Synthesis of spore proteins during development of *Dictyostelium discoideum*

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The pattern of synthesis of the spore coat proteins during development of *Dictyostelium discoideum* has been determined by using immunoprecipitation with spore protein antibody. SP170, SP103, 'SP94', SP82, SP76 and SP55 are all first synthesized just prior to the 'Mexican hat' stage of development (16–18 h), but the synthesis of SP72 is delayed. This protein is apparently synthesized as a precursor, P66, which is modified during spore maturation to yield SP72. The nature of the modification is unknown. At their peak period of synthesis during early culmination (18–20 h), the spore coat proteins account for 5–9% of total protein synthesis. Shortly after synthesis, these proteins are inserted into the spore coat, where all except SP103 become disulphide-cross-linked during the period 24–30 h. SP3 does not accumulate until disulphide-cross-linking of the major spore coat proteins occurs and is itself disulphide-cross-linked into the spore coat. Several additional proteins that are accumulated during development have also been identified, namely P31, P25, P21 and P18. P25 first appears at 18–20 h and then continues to be made throughout development. P31 synthesis begins at 12–14 h and then largely ceases after approx. 20 h of development. The genes for both P21 and P18 are first expressed early in development, starting at 9–12 h. P21 synthesis ceases at approx. 14 h, but P18 continues to be synthesized throughout the rest of development. The marked differences in the time period of accumulation of these proteins compared with the co-ordinated syntheses of SP170, SP103, 'SP94', SP82, SP76 and SP55 provide a useful system for analysis of the mechanism of temporal gene expression during development.

When deprived of nutrients, *Dictyostelium discoideum* amoebae become cohesive and aggregate via chemotaxis to form multicellular pseudoplasmodia. After a variable period of migration (the 'slug' stage) each pseudoplasmodium culminates to produce a fruiting body consisting of only two major cell types, spore and stalk cells. Most of our knowledge of the regulation of gene expression during development in this organism has been derived from studies of enzymic (Loomis, 1975; Loomis *et al.*, 1976; Town & Gross, 1978), antigenic (Forman & Garrod, 1977; Kay *et al.*, 1978) and overall polypeptide composition changes (Alton & Lodish, 1977; Alton & Brenner, 1979; Landfear & Lodish, 1980; Coloma & Lodish, 1981) that occur during this phase of the life cycle.

Nevertheless, an understanding of the detailed molecular mechanisms involved in the regulation of developmental gene expression requires the study of specific, relatively major protein products if modern analytical techniques are to be used to full advantage. Unfortunately, of the several thousand genes which are expressed for the first time during development (Blumberg & Lodish, 1980), only 500 or so proteins are accumulated to a relatively abundant level and few of these are specific to either spore or stalk cells (Alton & Brenner, 1979; Coloma & Lodish, 1981). However, in 1979, Orlowski & Loomis detected a group of abundant polypeptides with relative molecular masses (M_r) of 200 000 (SP200), 96 000 (SP96), 70 000 (SP70), 68 000 (SP68) and 60 000 (SP60) which were present in the coats of mature spores. Three of these polypeptides (SP96, SP70 and SP60) are synthesized only by pre-spore cells, being undetectable in either pre-stalk cells or mature stalk (Devine *et al.*, 1982). The pattern of accumulation of the other spore coat proteins has not been reported.

Abbreviation used: SDS, sodium dodecyl sulphate.

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Recently we have re-examined the spore coat proteins of *D. discoideum* (Wilkinson & Hames, 1982) and have identified components with M_r values of 170 000 (SP170), 103 000 (SP103), 97 000 (SP97), 90 000 (SP90), 82 000 (SP82), 76 000 (SP76), 72 000 (SP72), 55 000 (SP55), 39 000 (SP39), 33 000 (SP33), and 3 400 (SP3). Unlike the other spore coat proteins, SP103 is not disulphide-cross-linked in the spore coat. It corresponds to SP96 described by Orlowski & Loomis (1979), which exhibits the same characteristic. In the present study we have used an antibody raised against the spore coat proteins to define their temporal pattern of synthesis during development. In addition, we have identified several other proteins whose synthesis is developmentally regulated.

Methods

Growth and development of D. discoideum

D. discoideum AX2 amoebae were grown in HL5 medium, shaken at 22°C, and allowed to develop on Millipore filters as described previously (Wilkinson & Hames, 1982).

