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Developmental Regulation of the α-Mannosidase Gene in
Dictyostelium discoideum: Control Is at the Level of
Transcription and Is Affected by Cell Density

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In Dictyostelium discoideum, there is a group of genes that are expressed following starvation and when
exponentially growing cells reach high densities. We have examined the expression of one of these genes,
α-mannosidase. Using an α-mannosidase cDNA probe in Northern (RNA) blot analysis, we have shown that the
previously observed increase in α-mannosidase enzyme-specific activity during development is due to an
increase in the levels of α-mannosidase mRNA. mRNA levels reach a maximum by 8 h of development and then
begin to decline by 14 to 22 h. Using nuclear run-on analysis, we have found that this gene is regulated at the
level of transcription. We also examined the effects of cell-cell contacts, cyclic AMP levels, and protein synthesis
on expression of this gene and found that they were not critical in regulating its expression. However, cell
density did play a major role in the expression of α-mannosidase. High cell density or the presence of buffer
conditioned by high-density cells was sufficient to induce expression of α-mannosidase, indicating that this is
one of the starvation response genes. Finally, the α-mannosidase gene was not expressed in aggregation-
negative mutant strain HMW 404.

The proper progression of development in the simple eukaryote Dictyostelium discoideum depends on a variety
of factors, including starvation and a relatively high cell density (14, 25). The 24-h developmental cycle is characterized by
orderly, temporal changes in gene expression. Molecular mechanisms responsible for a number of these changes have
been elucidated, and both transcriptional and posttranscriptional mechanisms have been identified (8, 19, 26–28,
39, 41).

We and others have described a group of genes (α-mannosidase, discoidin I, and pCZ22) expressed very early in
development when early log-phase cells growing on a bacterial food source are starved to initiate development (1,
4, 13, 22, 24, 34). These genes are also expressed in cells growing axenically at densities greater than 106 cells per ml
and in bacterially grown cells in late log phase (2, 7, 36). Recent evidence indicates that expression of these genes is
dependent on production of a secreted factor (PSF or prestarvation factor) that is continuously synthesized by
cells during vegetative growth and during early development (6, 7). PSF is heat and protease sensitive and binds to
concanavalin A, suggesting that it is a glycoprotein (6, 7, 35). In growing cells, the prestarvation response is triggered
when the concentration of PSF is high relative to the concentration of food bacteria. Bacteria interfere with the
ability of cells to detect or respond to PSF, possibly by binding to or down regulating a cell surface receptor for PSF
(6). This would explain why cells growing in axenic broth (in the absence of bacteria) are able to respond to this factor at
a lower cell density (6).

One of the earliest developmentally controlled gene products to accumulate following starvation of cells previously
grown on bacteria is the lysosomal enzyme α-mannosidase-1 (3, 21, 24, 32). Enzyme-specific activity of α-mannosidase is
low (<0.5 U/mg) in amoebae growing exponentially on bacteria but begins to increase on starvation and accumu-
lates linearly throughout the 24-h developmental cycle, reaching a plateau of 40 to 50 U/mg by 20 h (22, 25). In
addition, the specific activity of α-mannosidase is high in cells growing in axenic broth culture at titers greater than 1
× 105 cells per ml and in bacterially growing cells at 5 × 106 cells per ml, thus identifying this gene product as one of the
prestarvation response class (2, 7). Although α-mannosidase is not itself required for development, enzyme accumu-
lation fails to occur in aggregation-negative mutants (21, 22). This suggests that cells reaching high density in the presence
of bacteria or in axenic broth culture are already expressing the earliest stages of the developmental program.

The observed increase in specific activity of α-mannosidase during development and in axenic cells at high titer is
due to an increase in the amount of functional mRNA as determined by in vitro translation assays (21). Here we show
that this increase is due to an increase in the steady-state levels of mRNA as determined by Northern (RNA) blot
analysis with a cDNA probe specific for α-mannosidase. The mechanism accounting for this increase in mRNA is tran-
scriptional in nature. In addition, we show density-dependent expression of the α-mannosidase gene in growing cells,
which confirms that it belongs to the prestarvation response class of genes.

