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Genetic and Physiologic Modulation of the Prestarvation Response in *Dictyostelium discoideum*

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Throughout vegetative growth, *Dictyostelium* amoebae secrete an autocrine factor, prestarvation factor, PSF, which accumulates in proportion to cell density. During late exponential growth, PSF induces the expression of several genes whose products are needed for cAMP signaling and cell aggregation. Among these genes are discoidin-I and the 2.4-kb transcript of cyclic nucleotide phosphodiesterase (PDE). We have identified several parameters that modulate expression of one or both of these prestarvation response genes; all effects were monitored in cells growing exponentially on bacteria. Under these conditions, axenic mutants produce higher levels of PSF activity than wild-type cells. Consistent with the high PSF levels, the 2.4-kb PDE transcript is more abundant in axenic strains than wild-type cells at the same cell density. In contrast, the density-dependent induction of discoidin-I is greatly delayed in axenic strains, occurring only at the very end of exponential growth. Analysis of axenic strains of independent origin suggested that this negative effect on discoidin-I expression is attributable to the axenic mutations themselves. The effects of two environmental factors that inhibit the prestarvation response (the bacteria upon which the cells feed and a bacterial product, folic acid) were also analyzed. We found that folate does not account for the inhibitory effect of bacteria. Cells deficient in the G-protein β subunit, which is thought to be common to all heterotrimeric G-proteins in *Dictyostelium*, respond to PSF in the same manner as $G\beta^+$ cells, and this response is inhibited by bacteria. However, folate has no inhibitory effect on $g\beta^-$ cells, indicating that folate inhibition is mediated by a heterotrimeric G-protein. In cells lacking the catalytic subunit of protein kinase A, the prestarvation response is severely impaired, but about 3% of the pka^- cells manifest an apparently normal density-dependent induction of discoidin-I. This behavior and the heterogeneity of the prestarvation response in wild-type cells lead us to speculate that protein kinase A may not be required for PSF signal transduction per se, but rather may render the cells responsive to PSF. Based on analysis of adenylyl cyclase mutants (aca^-), the effect of protein kinase A is not cAMP-dependent.

INTRODUCTION

Amoebae of the eukaryotic microorganism *Dictyostelium discoideum* feed on bacteria, growing exponentially until the bacterial food supply is depleted. Starvation initiates the developmental phase of the life cycle. Using cAMP as a chemotactic signal, the cells aggregate into multicellular masses, which undergo differentiation and morphogenesis to yield fruiting bodies (reviewed in Loomis, 1982). Throughout growth, the cells secrete an autocrine factor that accumulates in proportion to cell density (Clarke *et al.*, 1987, 1988). This factor (prestarvation factor, or PSF) triggers the expression of several genes whose products are important for cell aggregation (Rathi and Clarke, 1992). This change in gene expression, which

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occurs late during exponential growth, is called the prestarvation response.

The onset of the prestarvation response is inhibited by the presence of the bacteria (*Klebsiella aerogenes*) on which the cells feed, the extent of inhibition being proportional to the concentration of bacteria (Clarke *et al.*, 1987). As a culture of *Dictyostelium* cells grows, PSF accumulates and bacteria are consumed, leading to the condition (high PSF/bacteria ratio) that triggers the prestarvation response. For wild-type (NC4) cells, this condition is reached three to four generations before the bacterial concentration becomes too low to support further exponential growth (Clarke *et al.*, 1987; Rathi *et al.*, 1991). Thus, the PSF signal transduction pathway allows cells not only to monitor their own density, but also to anticipate the depletion of their food supply. Both parameters are relevant to the cells in determining whether and when to begin producing the gene products needed for cell aggregation.

Although production of PSF declines at the onset of starvation (Rathi and Clarke, 1992), certain of the genes initially induced by PSF are expressed at even higher levels during early development. For example, two components of the cAMP signaling system required for cell aggregation, the cAMP receptor (cAR1) and the aggregation-specific form of cyclic nucleotide phosphodiesterase (2.4-kb PDE transcript), are expressed at low levels during late exponential growth in response to PSF (Rathi and Clarke, 1992), and then at higher levels in starving cells, stimulated at least in part by cAMP pulses (Franke *et al.*, 1987; Faure *et al.*, 1990; Saxe *et al.*, 1991). Thus, the low level of expression induced by PSF may render the cells minimally competent for cAMP signaling, such that, upon starvation, a positive feedback loop can be set in motion by cAMP.

Discoidin-I, a lectin important in the formation of head-to-tail streams by aggregating cells (Crowley *et al.*, 1985), displays a different pattern of regulation. Discoidin-I is induced by PSF during late exponential growth (Clarke *et al.*, 1987; Rathi *et al.*, 1991), and its production continues to be stimulated during early development by CMF, a factor secreted by starving cells (Mehdy and Firtel, 1985; Gomer *et al.*, 1991). At the time of aggregation, discoidin-I expression is down-regulated by cAMP (Williams *et al.*, 1980; Vauti *et al.*, 1990). In *Dictyostelium* mutants capable of growth on liquid nutrient medium (*axenic* mutants), discoidin-I expression is also up-regulated by growth on axenic medium (Kayman *et al.*, 1988; Vauti *et al.*, 1990). Thus, the regulatory patterns of prestarvation response genes are quite complex, with factors other than PSF capable of modulating their expression, especially under conditions of starvation or axenic growth.

In the present study, we have analyzed the effects of several genetic and physiologic modulators of prestarvation response gene expression in cells growing exponentially on bacteria. Some components were selected for examination because of their known effects on discoidin-I expression in other contexts, e.g., during starvation or axenic growth. For example, folic acid has been shown to repress transcription of reporter genes driven by the discoidin Iγ promoter under axenic growth conditions (Blusch *et al.*, 1992). We examined the inhibitory effect of bacteria and the possible contribution of folate to this inhibition. We also tested heterotrimeric G-proteins and the cAMP-dependent protein kinase (protein kinase A) for possible roles in the PSF signal transduction pathway, because both types of proteins are essential for cell aggregation (Mann and Firtel, 1991; Mann *et al.*, 1992; Lilly *et al.*, 1993). To elucidate the contributions of the heterotrimeric G-proteins and of protein kinase A, we examined mutant strains in which individual genes of interest had been inactivated. These mutant strains and their parents carried additional mutations enabling them to grow axenically, so the effects of the axenic genotype were also defined. Our studies showed that the prestarvation response is a complex process affected by a variety of cellular (genetic) and environmental variables.

MATERIALS AND METHODS

Cells and Culture Conditions

Wild type *Dictyostelium discoideum* cells (strain NC4) were used in all experiments unless otherwise noted. The cells were grown on a suspension of *K. aerogenes* as previously described (Clarke *et al.*, 1987). In brief, the bacteria were allowed to grow on SM (Loomis, 1975) nutrient agar plates at 22°C for 3 days before they were rinsed from the plates with 17 mM KH_2PO_4/K_2HPO_4 buffer (pH 6.5), hereafter called "phosphate buffer". The bacterial suspension was adjusted to a standard concentration; a 1× bacterial suspension contained approximately 3×10^{10} bacteria/ml (Rathi *et al.*, 1991). *Dictyostelium* spores or amoebae were inoculated into the bacterial suspension at a density of 10^3-10^4 cells/ml and incubated at 22°C on a rotary shaker (180 rpm) until they reached the desired cell density. Cell density was determined by counting the cells in a hemacytometer.

Several mutant strains were also examined. Axenic mutants were AX2, $AX3_L$, $AX3_K$, AX4, and a new axenic derivative of NC4, called NC4A2, isolated in David Knecht's laboratory (University of Connecticut, Storrs, CT) by prolonged selection in HL5 (D. Knecht, personal communication). Other mutants derived from these axenic strains were deficient in a specific protein owing to inactivation of that gene by homologous recombination. The targeted genes included those encoding the G-protein α subunits Gα1 (Kumagai *et al.*, 1991), Gα2 (Kumagai *et al.*, 1991), Gα3 (M. Brandon, personal communication), and Gα4 (Hadwiger *et al.*, 1994), the G-protein β subunit (Lilly *et al.*, 1993), the catalytic subunit of protein kinase A (Mann and Firtel, 1991; Mann *et al.*, 1992), and the early form of adenylate cyclase, ACA (Pitt *et al.*, 1992). We also used K2, a derivative of AX4 in which the gene for CMF has been inactivated by antisense mutagenesis (Jain and Gomer, 1994).

Preparation of Conditioned Buffer and Partial Purification of PSF

First, the bacterial suspension that serves as a food source for *Dictyostelium* cells was prepared. One liter of SM nutrient medium

was inoculated with *K. aerogenes* and incubated overnight at 22°C on a rotary shaker at 300 rpm. The next day the overnight culture was added to a fermenter (New Brunswick BIOFLO II; Edison, NJ) along with 4 l of SM nutrient medium, 500 ml of 50% dextrose, and 250 ml of 20× M-9 salts (Miller, 1972). Fermentation was started with agitation 400 rpm, air flow 4 l/min, and pH 6.5. The pH was adjusted periodically by adding concentrated ammonium hydroxide (15 M), and foaming was suppressed with antifoam spray (Thomas Scientific, Swedesboro, NJ). The optical density was monitored, and, at $OD_{660\ nm}$ = 1.0, the agitation and air flow were increased to 600 rpm and 6 l/min, respectively. The bacteria were harvested when the $OD_{660\ nm}$ reached 8–10. Using a Filtron Minisette System (Northborough, MA) equipped with five Omega 1000K membranes (Filtron, Northborough, MA), the bacteria were concentrated to 0.5 l, then rinsed with 12 l of phosphate buffer, and finally concentrated to a vol of 2 l. The Minisette membranes were rinsed with 3 l of phosphate buffer, and the rinse was combined with the bacterial suspension. The concentration of the bacterial suspension was adjusted to 1× with additional phosphate buffer, yielding a final vol of 9–10 l.

