing would yield an increase in the amount of translatable mRNA. Another reasonable explanation might be that the lower translation efficiency of dormant spore RNA is due to the presence of an inhibitor in the cell free system. There is not sufficient data available to definitively support either of these hypotheses at this time.

The in vivo translation products of the mRNA stored in M. race-mosus sporangiospores were compared to proteins synthesized during sporangiospore formation by polyacrylamide gel electrophoresis. The total protein composition of sporangiospores was found to change little during the first 60 min of germination. However, the composition of proteins synthesized during spore formation vis-a-vis spore germination did differ significantly. Several proteins were synthesized in significant amounts during spore formation but were not made at all during early germination. A few of these proteins appear to be specific to dormant spores although their function is not known. Translatable mRNA for only one of these "sporulation proteins" was detected in dormant spores and this was degraded or inactivated within the first 30 min of germination.
Four proteins were synthesized during the first 30 min of spore germination but not during spore formation. Translatable mRNA for 2 of these proteins was detected in dormant spores while mRNA for a 3rd protein was detected within 15 min of the start of germination. Developing sporangiospores thus appear to have the ability not only to store mRNA in a stable form but to selectively translate mRNA's specific for proteins required during spore maturation and to avoid translating stored mRNA's specific for proteins required during early germination.

The burst of protein synthesis during early germination was not simply the result of increased synthesis of all proteins. As mentioned above, several "sporulation proteins" were not synthesized in early germination whereas the synthesis of 4 new proteins was detected. These examples represent qualitative changes in protein synthesis. However, the majority of the changes in protein synthesis observed in this study were quantitative, that is, increases or decreases in the relative rates of synthesis of individual proteins. Many of the changes in rates of synthesis of proteins were accompanied by a corresponding change in the levels of translatable mRNA in the spore. The regulation of synthesis for these proteins is at the level of transcription (RNA synthesis) or post-transcription (degradation or inactivation). Indeed, mRNA degradation or inactivation appears to play a major role in the regulation of synthesis of several proteins during early germination (see below).
The synthesis of 3 proteins was demonstrated to be regulated, at least in part, at the translational level. The level of translatable mRNA for 2 of these proteins remained constant while their in vivo rate of synthesis declined. The level of mRNA for the 3rd protein was found to decrease while its rate of synthesis actually increased. These data imply that the affinity of certain mRNA's for ribosomes can be altered. Thus differential gene expression in early germination may be effected by both quantitative and qualitative adjustments.

How do *Mucor* sporangiospores maintain dormancy and what is the trigger for germination? We propose that dormancy is maintained, at least in part, by blocking the initiation of protein synthesis. This is accomplished by masking stored mRNA making it unavailable for translation. Once germination is initiated by the presence of water and nutrients, the mRNA's are unmasked and become available to ribosomes. This could explain the rapid recruitment of ribosomal sub-units into polysomes seen during the first 10 min of germination in the absence of significant RNA synthesis. A situation analogous to this has been demonstrated in developing sea urchin embryos (Stavy and Gross, 1969). In this system, maternal mRNA is stored in oocytes in a form which is not translatable in vitro. Upon fertilization, RNA purified from developing oocytes up through the blastula stage stimulate increasing incorporation of radioactivity into protein in vitro, even when RNA synthesis is completely inhibited by actinomycin-D. Throughout early development, maternal mRNA's are unmasked, making them available to translation.
Poly(A)$^+$ RNA in dormant *Mucor* spores is stored in nonpolysomal (< 80S) RNP's containing about 10 major proteins. When germination is initiated, the RNP's become associated with polysomes within 10 min. It seems reasonable that the masking of stored mRNA may be effected by the protein composition of RNP's either by physically blocking the translation initiation codon or by burying it within the secondary structure of the folded mRNA. The loss or modification of certain proteins could result in the unmasking.

In a study on chick embryonic muscle, free and polysomal RNP's were purified (Jain and Sarkar, 1979). Free RNP's were found to contain up to 10 proteins while actively translated polysomal-derived RNP's were protein deficient, containing only 2 proteins. These 2 proteins were common to free and polysomal RNP's and were thought to be associated with the poly(A) region of mRNA. The other 8 proteins, present only in free RNP's, were thought to be associated with non-poly(A) regions of the mRNA. In this system, RNP proteins appear to play a role in determining which mRNA species are translated.

