JOURNAL OF BACTERIOLOGY, Jan. 1974, p. 196-202

Vol. 117, No. 1
Printed in U.S.A.

Polysomes, Ribonucleic Acid, and Protein Synthesis During Germination of *Neurospora crassa* Conidia

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Received for publication 13 August 1973

The level of polysomes in ungerminated conidia of *Neurospora crassa* depends on the method used to collect spores. Spores harvested and exposed to hydration contain 30% of their ribosomes as polysomes, whereas those not exposed to hydration contain only 3% of their ribosomes as polysomes. During the germination process, the percentage of the ribosomes which sediment as polysomes increases rapidly to a level of approximately 75% during the first 15 to 30 min of germination. This rapid increase has been shown to require a carbon source. During the first 30 min of germination, spores synthesize ribosomal ribonucleic acid (RNA) and heterogeneously sedimenting RNA, i.e., presumptive messenger RNA.

The life cycle of many organisms includes a period of dormancy or semidormancy characterized by reduced metabolic activity. This quiescent state is eventually broken by some stimulus which activates metabolism leading to growth. Examples of quiescence include fungal spores, bacterial spores, pollen grains, plant seeds, and unfertilized eggs.

In all such systems, an important question is whether the transition to active growth requires concomitant ribonucleic acid (RNA) synthesis or can be supported by prepackaged RNA. In most studies to date, the presence of prepackaged RNA in the dormant cell has been indirectly assayed by two methods: (i) demonstration of polysomes in the dormant cell or (ii) demonstration that dormancy can be broken even though new RNA synthesis is blocked.

Evidence of polysomes in ungerminated fungal spores is documented for uredospores of *Uromyces phaseoli* (11), lyophilized basidiospores of *Schizophyllum commune* (8), conidia of *Botryodiplodia theobromae* (1), and macroconidia of *Fusarium solanis* (2). On the other hand, an absence of polysomes in ungerminated spores has been reported for *Aspergillus oryzae* (6) and *Neurospora crassa* (3). Thus, some fungal spores have a potential store of prepackaged messenger RNA (mRNA) in the form of polysomes and some do not.

Furthermore, the early stages of germination have been demonstrated to occur in the apparent absence of RNA synthesis in *B. theobromae* (1), *Peronospora tabacina* (4, 5), and *Alternaria solani* (5). In contrast, RNA synthesis is apparently necessary for germination in *A. nidulans* (5) and *N. crassa* (5). However, the results of Inoue and Ishikawa (7) indicate that new RNA synthesis in *N. crassa* is not absolutely necessary for germination. The reason for this discrepancy is unknown. The overall results with inhibitors of RNA synthesis suggest again that some fungal spores have a potential store of RNA whereas others may not.

This study was undertaken (i) to determine polysome levels during early stages of spore germination, (ii) to determine the species of RNA, if any, which become associated with polysomes during spore germination, and (iii) to determine how protein synthesis is controlled during the transition from dormancy (ungerminated spore) to active growth (germinated spore).

MATERIALS AND METHODS

Harvest and culture conditions. *N. crassa* wild-type strain 74A was used in all experiments. To obtain conidia, the fungus was grown in 250-ml Erlenmeyer flasks containing 50 ml of agar medium (2% sucrose-minimal salt solution [11]). Inoculated flasks were incubated for 2 days at 33 C and then placed at room temperature. Conidia were routinely harvested between 7 and 10 days after inoculation by two methods: (i) washing the flasks with sterile water, followed by filtration of the conidia through glass wool to remove mycelial debris and agar pieces, or (ii) by inverting a flask containing conidia over a 15-ml membrane filter chimney (Millipore Corp.) equipped

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with a membrane filter. With gentle tapping of the flask and with suction applied to the filter apparatus, conidia were collected on the membrane filter. The first method yields hydrated or "wet" spores, whereas the second method yields nonhydrated or "dry" spores. Wet spores were hydrated for 10 to 15 min during preparation for inoculation.

