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Regulation of protein synthesis during spore germination in *Dictyostelium discoideum*

(transcriptional control/*in vitro* translation/mRNA/two-dimensional gel electrophoresis)

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ABSTRACT Spore germination in the slime mold *Dictyostelium discoideum* is a particularly suitable paradigm for studying the regulation of gene expression because developmentally regulated changes in both protein and mRNA synthesis occur during the synchronous transition from dormant spore to growing ameba. To investigate the regulation of protein synthesis during germination, we labeled activated spores with [^{35}S]methionine at 1-hr intervals during germination, until amebas emerged (at 3 hr). The labeled proteins were resolved by two-dimensional polyacrylamide gel electrophoresis. Six classes of proteins were distinguished, depending on the time of onset and duration of their synthesis: (*i*) proteins made only during the first hour of germination, (*ii*) proteins made during the second hour, (*iii*) proteins made during the third hour, (*iv*) those synthesized only between 1 and 3 hr after activation, (*v*) peptides made only between 0 and 2 hr after activation, and (*vi*) proteins that were made throughout germination. mRNA isolated from dormant spores and from spores at different stages of germination was translated in a wheat germ cell-free protein-synthesizing system, and the proteins made *in vitro* were compared to those synthesized *in vivo*. The majority of the changes in the pattern of protein synthesis that occurred during the different stages of germination were attributable to the presence or absence of translatable mRNA. It is concluded that the synthesis of a majority of the proteins during spore germination is transcriptionally controlled.

The cellular slime mold is an exceptionally suitable organism for quantitative studies of the interactions occurring during morphogenesis (1). Distinct stages in the process of spore germination in *Dictyostelium discoideum* are recognized: activation, postactivation lag, swelling, and emergence of amebas (2–5). Germination is a synchronous developmental process which is completed within 3.5 hr after activation of dormant spores (2, 3, 6, 7). Interference with RNA or protein synthesis (or both) stops germination, indicating that concomitant synthesis of both RNA and protein is required for normal germination (4, 6).

One of the main problems of developmental biology is to determine the nature of the mechanisms that control the activation and expression of developmentally regulated genes. One way of studying the regulation of gene expression is to recognize those genes whose activation and eventual expression as proteins is regulated. We have studied this during spore germination, and the results suggest that protein synthesis is regulated at the transcriptional level.

MATERIALS AND METHODS

Spore Germination. Spores of wild-type *D. discoideum* strain B were isolated, stored, and activated as described (7, 8).

Isolation of RNA. Total RNA was isolated from dormant spores and from spores as described (9).

Translation of RNA in Wheat Germ Cell-Free System. The system described by Roberts and Paterson (10) was used with a few modifications. The final concentration of constituents in the reaction mixture were as follows: 2.4 mM ATP, 0.96 mM GTP, 24 mM creatine phosphate, 2.4 mM dithiothreitol, 14 mM Hepes-KOH (pH 7.3), 1.2 mM spermidine, 100 μg of creatine phosphokinase per ml (Calbiochem), 60 mM potassium acetate, 3.0 mM magnesium acetate, 100 μM each of the unlabeled amino acids, and 20 μl of wheat germ extract in a total volume of 50 μl of reaction mixture. Twenty micrograms of total cell RNA was added, and the reaction mixture was incubated for 90 min at 25°C. The label incorporated into trichloroacetic-acid-insoluble protein was determined. Total incorporation is proportional to the amount of RNA added and is therefore a reflection of the mRNA content of the sample.

When [^{35}S]methionine (10 μCi/50 μl of reaction mixture, 1000 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels) was used in the *in vitro* reaction, the complete amino acid mixture lacked only methionine; when [^{14}C]leucine (0.5 μCi, 250 μCi/mmol) was used, the amino acid mixture lacked only leucine.

The translation products and the samples from *in vivo* labeled germinating spores were precipitated with acetone by the method described by Alton and Lodish (9). Acetone precipitates were solubilized in 62.5 mM Tris·HCl, pH 6.8/0.5% 2-mercaptoethanol prior to dilution with 10 vol of lysis buffer.