Conditioned buffer (CB) was prepared by growing *Dictyostelium* cells to a density of 6×10^6 cells/ml on 10 l of the 1× bacterial suspension. Centrifugation was used to remove the *Dictyostelium* cells (200 × *g*, 10 min) and then the bacteria (13,000 × *g*, 20 min). Tween-20 (0.002%) and dithiothreitol (DTT) (0.1 mM) were added to the final concentrations shown, and the supernatant was clarified by filtration (Millipore DV membrane, 0.65-μm pore size; Bedford, MA). The Filtron Minisette system, fitted with three Omega 10K membranes, was used to concentrate the solution to 500 ml and to replace the phosphate buffer with buffer A (20 mM potassium phosphate [pH 7.0], 50 mM potassium chloride, 1 mM DTT, and 0.002% Tween-20).

PSF was enriched by affinity chromatography on concanavalin A (ConA)-Sepharose (Sigma Chemical, St. Louis, MO). The affinity resin (10 ml packed vol) was equilibrated with buffer A and then mixed with the concentrated CB (500 ml) by gentle rotation at 4°C for 1 h. The resin was collected, washed with 100 ml buffer A, and collected again by gentle centrifugation or filtration. Bound molecules were eluted by mixing the resin with 30 ml of buffer B (buffer A plus 0.5 M methyl-α-D-mannopyranoside and 0.5 M sodium chloride) at 4°C for 1 h. The eluate was separated from the resin by centrifugation, and the elution was repeated with another 30 ml of buffer B. Protease inhibitors TLCK (10 mg/ml final concentration), chymostatin (2 mg/ml), leupeptin (2 mg/ml), and EDTA (1 mM) were added to the pooled eluate. The ConA eluate was concentrated to ~1.5 ml using a Centriprep-10 unit (Amicon, Beverly, MA).

Gel filtration chromatography was carried out using a Sephacryl S300 column (1.4 × 33 cm) equilibrated with running buffer (20 mM potassium phosphate buffer, 1 mM DTT, 1 mM EDTA, 0.5 M NaCl, 0.002% Tween-20 [pH 7.0]), usually plus carrier protein (0.1 mg/ml bovine serum albumin). One-half milliliter of concentrated ConA eluate was pumped onto the column followed by running buffer at a rate of 1 ml/min. One-half milliliter fractions were collected and assayed for PSF activity and, if the column had been run in the absence of carrier protein, for protein. The fractions containing high levels of PSF activity were pooled. The peak of PSF activity eluted at the same position as the 66,000 molecular weight marker.

The Sephacryl pool was subjected to antibody affinity chromatography to remove contaminants bearing the *Dictyostelium* "common antigen", a highly immunogenic epitope present on many *Dictyostelium* glycoproteins (Freeze *et al.*, 1984; Knecht *et al.*, 1984). An appropriate antiserum (a polyclonal rabbit serum against the lysosomal enzyme α-mannosidase) was generously provided by Dr. J.A. Cardelli (Mierendorf *et al.*, 1983). Antibodies present in 1 ml of this antiserum were conjugated to 4 ml protein A-Fast Flow Sepharose (Sigma Chemical) using the direct coupling method described by Harlow and Lane (1988). After being equilibrated with a buffer containing 20 mM potassium phosphate, 0.5 M sodium chloride, and 0.002% Tween-20 (pH 7.0), the antibody-bearing resin was mixed with the the Sephacryl S300 pool and incubated overnight at 4°C. The next day the supernatant (partially-purified PSF) was recovered by centrifugation and, after being assayed, was stored frozen at −20°C in 0.5 ml aliquots. In some instances, PSF was separated from the carrier protein after Sephacryl column chromatography (using ConA resin as described above) and then absorbed with the immobilized antibodies. This protocol allowed the protein concentration and composition of the partially purified PSF to be determined. The final product was approximately 100-fold enriched in PSF activity relative to the starting CB and contained about 3% of the initial activity.

Quantitation of PSF Activity

PSF was assayed by its ability to cause low density, exponentially growing NC4 cells to produce discoidin-I, as detected by an indirect immunofluorescence assay using a rabbit antiserum specific for this protein (Rathi and Clarke, 1992). NC4 cells were grown overnight on a 1× suspension of *K. aerogenes* and harvested at a density of 1–2 × 10^5 cells/ml. Under these conditions, the cells produce discoidin-I only if exogenous conditioned buffer or PSF has been added, and, as shown in RESULTS, the fraction of cells that produce discoidin-I is linearly related to the amount of PSF added. One unit of PSF activity is defined as the amount sufficient to induce discoidin-I production in 50% of the cells in a 1-ml assay vol. Further details of the assay have been provided elsewhere (Rathi and Clarke, 1992).

A small-scale version of this assay, suitable for the analysis of large numbers of samples, was developed during the present study. One-milliliter cultures were grown on a 1× bacterial suspension in 16-mm culture tubes on a rotary shaker. The cells were washed by differential centrifugation in microcentrifuge tubes and then transferred to individual wells of a 16-well chamber slide (Nunc, Naperville, IL). The cells were allowed to adhere to the glass surface for 5 min, then fixed in 2% buffered formaldehyde (30 min) and permeabilized with 100% methanol (10 min), all at room temperature. The fixed cells were washed with 20 mM Tris-HCl, 150 mM NaCl (pH 7.5) and immunostained for discoidin-I.

Other Assays

Folate deaminase activity was measured spectrophotometrically as described by Bernstein *et al.* (1981). Protein was determined using the Coomassie Protein Assay (Pierce, Rockford, IL).

Northern Blot Analysis

Total cellular RNA was isolated using TRIzol Reagent (Life Technologies, Gaithersburg, MD) according to the manufacturer's instructions. Samples were electrophoresed in a 1.3% agarose/formaldehyde gel and transferred by capillary blotting onto Hybond N membrane (Amersham, Arlington Heights, IL). Each lane contained 5 μg of RNA. The DNA restriction fragments used as probes for calmodulin, discoidin-I, and cyclic nucleotide phosphodiesterase (PDE) were as previously described (Rathi *et al.*, 1991; Rathi and Clarke, 1992). The probes were labeled with [α^{32}P]dATP by nick translation (Life Technologies kit), and unincorporated nucleotides were removed by passage through a NICK column (Pharmacia, Piscataway, NJ). Hybridization was performed in Rapid-hyb buffer (Amersham) according to the manufacturer's instructions. The blots were washed twice for 10 min at room temperature in 2× SSC, 0.1% sodium dodecyl sulfate, then 15 min at 65°C in 1× SSC, 0.1% sodium dodecyl sulfate.

RESULTS

A Quantitative Assay for PSF Activity

We have defined conditions for NC4 cells (growing at low density on a standard 1× concentration of *K.*

aerogenes) in which the cells manifest no detectable expression of discoidin-I mRNA or protein unless conditioned medium or partially purified PSF is added (Clarke *et al.*, 1987; Rathi *et al.*, 1991). Under these conditions, detailed in MATERIALS AND METHODS, the fraction of the cell population induced to produce discoidin-I protein is essentially linear with respect to the amount of partially purified PSF added over a three- to fourfold range (Figure 1). This assay for PSF activity avoids starvation-dependent signals and any effects of axenic growth. However, PSF has not been completely purified, so the response may involve multiple components. It is noteworthy that the assay detects the response of individual cells rather than the average behavior of a mixed population. Thus, the data in Figure 1 show that, as the PSF level increases, the fraction of discoidin-producing cells increases. This observation reveals population heterogeneity with respect to PSF sensitivity that would not have been evident with assays based on cell populations.

The Effect of the Axenic Mutations on the Prestarvation Response

Wild-type and Axenic Strains Differ in the Timing and Uniformity of Discoidin-I Induction. The broad linear range of response of wild-type *Dictyostelium* cells to PSF, illustrated in Figure 1, is not manifested by strains carrying the mutations that permit axenic growth. This difference is evident in Figure 2, which shows the prestarvation response, as detected by discoidin-I immunofluorescence, in wild-type and axenic *Dictyostelium* strains during exponential growth on bacteria. For the wild-type strain NC4, most cells (80%) began expressing discoidin-I between densities of 7×10^5 cells/ml (10% of the cells positive) and 5×10^6 cells/ml (90% of the cells positive), a sevenfold density range. In the axenic mutant AX2, also growing on bacteria, the onset of discoidin-I synthesis was delayed, occurring two to three cell generations later than it did in NC4 cells, or almost at the end of the exponential phase of growth. Furthermore, the response occurred over a much narrower range of cell density, with 90% of the cells becoming discoidin-positive at densities between 5×10^6 and 1×10^7 cells/ml. All of the other axenic strains we examined ($AX3_K$, $AX3_L$, AX4, and their mutant derivatives) manifested delays similar to that shown for AX2, suggesting that this behavior correlated with the axenic genotype. To test this hypothesis, we examined the behavior of an independently isolated axenic strain, NC4A2, which was recently derived from NC4 in D. Knecht's laboratory by prolonged selection in HL5 (according to the method of Morrison and Harwood, 1992). This strain was also delayed in discoidin-I induction, closely resembling AX2 (Figure 2), confirming that this behavior is linked to the capacity for axenic growth.

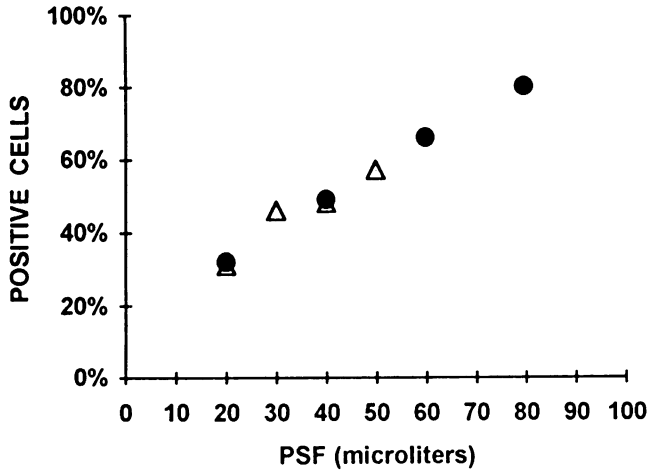

Figure 1. PSF assayed by its effect on discoidin-I production in exponentially growing wild-type cells. Low density NC4 cells growing on a 1× suspension of *K. aerogenes* (4 ml vol) were incubated overnight with the indicated amounts of added PSF. The next day, the fraction of the cells producing discoidin-I was determined by indirect immunofluorescence. For each data point, 300–400 cells were scored. The background value (the fraction of cells positive in the absence of any added PSF) was subtracted (5% and 7% in these two experiments). The open and closed symbols show data from two different experiments. The PSF-containing sample was the ConA eluate (protein concentration 42 μg/ml). Over the range shown, a linear relationship was observed between the PSF concentration and the fraction of cells positive for discoidin-I.