Of course, masking of mRNA could take place at other levels. For example, mRNA in dormant spores could be stored in the cell nucleus. The inhibition of translation could result from blocking transport of the mRNA to the cytoplasmic ribosomes. Capping or some other covalent modification of the mRNA after initiation of germination could play a role in this type of control mechanism.

*M. racemosus* sporangiospores demonstrated the ability to regulate protein synthesis at the level of translation. *Mucor* yeasts and
hyphae have been shown to have the ability to increase the poly-
peptide chain elongation rate in response to an increase in growth
rate (Ross and Orlowski, 1982 A and B). The degree of phosphorylation
of ribosomal protein S-6 was directly correlated to growth rate in
Mucor yeasts and hyphae (Larsen and Sypherd, 1980) and could possibly
play a role in this translational regulation. Another likely candi-
date in this type of model is elongation factor EF-1α. Both EF-1α
and S-6 are present in dormant spores in the unmodified state, but
are modified to varying degrees in actively growing vegetative cells
(Hiatt et al., 1982). These modifications could serve to activate
ribosomal activity and help regulate the percentage of active ribo-
somes and the polypeptide chain elongation rate. Protein modifica-
tions could also be involved in regulating the translation of certain
mRNA's by altering their affinity to ribosomes.

Several of the stored mRNA's in dormant sporangiospores are
degraded or inactivated during the first 30 min of germination,
presumably causing the observed decreases in the extent of their
in vivo translation. One wonders why the spore should expend energy
to store high levels of a mRNA which is fated to be discarded.

One possible explanation for this phenomenon may be related to
the fact that Mucor is dimorphic. The spores have two potential
morphologic fates: yeasts or hyphae. The spores may maximize their
developmental options by storing messages essential to the formation
of both cell types. Once a morphogenetic commitment has been made,
mRNA degradation may be the most expedient means of effecting the
appropriate differential gene expression. A comparison of protein extracts from CO$_2$-grown yeast cells and aerobic hyphae revealed that most differences in protein composition are quantitative (Figure 15). Several proteins, especially 33, 35, 45 and 30, are present at much higher relative concentrations in yeast cells. Translatable mRNA levels for proteins 33, 35, and 45 are relatively high in dormant spores. The spores have the potential to begin high levels of protein synthesis from these mRNA's. Under aerobic germination conditions, relatively low levels of these proteins are required, so excess mRNA can be degraded or inactivated. Perhaps under a CO$_2$ atmosphere, these mRNA's are maintained and translated at high levels.

An alternative explanation is related to the spore's ability to store mRNA in a stable form. Once the storage and packaging mechanism is activated in the maturing spore, a part of mRNA's synthesized, regardless of function, could be stored in the spore. The 4 proteins mentioned above (30, 33, 35, and 45) are all present at relatively high concentrations in dormant spores requiring the presence of large quantities of their respective mRNA's at some point during spore maturation. These mRNA's may then simply be stored fortuitously along with all other mRNA's synthesized at that time. Once germination is initiated, mRNA's for proteins whose activity is not required would be degraded, or inactivated. More data are needed before a complete understanding of mRNA degradation/inactivation is forthcoming.

Approximately 500 Mucor proteins were resolved by 2-dimensional electrophoretic analysis. These proteins presumably comprise only a
fraction of the coding capacity of the *Mucor* genome and represent those proteins present in the highest concentrations in *Mucor* spores. Changes in less abundant, but perhaps functionally important, *Mucor* proteins were not detected in this analysis.

The functions of the proteins resolved on 1-D and 2-D gels were not identified. Therefore, no specific cause-effect relationships could be established between the qualitative and quantitative changes in gene expression observed and the morphologic events of early germination. For example, the protein designated 60 was synthesized during early germination but not during spore maturation. This protein was detected in cell extracts of hyphae but not of yeasts, implying that protein 60 could play a role in the determination of cell morphology. Knowing the molecular function of this particular protein might enable one to explain its role in morphogenesis. Future studies that focus on identifying the functions of such morphology-specific proteins will be essential to our understanding of development.