Two-Dimensional Gel Electrophoresis. The first dimension was equilibrium-isoelectric focusing (11) with an ampholine combination consisting of pH range 3.5–10 and 5–7, in a ratio of 2:3, respectively. This ampholine combination gives a wide range of pH gradient that is linear between approximately pH 4.0 and 7.8. The second dimension was performed in Na-DodSO$_4$/10% polyacrylamide gels. After electrophoresis, the gels were fixed overnight in 12% acetic acid/5% methanol and prepared for fluorography as described (12). The gels were loaded with equivalent amounts of protein and were exposed to the equivalent of 10% of the cpm of ^{35}S added to the gel for 24 hr. This procedure ensures that the intensity of the protein spot seen is a measure of the relative rate of synthesis of that protein. Other characteristics and the validity of comparing the synthesis of proteins in different gels have been presented (9).

Hybridization of RNA to [^{3}H]Poly(U). RNA samples were made 0.15 M NaCl/0.15 M sodium citrate (1 × SSC buffer) and incubated at 45°C for 15 min in the presence of 5.0 μCi of [^{3}H]poly(U) (Miles, 300–500 μCi/mmol of polymer). The 50-μl reaction volume was then diluted 1:20 with 2 × SSC buffer. Twenty-five micrograms of RNase A (Worthington) was added per sample and incubation was continued for 1 hr at 25°C. Trichloroacetic-acid-insoluble radioactivity was determined after collection on Whatman GF/C filters.

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Comparison of Proteins Present in Different Gels. It was especially important to compare the proteins present in different gels to determine whether proteins were the same or different in separate gels. For this purpose, the negatives obtained directly from the exposure of the gels to the x-ray film were placed on top of each other and the protein patterns were compared. In another method the transparencies of prints of two or more gels were superimposed. In this way, protein spots that were common to two gels were superimposed and the spots that were not the same were easily distinguished.

RESULTS

Pattern of Proteins Synthesized During Spore Germination. In this experiment, wild-type *D. discoideum* spores were labeled with [^{35}S]methionine for 1-hr periods during germination, and the extracts obtained after sonication of the cells were analyzed by two-dimensional polyacrylamide gel electrophoresis. As seen in Fig. 1, six classes of protein, distinguished by their temporal synthesis, were made during spore germination. In the following description are listed some of the more prominent proteins demonstrated by gel analysis. Other not so obvious spots can also be seen by careful examination of the gels. Approximately 300 unique proteins were observed.

(*i*) This group contains proteins made only during the first hour of germination. These are proteins numbered 1–9 in Fig. 1*A*, which are circled. Only nine proteins are specific for hours 0–1. Some of the circled spots are not visible in the photograph, but can be seen in the negative. They are circled for completeness.

(*ii*) These are proteins made during the second hour. Some of these are numbered 10–14 in Fig. 1*B*, which are also circled. There are actually nine proteins specific for this time, some of which are difficult to see in the photograph of the gel.

(*iii*) This group contains proteins made during the third hour. Numbers 15–27 circled in Fig. 1*C* are some representative examples of these. There are actually 30 proteins specific for 2–3 hr.

(*iv*) Another group of proteins is labeled only during the first through third hour after activation, and is not made before that time. Proteins numbered 46–53, which are enclosed in open triangles, and actin, in Fig. 1 *B* and *C* are examples of this group.

(*v*) Another set of proteins is labeled during the first 2 hr after activation but not later. Proteins numbered 54–63, which are surrounded by the open squares in Fig. 1 *A* and *B*, are representatives of this group.

(*vi*) A large number of proteins is made throughout germination. In Fig. 1 these are the numbered proteins (28–45) that are not designated by one of the above symbols. The majority of proteins falls into this category.

***In Vitro* Protein Synthesis.** The question we want to answer is whether the changes in protein synthesis observed during germination result either from the synthesis of new messengers or from selective translation of a constant pool of mRNA. For this purpose, we isolated total RNA from dormant and from germinating spores and tested these RNAs for their ability to stimulate the synthesis of protein in a wheat germ cell-free extract.