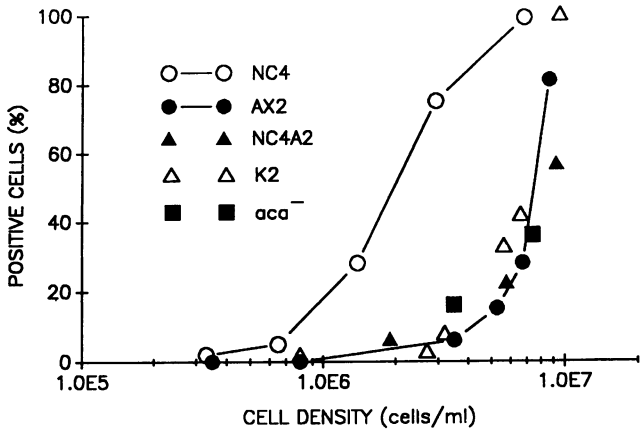

Figure 2. Density-dependent induction of discoidin-I synthesis in wild-type and axenic cells. Cells growing on 1× bacterial suspensions were sampled at intervals throughout exponential growth and assayed for discoidin-I production. The fraction of cells producing discoidin-I is plotted as a function of cell density. The strains examined here were the wild-type NC4, and the axenic strains AX2, NC4A2, K2 (a CMF^- derivative of AX4), and a strain deficient in adenylyl cyclase activity. All of the axenic strains manifested similar delays in the density-dependent induction of discoidin-I.

The Late Induction of Discoidin-I in Axenic Strains Does Not Depend on CMF. Because discoidin-I induction occurred so late in the axenic strains, we considered the possibility that this induction might not be due to PSF at all, but rather to CMF, a factor secreted by starving cells that also regulates discoidin-I expression (Gomer *et al.*, 1991; Clarke *et al.*, 1992; Jain *et al.*, 1992). A CMF^- antisense strain (K2), generated by Jain and Gomer (1994), was examined. Cells were shifted from low density axenic culture to bacterial suspension culture and sampled at intervals as they grew from low to high density. (In parallel with this experiment, some of the K2 cells were plated with bacteria on nutrient agar, and their aggregation-minus phenotype verified that they were continuing to express the antisense transcript). As shown in Figure 2, discoidin-I expression in K2 cells during exponential growth on bacteria was similar to that of other axenic strains. Thus, the induction of discoidin-I in the axenic strains is not mediated by CMF.

Axenic Strains Produce Higher Levels of PSF Activity than Wild-type Cells. We next analyzed whether the delay in discoidin-I induction reflected diminished production of PSF or reduced responsiveness to PSF on the part of the axenic strains. $AX3_K$ and NC4 cells were grown on 1× bacterial suspensions until they had each reached a density of 6×10^6 cells/ml. From each culture, the cells and bacteria were removed by centrifugation, leaving conditioned buffer. To measure PSF activity, each batch of conditioned buffer was used to dilute a fresh 4× suspension of bacteria to 1× final concentration. These bacterial suspensions were then used to support the overnight growth of low density NC4 or $AX3_K$ cells, and the fraction of cells producing discoidin-I was measured. This assay indicated that the level of PSF activity produced by $AX3_K$ cells was two- to threefold greater than that produced by NC4 cells (Figure 3, assay shown at left; compare to Figure 1). Furthermore, when exposed to identical concentrations of PSF, fewer $AX3_K$ cells produced discoidin I than did NC4 cells (Figure 3, assay at left vs. assay at right). Thus, bacterially grown axenic cells produced higher levels of PSF than did wild-type cells, but, as measured by discoidin-I synthesis, were on the average more refractory in their response.

Not All Genes Subject to the Prestarvation Response Are Regulated Identically. We investigated whether the axenic genotype also affected the expression of another PSF-regulated gene, that for cyclic nucleotide PDE. The single PDE gene generates three mRNAs from different promoters (Franke and Kessin, 1992): a 1.9-kb vegetative transcript, a 2.4-kb aggregation transcript (induced by PSF; Rathi and Clarke, 1992), and a 2.2-kb late transcript.

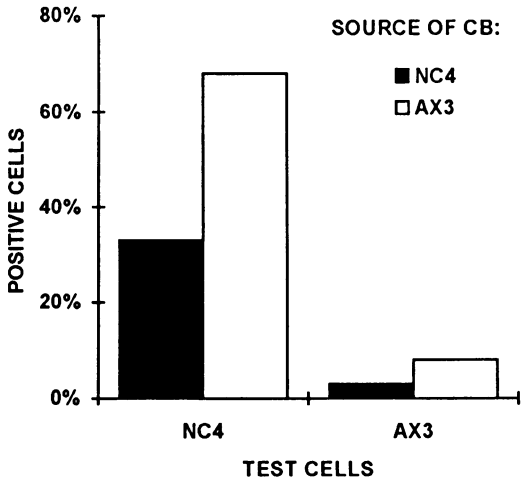

Figure 3. Production of and response to PSF by axenic and wild-type cells. Conditioned buffer (CB) was collected from $AX3_K$ and NC4 cells grown on 1× bacterial suspensions and harvested when the cell densities reached 6×10^6 cells/ml. (Left) The PSF activity in each batch of CB was assayed for its ability to cause low density NC4 cells to produce discoidin-I (our standard assay, illustrated in Figure 1). More PSF activity was present in the buffer conditioned by $AX3_K$ cells. (Right) The same batches of CB were also tested for their effects on $AX3_K$ cells. The axenic cells barely responded to PSF levels that caused a strong response in NC4 cells.

Northern blot analysis was used to detect the first two transcripts during late exponential growth of cells on bacteria.

We compared PDE transcript levels in NC4 and AX2 cells harvested at 5 and 7.5×10^6 cells/ml, densities at which the fraction of discoidin-I-producing cells is about threefold lower in axenic than wild-type strains (see Figure 2). As shown in Figure 4, the 2.4-kb PDE transcript was actually higher in AX2 than NC4 cells at these cell densities, as would be expected from the higher levels of PSF produced by axenic cells. The same blot was reprobed to detect calmodulin mRNA, a transcript that is expressed at constant levels throughout growth and development (Liu *et al.*, 1992). This probe verified that equivalent amounts of mRNA had been loaded in each lane. Thus, the relative levels of the PDE transcript in wild-type and axenic cells correlated with the concentration of PSF.

For comparison with the immunofluorescence data, which had indicated an opposite behavior for discoidin-I, the same blot was also probed to detect discoidin-I mRNA. Discoidin-I mRNA levels in the two cell types corresponded well with the fraction of discoidin-positive cells detected by antibody staining. Higher levels of discoidin-I mRNA were present in NC4 than in AX2 cells at the same cell density, and there was a marked density-dependent increase in discoidin-I mRNA for AX2 cells during this brief interval of late exponential growth. These data indicate that discoidin-I synthesis is repressed at the transcrip-

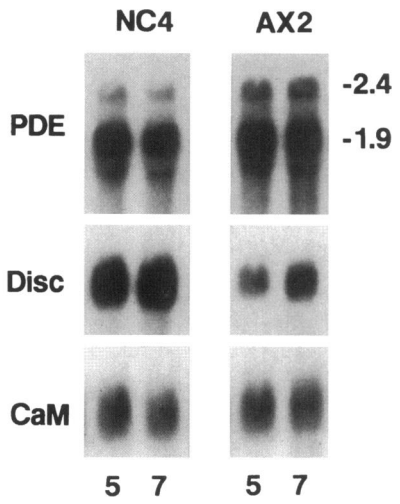

Figure 4. Northern blot comparing levels of the 2.4-kb PDE transcript and discoidin-I mRNA during late exponential growth in wild-type and axenic cells. Total RNA was prepared from NC4 and AX2 cells growing on 1× suspensions of bacteria and harvested at high cell densities (5 and 7.5 × 10^6 cells/ml). The RNA was size-fractionated by gel electrophoresis and transferred to a membrane. The blot was first probed to detect PDE transcripts, and then re-probed for discoidin-I and calmodulin. The results indicated that the 2.4-kb PDE transcript was expressed at higher levels in axenic cells than wild-type cells at the same cell density, in contrast to the behavior of discoidin-I.

tional level in bacterially grown axenic strains until late exponential phase, although there is no repression of the 2.4-kb PDE transcript. Thus, the axenic genotype does not in fact render cells less sensitive to PSF, but overlays a negative regulatory mechanism on the expression of the discoidin-I genes; this mechanism can be overcome by PSF, but only at very high levels.

The Effects of Bacteria and of Folate (a Bacterial Product) on the Prestarvation Response

Folate Modulates the Prestarvation Response in Wild-type as well as Axenic Cells. The possible role of folate in the prestarvation response was examined in several ways. Nellen's group had demonstrated that the expression of reporter genes driven by the discoidin-Iγ promoter could be repressed in axenically growing cells by the addition of 1 mM folate to the axenic growth medium (Blusch *et al.*, 1992). We determined that folate also reduces discoidin-I synthesis in wild-type cells growing on bacteria. For NC4 cells growing on a 1× suspension of *K. aerogenes*, discoidin-positive cells are normally first detected when the population reaches a density of ~3 × 10^5 cells/ml (Figure 2; also see Rathi *et al.*, 1991). In 1 mM folate, induction did not commence until the cells had reached a density of ~2 × 10^6 cells/ml (Figure 5). Furthermore, addition of 1 mM folate to low density NC4 cells blocked discoidin-I production completely at PSF levels near saturating for cells in the absence of folate (our unpublished observations). Thus, the inhibitory effect of folate on discoidin-I expression does not require an axenic genotype, and, in agreement with Nellen's results, folate and PSF appear to have antagonistic effects on discoidin-I induction.