Research in many other areas is needed to help elucidate the mechanisms integral to spore germination. The stored mRNA of dormant spores must be further characterized in terms of its intracellular location and the types of processing which it may undergo (e.g., capping, polyadenylation, methylation, intron excisions etc.). The protein composition of free and polysomal RNP's needs to be characterized in more detail, perhaps establishing its role in masking, processing, and transport of mRNA. Finally, a study
investigating the germination of sporangiospores into yeast cells under a CO\(_2\) or a N\(_2\) atmosphere would provide useful information about the spore's ability to implement alternative genetic programs in response to differing environmental conditions.

In summary, *Mucor racemosus* sporangiospores were found to contain stored mRNA which is translated immediately upon the initiation of germination by exposure to aqueous nutrients. A large fraction of this mRNA, stored in the form of nonpolysomal RNP's, is functional mRNA but is in some manner prevented from being translated by the ribosomes. Developing sporangiospores selectively translate mRNA's specifying proteins integral to spore maturation and avoid translating stored mRNA's specifying proteins characteristic of early germination. Spores also display differential gene expression during aerobic germination. This may be effected by quantitative as well as qualitative adjustments in protein synthesis. The regulation of gene expression during early germination is mainly at the level of transcription although clear examples of post-transcriptional and translational regulation also exist.
Literature Cited


APPENDIX
<table>
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<th>Time (min)</th>
<th>ymoles of leucine</th>
<th>ymoles of Amino Acids</th>
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Sporangiospores of strain Leu 2A were harvested directly into YPG medium and incubated with shaking at room temperature under air. Samples (2 ml) were withdrawn at the times indicated in column 1 and the amino acid pools extracted and analysed as described in the text. Values were normalized to the dry weight of each spore sample.

*This value was below the resolution limit of the amino acid analyzer (.005 ymole). No peak was seen on the data printout.
Table 2.
The Effect of Sporangiospore Harvest Conditions on the Percentage of Ribosomes Associated with mRNA

<table>
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<th>Harvest Conditions</th>
<th>Percentage of Ribosomes Associated with mRNA</th>
<th>Percent Spore Viability</th>
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<tr>
<td>Heptane</td>
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<td></td>
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<tr>
<td>Freon</td>
<td>45*</td>
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<td>Cycloheximide (200 µg/ml)</td>
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<tr>
<td>Cycloheximide (200 µg/ml) plus</td>
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<tr>
<td>verrucarim (25 µg/ml)</td>
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<td>Na Azide (10 mM) plus Na Flouride (10 mM)</td>
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Dormant sporangiospores were harvested under the conditions indicated in column 1 and the percentage of ribosomes associates with mRNA determined as described in the text. To determine the effect of antibiotics and inhibitors on the percentage of ribosomes associated with mRNA, sporangiospores were harvested into sterile water containing the indicated substance(s). The age of the sporangiospore culture refers to the time allowed for sporulation to occur on YPG agar plates before harvesting. The percent viability was determined by assaying spore cultures for germ tube emergence after 24 hours incubation in YPG.

*Microscopic examination revealed hyphal fragment contamination in the freon harvested spore preparation.
Equilibration of \([^{32}\text{P}]\)Phosphate into Nucleotide Pools During Germination of Sporangiospores of *M. racemosus*.

<table>
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<tr>
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<th>(\text{CPM}^{32}\text{P} ) mg Dry Weight</th>
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<td>1,182</td>
<td>2,749</td>
</tr>
<tr>
<td>15</td>
<td>.49</td>
<td>4,707</td>
<td>9,606</td>
</tr>
<tr>
<td>20</td>
<td>.55</td>
<td>8,468</td>
<td>15,396</td>
</tr>
<tr>
<td>40</td>
<td>.56</td>
<td>21,327</td>
<td>38,083</td>
</tr>
</tbody>
</table>