Radiosotopic labelling and extraction of developing cells

Synchronously developing cells were radio-labelled at intervals in development with 100–150 μ Ci of [35 S]methionine as described previously (Wilkinson & Hames, 1982), except that the radioisotope was added to individual one-sixteenth Millipore-filter segments, each equivalent to approx. 3×10^6 amoebae. Pulse-labelled cells were harvested in ice-cold water and divided into portions which were used immediately for either plasma-membrane isolation or extraction of total proteins with SDS/mercaptoethanol buffer [1% SDS/5% (v/v) 2-mercaptoethanol/50 mM-Tris/HCl, pH 6.8] containing a proteinase-inhibitor mixture (see below) or sequential extraction with SDS buffer (1% SDS/50 mM-Tris/HCl, pH 6.8) and then SDS/mercaptoethanol buffer to separate non-cross-linked from disulphide-cross-linked proteins respectively (Wilkinson & Hames, 1982).

Isolation of plasma membranes

Developing cells were lysed by a modification of the method of Gilkes & Weeks (1977) using disruption with glass beads, and then plasma membranes were isolated from the cell lysate by a modified two-phase polymer system (Brunette & Till, 1971). Given that only small numbers of cells were radiolabelled in development, vegetative amoebae were included to provide a plasma-membrane carrier during the isolation of radiolabelled membranes. The detailed methodology was as follows. Approx. 4×10^7 *D. discoideum* AX2

amoebae in the exponential phase of growth were harvested by centrifugation at 17 000 g for 3 min, washed once with ice-cold distilled water, and pooled with the freshly harvested, pulse-labelled developing cells. The mixture was resuspended in 0.5 ml of ice-cold PM buffer (1 mM-ZnCl₂/5 mM-Tris/HCl, pH 6.8) containing a proteinase-inhibitor mixture [0.1 mM-phenylmethanesulphonyl fluoride/0.2 mM-HgCl₂/0.1 mM-benzamidine/leupeptin (0.4 mg/ml)/pepstatin (0.4 mg/ml)/antipain (0.4 mg/ml)] and stirred vigorously with 0.15 g of glass beads for 10 min. At this time, cell lysis was 95% complete. The lysate was transferred to a 3 ml Pyrex tube, together with 0.5 ml of PM buffer wash of the glass beads, and centrifuged at 500 g for 10 min. The supernatant was discarded and the pellet was resuspended in 0.5 ml of ice-cold 'upper phase' and mixed with 0.5 ml of ice-cold 'lower phase'; the 'upper' and 'lower' phases were prepared as described by Brunette & Till (1971). After centrifugation at 10 000 g for 10 min, the material banding at the two-phase interface was recovered, diluted with 1 ml of ice-cold PM buffer and re-centrifuged at 17 000 g for 10 min. The pellet was washed with 1 ml of ice-cold PM buffer and then extracted with 50 μ l of SDS/mercaptoethanol buffer at 100°C for 5 min. Samples were then stored in liquid N₂ prior to immunoprecipitation with spore protein antibody as described below.

Immunoprecipitation of spore coat proteins

Immunoprecipitation of spore proteins which had been pulse-labelled at various stages of development of *D. discoideum* AX2 cells was carried out using a spore-protein antibody as described elsewhere (Delaney *et al.*, 1983). In summary, cell extracts were prepared in SDS/mercaptoethanol buffer, incubated with spore-protein antibody previously cross-absorbed with excess *D. discoideum* amoebal protein, and then the immunoglobulin G-antigen complexes were recovered by adsorption to Protein A-Sepharose. After washing the Protein A-Sepharose by centrifugation, the antigen was eluted by the addition of 50 μ l of 2% SDS/5% 2-mercaptoethanol/50 mM-Tris/HCl, pH 6.8, and heating at 100°C for 5 min. To ensure quantitative analysis of the immunoprecipitated antigen by SDS/polyacrylamide-gel electrophoresis, this whole mixture was loaded into the corresponding sample well of the gel and electrophoresis carried out as described below.

SDS/polyacrylamide-gel electrophoresis and fluorography

Electrophoretic analysis of cell extracts and immunoprecipitates was carried out on 6–18% linear-gradient polyacrylamide slab gels using the SDS/discontinuous-buffer system of Laemmli (1970). Fluorography was performed as described

by Bonner & Laskey (1974), with Fuji RX X-ray film pre-flashed to 0.15 A_{340} unit.