The Deamination of Folate Is Not Necessary for the Prestarvation Response. The onset of discoidin-I production during the prestarvation response is inhibited by the bacteria (*K. aerogenes*) on which the cells are feeding, the extent of inhibition being proportional to the concentration of bacteria (Clarke *et al.*, 1987). The bacteria do not inactivate secreted PSF or suppress its production, and buffer conditioned by high density bacterial cells does not possess inhibitory activity (Clarke *et al.*, 1988). An attractive possibility, suggested by Blusch and coworkers (1992) and not excluded by our earlier experiments, is that folate released from bacterial cells accounts for the inhibitory effect of bacteria. In this case, the antagonistic effects of PSF and folate might reflect the destruction of folate by an enzymatic activity of PSF, presumably folate deaminase. We tested this hypothesis.

We first examined the inhibitory activity of several folate analogues. Pterin, lumazine, PABA, and L-glutamic acid, all of which are folate precursors, had no effect on discoidin-I production (our unpublished observations). However, the addition of either aminopterin or methotrexate to high density NC4 cultures caused an even greater inhibition of discoidin-I production than did folate (Table 1). Aminopterin and methotrexate are noncompetitive inhibitors of folic acid at its receptor(s), but are not substrates for folate deaminase (Nandini-Kishore and Frazier, 1981). The finding that these analogues were more effective inhibitors of the prestarvation response than folate sup-

Table 1. Inhibition by folate and folate analogues of discoidin-I synthesis in vegetative wild-type cells

Addition	Total cells	Positive cells
None	397	54%
Folate	348	36%
Methotrexate	373	2%
Aminopterin	360	1%

NC4 cells were grown on a 1× bacterial suspension to a density of 1 × 10^6 cells/ml in the absence of added folate, in the presence of 1 mM folate, or in the presence of 1 mM methotrexate or 1 mM aminopterin, two drugs that act as competitive inhibitors of folate at the folate receptor. Samples from each culture were assayed for discoidin-I production by indirect immunofluorescence. The table shows the total number of cells that were counted and the percentage that were positive for discoidin-I protein.

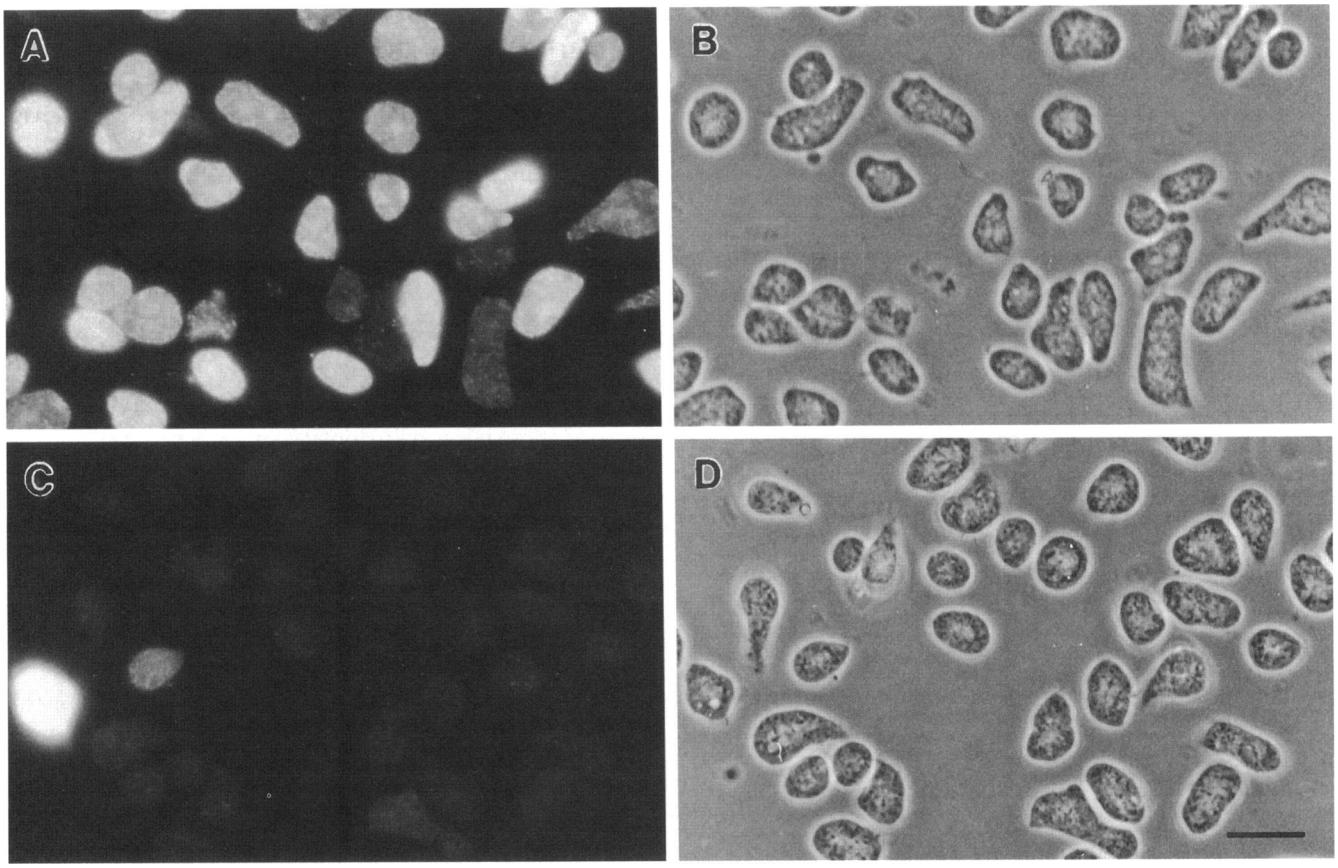

Figure 5. The effect of folate on discoidin-I production by wild-type cells. NC4 cells were grown on a 1× suspension of *K. aerogenes* in the absence (A and B) and presence (C and D) of 100 μM folic acid. The cells were harvested at a density of 2×10^6 cells/ml and assayed by indirect immunofluorescence for discoidin-I production. At this density, more than half of the cells in the control population were positive for discoidin-I (A), and virtually all of the cells exposed to folate were negative (C). The heterogeneous nature of the response was evident under both conditions. Phase contrast and fluorescence micrographs of the two populations are shown. Bar, 10 μm.

ported the view that PSF might possess folate deaminase activity, and that this might be the key to its effects.

We next examined the effect of 8-azaguanine, an inhibitor of folate deaminase activity (De Wit, 1982). A spectrophotometric assay (see MATERIALS AND METHODS) indicated that folate deaminase activity was indeed present in conditioned buffer and partially purified PSF, and showed that this activity could be blocked by the addition of 50 μM 8-azaguanine. However, even at 200 μM, 8-azaguanine had no effect on discoidin-I production (our unpublished observations), arguing against an involvement of folate deaminase. As an additional test, we assayed fractions from each step of our PSF purification procedure for folate deaminase activity. We found that PSF activity and folate deaminase activity co-purified through the initial PSF fractionation steps, but were resolved by gel-filtration chromatography. Figure 6 shows the separation of these two activities on a Sephacryl S300 column. We concluded that PSF does not possess folate deaminase activity and that such activity is not required for the prestarvation response.

The Inhibitory Effect of Bacteria Is Not Dependent on Any Unstable Bacterial Product. A subsequent experiment addressed the more general question of whether any unstable, continuously replenished bacterial product is required for the inhibitory action of bacteria. For this analysis, we compared the effects of living and dead bacteria. *K. aerogenes* cells were suspended at 1× concentration in phosphate buffer, then microwaved for the minimum length of time required to kill them. (Viability was assayed by plating the heat-treated bacteria on nutrient agar). *Dictyostelium* cells were grown at low cell density on 1/2×, 1×, and 2× suspensions of living and heat-killed bacteria. All cultures were harvested at a density of 2×10^5 cells/ml after overnight exposure to identical concentrations of added PSF, and the cells were assayed for discoidin-I production.

The results (Figure 7) indicated that heat-killed bacteria, like living bacteria, suppressed discoidin-I

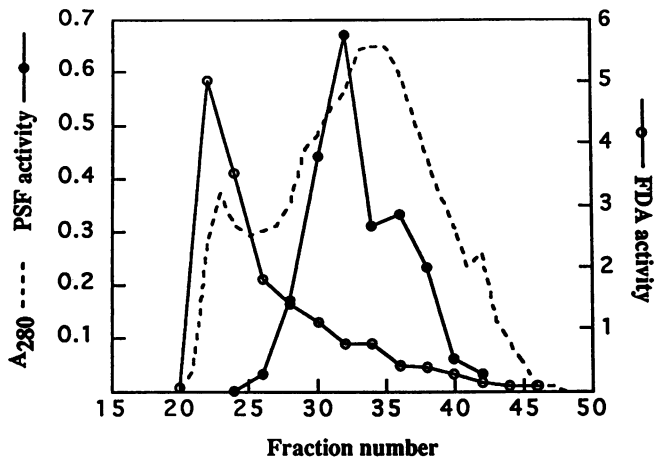

Figure 6. Separation of PSF and folate deaminase activities by gel filtration chromatography. One-half milliliter of concentrated ConA eluate was chromatographed on a 50-ml Sephacryl S300 column. One-half milliliter fractions were collected and assayed for PSF and folate deaminase (FDA) activity. PSF activity is plotted as the fraction of cells producing discoidin-I. FDA activity is plotted as U/ml. One unit (U) of FDA activity is defined as a 0.01 OD unit/min rate of change in the absorbance of 1 ml of 50 μM folate at 280 nm. The distribution of total protein, plotted as A280 nm, is also shown. On this gel filtration column, the peak of PSF activity eluted at the same position as the serum albumin size marker (66 kDa), well separated from the peak of FDA activity.

production, the extent of inhibition being a function of the concentration of bacteria. This outcome ruled out the possibility that an unstable bacterial product is the active agent. Remarkably, the inhibitory activity of heat-killed bacteria was greater than that of the same concentration of living bacteria. Inspection of cells from the two types of cultures by phase contrast microscopy suggested an explanation for this difference: the heat-killed bacteria adhered to the surface of *Dictyostelium* cells more tightly than did living bacteria. After being washed four times by sedimentation and resuspension in buffer, *Dictyostelium* cells grown on heat-killed bacteria still had many bacteria clinging to their surface, whereas the same number of washes removed essentially all living bacteria. Thus, the binding of bacteria to *Dictyostelium* cells may contribute to their inhibitory activity. As described in the DISCUSSION, some preliminary data from phagocytosis mutants also lend support to this hypothesis.