For germination studies, conidia were placed in Vogel 2% sucrose-minimal medium at a density of ~5 $\times$ 10^6/ml and incubated at 33 C in a shaking water bath.

Labeling conditions. Conidia at the required stage were labeled with the appropriate isotopes; details are given in figure legends. Isotopes used were L-[4,5-^{3}H]leucine, specific activity 19 Ci/mmol (Amersham/Searle); [2-^{14}C]uracil, specific activity 52 Ci/mol (Schwarz BioResearch); ^{14}C uniformly labeled amino acid mixture, specific activity 270 Ci/mol (Amersham/Searle); [8-^{3}H]adenine, specific activity 17 Ci/mmol (Schwarz Bioresearch).

Polysome isolation. Ungerminated or germinated conidia were collected for polysome isolation by filtration onto a membrane filter (Millipore Corp.). The resulting pad of conidia was placed in a chilled mortar (4 C) with an equal volume of acid-washed sea sand (Fischer Scientific Co.) and a few drops of homogenization buffer: 0.01 M KCl-0.01 M $MgCl_2$-0.03 M triethanolamine (TEA) (pH 7.5). After 30 s of grinding, 3 to 4 ml of buffer was added and the homogenate was ground for an additional 15 s. The homogenate was then centrifuged for 10 min at 10,000 to 15,000 rpm at 4 C. Volumes of the resulting supernatant containing 3 to 15 absorbancy units (at 260 nm) (A_{260}) were layered onto 12.5-ml 10 to 40% (wt/wt) linear sucrose gradients (ribonuclease-free sucrose). The gradients were centrifuged for 90 min at 35,000 rpm in an SW41 rotor at 4 C. The gradient tubes were punctured at the bottom, and the gradients were displaced upwards at a constant rate when 50% (wt/wt) sucrose was pumped in with a LKB peristaltic pump. The fluid passed through a 1-cm flow cell and was continuously monitored at 260 nm with a Gilford model 2000 recording spectrophotometer. When necessary, gradient fractions were collected with a Gilson Micro Fractionator. Radioactive fractions were precipitated by adding 100 μg of bovine serum albumin and then making the fractions 10% with trichloroacetic acid. Fractions were stored at 4 C for 2 h and either filtered directly onto membrane filters (No. 25 Schleicher & Schuell, Inc.) or heated to 90 C before filtering. The precipitates were collected on filters washed with 5% trichloroacetic acid containing 0.1 g of cold amino acid or nucleotide per ml. Filters were then washed with 5% trichloroacetic acid and 95% ethanol (both containing 0.1 g of cold amino acid or nucleotide per ml), dried, and counted in a Beckman LS100 or Beckman LS230 scintillation counter.

Isolation and characterization of polysomal RNA. RNA was extracted by the method of Perry et al. (10). The polysome fractions from a sucrose gradient were pooled and precipitated overnight at −20 C with 2.5 volumes of absolute ethanol after the addition of *Neurospora* carrier RNA (12 A_{260} units). Polysomes and carrier RNA were collected by centrifugation at 10,000 rpm for 10 min and then suspended in tris(hydroxymethyl)aminomethane (Tris)-ethylenediaminetetraacetic acid (EDTA)-sodium dodecyl sulfate (SDS) buffer (0.01 M Tris buffer [pH 7.4] containing 1 mM EDTA, 0.1 M NaCl, and 0.5% SDS). The resuspended polysomes were mixed with an equal volume of chloroform-phenol-isoamyl alcohol (48:48:4) saturated with acetate-NaCl-EDTA buffer (0.01 M sodium acetate [pH 6.0] containing 0.1 M NaCl and 1 mM EDTA) and shaken at room temperature for 5 to 10 min. After low-speed centrifugation, the aqueous phase was removed and extracted again with chloroform-phenol-isoamyl alcohol. The RNA was precipitated from the aqueous phase with 2.5 volumes of absolute ethanol at −20 C.