Sporangiospores were harvested directly into YPG medium containing \([^{32}\text{P}]\)phosphate and incubated by shaking at room temperature under air. Samples (2 ml) were withdrawn at the times indicated in column 1. Nucleotide pools were extracted from the spores and analysed for ATP by the luciferin-luciferase assay and for radioactivity by paper chromatography and scintillation spectroscopy as described in the text. All values were normalized to the dry weight of the spore sample.
Table 4.
Sucrose Density Gradient Analysis of $^{32}$P-Labeled RNA Purified From Germinating Sporangiospores of M. racemosus.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Total CPM $^{32}$P $4,A_{260}$ of RNA</th>
<th>CPM $^{32}$P Found in Individual RNA Size Classes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>4-6S peak 18S peak 25S peak</td>
</tr>
<tr>
<td>5</td>
<td>2,720</td>
<td>Background Background Background</td>
</tr>
<tr>
<td>10</td>
<td>11,790</td>
<td>11,790 (100) Background Background</td>
</tr>
<tr>
<td>15</td>
<td>39,849</td>
<td>14,226 (35.7) 7,412 (18.6) 18,211 (45.7)</td>
</tr>
<tr>
<td>20</td>
<td>102,695</td>
<td>57,406 (55.9) 25,263 (24.6) 20,026 (19.5)</td>
</tr>
<tr>
<td>40</td>
<td>644,004</td>
<td>146,832 (22.8) 144,257 (22.4) 352,914 (54.8)</td>
</tr>
<tr>
<td>60</td>
<td>1,168,085</td>
<td>263,987 (22.6) 241,794 (20.7) 662,304 (56.7)</td>
</tr>
</tbody>
</table>

Sporangiospores were harvested into YPG medium containing $^{32}$Pphosphate and incubated with shaking at room temperature under air. RNA was purified from spore samples and separated into discreet size classes by means of sucrose density gradient centrifugation. Gradients were fractionated on a density gradient fractionator and fractions assayed for $A_{260}$ and CPM of $^{32}$P incorporated. RNA purification, sucrose density gradient centrifugation and fraction analysis are described in the text. $4\,A_{260}$ of each RNA sample was loaded onto the gradients. The numbers in parentheses refer to the percentage of the total CPM found in each individual RNA size class.
Table 5.
Specific Activity of Proteins Pulse Labeled with L-\(^{35}\)S\)methionine During Germination of *M. racemosus* Sporangiospores

<table>
<thead>
<tr>
<th>Pulse Period</th>
<th>CPM in TCA ppt. (^{1}) µl Extract</th>
<th>µg Protein µl Extract</th>
<th>CPM µg Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 - 15 min</td>
<td>1,120</td>
<td>3.3</td>
<td>340</td>
</tr>
<tr>
<td>15 - 30 min</td>
<td>5,080</td>
<td>6.8</td>
<td>747</td>
</tr>
<tr>
<td>30 - 45 min</td>
<td>7,000</td>
<td>8.0</td>
<td>875</td>
</tr>
<tr>
<td>45 - 60 min</td>
<td>6,940</td>
<td>6.4</td>
<td>1084</td>
</tr>
</tbody>
</table>

Spores were harvested into YPG medium and incubated with shaking at room temperature under air. L\(^{35}\)S\)methionine was added to the cultures at the appropriate time. Four consecutive 15-min pulse periods were used starting with initiation of germination by exposure of the spores to YPG. Pulse periods were stopped by the addition of cycloheximide. Proteins were extracted from sporangiospores and the specific activity determined as described in the text.

\(^{1}\)Abbreviations: Counts per minute of radioactivity in trichloroacetic acid precipitable material.
Table 6.
Size of Amino Acid Pools in Germinating Sporangiospores of M. racemosus 1216B.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>µmoles of Amino Acids</th>
<th>Dry Weight (mg)</th>
<th>µmoles of Amino Acids mg Dry Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>.83</td>
<td>2.34</td>
<td>.35</td>
</tr>
<tr>
<td>30</td>
<td>.67</td>
<td>2.27</td>
<td>.30</td>
</tr>
<tr>
<td>45</td>
<td>.59</td>
<td>2.43</td>
<td>.24</td>
</tr>
<tr>
<td>60</td>
<td>.65</td>
<td>2.53</td>
<td>.26</td>
</tr>
</tbody>
</table>