These results indicated that neither folate nor any other unstable bacterial product can account for the inhibitory effect of bacteria on the prestarvation response. As described in the next section, this conclusion was specifically verified for folate by the analysis of signal transduction mutants.

The Effects of Signal Transduction Mutations on the Prestarvation Response

Heterotrimeric G-Proteins Are Not Required for the Prestarvation Response or Its Inhibition by Bacteria, but Are Essential for the Effect of Folate. Many of the signal transduction pathways in *Dictyostelium*, including at least two that affect discoidin-I synthesis, utilize heterotrimeric G-proteins (see DISCUSSION). We examined null mutants for each of the G-protein α subunit genes *gα1*, *gα2*, *gα3*, and *gα4* to determine whether any of these Gα subunits is needed for transducing the PSF signal. Mutant cells were grown on a 1× suspension of bacteria under standard conditions, and discoidin-I production was monitored as a function of increasing cell density or as a function of the addition of varying amounts of exogenous PSF to low density cells. In both situations, the behavior of mutant cells lacking one of the Gα subunits was indistinguishable from that of the control $AX3_K$ cells (our unpublished observations). Consequently, none of these Gα subunits can be required for transduction of the PSF signal (or for the effects of the axenic mutations on discoidin-I expression).

We next examined a strain in which the gene for the G-protein β subunit had been disrupted. Only a single Gβ subunit gene has been detected in *Dictyostelium*, suggesting that this subunit is common to all *Dictyostelium* heterotrimeric G-proteins (Lilly *et al.*, 1993). Like the $g\alpha^-$ mutants, $g\beta^-$ cells manifested the densi-

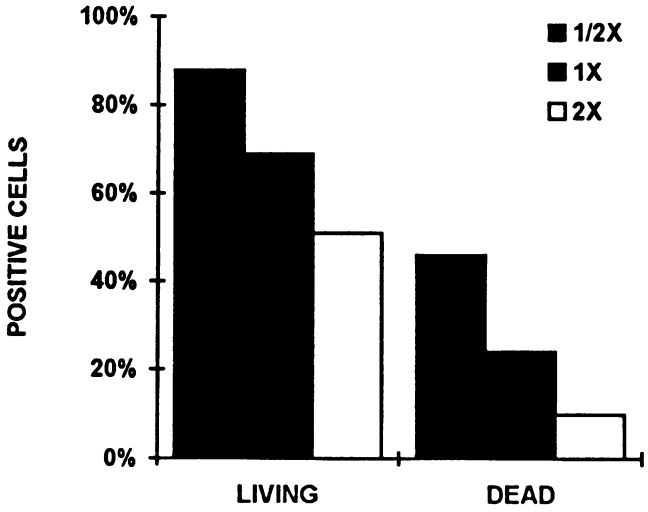

Figure 7. Inhibition of the prestarvation response by living and heat-killed bacteria. NC4 cells were grown overnight on different concentrations (1/2×, 1×, and 2×) of living or heat-killed *K. aerogenes*. Each culture contained the same amount of added PSF. The cells were harvested the next morning at a density of 2×10^5 cells/ml and assayed for discoidin-I production. The fraction of cells positive for discoidin-I is shown. Both living and dead bacteria suppressed the cells' response to PSF in a density-dependent manner, with the dead bacteria having a greater inhibitory effect.

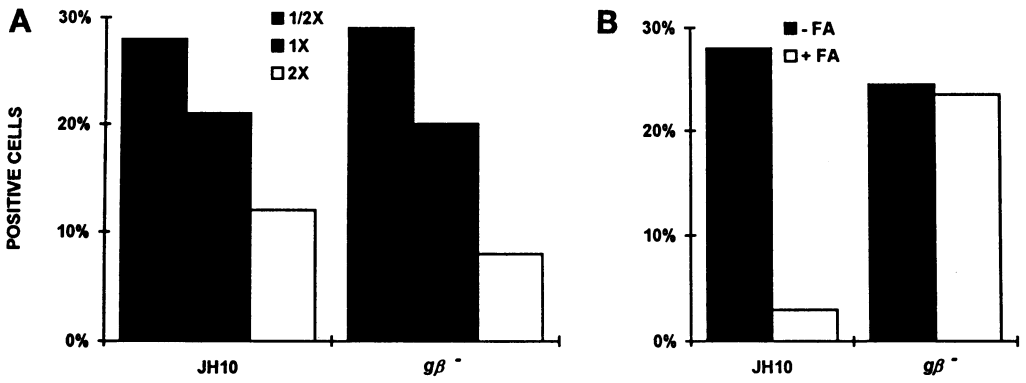

Figure 8. Properties of the prestarvation response in cells lacking the G-protein β subunit. (A) Effect of bacteria. The $g\beta^-$ cells and the parental control JH10 cells were grown overnight on 1/2×, 1×, and 2× bacterial suspensions. Each culture was supplied with the same concentration of added PSF. The cells were harvested the next morning at a density of ~2 × 10^5 cells/ml and assayed for discoidin-I. The mutant and control cells were very similar in the timing and extent of density-dependent induction of discoidin-I, and in the inhibition of this response by bacteria. (B) Effect of folate. The mutant and control cells were grown overnight on 1/2× bacterial suspensions in the presence and absence of 1 mM folic acid (FA). All cultures contained an equal concentration of added PSF. The cultures were harvested at a density of ~2 × 10^5 cells/ml and assayed for discoidin-I. Although folate blocked discoidin-I expression in the JH10 cells, it had no effect on the $g\beta^-$ cells. In both experiments, cells were assayed for discoidin-I production by indirect immunofluorescence, and the percentage of the cells positive for discoidin-I is plotted.

ty-dependent induction of discoidin-I very late during exponential growth, as is characteristic of axenic strains. In addition, low density mutant cells exhibited a normal dose-dependent response to added PSF, and this response was inhibited by the presence of bacteria (Figure 8A). Thus, the Gβ subunit is not required to enable *Dictyostelium* cells to respond to PSF, nor to allow bacteria to inhibit this response.

The prestarvation response of $g\beta^-$ cells did differ from that of control cells in one important respect, namely, a lack of inhibition by folate. Figure 8B shows induction of discoidin-I synthesis in $g\beta^-$ cells and in their axenic parent strain, JH10, in the presence and absence of 1 mM folate. Folate had no effect on the mutant cells, whereas the control cells exhibited normal sensitivity to folate. This result shows that the inhibitory effect of folate on discoidin-I production requires a heterotrimeric G-protein. It also clearly demonstrates that folate cannot account for the inhibitory action of bacteria, which persists in cells that are insensitive to folate.

Protein Kinase A Function May Determine the Sensitivity of Individual Cells to the Prestarvation Response. The cAMP-dependent protein kinase, or protein kinase A, is required for aggregation of *Dictyostelium* cells (Mann and Firtel, 1991; Mann *et al.*, 1992) and for expression of at least one component of the cAMP signaling pathway, the PDE inhibitor (G. Podgorski, personal communication). We therefore examined a mutant strain in which the gene for the catalytic subunit of protein kinase A had been disrupted (Mann and Firtel, 1991; Mann *et al.*, 1992). Mutant cells were grown on a 1× suspension of bacteria and tested for discoidin-I production at low and high cell densities, using the parental strain JH10 as a control. Few pka^- cells responded to PSF. Even at very high densities (7 × 10^6 to 1 × 10^7 cells/ml), only about 3% of the pka^- cells were positive for discoidin-I by immunofluorescence. To test whether the pka^- cells might respond normally to higher PSF levels, pka^- and JH10 cells were grown to high density in the presence of buffer that had been conditioned by high density AX2 cells. This effectively doubled the PSF concentration to which the cells were exposed. Under these conditions, the control cells were all positive (99%) by the time they had reached a density of 7 × 10^6 cells/ml, but the pka^- cells were still only 3% positive at this density (Figure 9B and C). We also examined cells from a high density culture that had been shifted to starvation conditions for 6 h; starvation produced no increase in the fraction of discoidin-positive cells.

Although most pka^- cells were unresponsive, the behavior of the small number of discoidin-positive cells suggested that these cells were in fact responding to PSF. First, buffer conditioned by high density pka^- cells contained normal levels of PSF activity when tested on low density NC4 cells (our unpublished observations), so PSF production was not affected by the protein kinase A deficiency. Second, induction was dependent on cell density, because all cells in a low density culture were negative (Figure 9A). Third, the fraction of positive cells in a high density culture was diminished when the cells were grown on a higher concentration of bacteria, as is typical for the prestarvation response (our unpublished observations). Fourth, the ability of a small fraction of the cells to be induced (about 3%) persisted in a clonal isolate of the original population; it is this clonal isolate that is shown in all panels of Figure 9. Thus, the difference between responsive and nonresponsive cells did not appear to be genetic.