MATERIALS AND METHODS

Organisms, growth, and developmental conditions. D. discoideum Ax3 or HMW 404 was grown in association with
Klebsiella aerogenes on SM agar medium at 21°C (42).
Vegetative amoebae were collected at a titer of $7 \times 10^6$ cells per cm$^2$ (approximately four generations before stationary phase), resuspended in cold morpholineethanesulfonic acid (MES)-PDF (8 mM MES, 0.7 mM CaCl$_2$, 20 mM MgCl$_2$, and 0.3 mM streptomycin sulfate [pH 6.5]), washed free of bacteria by repeated centrifugations (400 g resuspended in starvation buffer (25 mM Na$_2$HPO$_4$, 8.8 mM KH$_2$PO$_4$) at a density of $5 \times 10^6$ cells per ml. Filters were incubated at 23°C under constant illumination, and cells were harvested at various times during development. Suspension cultures were shaken at 120 rpm in 21°C water baths, and cells were harvested at the indicated times. Alternatively, cells were harvested from bacteria and resuspended in growth medium (TM) at a titer of $5 \times 10^6$/ml and then collected at various times following resuspension (9). Cells of the wild-type strain NC4 were grown on a suspension of K. aerogenes. Conditions for cell growth and preparation of conditioned buffer were as previously described (36).

**Enzyme activity determination.** At the indicated times, $10^7$ cells were harvested and resuspended in 500 µl of 0.5% Triton X-100. α-Mannosidase-1 activity was assayed by its ability to hydrolyze the substrate p-nitrophenyl-α-D-mannopyranoside (24). Specific activity is expressed as nanomoles of substrate hydrolyzed per minute per milligram of total protein. Protein concentrations were determined by using the Pierce BCA protein assay system with bovine serum albumin (BSA) as the standard.

**RNA extractions and Northern blot analysis.** At the indicated times, $10^8$ cells were harvested and RNA was extracted as previously described (4). RNA samples were fractionated on 1% formaldehyde agarose gels, the gels were rinsed in water, and the RNA was transferred to GeneScreen Plus nylon membranes by overnight capillary transfer in 10× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). Following transfer, the filters were baked at 80°C in a vacuum oven for 2 h. The membranes were stained with 0.5% methylene blue–0.3 M sodium citrate (pH 7.5) for 2 min and then examined to confirm that equal amounts of RNA were loaded and transferred for each sample (38). Prior to hybridization, the stain was removed by washing in 0.2× SSC–1% sodium dodecyl sulfate (SDS) for 20 min. Filters were then prehybridized in 1× P buffer (0.2% BSA, 0.2% polyvinylpyrrolidone, 0.2% Ficoll, 50 mM Tris [pH 7.5], 0.1% sodium PP) containing 1% SDS and 1 M NaCl for 4 h at 65°C. The probe used for Northern blot analysis was a cDNA of α-mannosidase (the details of this cDNA probe and its isolation will be presented in a separate publication which is currently in preparation) which was nick translated with $[^{32}P]dATP$ and was added at a concentration of $10^6$ cpm/ml of hybridization buffer. Hybridizations were performed overnight at 65°C and then followed by two washes (10 min each) in 2× SSC at room temperature and then 2 washes (30 min each) in 2× SSC–1% SDS at 65°C. Filters were wrapped in Saran wrap and exposed to Kodak XAR-5 film at $-70°C$.

**Determination of protein synthesis.** Following removal from bacteria, $1.5 \times 10^7$ cells were harvested and resuspended at a density of $5 \times 10^6$ cells per ml in starvation buffer alone or in starvation buffer containing 400 µg of cycloheximide per ml. Cells were then pulsed with 300 µCi of $[^{35}S]$methionine for 30 min or 1 h, after which time $5 \times 10^6$ cells were harvested and resuspended in 100 µl of 0.5% Triton X-100. Incorporation of radioactive amino acids was determined by hot trichloroacetic acid precipitation of the lysates: the precipitates were collected on filters, and radioactivity was measured by scintillation counting. Alternatively, samples were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE). Following electrophoresis, the gels were fixed in En3Hance and were then dried on a Bio-Rad gel dryer and exposed to Kodak XAR-5 film at $-70°C$. Laser densitometric analysis of these gels with an LKB 2202 Ultrascan attached to an Apple II computer was used to calculate incorporation of labeled amino acids into total proteins. The values obtained for samples treated with cycloheximide were compared with the values obtained for control cells, which represented 100% total incorporation of radiolabeled amino acids.

**Pulse-chase and immunoprecipitation.** Following removal from bacteria, cells were resuspended in either starvation buffer alone or starvation buffer containing 2 mM EDTA. At 4 h of development, $10^7$ cells were pulsed with 800 µCi of $[^{35}S]$methionine for 30 min and then chased with starvation buffer containing 1 mM unlabeled methionine for 30 min, 2 h, and 4 h. Cells were harvested immediately following the pulse and at the indicated chase times and resuspended in 0.5% Triton X-100. Equal amounts (counts per minute) of radiolabeled cellular proteins were immunoprecipitated with α-mannosidase-specific antibodies and subjected to SDS-PAGE and autoradiography as previously described (32).