Sporangiospores were harvested directly into YPG medium and incubated with shaking at room temperature under air. Samples (2 ml) were withdrawn at the times indicated in column 1 and the amino acid pools extracted and analysed as described in the text. Values were normalized to the dry weight of each spore sample.
Table 7.
Specific Activity of L-[^35]S)methionine in Methionine Pools of Germinating Sporangiospores of M. racemosus

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>μmoles Methionine mg Dry Weight</th>
<th>CPM of L-[^35]S)methionine mg Dry Weight</th>
<th>CPM of L-[^35]S)methionine μmole Methionine</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>1.8 x 10^-3</td>
<td>1.25 x 10^5</td>
<td>6.9 x 10^7</td>
</tr>
<tr>
<td>30</td>
<td>2.5 x 10^-3</td>
<td>1.22 x 10^5</td>
<td>4.9 x 10^7</td>
</tr>
<tr>
<td>45</td>
<td>2.2 x 10^-3</td>
<td>1.17 x 10^5</td>
<td>5.3 x 10^7</td>
</tr>
<tr>
<td>60</td>
<td>1.9 x 10^-3</td>
<td>0.62 x 10^5</td>
<td>3.3 x 10^7</td>
</tr>
</tbody>
</table>

Sporangiospores were harvested into YPG medium and incubated with shaking at room temperature under air. L-[^35]S)methionine was added to separate cultures for 4 consecutive 15 min. pulse periods ending with the times indicated in column 1. Amino acid pools were extracted and quantitated with an amino acid analyzer. Radioactivity within the methionine pool was assayed using paper chromatography, autoradiography, and scintillation spectroscopy.
Table 8.
Incorporation of L-[\textsuperscript{35}S]methionine into Hot Trichloroacetic Acid-Insoluble Material in a Rabbit Reticulocyte Cell-Free Protein-Synthesizing System Programmed with M. racemosus RNA.

<table>
<thead>
<tr>
<th>Source of RNA</th>
<th>CPM of L-[\textsuperscript{35}S]methionine (X 10\textsuperscript{5})</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5 \textmu lter Reaction Mixture</td>
</tr>
<tr>
<td>Dormant Spore</td>
<td>4.29</td>
</tr>
<tr>
<td>15 min</td>
<td>7.06</td>
</tr>
<tr>
<td>30 min</td>
<td>6.26</td>
</tr>
<tr>
<td>45 min</td>
<td>7.11</td>
</tr>
<tr>
<td>60 min</td>
<td>6.29</td>
</tr>
<tr>
<td>-RNA</td>
<td>0.56</td>
</tr>
<tr>
<td>Globin mRNA</td>
<td>7.83</td>
</tr>
</tbody>
</table>

Total cellular RNA was purified from dormant spores and from germinating spores following 15, 30, 45 and 60 min of germination in YPG medium under air. Twenty \textmu g of Mucor RNA and 1 \textmu g of globin mRNA were included in separate 30-\textmu lter cell-free protein synthesis reaction mixtures and incubated for 60 min. Samples (5 \textmu lters) were withdrawn and assayed for CPM of L-[\textsuperscript{35}S]-methionine incorporated into protein. RNA purification, conditions for cell-free protein synthesis, and assay of protein products are described in detail in the text.
Figure 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 μlitters) 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.
$10^3 \times \text{CPM} / 100 \mu\text{liters}$
Figure 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.
Figure 3. Incorporation of $[^{3}\text{H}]$uracil and $[^{32}\text{P}]$phosphate into cold trichloroacetic acid-insoluble material in germinating sporangiospores of *M. racemosus*. Spores were harvested directly into YPG medium containing $[^{3}\text{H}]$uracil or $[^{32}\text{P}]$phosphoric acid and incubated with shaking at room temperature under air. Samples (100 μliters) 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: (●), $[^{3}\text{H}]$uracil label; (■), $[^{32}\text{P}]$phosphate label.
Figure 4. Effect of growth inhibitors on incorporation of $[^3H]$-uracil or $[^32P]$phosphate into cold trichloroacetic acid-insoluble material in germinating sporangiospores of *M. racemosus*. Spores were harvested directly into YPG medium containing either $[^32P]$-phosphate and actinomycin D plus daunomycin or $[^3H]$uracil and sodium azide plus sodium fluoride. Spore suspensions were incubated with shaking at room temperature under air. Samples (100 µliters) 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: (●), $[^32P]$phosphate label; (---), $[^3H]$uracil label.
$10^5 \times \text{CPM}^{(32P)/100 \text{ milliters}}$