Finally, Northern blot analysis indicated that discoidin-I and PDE expression in pka^- cells were both regulated transcriptionally (Figure 10). High density, bacterially grown cells were examined under the same conditions that had been used in the experiment

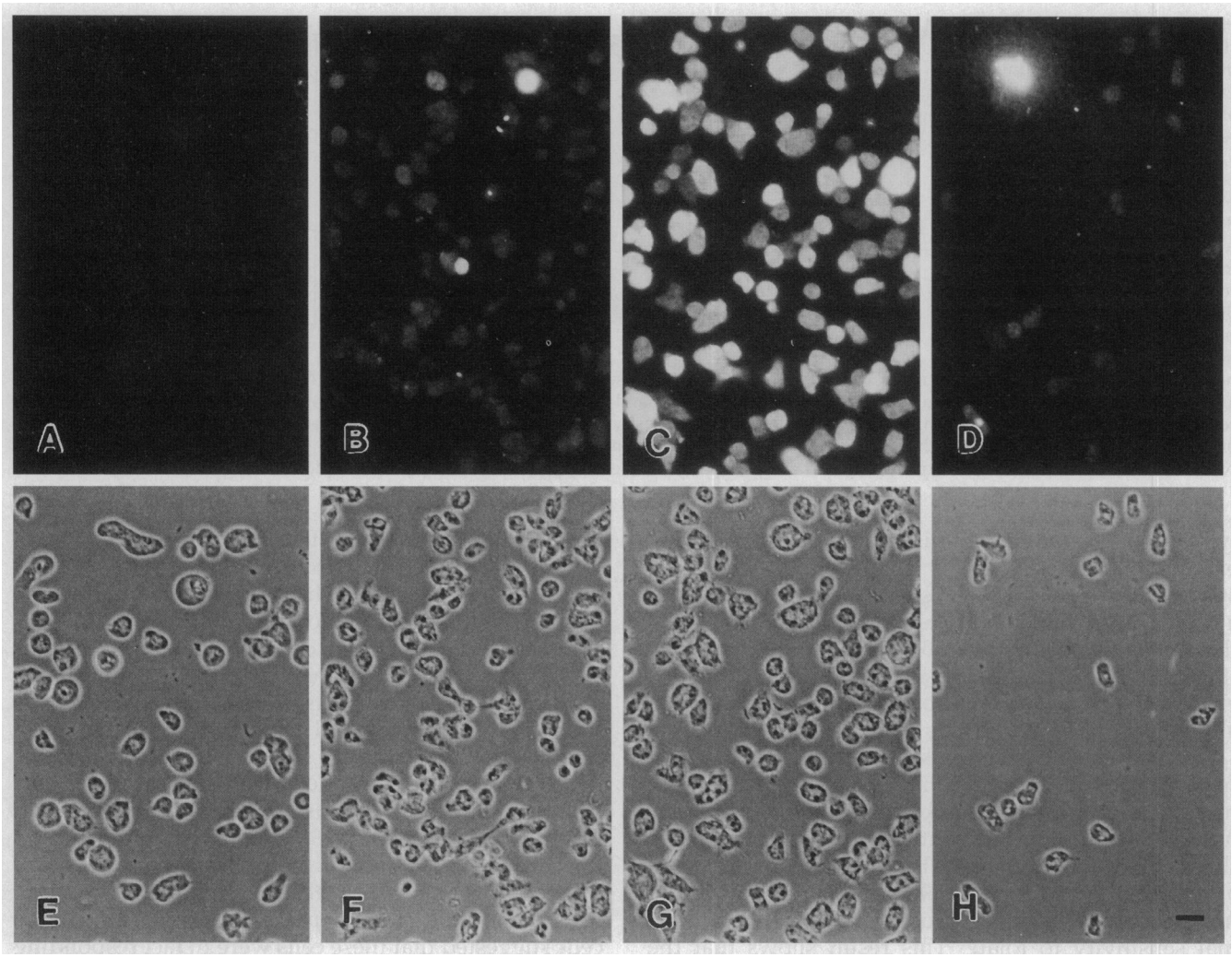

Figure 9. Density-dependent expression of discoidin-I protein in *pka*$^-$ cells. The first three panels in each row show mutant and control cells grown on 1× bacteria. (A and E) Low density *pka*$^-$ cells (2×10^5/ml). (B and F) High density *pka*$^-$ cells (7×10^6/ml), grown in the presence of buffer conditioned by high density AX2 cells. (C and G) High density JH10 cells (7×10^6/ml), grown in the presence of buffer conditioned by high density AX2 cells. The fourth panel in each row (D and H) shows axenically grown *pka*$^-$ cells at high cell density (5×10^6/ml). Whether grown on bacteria or axenic medium, only 3% of the *pka*$^-$ cells manifested a density-dependent induction of discoidin-I protein. Bar, 20 μm.

shown in Figure 4. In *pka*$^-$ cells, the PDE probe did not detect any of the 2.4-kb PSF-regulated transcript, although the 1.9-kb transcript was present at normal levels. When the same blot was re-probed for discoidin-I, no signal was detected under standard conditions, although prolonged exposure revealed a very faint signal. As before, the blot was also re-probed for calmodulin, to confirm that equal amounts of RNA had been loaded in each lane. Note that the expression of discoidin-I by a small fraction of the population, clearly evident when single cells were assayed by antibody staining, was almost undetectable by this technique. Therefore, the possibility remains open that a small fraction of the cells were also expressing the less abundant 2.4-kb PDE transcript.

These striking results obtained with bacterially grown cells prompted us also to examine *pka*$^-$ cells that were growing axenically. The *pka*$^-$ cells were sampled during late exponential growth on HL5 medium, at a density 5×10^6 cells/ml. Just as we had observed for cells growing on bacteria, only a small fraction of the axenically growing *pka*$^-$ cells (~3%) were positive for discoidin-I (Figure 9D). Their staining was very intense, and many of the positive cells were surrounded by a cloud of stained material on the substratum, suggesting that these cells were producing and secreting high levels of discoidin-I protein. However, under axenic as well as bacterial growth conditions, the fraction of the *pka*$^-$ population that responded to PSF was very small. Thus, axenic growth

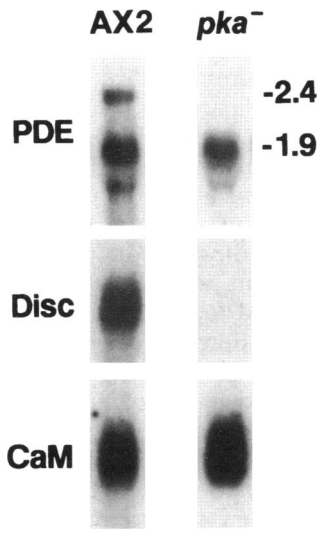

Figure 10. Northern blot of PSF-regulated transcripts in *pka*⁻ cells. AX2 and *pka*⁻ cells were grown on 1× bacteria and harvested at a density of 7.5×10^6 cells/ml. A Northern blot of total RNA was probed to detect PDE transcripts and discoidin-I. The *pka*⁻ cells accumulated normal levels of the 1.9-kb PDE transcript, but the PSF-induced 2.4-kb transcript was undetectable. The discoidin-I transcript, although not visible here, gave a faint signal upon prolonged exposure. The blot was also probed for calmodulin mRNA to confirm that equal amounts of RNA had been loaded for each sample.

conditions did not render a larger fraction of the *pka*⁻ population responsive to PSF, nor induce discoidin-I synthesis by some independent mechanism.

Our data indicate that protein kinase A plays an important role in the prestarvation response. However, the results suggest to us that protein kinase A is not directly required for transduction of the PSF signal (because some cells in a clonal population do appear to be responding to PSF). Rather, we speculate that the protein kinase A is required for cellular events manifested at the population level as a high fraction of cells able to carry out the prestarvation response under the appropriate conditions (see DISCUSSION).

Cyclic AMP Is Not Involved in the Prestarvation Response. The finding that protein kinase A is important in the prestarvation response raised the question of whether cAMP is also involved, as the activator of this enzyme. In aggregating *Dictyostelium* cells, adenylyl cyclase is the product of the *aca* gene, and a mutant strain has been generated in which this gene has been disrupted (Pitt *et al.*, 1992). We examined the *aca*⁻ strain for density-dependent production of discoidin-I and found that its behavior was like that of other axenic strains (see Figure 2), rather than like the *pka*⁻ cells. Thus, normal adenylyl cyclase activity is not required for the function of protein kinase A in the prestarvation response

DISCUSSION

The Effects of the Axenic Genotype on the Prestarvation Response

Elucidation of regulatory mechanisms affecting the prestarvation response has gained new significance with the use of the discoidin-Iγ promoter to control expression of heterologous DNA sequences in *Dictyostelium* cells (Blusch *et al.*, 1992; Liu *et al.*, 1992; Wetterauer *et al.*, 1993; Luo *et al.*, 1994). This inducible promoter is commonly utilized in strains having an axenic genotype, because of the ease of selecting transformants from axenically growing populations. For similar reasons, the large number of *Dictyostelium* mutants in which a specific gene has been inactivated by gene targeting techniques are all derived from axenic parents. It is therefore important to extend the analysis of the prestarvation response, heretofore characterized in wild-type cells, to axenic strains.

This study revealed substantial differences in the prestarvation response between wild-type and axenic strains, even when the two types of cells were both examined during exponential growth on bacteria. Axenic strains produced two- to threefold higher levels of PSF activity than NC4 cells under these conditions. Nonetheless, all strains capable of axenic growth manifested a substantial delay in the density-dependent induction of discoidin-I expression during growth on bacteria. This was true not only for AX2 and AX3 and their derivatives, but also for an independently isolated axenic variant of NC4, strongly suggesting that this property is linked to the mutations that allow axenic growth. However, a second PSF-regulated transcript, the 2.4-kb PDE transcript, was more strongly induced in AX2 than in NC4 at the same cell density, consistent with the higher PSF levels produced by the AX2 cells. Thus, the delayed expression of discoidin-I in axenic strains appears to be due to an additional level of negative regulation conferred, perhaps specifically, on this gene family by the axenic genotype. Negative regulation of discoidin-I is also evident in cells growing axenically; it is reflected in a pronounced decline in discoidin-I mRNA levels as the cells reach the end of exponential growth and approach stationary phase (our unpublished observations).