The data presented in Table 1 show that stimulatory activity is found in dormant spores (see also ref. 7) and that this activity increases during germination. The activity was measured both as the ability of the RNA to stimulate the incorporation of radioactive leucine into protein and by its poly(A) content. The stimulatory activity was only approximately 3-fold elevated at 3 hr, but the poly(A) content was increased 10-fold. This might mean that short poly(A) chains were extended during germination or that the poly(A) tail on 3-hr mRNA was larger than on earlier mRNA.

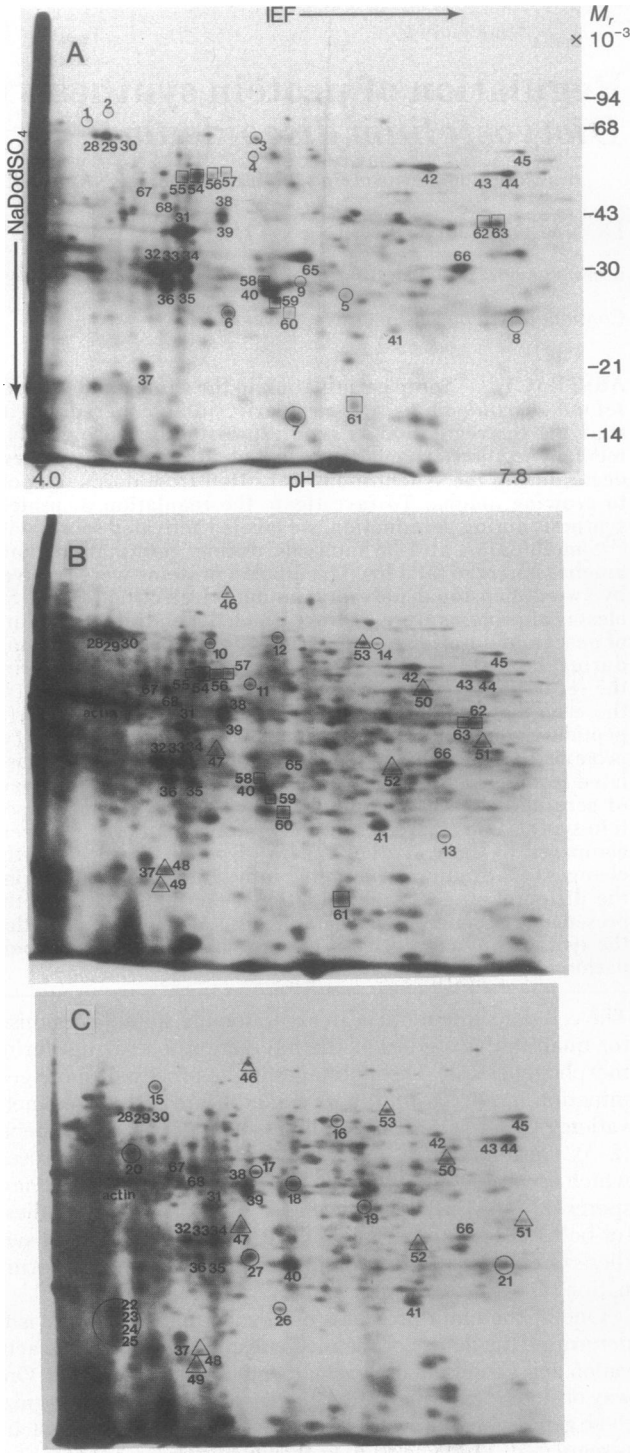

FIG. 1. Two-dimensional gel of proteins synthesized during germination. Germinating spores were labeled for 1-hr periods with [^{35}S]methionine, (200 μCi/ml, 1000 Ci/mmol; Amersham), and the extracts obtained after sonication were electrophoresed. Spores labeled 0–1 hr (*A*), 1–2 hr (*B*), and 2–3 hr (*C*) after activation are shown. The protein spots that are circled are those that are specific for that labeling time period in each gel and are not made at other times. Those proteins that are made only 0–2 hr after activation and not labeled later are denoted by the open squares (*A* and *B*). The proteins that are exclusively synthesized 1–3 hr after activation are enclosed in open triangles (*B* and *C*).