$10^4 \times \text{CPM}^{(3H)/100 \text{ milliters}}$

MINUTES
Figure 5. Size of ATP pools in germinating sporangiospores of M. racemosus. Spores were harvested directly into YPG medium and incubated with shaking under air. Samples (2 ml) were withdrawn at the times indicated and filtered. ATP pools were extracted as described in the text. Pool sizes were determined by the luciferin-luciferase assay system and the values normalized to dry weight of the sample. Ordinate: µg ATP/mg Dry Weight. Abscissa: time after exposure of spores to medium.
Figure 6. Fractionation of RNA from dormant sporangiospores of *M. racemosus* on an oligo(dT)-cellulose column. The RNA was purified, methylated with $[^3H]$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 eluant fractions was measured and plotted versus fraction number. Peak A represents RNA not retained by the column (non-polyadenylated RNA). Peak B represents RNA initially bound by the column and released at low ionic strength [poly(A)+RNA].
Figure 7. 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 summed for the "bulk" RNA fractions and poly(A)+RNA fractions, was normalized to A\(_{260\text{nm}}\) and was plotted versus time of germination. Symbols: (●), "bulk" RNA; (■), poly(A)+RNA.
10^5 \times \text{CPM} / \text{A}_{260} \text{ ("BULK" RNA)}

10^4 \times \text{CPM} / \text{A}_{260} \text{ (POLY A+ RNA)}

\text{MINUTES}

\text{20}

\text{15}

\text{10}

\text{5}

\text{0}

\text{0}

\text{10}

\text{20}

\text{30}

\text{40}

\text{50}

\text{60}
Figure 8. 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. A $A_{254\text{nm}}$ (........) 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.
Figure 9. 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 unlabelled *Mucor* marker RNA. $A_{254\text{nm}}$ (.........) and radioactivity (______) were measured in each fraction. RNA molecules of known size are identified with arrows.
Figure 10. Incorporation of $L[^{35}S]$methionine into hot trichloroacetic acid-insoluble material in germinating sporangiospores of *M. racemosus*. Spores were harvested directly into YPG medium containing $L[^{35}S]$methionine and incubated with shaking at room temperature under air. Samples (100 µliters) 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: (●), spores germinated in YPG; (---) spores germinated in YPG plus cycloheximide.
$10^4 \times \text{CPM} / 100 \mu\text{liters}$

MINUTES

0 10 20 30 40 50 60

0 1 2 3 4
Figure 11. Coomassie blue stained 2-D polyacrylamide gels of proteins extracted from germinating sporangiospores of *M. racemosus*. Spores were harvested into sterile water containing cycloheximide (dormant spores) or directly into YPG medium and incubated with shaking at room temperature under air. Proteins were extracted and electrophoresed, as described in the text. Gel A: 200 μg of protein extracted from dormant spores. Gel B: 200 μg of protein extracted from spores germinated in YPG medium for 60 min. Molecular weight markers: bovine serum albumin (BSA), 66 kilodaltons; ovalbumin (OVA), 45 kilodaltons; and trypsinogen (TRP), 24 kilodaltons.
pH 4.5