The extracted RNA was redissolved in Tris-NaCl-EDTA buffer and reprecipitated with 2.5 volumes of absolute ethanol at −20 C. This RNA (free from phenol) was dissolved in Tris-NaCl-EDTA-SDS buffer, layered on a 12.5-ml 5 to 20% (wt/wt) sucrose gradient (Tris-NaCl-EDTA-SDS buffer), and centrifuged in an SW41 rotor at 25,000 rpm for 16 h or at 22,000 rpm for 16.5 h.

RESULTS

Figure 1A depicts the level of polysomes present in ungerminated spores of *N. crassa* and the activity of these polysomes in protein synthesis. Single ribosomes predominate in the ungerminated spores; however, the small percentage of polysomes present contain the same range of polysome size as found after germination. Furthermore, the incorporation of radioactive amino acids into protein indicates that these polysomes are active in protein synthesis. Figure 1B shows that after exposure to ribonuclease (RNase) the polysomes are completely converted to monosomes. Likewise, the radioac-

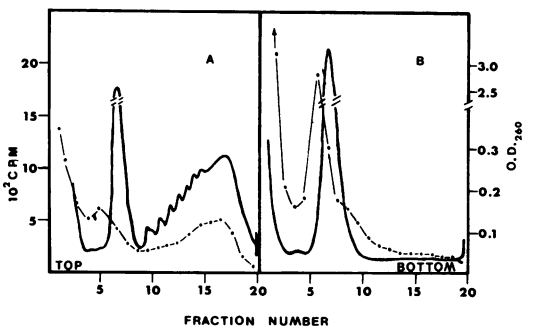

FIG. 1. *Ribosome content of ungerminated spores. Spores were harvested with deionized water (wet spores) from 7-day-old cultures and labeled with 0.25 μCi of ^{14}C-labeled amino acids per ml for 5 min before the isolation of polysomes. (A) Polysomes from ungerminated spores. (B) After treatment with 5 μg of boiled pancreatic ribonuclease per ml for 30 min at 4 C. (——) A_{260}; (●-----●) counts per minute.*

tivity coincident with the polysomes is shifted to the monosome region. Upon initiation of the germination process, the percentage of ribosomes present as polysomes increases rapidly during the first 30 min (Fig. 2). If wet spores are placed in the germination medium, the percentage of polysomes varies from 20 to 40%, with a mean value of 30%. Upon germination, this value increases to approximately 70% within the first 15 min. A further increase to 80 to 85% occurs during the ensuing 45 min, and the percentage of polysomes remains at this level at least through the first 3 h of germination.

If dry spores are placed in the germination medium, the level of polysomes is only 3%. This low level of polysomes in dry spores compared to wet spores is found even though the spores are treated identically, with the exception that wet spores were exposed to distilled water for 10 to 15 min before homogenization. Upon germination, the percentage of polysomes rises rapidly, reaching the same level as hydrated spores (Fig. 2). Thus hydration of ungerminated spores evokes an increase in polysomes which is stimulated further in germination medium.

To characterize further the transition from ungerminated to germinated spores, cultures were labeled with [^{3}H]leucine and harvested at various times during the first 2 h of germination. Subsequently, the spore extracts were characterized as to the level of polysomes and the amount of protein associated with the polysomes (Fig. 3). Again the rise in the percentage of polysomes follows that shown in Fig. 2. Table 1 shows how the specific activity (counts per minute per polysome A_{260}) of the polysomes changes during germination. The specific activity increases threefold between 30 and 60 min into germination.