Table 1. *In vitro* translation of RNA from germinating spores and hybridization of the RNA to [^{3}H]poly(U)

Source of RNA	Specific stimulatory activity*	Hybridization with [^{3}H]poly(U)†
Dormant spores	147	102
At 1 hr	177	450
At 2 hr	273	622
At 3 hr	367	1044

RNAs isolated at the indicated times after activation of dormant spores were tested for mRNA activity in the wheat germ cell-free protein-synthesizing system. [^{14}C]Leucine was used.
* cpm of [^{14}C]leucine incorporated per μg of RNA.
† cpm in RNase-resistant hybrid per μg of RNA.

RNA obtained from dormant spores (0 hr) and from spores at 1 hr (swollen stage), 2 hr (emerging amebas stage), and 3 hr after activation (emerged amebas stage) were translated in wheat germ extracts). The [^{35}S]methionine-labeled products of the translation were subjected to electrophoresis (Fig. 2).

The *in vivo* and *in vitro* labeling patterns appear to be similar. Thus, proteins 6, 7, and 9 were made *in vivo* exclusively from 0 to 1 hr after activation, and their *in vitro* patterns of synthesis are consistent with this observation. However, although the mRNA for protein 6 was found both in spores (Fig. 2*A*) and in activated spores at 1 hr (Fig. 2*B*), the messenger for protein 7 was present only in the spore and, conversely, the mRNA for protein 9 was found only in cells at 1 hr after activation. Thus it is possible that proteins made from 0 to 1 hr after activation include some that were made from RNA present in the spores prior to activation and others by mRNA made afterwards.

The mRNA for protein 14, which was synthesized 1–2 hr after activation, was found only in 1-hr (Fig. 2*B*) and 2-hr (Fig. 2*C*) mRNA but not at 3 hr (Fig. 2*D*). It was not found in spore RNA translation products. Similarly, the developmentally regulated proteins, 15–19 and 22–26, which were made 2–3 hr after activation, were found in translation products programmed with preparations of RNA isolated from activated spores solely at either 2 or 3 hr or both (Fig. 2 *C* and *D*). mRNAs coding for the proteins 28–36 and 38–45 that were made at all stages of development were isolated from spores at all times throughout germination. Other proteins—e.g., 52 and 53 (1- to 3-hr proteins, Fig. 2 *C* and *D*), 58 (0- to 2-hr proteins, Fig. 2 *A–C*), and 67 and 68 (whose concentration at 0–1 hr was low but increased dramatically later on, Fig. 2)—were all also regulated transcriptionally. Thus, the concentration of mRNA presumably determines the amount of protein made at the indicated times. A number of proteins that were not observed *in vivo* were synthesized *in vitro* from dormant spore RNA. These are indicated by the arrows in Fig. 2*A*.

We were unable to find proteins 65 and 66, which are major proteins *in vivo*, among the *in vitro* translation products. We do not know the reason for this, but it is possible that the protein is processed from a larger precursor *in vivo* and it is not pro-