**Nuclear run-on analysis.** Cells were harvested at the indicated times, nuclei were isolated, and nuclear run-on analysis was performed as previously described (33). Radiolabeled transcripts were then hybridized to linearized plasmids which had been slot blotted to nitrocellulose filters. Plasmids used were pSC79 (Dictyostelium actin genomic clone, kindly provided by C. Singleton), pM4 (α-mannosidase cDNA in pBluescript vector), and pBluescript alone. Hybridizations and washes were performed as outlined in the section concerning Northern blot analysis. Filters were exposed to Kodak XAR-5 film at $-70°C$, and autoradiographic signals were analyzed by laser densitometric analysis for quantitation of transcription levels. Transcription levels for α-mannosidase were normalized by comparing signals obtained at each time point with the signals obtained for actin. These normalized values were then compared with the value obtained at 0 h (vegetative growth), which was assigned a value of 1. These results were used to calculate the observed fold increase in α-mannosidase transcription.

**RESULTS**

Expression of the α-mannosidase gene under standard developmental conditions. Previous reports have shown that the specific activity of the lysosomal enzyme α-mannosidase is low in amoebae grown on bacteria but increases on starvation and accumulates linearly throughout the 24-h developmental cycle (24). This increase in specific activity correlates with an increase in biosynthesis of the 140-kDa enzyme precursor polypeptide and is paralleled by an increase in functional mRNA during development (21). We wanted to determine if this increase in functional mRNA also correlated with an increase in levels of total α-mannosidase mRNA. Figure 1A shows that the specific activity of α-mannosidase increased linearly during the developmental cycle and that the kinetics of accumulation of α-mannosidase were similar for cells developing in suspension and on filters. However, there was a decrease in α-mannosidase specific activity in cells developing in suspension between 14 and 22 h of development. We have previously observed an en
FIG. 1. Regulation of α-mannosidase during standard development. AX3 cells growing on a bacterial lawn were harvested, washed free of bacteria, and either plated for development on filters or resuspended in starvation buffer for suspension development. At the indicated times, cells were harvested, and cellular specific activity of α-mannosidase was determined (A) or RNA was extracted and used in Northern blot analysis with a radiolabeled α-mannosidase cDNA probe (B). Ten micrograms of total RNA was loaded onto each lane, and filters were stained after transfer of RNA as described in Materials and Methods. The top portion of panel B shows the result of Northern blot analysis with the α-mannosidase cDNA probe, and the bottom portion of the panel shows the stained membrane after RNA transfer to confirm equal loading of sample in each lane.

enhanced rate of α-mannosidase secretion during starvation in suspension which may account for this decrease (3).

Northern blot analysis of the RNA from these cells with a radiolabeled α-mannosidase-specific cDNA probe revealed hybridization to a single mRNA species of 3.6 kb (Fig. 1B) which was not present at 0 h (vegetative growth) and which increased in abundance in parallel with the increase in α-mannosidase specific activity during development. As determined by laser densitometric analysis, mRNA levels reached a maximum (20- to 30-fold increase) by 8 h of development and began to decline by 14 to 22 h. We also observed that levels of α-mannosidase mRNA began to increase by as early as 30 min following starvation (data not shown). The kinetics of accumulation of α-mannosidase mRNA were the same for both filter and suspension development conditions. Therefore, suspension development conditions were used to examine α-mannosidase expression because of the relative ease with which developmental conditions can be manipulated in suspension.

Effects of cell-cell contacts, cAMP, and protein synthesis on α-mannosidase expression. We next examined the effects of a variety of factors known to be important in developmental
gene regulation in D. discoideum on the accumulation of α-mannosidase mRNA. For example, the generation of intracellular signals in response to pulses of cyclic AMP (cAMP) plays a major role in regulating the expression of many genes during development (10, 15, 45). In order to examine the role of cAMP in α-mannosidase gene expression, cells previously growing on bacteria were placed in starvation buffer and exposed to cAMP pulses or continuously high levels of cAMP as described in Materials and Methods. Figure 2 shows that the kinetics of accumulation of α-mannosidase enzyme activity were essentially the same for cAMP-pulsed cells, for cells exposed to continuously high levels of cAMP, and for cells placed in starvation buffer alone. To determine if the specific activity increases also correlated with increases in mRNA levels, cells were treated as described above, and RNA was extracted and subjected to Northern blot analysis. Figure 3A shows that the accumulation of α-mannosidase activity correlated with increased levels of mRNA and that neither cAMP pulses nor the continuous presence of cAMP had any effect on the accumulation of α-mannosidase mRNA.