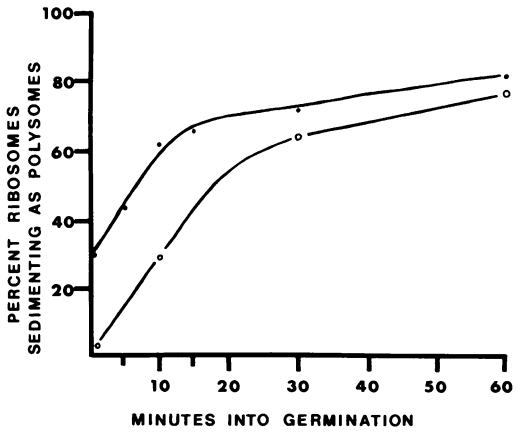

FIG. 2. *Increase in the percentage of polysomes during the first hour of germination. Wet spores (○), points are mean values of at least three determinations; dry harvested spores (●), average of two determinations.*

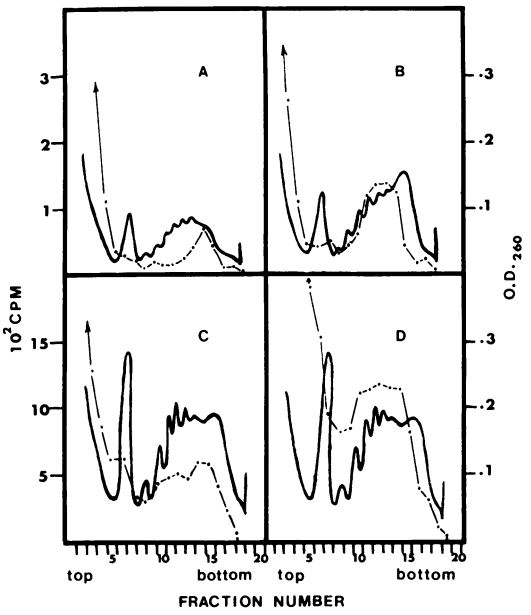

FIG. 3. *Polysomes and protein synthesis during first 2 h of germination. Four cultures were inoculated at 6.3 × 10^6 wet spores/ml and pulse labeled with 0.1 μCi of [^{3}H]leucine per ml (for 2 min) at 15, 30, 60, and 120 min into germination. (A) 15, (B) 30, (C) 60, and (D) 120 min into germination at 33 C. (——) A_{260}; (●——●) [^{3}H]leucine in hot trichloroacetic acid-insoluble material.*

TABLE 1. *Specific activity of polysome changes during germination*

Stage (min)	[H^3]Leucine[a] counts/min	Optical density of polysomes	Sp act[b] (×10^3)
1. 15	257	0.104	2.47
2. 30	938	0.394	2.40
3. 60	4,631	0.616	7.50
4. 120	9,259	1.10	8.40

[a] Fraction 8 to 20.

[b] Specific activity, counts per minute per optical density.

When isolating polysomes per se or polysomes labeled with precursors of protein and/or RNA, it is important to demonstrate that the isolated constituents fulfill the following criteria. (i) Polysomes are sensitive to RNase, EDTA or low magnesium, and puromycin, (ii) polysomes do not arise by nonspecific aggregation, (iii) polysomes profiles do not arise from RNase degradation, (iv) RNA associated with polysomes is an integral part of the polysome and does not

arise by the adventitious attachment of cellular RNA during the isolation procedure.

Figure 4 shows the effects of exposing polysomes to RNAse, puromycin, and low magnesium. RNase digestion of polysomes (Fig. 4B) isolated from germinated spores leads to a complete degradation of polysomes into monosomes. On the other hand, puromycin (Fig. 4D) or low-magnesium treatment of polysomes (Fig. 4C) leads to the complete conversion of monosomes and polysomes into their subunits. Addition of labeled monosomes to the 10K supernatant followed by co-centrifugation indicates that, at least under these conditions, no label is found in the polysome region of the gradient (Fig. 5C and D). The possibility of degradation is more difficult to rule out but the following comments are relevant. First, the profiles themselves do not suggest degradation. Secondly, degradation of polysomes whose nascent polypeptides had been labeled in vivo would lead to a peak in radioactivity coincident with the monosome absorbance peak. No such peak is observed in Fig. 3A, B, and D, although a shoulder of radioactivity in the monosome region of Fig. 3C may indicate limited degradation during isolation. Addition of labeled total cell RNA to the 10K supernatant, followed by sucrose gradient sedimentation, shows that little, if any, labeled RNA is associated with the polysomes (Fig. 5A and B).