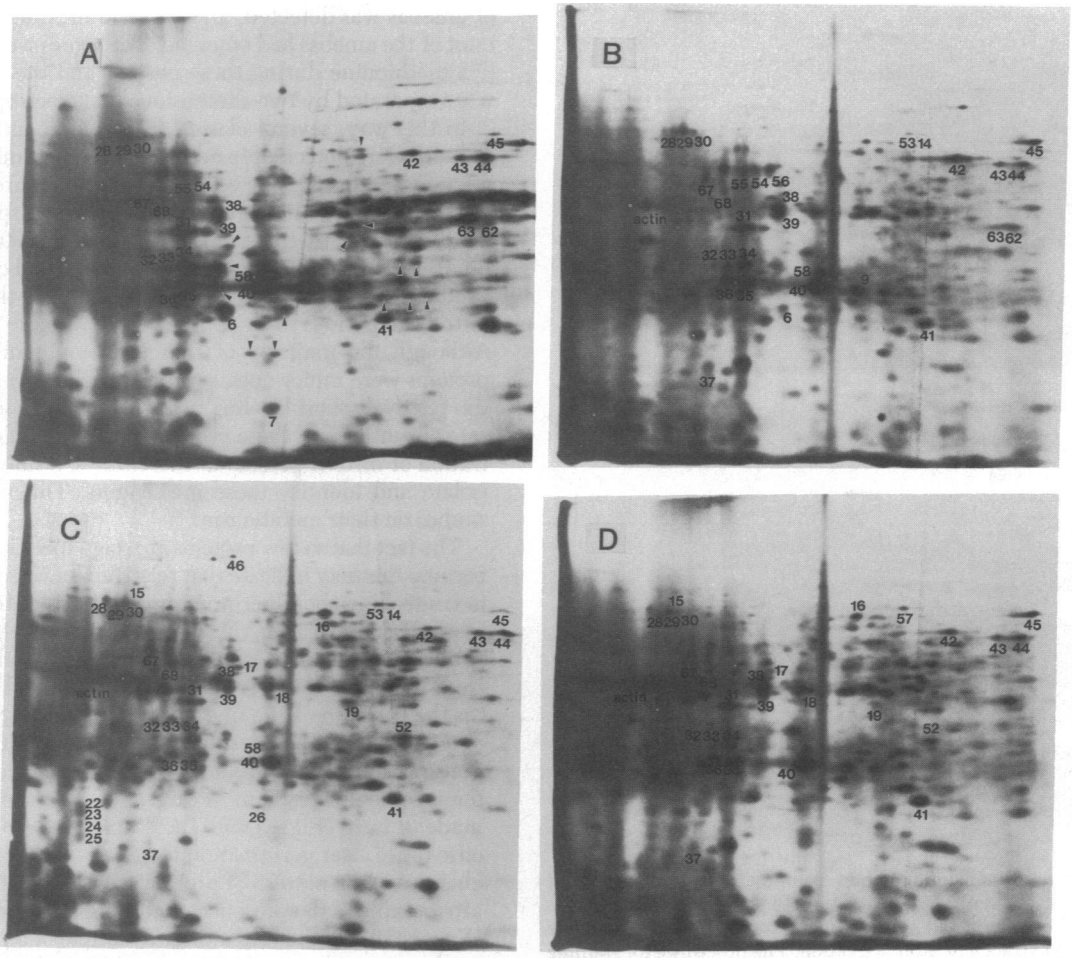

FIG. 2. Two-dimensional gel of *in vitro* synthesized proteins directed by RNA isolated from dormant spores (*A*) and 1 hr (*B*), 2 hr (*C*), and 3 hr (*D*) after activation. The numbered protein spots correspond to those made *in vivo* and denoted in Fig. 1. The arrows in *A* point to proteins synthesized *in vitro* from spore RNA, but not made *in vivo*.

cessed *in vitro*, that the mRNA may have a low affinity for the wheat germ ribosomes, or that it may be exceptionally labile and is degraded during isolation.

Relative Quantities of Proteins Synthesized During Germination. We wanted to determine whether the rates at which individual proteins were synthesized during germination were accurate reflections of the total proteins present in the cell at this time. For this purpose we compared the synthesis of proteins during germination (determined by incorporation of [^{35}S]methionine, Fig. 1) to those proteins present in the germinating spores at these times (determined by silver staining, Fig. 3). All proteins react with silver, and it is reported that as little as 10 ng of protein can be visualized (13).

Fig. 3*A* is an electropherogram of proteins isolated from spores 1 hr after activation; Fig. 3*B*, that after 3 hr. (The pattern observed at 1 hr is essentially the same as that of dormant spores, and this analysis is presented because the photograph was of better quality.) The results give further insight into regulation of protein synthesis during germination.

Many of the proteins that were developmentally regulated (Fig. 1) were present in dormant spores as well as 3 hr after activation (e.g., actin and proteins 22, 23, 24, and 25). Some heavily labeled proteins (Fig. 1) and many developmentally regulated ones were not found in stained gels or were only very minor proteins in terms of fraction of total cell protein (e.g., proteins 6, 7, 37, 38, 39, 41, 61, and 65). This indicates that they are present in very low concentration. In contrast, many of the stained proteins (Fig. 3) were not synthesized during germination. Protein 48, a developmentally regulated protein, was the only protein whose increase in incorporation of label *in vivo* (Fig. 1 *B* and *C*) was correlated with an increase in protein content (Fig. 3*B*).