Results

When analysed by one-dimensional SDS/polyacrylamide-gel electrophoresis, spore coat proteins SP97 and SP90 usually co-migrate as a single band, 'SP94' (Wilkinson & Hames, 1982). Likewise SP76 and SP72 also often co-migrate. Only by two-dimensional polyacrylamide-gel electrophoresis can these distinct polypeptides be resolved (Wilkinson & Hames, 1982). However, since SP97 and SP90 are not routinely resolved even by two-dimensional polyacrylamide-gel electrophoresis, the sole advantage of this technique is the separation of SP76 and SP72. On the other hand, the marked acidity of many of the spore coat proteins often causes some of these to be lost by migration off the end of the first-dimensional isoelectric-focusing gel. Non-equilibrium pH-gradient electrophoresis avoids this problem, but results in rather diffuse spots and streaking for some spore coat proteins (Devine *et al.*, 1982) making quantification difficult. Overall, therefore, one-dimensional SDS/polyacrylamide-gel

electrophoresis was chosen as the preferred method for a quantitative analysis of spore-coat-protein synthesis as described in the present paper.

In order to determine the time course of spore-coat-protein accumulation, *D. discoideum* AX2 cells were pulse-labelled with [35 S]methionine at 2 or 3 h intervals throughout development until fruiting-body formation. Since it is technically not feasible to pulse-label fruiting bodies, labelling at later time points could only be achieved by adding [35 S]-methionine at 20 h and then harvesting at 22 h, 24 h, etc., that is, after 22 h the labelling was cumulative. Although these later samples are invaluable in the analysis of both the profile of proteins synthesized and the kinetics of disulphide-cross-linking of the spore coat proteins (see below), the cumulative-labelling protocol results in a non-linear increase in the incorporation of [35 S]methionine after 24 h (Wilkinson, 1983). Therefore accurate estimates of the rate of spore-coat-protein synthesis relative to total protein synthesis can only be obtained up to 24 h and hence only these results are shown (Fig. 1a, histogram). Two separate peaks of spore-protein synthesis were observed, a minor peak just after the formation of tight cell aggregates (9–12 h) and a

Fig. 1. Kinetics of spore-coat-protein synthesis during development

(a) *D. discoideum* cells were pulse-labelled with [35 S]methionine for 3 h periods up to 12 h development and then for 2 h periods thereafter until fruiting-body formation. SDS/mercaptoethanol buffer extracts of unfractionated labelled cells or of purified plasma membranes or SDS buffer and SDS/mercaptoethanol buffer sequential extracts of labelled cells were immunoprecipitated with spore-protein antibody to determine the percentage of total protein synthesis which represents spore-coat-protein synthesis during each time period of development (histogram), the percentage of newly synthesized plasma membrane protein which is spore coat protein (●) and the percentage of immunoprecipitated spore coat protein which is disulphide-cross-linked (O). (b) A second experiment analogous to that described in (a) but in which only the total protein synthesis which represents immunoprecipitable spore coat protein is plotted (histogram). In both (a) and (b), for all pulse-label periods up to and including 20–22 h, the period of labelling *in vivo* with [35 S]methionine is given by the histogram. For the time period 22–24 h the [35 S]methionine was added at 20 h. The relative rate of spore-coat-protein synthesis from 22 to 24 h was then determined by correcting for the known rate of spore-coat-protein synthesis during the period 20–22 h.

major peak corresponding to early culmination (18–20h). Initiation of this major period of spore-protein synthesis occurred at 16–18h. The proportion of total protein synthesis which represented spore-specific protein synthesis during culmination was approx. 5% in the experiment shown in Fig. 1(a), but depends on the synchrony of development and the exact time of labelling. In other experiments, one of which is shown in Fig. 1(b), the value was approx. 9%. The newly synthesized spore coat proteins enter the plasma membrane and then pass into the spore coat where several of them become disulphide-cross-linked (Fig. 1a). Disulphide-cross-linking of the spore coat proteins was not appreciable until after the fruiting body had been formed

(24h) and was not complete until 30h (Fig. 1a). Therefore there is a lag of approx. 6–8h between the onset of spore coat synthesis at early culmination and the initiation of disulphide cross-linking of these proteins in the spore coat. Even 42h after cross-linking is complete, some spore coat proteins are still present in the spore plasma membrane (Wilkinson, 1983).