Several developmentally regulated genes have been shown to require protein synthesis for their expression and therefore may require the de novo synthesis of factors necessary for their induction (40). As indicated in Fig. 3B, cells starved in the presence of cycloheximide accumulated less α-mannosidase mRNA (approximately 40 to 50% of control levels as determined by laser densitometric analysis) than did control cells, although the kinetics of accumulation were similar. Figure 2 indicates that there was no accumulation of α-mannosidase enzyme activity during development, which is consistent with cycloheximide inhibition of protein synthesis. Additionally, protein synthesis rates were determined by measuring hot trichloroacetic acid-precipitable counts in cell lysates and by analysis of labeled proteins by using SDS-PAGE as described in Materials and Methods (data not shown). Using these approaches, we determined that greater than 95% of protein synthesis was inhibited by the concentration of cycloheximide that we used. Additional
experiments using 500 μM emetine revealed the same effect on α-mannosidase mRNA induction during development that we observed for cycloheximide, indicating that the reduced level of message seen was not due to toxic effects of cycloheximide or to nonspecific inhibition of transcription by this inhibitor (data not shown). These results show that while protein synthesis is required for the full induction of α-mannosidase, low levels of induction occur in the absence of protein synthesis.

Cell-cell contacts may also affect developmental gene expression in *D. discoideum* (23). We examined the expression of α-mannosidase in the absence of cell-cell contacts by harvesting cells growing on bacteria and resuspending them in starvation buffer containing 2 mM EDTA, which has been shown to disrupt cell-cell contacts early in the developmental cycle (11). Figure 2 shows that the presence of EDTA resulted in the inability of cells to accumulate α-mannosidase enzyme activity during development. However, cells accumulated α-mannosidase mRNA with kinetics similar to those of control cells (Fig. 3C). Therefore, cell-cell contacts are not important for the induction of α-mannosidase mRNA synthesis.

To determine whether EDTA generally reduces protein synthesis or specifically inhibits the accumulation of active α-mannosidase enzyme, cells growing on bacteria were harvested and resuspended in starvation buffer containing or lacking 2 mM EDTA. Cells were subjected 4 h later to radiolabeling and pulse-chase procedures followed by immunoprecipitation of α-mannosidase (see Materials and Methods). Figure 4 indicates that in cells placed in starvation buffer alone, α-mannosidase was synthesized as a 140-kDa precursor which was processed through an 80-kDa intermediate to a mature form of the enzyme consisting of equimolar amounts of 58- and 60-kDa subunits, which is in agreement with previous results (5, 37). A small percentage of the enzyme precursor was secreted into the medium (Fig. 4, bottom panels). However, in the presence of EDTA (Fig. 4, right panels) α-mannosidase precursor was inefficiently processed to mature forms and instead virtually all of the precursor was secreted into the medium. The level of precursor produced in these cells was lower than that seen in control cells, but we attribute this to a slight reduction in overall protein synthesis in cells treated with EDTA (data not shown). Therefore, while EDTA did not affect the synthesis of α-mannosidase mRNA or protein, it did have a dramatic effect on the processing and transport of this enzyme.

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**FIG. 3. Roles of various effectors on α-mannosidase mRNA accumulation.** Ax3 cells were prepared as described in the legend to Fig. 1 and resuspended in starvation buffer alone (SUSPENSION) or placed in continuously high cAMP conditions (Cont.) or cAMP pulse conditions (Pulses) as described in the legend to Fig. 2 (A). Cells were also resuspended in starvation buffer containing 400 μg of cycloheximide per ml (CHX), resuspended in axenic growth medium (TM) (B), or placed in starvation buffer containing 2 mM EDTA (C). Cells were harvested at the indicated times, and RNA was extracted and used in Northern blot analysis as described in the legend to Fig. 1.

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α-Mannosidase expression is affected by cell density. Previous reports have shown that α-mannosidase enzyme specific activity levels increase as cells growing axenically reach titers greater than 1 × 10⁶ cells per ml or when cells growing on a suspension of bacteria reach titers of 5 × 10⁵ cells per ml (2, 6). This suggests that α-mannosidase is regulated by PSF, a *D. discoideum* autocrine factor that is known to affect the expression of other early developmental genes (36). Therefore, we wanted to determine if the α-mannosidase gene would show the same pattern of expression as other prestarvation response genes. To facilitate comparison with earlier studies, NC4 cells were used, although other experiments in this study used Ax3, a derivative of NC4 that is able to grow on liquid nutrient medium (43). α-Mannosidase regulation during development has been shown to be similar at the protein level in both NC4 and Ax3 cells (2, 7, 21).