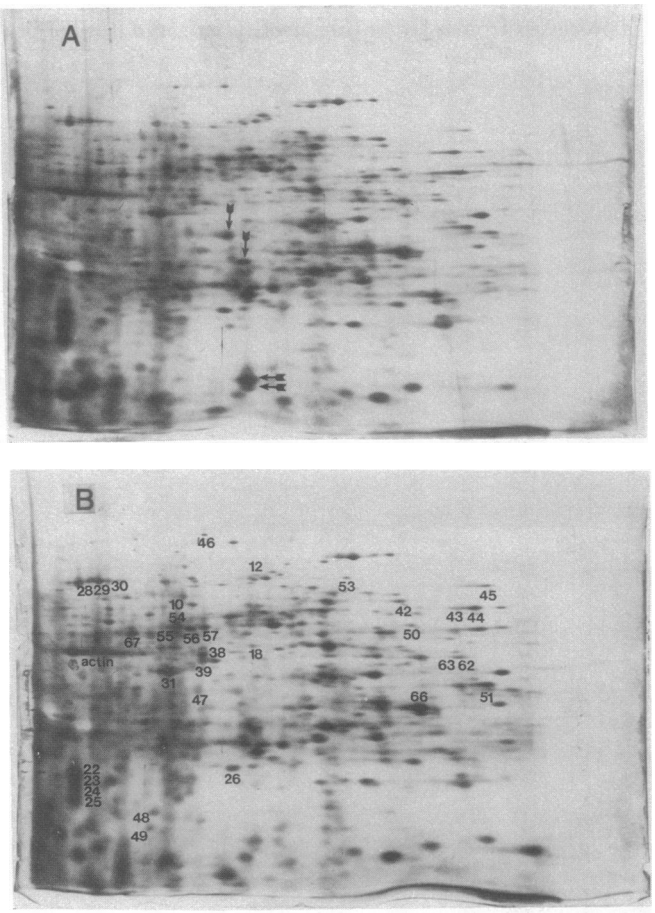

FIG. 3. Two-dimensional gel of proteins synthesized during germination: silver stain. The extracts used in the electrophoresis were the same as those described in Fig. 1. (*A*) Extract of spores 1 hr after activation; (*B*) extract 3 hr after activation. The procedure for staining has been described (13). The numbered protein spots correspond to those made *in vivo* and denoted in Fig. 1. The arrows in *A* point to proteins that are not present at 3 hr (*B*).

Some heavily labeled spots considered individual proteins in Fig. 1 may actually be multiple proteins of similar molecular weight and isoelectric point. Two examples are proteins 31 and 40, and there are many more than the five proteins in the area denoted as proteins 32–36 in Fig. 1.

There were very few changes in total protein content during germination. As mentioned above, protein 48 content increased. The only other significant changes were four other major spore proteins that decreased or completely disappeared during germination. These are indicated by the arrows in Fig. 3*A*.

DISCUSSION

Spore germination in the slime mold *D. discoideum*, a stage in the developmental cycle that has been relatively neglected in recent years, is a particularly suitable model system for studying the regulation of gene expression because developmentally regulated changes in both protein and mRNA synthesis occur during the synchronous transition from dormant spore to growing amebas (6, 7). We have been able to detect approximately 300 proteins that are synthesized during spore germination. Germination was divided into three hourly periods: (*i*) 0–1 hr, during which swelling occurred; (*ii*) 1–2 hr, during which swelling continued until the first evidence of emergence of amebas was detected; and (*iii*) 2–3 hr, during which time most of the amebas had emerged. The spores were labeled with [^{35}S]methionine during these periods and the proteins made were separated by two-dimensional gel electrophoresis.