SDS/polyacrylamide-gel electrophoretic analysis of the proteins immunoprecipitated at each development stage indicated that the major peak of spore-protein synthesis at early culmination (Fig. 1) represents the synthesis of spore coat proteins SP170, SP103, SP94, SP82, SP76 and SP55 (Fig. 2a). The increase in radioactivity of the

Fig. 2. *SDS/polyacrylamide-gel electrophoresis analysis of spore coat protein synthesis during development* SDS/mercaptoethanol buffer extracts were prepared from cells pulse-labelled with [^{35}S]methionine at different stages of development and then immunoprecipitated with spore-protein antibody. These were electrophoresed on 6–18% linear-gradient polyacrylamide slab gels and the proteins detected by fluorography. (a) Immunoprecipitates of total [^{35}S]methionine-labelled proteins corresponding to the samples which form the histogram in Fig. 1(a) plus samples labelled with [^{35}S]methionine at 20h and harvested at 30h, 52h and 72h. (b) Immunoprecipitates which correspond to the samples which form the histogram in Fig. 1(b), plus a sample labelled with [^{35}S]methionine at 20h and harvested at 52h. Only the M_r region 66 000–103 000 is shown to illustrate the apparent conversion of P66 to SP72. The M_r values for these proteins were estimated from the mobility of co-electrophoresed [^{14}C]methylated marker polypeptides (Amersham International Ltd.) as described previously (Wilkinson & Hames, 1982).

Fig. 3. Kinetics of synthesis of individual spore coat proteins

Different exposures of the fluorograph shown in Fig. 2(a) were scanned with a Joyce-Loebl microdensitometer. The area under the peaks falling within the linear response range (1.8–6.7 nCi·days) was estimated by weighing (Madley & Hames, 1981). The percentage of total radiolabelled protein that is represented by each of the major spore coat proteins is shown for the period 14–24 h of the development.

spore-coat-protein bands observed on the polyacrylamide gel after 24 h reflects the fact that, for the late sampling times, the fluorographic profiles reflect cumulative radiolabelling (see above). The relative rate of synthesis of individual spore coat proteins was determined by quantitative scanning densitometry (Fig. 3). Although the detailed kinetics of accumulation varied between proteins, the time of initiation of synthesis and the period of peak synthesis was identical for SP170, SP103, SP94, SP82, SP76 and SP55.

In contrast with the other major spore coat proteins, SP72 was not accumulated until after fruiting-body formation. This is clearly seen in the pulse-labelling experiment shown in Fig. 2(b). SP72 appears to be derived from a precursor of M_r 66 000 (P66) which is accumulated at culmination (20–22 h) and then modified to SP72 during spore maturation. The evidence for this is two-fold. First, P66 was synthesized prior to the accumulation of SP72. Since for the time points after 20 h the radiolabelling profile is cumulative (see above), P66 definitely disappeared as SP72 appeared. Secondly, cross-absorption of the spore-protein antibody with an extract from 18 h-stage cells suppressed the immunoprecipitation of all the spore coat proteins

Fig. 4. Analysis of SP72

SDS/mercaptoethanol buffer extracts from spores labelled with [35 S]methionine from 20 h to 72 h of development were immunoprecipitated with spore-protein antibody previously cross-absorbed with an antigen excess of SDS/mercaptoethanol buffer extract from vegetative amoebae (lane a), cells at 18 h of development (lane b) or cells at 24 h of development (lane c). In each case the extracts for cross-absorption were prepared by heating cells in SDS/mercaptoethanol buffer at 100°C followed by alkylation with iodoacetamide in the presence of 8.5 M-urea, and then acetone-precipitated and solubilized in SDS buffer (Wilkinson & Hames, 1982).

except SP72 (Fig. 4, lane b), whereas cross-absorption with an extract from 24 h-stage cells suppressed the immunoprecipitation of SP72 also (Fig. 4, lane c). Since P66 was the only immunoprecipitable [35 S]methionine-labelled polypeptide which was absent at 18 h but present at 24 h of development (Figs. 2a and 2b), and SP72 was absent at both time points, this argues for immunological cross-reactivity between P66 and SP72.