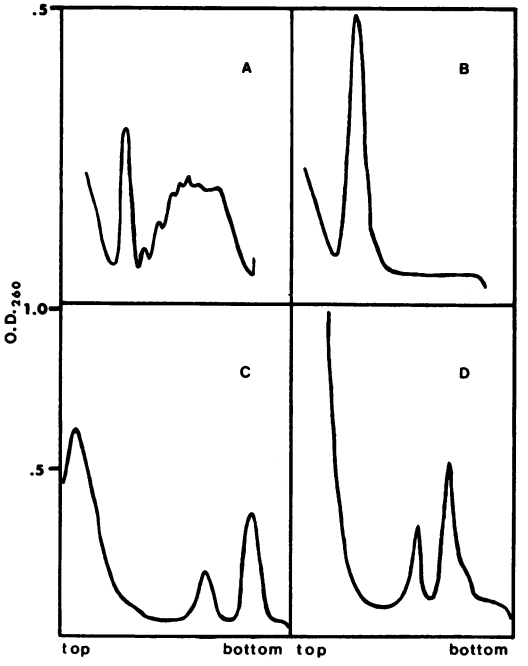

FIG. 4. *Effects of RNase, low Mg^{2+}, and puromycin on germinated conidial polysomes. (A) Polysome profile. (B) After exposure of 10,000-rpm supernatant to 5 μg of boiled pancreatic RNAse per ml for 15 min at 33 C. (C) Sample of 10,000-rpm supernatant run on a 5 to 30% (wt/wt) sucrose gradient (sucrose made up in 0.03 M TEA [pH 7.5]; 0.1 M KCl-10^{-5} M $MgCl_2$) for 4.5 h at 40,000 rpm (SW41 rotor, 4 C). (D) Sample of 10,000-rpm supernatant incubated with puromycin at 4 C for 30 min. Conditions of incubation were 0.5 M KCl-0.05 M TEA [pH 7.5]-0.01 mM $MgCl_2$-1 mM puromycin.*

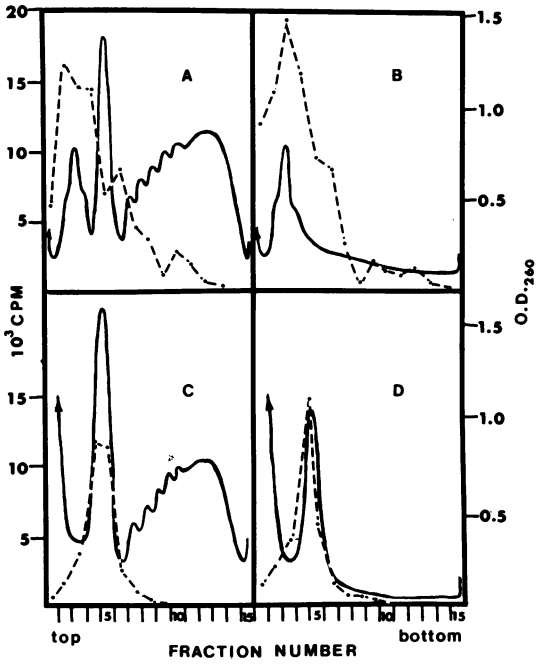

FIG. 5. *Association of monosomes and total cell RNA with polysomes. (A) Total cell RNA co-centrifuged with unlabeled 10,000-rpm supernatant; (B) total cell RNA centrifuged in a 10 to 40% gradient; (C) labeled monosomes co-centrifuged with unlabeled 10,000-rpm supernatant; (D) centrifugation of labeled monosomes on 10 to 40% gradient.* (——) A_{260}; (●-----●) [^3H]*uridine.*