A
66K-
45K-
24K-

B
Figure 12. SDS/polyacrylamide gel electrophoresis of proteins extracted from germinating sporangiospores of *M. racemosus*. Proteins of dormant spores were prelabeled by allowing sporulation of a hyphal culture for 7 days on YPG medium containing either L[^35S]-methionine or[^35S]sulfate. Proteins of germinating spores were pulse labeled with L[^35S]methionine during four consecutive 15-min pulse periods during the first 60 min of germination. Proteins were extracted from sporangiospores and separated by SDS/polyacrylamide gel electrophoresis as described in the text. Gel A: Coomassie blue stained gel of protein extracted from dormant spores (lane 1) and following each of the four consecutive pulse periods; lane 2, 0-15 min; lane 3, 15-30 min; lane 4, 30-45 min; lane 5, 45-60 min, (40 μg of protein in each lane). Gel B: Autoradiograph of a dried gel of the same protein extracts as in Gel A (35,000 CPM in each lane, 24 day exposure time) Molecular weight markers are the same as in Figure 11.
Figure 13. Autoradiographs of 2-D polyacrylamide gels of pre-labeled proteins from sporangiospores of *M. racemosus* 1216 B. Spores prelabeled with L[^35]S-methionine were harvested directly into sterile water containing cycloheximide or into YPG containing an excess of unlabeled L-methionine and incubated for 60 min with shaking at room temperature under air. Protein extracts (200 µg) were loaded onto the first dimension of 2-D gels. Gel A, pre-labeled proteins of spores harvested into sterile water with cycloheximide. Gel B, prelabeled proteins of spores following 60 min of germination in YPG. Molecular weight markers are the same as in Figure 11. Protein 34 is one example of post-translational processing of a pre-existing protein (Gel A and B).
Figure 14. Autoradiographs of 2-D polyacrylamide gels of proteins pulse-labeled during germination of *M. racemosus* sporangiospores. Proteins of germinating sporangiospores were pulse-labeled with L-[35S]methionine during four consecutive 15-min pulse periods in the first 60 min of germination. Proteins were extracted and electrophoresed as described in the text. Protein samples (200 μg) containing 8.0 x 10⁴ to 1.6 x 10⁶ CPM were loaded onto the first dimension of 2-D gels. Gel A: 0-15 min pulse. Gel B: 15-30 min pulse. Gel C: 30-45 min pulse. Gel D: 45-60 min pulse. Molecular weight markers are the same as in Figure 11.
Figure 15. Coomassie blue stained 2-D polyacrylamide gels of protein extracted from hyphae and yeast cells of *M. racemosus* 1216 B. Hyphae were grown from sporangiospores \(2 \times 10^5/\text{ml}\) incubated 16 hours with shaking at room temperature under air. Yeasts were grown from sporangiospores \(2 \times 10^5/\text{ml}\) incubated 40 hours with shaking at room temperature under CO\(_2\). (Cells were prepared, proteins extracted, and electrophoresed as described in the text). Two hundred \(\mu\)g of protein were loaded onto the first dimension of 2-D polyacrylamide gels. Gel A: hyphal proteins. Gel B: yeast proteins. Molecular weight markers are the same as in Figure 11.
Figure 16. Protein turnover in germinating sporangiospores of *M. racemosus*. Spores, whose proteins had been prelabeled with L-[\textsuperscript{35}S]methionine, were harvested directly into YPG medium containing an excess of non-radioactive L-methionine, filtered and washed rapidly with YPG containing non-radioactive L-methionine. The spores were resuspended in YPG containing non-radioactive L-methionine and incubated with shaking at room temperature under air. Samples (100 \, \mu\text{liters}) were collected every 5 min and treated as described in the text. Ordinate: radioactivity remaining. Abscissa: time after exposure of spores to medium.
Figure 17. Incorporation of L-[\textsuperscript{35}S]methionine into hot trichloroacetic acid-insoluble material in a rabbit reticulocyte cell-free protein synthesizing system as a function of input \textit{M. racemosus} RNA. Total cellular RNA was purified from dormant sporangiospores. Various amounts of the purified RNA were included in separate 30-\textmu liter cell-free protein synthesis reaction mixtures and incubated for 60 min. Samples (3 \textmu l) were collected following incubation and the amount of radioactivity incorporated was determined. Conditions for RNA purification, composition of cell-free reaction mixtures, and treatment of samples following incubation are discussed in the text. Ordinate: radioactivity incorporated. Abscissa: \textmu g of RNA included in cell free reaction mixture.
$10^5 \times \text{CPM/3 \mu l}$