Longer fluorographic exposure of the gel shown in Fig. 2(a) revealed the accumulation profiles of more minor proteins (Fig. 5). At 9–12 h of development, two proteins with M_r values at 18 000 (P18) and 21 000 (P21) began to be synthesized. Whereas P18 continued to be accumulated throughout the rest of development, the synthesis of P21 largely ceased at 12–14 h, thus accounting for the minor peak of protein synthesis observed at 9–12 h (Fig. 1). P18 is spore-specific, but is not a spore coat protein

Fig. 5. Accumulation of minor spore proteins during development
This Figure shows a longer fluorographic exposure of the gel shown in Fig. 2(a).

(Wilkinson, 1983). The cell-type specificity of P21 is unknown at present, since it is synthesized only early in development (Fig. 5) and hence not labelled in spore and stalk-cell extracts. Additional proteins with M_r values 31000 (P31) and 25000 (P25) accumulated starting at 12–14h and 18–20h respectively (Fig. 5). P31 synthesis largely ceased at approx. 20h, whereas P25 continued to be made throughout the rest of development. Actin was non-specifically precipitated at most time points.

The low- M_r spore coat oligopeptide detected in our previous study (Wilkinson & Hames, 1982), SP3, is only poorly labelled with [35 S]methionine and hence is most easily studied by Coomassie Blue staining. Unfortunately, at the levels of spore coat protein needed to detect SP3 by staining, the amount of antiserum required for immunoprecipitation studies is prohibitive. However, the accumulation of SP3 can be monitored by sequential extraction of developing cells with SDS buffer and then SDS/mercaptoethanol buffer followed by SDS/polyacrylamide-gel electrophoresis and Coomassie Blue staining. The data indicate that SP3 accumulates during the period of disulphide-cross-linking of the bulk of the spore coat proteins and is itself disulphide-cross-linked in the spore coat (Fig. 6). Finally, the minor spore coat proteins SP39 and SP33 identified in our previous study (Wilkinson & Hames, 1982) are also only poorly radiolabelled with [35 S]methionine. However, being minor proteins, these cannot be examined by Coomassie Blue staining, unlike SP3. Therefore their pattern of synthesis cannot be deduced from the present data.

Discussion

As demonstrated elsewhere (Orlowski & Loomis, 1979; Devine *et al.*, 1982; Wilkinson & Hames, 1982), the spore coat proteins are major, spore-specific proteins and are therefore likely to prove useful for detailed studies of the developmental regulation of gene expression in *D. discoideum*. The results presented here show that the genes for most of the major spore coat proteins (SP170, SP103, SP94, SP82, SP76 and SP55) are expressed coordinately, beginning at 16–18h of development, spore-coat-protein synthesis then reaching a peak during early culmination (18–20h) and thereafter declining. Two previous studies have also investigated the temporal pattern of spore-coat-protein synthesis, but in each case only a limited number of spore coat proteins have been examined. In good agreement with our data, Devine *et al.* (1982) found that three of the major spore coat proteins [SP96, SP70 and SP60; probably corresponding to SP103, SP82 and SP55 of our nomenclature (Wilkinson & Hames, 1982; Wilkinson, 1983)] are first synthesized at 14–16h of development, with the peak synthetic rate occurring at 18–20h. Similarly Lam & Siu (1981) showed that fucosylation (and by implication, synthesis) of three presumptive spore coat proteins (gp103, gp80 and gp72) also occurs at early culmination. However, since only some of the spore coat proteins are fucosylated (Wilkinson & Hames, 1982), not all the spore coat proteins were analysed by these workers. The appearance of major phosphoproteins, pp95 and pp74 during late

Fig. 6. Accumulation of SP3

D. discoideum cells were extracted during spore maturation according to the rapid sequential extraction protocol (Wilkinson & Hames, 1982). The resulting non-cross-linked and disulphide-cross-linked proteins were analysed by electrophoresis on a 6–18% gradient slab gel followed by staining with Coomassie Blue. The first four lanes show the non-cross-linked spore proteins at 24, 40, 48 and 72 h, whereas the next four lanes show the disulphide-cross-linked spore proteins at the same time points. The apparent modification of P66 to form SP72 is not observed in an experiment such as this, since P66 is not disulphide-cross-linked and is sufficiently minor that it cannot be identified amongst the other non-cross-linked proteins.

development (Coffman *et al.*, 1981), which correspond to the spore coat proteins SP103 and 'SP74' (Delaney *et al.*, 1983), is also consistent with the results of the present work. Other investigations have revealed polypeptides with similar M_r values and period of synthesis to the spore coat proteins described here (Siu *et al.*, 1977; Parish & Schmidlin, 1979; Burrige & Jordan, 1979), but these have not been identified as spore coat proteins.