To determine what species of newly synthesized RNA become associated with polysomes during the first 30 min of germination, polysomal RNA was extracted from cultures pulse labeled with [^{3}H]adenine from 0 to 5 min, 10 to 15 min, and 25 to 30 min into germination at 33 C (Fig. 6). Figure 6A shows the region of the sucrose gradient used for the extraction of polysomal RNA. Figure 6B shows that, during the first 5 min of germination, [^{3}H]adenine is incorporated predominately into 28 + 18*S* rRNA and secondarily into RNA which sediments between 6 and 15*S*, i.e., presumptive mRNA. Essentially the same pattern is exhibited by RNA labeled 10 to 15 min into germination (Fig. 6C). Between 25 and 30 min into

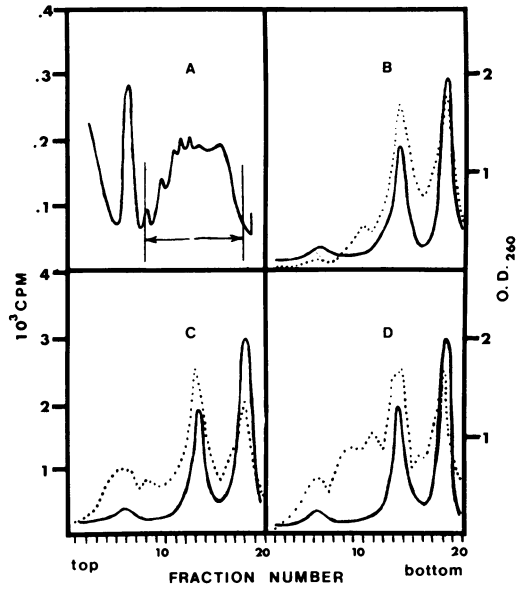

FIG. 6. *Species of RNA synthesized during first 30 min of germination. Three cultures were inoculated with 6.3 × 10^6 wet spores per ml. At 0 to 5 (B), 10 to 15 (C), and 25 to 30 min (D) into germination, cultures were pulse labeled with 5 μCi of [^{3}H]adenine per ml followed by polysome isolation. Polysomal RNA was extracted and run on 5 to 20% sucrose gradients (B–D). (A ← →) Region of polysome gradient collected for the extraction of polysomal RNA. Top of gradient is to the left, bottom is to the right. (——) A_{260}; (· · · ·) [^{3}H]adenine in trichloroacetic acid-insoluble material.*

germination (Fig. 6D) rRNA is being produced, and the nonribosomal, non-4*S* RNA is resolved into two peaks with *S* values of approximately 9 and 14. Furthermore, the amount of labeled 6 to 15*S* with respect to labeled rRNA increases with time into germination: 10% at 0 to 5 min, 20% at 10 to 15 min, and 39% at 25 to 30 min into germination.

To understand the mechanism(s) which controls the rapid shift in monosomes to polysomes upon germination, experiments were performed to determine what constituent of germination medium caused the observed shift. The percentage of polysomes was determined at 15, 30, and 60 min into germination of wet conidia germinated in minimal medium or deficient minimal medium. The deficient media are minimal medium minus carbon source (salt solution), minimal medium lacking salts (sucrose solution), and minimal medium lacking both salts and sucrose (distilled water). The results are shown in Fig. 7. Germination of conidia in a sucrose solution leads to an increase in a percentage of polysomes nearly identical to that found in spores germinated in minimal medium. However, germination in either the salt solution or distilled water results in a slight initial increase in the percentage of polysomes, followed by a gradual decline.