Nellen and co-workers have identified three regulatory elements in the promoter region of the discoidin-Iγ gene of AX2, these elements being defined by their effects on the expression of a reporter gene, chloramphenicol acetyltransferase. One of these elements, the dIE, was shown to be sufficient to confer developmental activation on the reporter gene (Vauti *et al.*, 1990). Subsequent collaborative studies indicated that in vegetative cells the dIE responds to partially purified PSF (Nellen and Clarke, unpublished observations). A second element, the dCRE, accounted for negative regulation by cAMP, and a third element, the dAE or dAXE, was sufficient for discoidin-I expression during growth on axenic medium (Vauti *et al.*, 1990; Blusch *et al.*, 1992). The latter element, which responds to axenic growth conditions, may also be the target of the inhibitory effects conferred by the axenic genotype. However, the axenic mutations are too poorly characterized to allow meaningful speculation about

possible mechanisms. The relevant genes have not been cloned, and their gene products are undefined. (See Clarke and Kayman, 1987, for a review of the axenic mutations). At present, the only certain conclusion is that discoidin-I is subject to multiple regulatory factors, both genetic and physiologic. Thus, the pattern of expression of a heterologous transcript controlled by a discoidin-I promoter is best defined in the transformed strain and under the growth conditions of the individual experiment.

Heterotrimeric G-Proteins and the Prestarvation Response

Several potential elements of the PSF signal transduction pathway were examined in the present study. Many signal transduction pathways in *Dictyostelium* are routed through heterotrimeric G-proteins (reviewed by Devreotes, 1994). There are eight G-protein α subunits expressed in *Dictyostelium* cells, each of which may have a distinct function. The Gα2 subunit is required for cAMP-mediated responses (Kumagai *et al.*, 1991). The Gα4 subunit is required for chemotactic responsiveness to folate (Hadwiger *et al.*, 1994). In addition, the subunits Gα1 (Kumagai *et al.*, 1991) and Gα3, whose functions are not yet known, are expressed at high levels during vegetative growth (Devreotes, 1994) and thus appeared to be possible transducers of the PSF signal. However, examination of null mutants for each of these four Gα subunits indicated that none was required for PSF-mediated induction of discoidin-I expression (for Gα2, also see Blusch *et al.*, 1995). Similarly, mutant cells lacking the Gβ subunit that is thought to be common to all heterotrimeric G-proteins in *Dictyostelium* (Lilly *et al.*, 1993) responded to PSF just as well as the parental JH10 cells, arguing that the PSF signal is not transduced by any heterotrimeric G-protein.

Analysis of cells deficient in the Gβ subunit clarified the relationship between two inhibitors of the prestarvation response, bacteria and the bacterial metabolite folate. Nellen's group had reported that exogenous folate could repress transcription from the discoidin-Iγ promoter under axenic growth conditions via both the dIE and the dAXE, and had suggested that this suppression might mimic that seen in the presence of bacteria (Blusch *et al.*, 1992). We showed here that folate inhibition also occurs in wild-type cells (and thus does not require the axenic genotype), and that it is mediated by a heterotrimeric G-protein, because folate has no effect on $g\beta^-$ cells. Collaborative studies with Drs. R. Firtel and W. Nellen (unpublished data) have determined that the G-protein α subunit involved in folate repression is Gα4, the same subunit that Hadwiger and co-workers (1994) identified as essential for chemotaxis toward folate. Importantly, our studies of the $g\beta^-$ cells demonstrated that the inhibitory effect of bacteria is not mediated by folate, because discoidin-I expression in the $g\beta^-$ cells was still suppressed by bacteria, and to the same extent as in the parental JH10 cells.

The basis of bacterial inhibition of the prestarvation response was further investigated. A comparison of the effects of living and dead bacteria indicated that both types are effective repressors of PSF-mediated discoidin-I production, ruling out any labile bacterial product as the active agent. In addition, there appeared to be a correspondence between the extent of inhibition and the binding of bacteria to the *Dictyostelium* plasma membrane. This correlation has also appeared in experiments using *Dictyostelium* dysphagia mutants. Dysphagia mutants are able to bind normally to bacteria, but are severely impaired in the ability to phagocytose them (Cohen *et al.*, 1994). Preliminary studies of these mutants have suggested that PSF-mediated discoidin-I synthesis is inhibited in these cells by bacteria in a concentration-dependent manner (our unpublished observations). These experiments were inconclusive because the mutant cells grow so poorly under our standard assay conditions (i.e., on bacterial suspensions), but the data are consistent with a mechanism in which binding alone is sufficient to account for the inhibitory effect of bacteria. This model will be tested more directly in future experiments by assaying bacterial cell envelopes and envelope components for inhibitory activity.

The Absence of Protein Kinase A Reveals a New Level of Complexity in the Prestarvation Response

An unexpected and intriguing result in the present study was the nature of the prestarvation response in cells lacking the catalytic subunit of protein kinase A. Most of the *pka*$^-$ cells showed no response to PSF, indicating that protein kinase A plays an important role in the prestarvation response. However, in a small fraction of the clonal cell population, discoidin-I synthesis was induced in what appeared to be a normal PSF-dependent manner. The responsive cells were easily detected by immunostaining of individual cells, although not by assays based on cell population, such as Northern blots.

The existence of both refractory and responsive cells in the same clonal population is a characteristic of the normal prestarvation response. A striking feature of PSF-mediated discoidin-I induction in wild-type cells, as detected for individual cells by immunostaining, is its non-uniformity (e.g., see Figure 5). At sufficiently high levels of PSF, all cells in a normal population produce discoidin-I. However, at intermediate levels of PSF, some cells respond strongly (i.e., bright immunofluorescence), some weakly, and others not at all. This same heterogeneity is observed in NC4 populations allowed to grow to high density, such that dis-

coidin-I production is triggered by the accumulation of endogenously produced PSF. Under these conditions (as shown in Figure 2), three to four cell generations elapse between the time the first and last cells in the NC4 population become positive. In each of these situations, for a given PSF concentration, some cells are capable of responding and others are not. Note that this difference persists across several cell generations and thus cannot be attributed, for example, to cell cycle stage at the time when a critical concentration of PSF is reached.

It is against this backdrop that the behavior of the *pka*$^-$ cells must be considered. Although only a small fraction of the *pka*$^-$ population initiates discoidin-I synthesis, these cells do so in a manner that resembles the behavior of normal cells. That is, the production of discoidin-I is density dependent, is stimulated by conditioned buffer, and is repressed by bacteria. All of these properties suggest that this fraction of the population is responding to PSF. If so, protein kinase A cannot be directly required for transducing the PSF signal. Instead, in its absence, most of the cell population may be trapped in a refractory state. This model would suggest that there are (at least) two steps required for the prestarvation response, an initial potentiation step involving protein kinase A, which renders the cells sensitive (or increases their sensitivity) to PSF, and a second step, the actual transduction of the PSF signal.

Of course, alternative models can be formulated. For example, protein kinase A might directly transduce the PSF signal, but there might exist an alternative, inefficient mechanism for inducing the expression of discoidin-I (and perhaps other prestarvation response genes) independently of PSF. Because neither conditioned buffer nor partially purified PSF is a single molecular species, this pathway could plausibly depend on some other component of conditioned buffer, accounting for the density effect. At present there are insufficient data to distinguish among these (and other) possibilities, although we are inclined to favor the first model because it is consistent with the behavior of the wild-type population. Whatever the mechanism, it is clear that the function of protein kinase A is not restricted to its essential roles in regulating differentiation during the developmental stage of the *Dictyostelium* life cycle (Harwood *et al.*, 1992; Mann and Firtel, 1993). In addition, it is required to enable growing cells to respond properly to PSF.

It is noteworthy that there was no increase in the fraction of discoidin-positive cells when the *pka*$^-$ cells were grown axenically. A different outcome might have been expected, because there is reported to be a second induction element for discoidin-I activated by axenic medium, the dAXE (Vauti *et al.*, 1990). Our result implies either that activation via the dAXE is absolutely dependent on protein kinase A, or that axenic induction of discoidin-I through this element does not occur independently of PSF. The positive cells did appear to be hyperinduced by the axenic medium, consistent with a further activation of cells already responding to PSF.

Our study adds to other observations (Mann and Firtel, 1993; Pitt *et al.*, 1993; Insall *et al.*, 1994) suggesting that in *Dictyostelium* cells, protein kinase A is capable of acting independently of cAMP. We found that the absence of a functional adenylyl cyclase gene does not impair the prestarvation response, in contrast to the severe consequences of the lack of protein kinase A activity. Thus, the function of protein kinase A in the prestarvation response is not dependent on cAMP. As previously suggested (Pitt *et al.*, 1993), the cAMP-independent activity of *Dictyostelium* protein kinase A might be a consequence of the loose association between its catalytic and regulatory subunits (Anjard *et al.*, 1992), or might indicate that there is another means of activating this enzyme.

Our analysis has not yet revealed the pathway by which the PSF signal is transduced. We are now approaching this question by seeking mutants that are deficient in the production of or response to PSF. We are using a mutant strain that lacks the G protein β subunit (and is therefore unable to develop) as the target for insertional mutagenesis ("REMI"; Kuspa and Loomis, 1994). Transformants are being screened to identify mutants that do not express discoidin-I during growth on bacteria. Among the discoidin-minus cells should be mutants defective in the prestarvation response. We hope that their analysis will help to elucidate the mechanism by which the cells become capable of responding to PSF, and to identify elements of the PSF signal transduction pathway, including PSF itself.

ACKNOWLEDGMENTS

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REFERENCES

Anjard, C., Pinaud, S., Kay, R.R., and Reymond, C.D. (1992). Overexpression of DdPK2 protein kinase causes rapid development and

affects the intracellular cAMP pathway of *Dictyostelium discoideum*. Development *115*, 785–790.

Bernstein, R.L., Tabler, M., Vestweber, D., and Van Driel, R. (1981). Extracellular folate deaminase of *Dictyostelium discoideum*. Biochim. Biophys. Acta *677*, 295–302.