The polysaccharide components of the spore coat are synthesized in defined prespore vesicles which are first acquired soon after aggregation, are retained throughout the slug stage and then increase in number and size during early culmination (Hohl & Hamamoto, 1976; Takeuchi, 1972). At the time of fruiting-body formation, the prespore vesicles fuse with the prespore plasma membrane so that their inner polysaccharide lining now faces the cell exterior and becomes the initial spore coat layer (Hohl & Hamamoto, 1976). As shown in the present study and described previously by Orlowski &

Loomis (1979), the spore coat proteins can be detected in the plasma membrane during culmination, apparently in transit to the spore coat. However, the mechanism of their assembly into the spore coat is unclear.

Disulphide-cross-linking of the major spore coat proteins occurs within the spore coat during spore maturation. Two other spore coat proteins, SP72 and SP3, appear at this time. The accumulation of SP72 coincides with the loss of P66. This, together with the immunological evidence presented here, suggests that P66 is the precursor of SP72. However, the nature of any modification is unclear. It is unlikely to be glycosylation, since P66 is present in pre-spore cell plasma membranes (Wilkinson, 1983), whereas most glycosylation of proteins in eukaryotes occurs soon after translation in the rough endoplasmic reticulum and Golgi apparatus prior to plasma-membrane insertion (Hubbard & Ivatt, 1981). Furthermore, although SP72 was not clearly resolved in our previous study (Wilkinson & Hames, 1982),

the evidence suggested that it is not extensively glycosylated in mature spores. One possibility is that the evidence suggested that it is not extensively glycosylated in mature spores. One possibility is that the conversion of P66 into SP72 involves covalent cross-linking to a second unidentified polypeptide. Inter-polypeptide cross-linking of this kind is known to occur (via tyrosine residues) during spore coat formation in *Bacillus subtilis* (Pandey & Aronson, 1979). The spore oligopeptide SP3 may arise by proteolytic processing of already-existing disulphide-cross-linked proteins (since SP3 is itself disulphide-cross-linked) or it may be an independent gene product accumulated late in development. These alternatives require further investigation.

The most unexpected finding of the present work is that at least two proteins which are precipitated by the spore-protein antibody, P18 and P21, begin to be synthesized in development at 9–12 h at the tipless-aggregate stage. Although P18 is detectable only in spores and not in stalk cells (Wilkinson, 1983), we have not yet examined its distribution between pre-spore and pre-stalk cells. If P18 is pre-spore-specific, it will represent the earliest indication of pre-spore cell differentiation so far identified on the basis of cellular polypeptide synthesis. The earliest histological sign of pre-spore cell differentiation is the appearance of pre-spore vacuoles in the aggregate coincident with tip formation (Forman & Garrod, 1977; Hayashi & Takeuchi, 1976). However, recent cell-fractionation (Sharpe *et al.*, 1982) and electron-microscopic (Schaap *et al.*, 1982) studies, as well as an analysis of spore-specific mRNA accumulation (Barkliss & Lodish, 1983) indicate that prespore cell differentiation may be initiated as early as 8 h, in agreement with the results presented here. Whether P21 is spore-specific is unknown.

Recent evidence suggests that short DNA repeat sequences, common to all genes of a co-ordinated gene set, may mediate the simultaneous expression of those genes (Kimmel & Firtel, 1979; Zuker & Lodish, 1981; Davidson *et al.*, 1983). The co-ordinate expression of the genes for SP170, SP103, SP94, SP82, SP76 and SP55 during development of *D. discoideum* provides a good opportunity to examine the mechanisms involved. Furthermore, since the spore coat proteins are synthesized only in pre-spore and not in pre-stalk cells, studies of this kind should also shed light on the cell-type specificity of gene expression during eukaryote development.

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