Protein and RNA synthesis in conidia germinating in the different media were monitored by in vivo isotope labeling. Table 2 shows the results of continuous labeling with [^{3}H]leucine and [^{14}C]uracil during the first hour of germination. Table 2 shows that, at least during the first 30 min into germination, the uptake and incorporation of [^{3}H]leucine into protein is equivalent in conidia germinated in minimal medium and sucrose but depressed in conidia germinated in salts or distilled water. During the second 30-min, uptake and incorporation decrease in conidia grown on any of the deficient media. On the other hand, the uptake and incorporation of [^{14}C]uracil into RNA are depressed during the first 30 min of germination and further depressed during the second 30 min when grown on any of the deficient media.

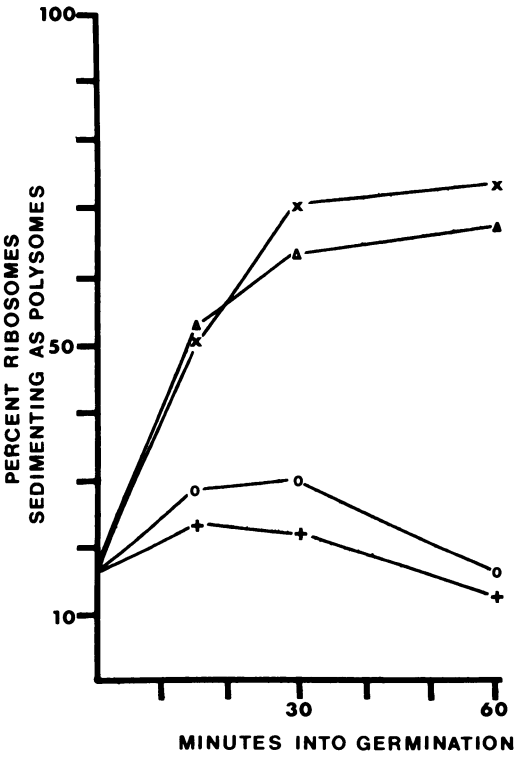

FIG. 7. *Polysome levels in wet spores germinated on minimal or minimal deficient media. Symbols: ×, minimal medium; Δ, sucrose-only medium; +, salts-only medium; O, distilled water.*

TABLE 2. *Results of continuous labeling during the first hour of germination*

Medium	[^{3}H]Leucine counts/min				[^{14}C]Uracil counts/min			
	0–30 min		0–60 min		0–30 min		0–60 min	
	Uptake	Incorporated	Uptake	Incorporated	Uptake	Incorporated	Uptake	Incorporated
Minimal	7,566	5,746	21,976	12,220	3,986	1,539	22,622	11,000
Sucrose	8,812	5,519	17,672	10,994	2,830	934	18,290	4,930
Salts	6,425	1,986	13,312	4,265	2,401	922	5,172	2,284
Water	6,091	724	7,986	1,349	2,580	181	2,552	242

DISCUSSION

During the first 15 min of germination, wild-type conidia of *N. crassa* undergo a dramatic shift in monosomes to polysomes, i.e., an increase in the percentage of the ribosomes which sediment as polysomes (percentage of polysomes). The magnitude of this increase depends upon the method utilized to obtain conidiospores. If wet spores are used, the increase is approximately 2.5-fold, whereas if dry spores are used the increase is approximately 10-fold. This difference is generated by the level of polysomes found in the wet versus dry harvested spores, 30 versus 3%, respectively.

Apparently a 15-min hydration period can stimulate an increase in percentage of polysomes. The mechanism for this stimulation is unknown, but hydration may simply release some stored product necessary for the activation of metabolism. Support for this mechanism comes from the work of Marrè (9) with plant seeds. Alternatively, hydration may permit better preservation of polysomes that exist in the ungerminated spores.

The absolute level of polysomes in ungerminated spores is difficult to determine. The 3% level found in dry, harvested spores may be an overestimate since the conidia are exposed to buffer (hydration) for a few seconds during the homogenization procedure. It is possible that this short period of hydration could result in the low level of polysomes found. Therefore, the question of whether conidia contain stored mRNA in the form of polysomes is still open. Inoue and Ishikawa (7) have demonstrated that proflavine, at a concentration which inhibits 80% of the incorporation of uridine into RNA, only inhibits germination by ~20%. They conclude that RNA synthesis is not necessary for germination up to 5 h. In contrast to these results, Holloman (5) found that inhibition of germination paralleled the inhibition of RNA synthesis by proflavine.