In this way, several classes of developmentally regulated proteins were detected. In addition, a large number of proteins were made throughout germination, which are probably indispensable for maintenance of the cell. Stage-specific proteins were detected; that is, proteins made at only 0–1 hr (total of nine), 1–2 hr (nine proteins), and 2–3 hr (total of 30 proteins). Proteins that were labeled exclusively during the 0- to 2-hr or 1- to 3-hr periods after spore activation were also observed. Although the majority of these developmentally regulated proteins were minor ones, several were heavily labeled during this relatively short labeling period of 1 hr. Consequently, if the rate of synthesis of these proteins is dependent on the concentration of mRNA present at these times, it may be possible to isolate and identify these messengers. This would expedite studies on their metabolism.

The fact that so few proteins are stage specific is encouraging because this may indicate that relatively few genes are involved in controlling spore germination. Analysis of the mutants defective in germination that we have isolated (8) may be useful in distinguishing those proteins that are developmentally critical from those that are developmentally regulated but not critical for the transition from one stage to the next.

mRNA isolated from spores and from the different stages of germination were translated in a wheat germ cell-free protein-synthesizing system. A careful comparison of the proteins made *in vitro* (Fig. 2) with those made *in vivo* (Fig. 1) at specific times after germination indicates that the majority of the changes in the pattern of protein synthesis that occurred were attributable to the presence or absence of translatable mRNA. We were unable to find a single example of a protein that was made *in vitro* from mRNA isolated from the cells but was not made *in vivo* in the corresponding cells, as was described for the developmental pathway leading to fruit construction (9, 14).

Therefore, protein synthesis is apparently transcriptionally controlled.

Two of the chief problems associated with the gel analysis of *in vitro* translation products are that not all proteins observed *in vivo* are made *in vitro* and the relative amounts of synthesis of some proteins *in vivo* and *in vitro* are also different. These findings indicate that there might be some selection, or preferential translation, of mRNAs by the wheat germ extract or that certain mRNAs are degraded during isolation. Furthermore, some proteins may be processed *in vivo* from larger precursors, and this may not occur in cell-free extracts. Consequently, the interpretation of the results must be tempered by these possible complications.

The identification of proteins only on the basis of their electrophoretic mobility in gels does not allow us to study function, and a major effort in the future will have to be the identification of these proteins. Actin is one protein that has been identified in the slime mold, and its synthesis has been shown to be regulated during fruiting body construction (9, 15). Its synthesis is also regulated during spore germination. It is not made during swelling of spores (Fig. 1*A*) but is synthesized in the subsequent two periods (Fig. 1 *B* and *C*). We have shown, by translation of the mRNA from dormant spores and from spores isolated prior to 1 hr after activation, that no translatable actin mRNA is present during this time. This finding gives support to the idea that the synthesis of actin is regulated by the level of mRNA present during germination and that no actin mRNA is made prior to 1 hr after activation of dormant spores.

It is possible to visualize unlabeled proteins on gels by use of a new, very sensitive method. Staining with cupric-silver is reported to be 100 times more sensitive than the Coomassie blue stain and can be used to detect, e.g., 10^{-2} pmol of albumin per mm^2 (13). A comparison of the same samples by radioautography and staining can give information concerning the turnover and net synthesis of proteins.

Interestingly, we found that there were very few changes in total protein content during the transition from spore to ameba (Fig. 3). Only one new protein was detected by silver stain (number 48, Fig. 3*B*) and only four major spore proteins disappeared during germination (arrows, Fig. 3*A*). Furthermore, many of the proteins whose synthesis is regulated are present in dormant spores as well as 3 hr after activation. This indicates that these proteins are probably not developmentally critical ones because only their synthesis is controlled; they are present and presumably would be functioning throughout germination. In addition, many proteins found in the spores and emerged amebas are evidently not synthesized during germination.

The most important finding is that many of the stage-specific proteins are not found in the stained gels. This is encouraging because it may mean that these proteins are critical to only a limited part of the developmental cycle. They would, therefore, be made when they are needed and might be in too low a concentration to be stained.

Although spore germination is morphologically a simple process, it is evident that on the molecular level complex changes accompany the sequence of morphological events. Further study of this process combined with the analysis of mutants defective at specific steps in the morphological sequence could provide ways of identifying key events and control mechanisms in the germination pathway.

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