Blusch, J., Alexander, S., and Nellen, W. (1995). Multiple signal transduction pathways regulate discoidin-I gene expression in *Dictyostelium*. Differentiation (*in press*).

Blusch, J., Morandini, P., and Nellen, W. (1992). Transcriptional regulation by folate: inducible gene expression in *Dictyostelium* transformants during growth and early development. Nucleic Acids Res. *20*, 6235–6238.

Clarke, M., Dominguez, N., Yuen, I.S., and Gomer, R.H. (1992). Growing and starving *Dictyostelium* cells produce distinct density-sensing factors. Dev. Biol. *152*, 403–406.

Clarke, M., and Kayman, S.C. (1987). The axenic mutations and endocytosis in *Dictyostelium*. Methods Cell Biol. *28*, 157–176.

Clarke, M., Kayman, S.C., and Riley, K. (1987). Density-dependent induction of discoidin-I synthesis in exponentially-growing cells of *Dictyostelium discoideum*. Differentiation *34*, 79–87.

Clarke, M., Yang, J., and Kayman, S.C. (1988). Analysis of the prestarvation response in growing cells of *Dictyostelium discoideum*. Dev. Genet. *9*, 315–326.

Cohen, C.J., Bacon, R., Clarke, M., Joiner, K., and Mellman, I. (1994). *Dictyostelium discoideum* mutants with conditional defects in phagocytosis. J. Cell Biol. *126*, 955–966.

Crowley, T.E., Nellen, W., Gomer, R.H., and Firtel, R.A. (1985). Phenocopy of discoidin-I-minus mutants by antisense transformation of *Dictyostelium*. Cell *43*, 633–641.

Devreotes, P.N. (1994). G protein-linked signalling pathways control the developmental program of *Dictyostelium*. Neuron *12*, 235–241.

De Wit, R.J.W. (1982). Two distinct types of cell surface folic acid-binding proteins in *Dictyostelium discoideum*. FEBS Lett. *150*, 445–448.

Faure, M., Franke, J., Hall, A.L., Podgorski, G.J., and Kessin, R.H. (1990). The cyclic nucleotide phosphodiesterase gene of *Dictyostelium discoideum* contains 3 promoters specific for growth, aggregation, and late development. Mol. Cell. Biol. *10*, 1921–1930.

Franke, J., and Kessin, R.H. (1992). The cyclic nucleotide phosphodiesterases of *Dictyostelium discoideum:* molecular genetics and biochemistry. Cell Signal. *4*, 471–478.

Franke, J., Podgorski, G.J., and Kessin, R.H. (1987). The expression of two transcripts of the phosphodiesterase gene during the development of *Dictyostelium discoideum*. Dev. Biol. *124*, 504–511.

Freeze, H.H., Mierendorf, R.C., Wunderlich, R., and Dimond, R.L. (1984). Sulfated oligosaccharides block antibodies to many *Dictyostelium discoideum* acid hydrolases. J. Biol. Chem. *259*, 10641–10643.

Gomer, R.H., Yuen, I.S., and Firtel, R.A. (1991). A secreted 80×10^3 M_r protein mediates sensing of cell density and the onset of development in *Dictyostelium*. Development *112*, 269–278.

Hadwiger, J.A., Lee, S., and Firtel, R.A. (1994). The Gα subunit Gα4 couples to pterin receptors and identifies a novel signaling pathway that is essential for multicellular development in *Dictyostelium*. Proc. Natl. Acad. Sci. USA *91*, 10566–10570.

Harlow, E., and Lane, D. (1988). Antibodies. A Laboratory Manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Harwood, A.J., Hopper, N.A., Simon, M.N., Bouzid, S., Veron, M., and Williams, J.G. (1992). Multiple roles for cAMP-dependent protein kinase during *Dictyostelium* development. Dev. Biol. *149*, 90–99.

Insall, R., Kuspa, A., Lilly, P.J., Shaulsky, G., Levin, L.R., Loomis, W.F., and Devreotes, P. (1994). CRAC, a cytosolic protein containing a pleckstrin homology domain, is required for receptor and G protein-mediated activation of adenylyl cyclase in *Dictyostelium*. J. Cell Biol. *126*, 1537–1545.

Jain, R., and Gomer, R.H. (1994). A developmentally regulated cell surface receptor for a density-sensing factor in *Dictyostelium*. J. Biol. Chem. *269*, 9128–9136.

Jain, R., Yuen, I.S., Taphouse, C.R., and Gomer, R.H. (1992). A density-sensing factor controls development in *Dictyostelium*. Genes Dev. *6*, 390–400.

Kayman, S.C., Birchman, R., and Clarke, M. (1988). *motA*1552, a mutation of *Dictyostelium discoideum* having pleiotropic effects on motility and discoidin I regulation. Genetics 118, 425–436.

Knecht, D.A., Dimond, R.L., Wheeler, S., and Loomis, W.F. (1984). Antigenic determinants shared by lysosomal proteins of *Dictyostelium discoideum*. J. Biol. Chem. *259*, 10633–10640.

Kumagai, A., Hadwiger, J.A., Pupillo, M., and Firtel, R.A. (1991). Molecular genetic analysis of two G α protein subunits in *Dictyostelium*. J. Biol. Chem. *266*, 1220–1228.

Kuspa, A., and Loomis, W.F. (1994). Transformation of *Dictyostelium*. Gene disruptions, insertional mutagenesis, and promoter traps. In: Methods in Molecular Genetics, vol. 3, New York: Academic Press, 3–21.

Lilly, P., Wu, L.J., Welker, D.L., and Devreotes, P.N. (1993). A G-protein β-subunit is essential for *Dictyostelium* development. Genes Devel. *7*, 986–995.

Liu, T., Williams, J.G., and Clarke, M. (1992). Inducible expression of calmodulin antisense RNA in *Dictyostelium* cells inhibits the completion of cytokinesis. Mol. Biol. Cell *3*, 1403–1413.

Loomis, W.F. (1975). *Dictyostelium discoideum*. A Developmental System, New York: Academic Press.

Loomis, W.F. (ed.) (1982). The Development of *Dictyostelium discoideum*, New York: Academic Press.

Luo, Q., Michaelis, C., and Weeks, G. (1994). Overexpression of a truncated cyclin B gene arrests *Dictyostelium* cell division during mitosis. J. Cell Sci. *107*, 3105–3114.

Mann, S.K.O., and Firtel, R.A. (1991). A developmentally regulated, putative serine/threonine protein kinase is essential for development in *Dictyostelium*. Mech. Dev. *35*, 89–101.

Mann, S.K.O., and Firtel, R.A. (1993). cAMP-dependent protein kinase differentially regulates prestalk and prespore differentiation during *Dictyostelium* development. Development. *119*, 135–146.

Mann, S.K.O., Yonemoto, W.M., Taylor, S.S., and Firtel, R.A. (1992). DdPK3, which plays essential roles during *Dictyostelium* development, encodes the catalytic subunit of cAMP-dependent protein kinase. Proc. Natl. Acad. Sci. USA *89*, 10701–10705.

Mehdy, M.C., and Firtel, R.H. (1985). A secreted factor and cyclic AMP jointly regulate cell-type specific gene expression. Mol. Cell Biol. *5*, 705–713.

Mierendorf, R.C., Cardelli, J.A., Livi, G.P., and Dimond, R.L. (1983). Synthesis of related forms of the lysosomal enzyme α-mannosidase in *Dictyostelium discoideum*. J. Biol. Chem. *258*, 5878–5884.

Miller, J.H. (1972). Experiments in Molecular Genetics, Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Morrison, A., and Harwood, A. (1992). Simple method of generating axenic derivatives of *Dictyostelium* strains. Exp. Cell Res. *199*, 383–386.

Nandini-Kishore, S.G., and Frazier, W.A. (1981). [^{3}H]methotrexate as a ligand for the folate receptor of *Dictyostelium discoideum*. Proc. Natl. Acad. Sci. USA *78*, 7299–7303.

Pitt, G.S., Brandt, R., Lin, K.C., Devreotes, P.N., and Schaap, P. (1993). Extracellular cAMP is sufficient to restore developmental gene expression and morphogenesis in *Dictyostelium* cells lacking the aggregation adenylyl cyclase (ACA). Genes Dev. *7*, 2172–2180.

Pitt, G.S., Milona, N., Borleis, J., Lin, K.C., Reed, R.R., and Devreotes, P.N. (1992). Structurally distinct and stage-specific adenylyl cyclase genes play different roles in *Dictyostelium* development. Cell *69*, 305–315.

Rathi, A., and Clarke, M. (1992). Expression of early developmental genes in *Dictyostelium discoideum* is initiated during exponential growth by an autocrine-dependent mechanism. Mech. Dev. *36*, 173–182.

Rathi, A., Kayman, S.C., and Clarke, M. (1991). Induction of gene expression in *Dictyostelium* by prestarvation factor, a factor secreted by growing cells. Dev. Genet. 12, 82–87.

Saxe, C.L., III, Johnson, R.L., Devreotes, P.N., and Kimmel, A.R. (1991). Expression of a cAMP receptor gene of *Dictyostelium* and evidence for a multigene family. Genes Dev. *5*, 1–8.

Vauti, F., Morandini, P., Blusch, J., Sachse, A., and Nellen, W. (1990). Regulation of the discoidin Iγ gene in *Dictyostelium discoideum*: identification of individual promoter elements mediating induction of transcription and repression by cyclic AMP. Mol. Cell. Biol. *10*, 4080–4088.

Wetterauer, B.W., Salger, K., and MacWilliams, H.K. (1993). Use of a transactive regulatory mutant of *Dictyostelium discoideum* in a eukaryotic expression system. Nucleic Acids Res. *21*, 1397–1401.

Williams, J.G., Tsang, A.S., and Mahbubani, H. (1980). A change in the rate of transcription of a eukaryotic gene in response to cyclic AMP. Proc. Natl. Acad. Sci. USA *77*, 7171–7175.