Because of the uncertainties involved in these inhibitor studies, the species of new RNA synthesized during the first 30 min were characterized. Absence of mRNA synthesis during the first 30 min of germination would be evidence that the rise in percentage of polysomes might occur via prepackaged RNA. On the other hand, detection of mRNA synthesis would leave open the possibility of a contribution of prepackaged RNA. The results show that, within the limits of the techniques used to extract and analyze RNA, RNA molecules with the sedimentation properties of mRNA are synthesized at all times during the first 30 min of germination. Furthermore, at least part of the increase in polysomes seen during the first 30 min is programmed by newly synthesized mRNA. On the other hand, the 10-fold increase in polysomes seen in dry spores germinated for only 10 min (Fig. 2) suggests that such a large increase may be programmed to some extent with preexisting mRNA. Experiments are now in progress which should provide data on the relative contributions of prepackaged and newly synthesized mRNA to the increase in polysomes.

It is also clear that ribosomal RNA (rRNA) is synthesized at all times during the first 30 min of germination, packaged into ribosomes, and incorporated into polysomes. The fact that labeled 18 and 28*S* rRNA appear in functional polysomes during a 5-min pulse indicates that new ribosome production is a major synthetic process during the early minutes of germination and that the level of ribosomes in the ungerminated spore may be limiting.

The fact that mRNA is synthesized from the very beginning of germination indicates that the concentration of these molecules in the ungerminated spores is limiting and possibly responsible in part for the low level of metabolism. Furthermore, it appears that the activation of RNA synthesis is one of the very early changes which occurs when the spores are induced to germinate.

Results shown in Fig. 7 clearly show that the important factor in germination medium responsible for the increase in polysomes is the carbon source. It may be that a limiting carbon

source is one factor which is responsible for the low metabolic activity of the ungerminated spore and, therefore, for the maintenance of the dormant or semidormant state. Cochrane et al. (2) have demonstrated in *Fusarium solani* a similar polysome increase in medium lacking ethanol, which is unable to support germ tube formation. Similarly in *Neurospora*, although the increase in polysomes occurs in conidia grown simply on sucrose, only a small percentage of the spores show germ tubes, and in those that do the germ tube never elongates beyond approximately one cell diameter. Thus, a carbon source is the only requirement for one of the earliest changes in germination, i.e., the increase in the percentage of polysomes, but it is not sufficient for at least one late change, i.e., normal emergence and growth of the germ tube.

The results presented in this paper suggest a mechanism by which polysome levels increase after germination and by which the low level of polysomes in the ungerminated conidia is achieved. Since hydration can elicit an increase in polysomes, it may be that dehydration is important in decreasing the level of polysomes during or after conidiogenesis. Thus, as the conidia are formed at the top of the aerial hyphae, they become removed from the source of water and nutrients. Furthermore, since sucrose has been shown to duplicate the rise in the percentage of polysomes found in minimal media, it may be that after the conidia are formed they are effectively cut off from a carbon source. Thus, a process of dehydration combined with a carbon source deprivation could lead to the shutdown of protein synthesis and the low level of polysomes observed. Germination then would simply be a reversal of this process, and RNA synthesis would be one of the very early synthetic processes which is turned on. This RNA is responsible in part at least for the rapid rise in the percentage of polysomes. The question of whether stored mRNA is utilized in this activation requires further experimentation.

ACKNOWLEDGMENTS

I wish to thank David Stadler for the use of his laboratory facilities and his constant encouragement. I also thank D. Stadler, B. D. Hall, and Paul Gladstone for reading the manuscript.

This research was supported by Public Health Service Postdoctoral Fellowship GM 52446-02 from the National Institute of General Medical Sciences.

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