$\mu g \text{RNA}$

The diagram shows a plot of $10^5 \times \text{CPM/3 \mu l}$ against $\mu g \text{RNA}$. The data points form a bell-shaped curve, indicating an optimal concentration of RNA at around 25 $\mu g$. The curve decreases beyond this point, suggesting a decline in activity.
Figure 18. Incorporation of L-[\textsuperscript{35}S]methionine into hot trichloroacetic acid-insoluble material in a rabbit reticulocyte cell-free protein synthesizing system as a function of incubation time. Total cellular RNA was purified from dormant sporangiospores of \textit{M. racemosus}. Twenty \(\mu\)g of RNA were included in a 30-\(\mu\)l cell-free protein synthesis reaction mixture and incubated for 60 min. Samples (5 \(\mu\)liters) were collected every 15 min and the amount of radioactivity incorporated was determined as described in the text. Ordinate: radioactivity incorporated. Abscissa: time of incubation of cell-free reaction mixture.
Figure 19. Polyacrylamide gel analysis of protein products of rabbit reticulocyte in vitro protein synthesizing system programmed with endogenous and exogenous (M. racemosus) RNA. Total cellular RNA was purified from dormant sporangiospores and from spore samples collected at 15-min intervals during the first 60 min of spore germination in YPG medium under air. Twenty μg of each Mucor RNA sample were included in separate 30-μl in vitro reaction mixtures which were incubated for 60 min. As a control, one reaction mixture contained no added RNA and another reaction mixture contained 1 μg of pure rabbit globin mRNA. In vitro protein products were precipitated with 80% acetone, resuspended in SDS sample buffer, and separated by SDS/polyacrylamide gel electrophoresis as described in the text. Quantities of in vitro labeled protein containing $2 \times 10^5$ CPM of L-[35S]methionine in each case were electrophoresed on a standard 1-D SDS/polyacrylamide slab gel. Gel A: autoradiograph of a dried 1-D gel; lane 1, no RNA added; lane 2, RNA from dormant spores, lane 3, RNA after 15 min of germination; lane 4, 30-min RNA; lane 5, 45-min RNA; lane 6, 60 min RNA; and lane 7, globin mRNA, Gel B: autoradiograph of a dried 2-D gel showing proteins synthesized in vitro in the absence of exogenous RNA. Molecular weight markers are the same as in Figure 11.
Figure 20. Two-dimensional isoelectric focusing/SDS polyacrylamide gel analysis of protein products from a rabbit reticulocyte in vitro protein synthesizing system programmed with *M. racemosus* RNA. Total cellular RNA was purified from dormant sporangiospores and from spore samples collected at 15-min intervals during the first 60 min of spore germination in YPG medium. Twenty μg of each *Mucor* RNA sample were included in separate 30-μl in vitro protein synthesis reaction mixtures which were incubated for 60 min using L-[³⁵S]methionine as the radioactive label. Protein samples (5 μliters) containing from $4.8 \times 10^5$ to $9.5 \times 10^5$ CPM were treated and electrophoresed as described in the text. The resulting gels were dried and autoradiographed. Gel A, Dormant spore; Gel B, 15 min; Gel C, 30 min; Gel D, 45 min; Gel E, 60 min. Molecular weight markers are the same as in Figure 11. The arrow on each gel identifies the endogenous protein product labeled in the absence of added RNA.
Vita

John Edgar Linz was born on January 27, 1954 in Flint, Michigan. He graduated from Flushing High School in 1972. He attended Albion College and received a Bachelor of Arts degree in Biology in 1976. John then entered graduate school at the University of Illinois and received a Masters of Science in Biology in 1979. John is currently enrolled at Louisiana State University and is a candidate for the Doctor of Philosophy degree in Microbiology with a minor in Biochemistry.
EXAMINATION AND THESIS REPORT

Candidate: John Edgar Linz

Major Field: Microbiology

Title of Thesis: The Regulation of Gene Expression during Aerobic Germination of Mucor racemosus Sporangiospores.

Approved:

[Signature]
Major Professor and Chairman

[Signature]
Dean of the Graduate School

EXAMINING COMMITTEE:

[Signature]

[Signature]

[Signature]

[Signature]

[Signature]

Date of Examination:

September 13, 1983