To test whether α-mannosidase expression was induced as part of the prestarvation response, RNA was extracted from exponentially growing low-density cells (3 × 10⁵ cells per ml) and high-density cells (5 × 10⁶ cells per ml). RNA was also extracted from low-density cells grown in the presence of buffer conditioned by high-density cells. Northern blot analysis of this RNA (Fig. 5) showed that α-mannosidase message was barely detectable with RNA from low-density cells (lane 1), whereas a strong signal was evident with RNAs from high-density cells (lane 3) and low-density cells grown in the presence of conditioned buffer (lane 2). The message levels were similar to that seen in cells that had been washed free of bacteria and starved for 4 h at high density (lane 4). These results confirm that α-mannosidase mRNA is induced in exponentially growing cells as part of the prestarvation response, and starvation is not required for this induction.

Experiments described in the previous section indicated that cycloheximide substantially reduced the induction of α-mannosidase mRNA. This effect could be due solely to the inhibition of PSF synthesis. If this were the case, addition of exogenous PSF (via conditioned buffer) should relieve this block. Figure 5B shows that conditioned buffer did not reverse the effects of cycloheximide. As previously observed, cycloheximide did not completely abolish the accumulation of α-mannosidase mRNA but greatly reduced the...
level of accumulation compared with that of control cells. Similar results have been obtained for other prestarvation response genes (35). Therefore, a lack of PSF synthesis does not sufficiently account for the inhibitory effect of cycloheximide on expression of these genes.

To establish that the cell density-dependent regulation of α-mannosidase expression was also common to Ax3 cells and that increases in cell density alone (as opposed to starvation) were sufficient to induce α-mannosidase, we harvested Ax3 cells previously growing on bacteria and resuspended them in either starvation buffer or TM at a density of 5 × 10^6 cells per ml. Figure 6 shows that α-mannosidase specific activity increased during standard development, as described above. Unexpectedly, when cells were resuspended in growth medium there was an increased level of α-mannosidase accumulation which was approximately 2.5 times higher by 8 h than that seen in starved cells. Northern blot analysis (Fig. 3B) indicated that this increase in enzyme activity correlated with an increase in mRNA levels. These results confirm that the accumulation of α-mannosidase mRNA occurs in high-density Ax3 cells in both growth and starvation conditions.

**α-Mannosidase expression is under transcriptional control.**

FIG. 5. Effects of cell density on α-mannosidase mRNA levels. NC4 cells were grown in suspension with bacteria as described in Materials and Methods. RNA was extracted and subjected to Northern blot analysis as described in the legend to Fig. 1. (A) Lane 1, RNA from cells growing at low density (3 × 10^5 cells per ml) in a bacterial suspension; lane 2, RNA from cells growing at low density in a bacterial suspension in the presence of conditioned buffer obtained from high-density cells (5 × 10^6 cells per ml); lane 3, RNA from cells growing at high density in a bacterial suspension; lane 4, RNA from cells resuspended in starvation buffer and harvested at 4 h of development. (B) Lane 1, RNA from cells growing at low density in a bacterial suspension in the presence of conditioned buffer from high-density cells and 400 μg of cycloheximide per ml; lane 2, RNA from cells growing at low density in a bacterial suspension in the presence of conditioned buffer from high-density cells (10 μg of total RNA was loaded for each lane).

FIG. 6. Cell density affects the accumulation of α-mannosidase enzyme activity. Ax3 cells were prepared as described in the legend to Fig. 1 and resuspended in either starvation buffer (●) or axenic growth medium (○). At the indicated times, cells were harvested and cellular α-mannosidase specific activity was determined.
The increased accumulation of mRNA which we observed could be due to either transcriptional or posttranscriptional control, and both mechanisms have been observed for a variety of genes which are differentially regulated in *D. discoideum* (19, 26-28, 39, 41). To distinguish between the two mechanisms, we used the nuclear run-on assay to determine the transcription rate of the α-mannosidase gene (30). Figure 7A shows that while transcription of actin mRNAs remained relatively constant during development, transcription of α-mannosidase mRNA increased and reached a maximum level by 4 h of development. α-Mannosidase transcription in cells resuspended in axenic growth medium was two to threefold higher than in starving cells, which accounts for the increased levels of mRNA that we observed (Fig. 3). Peak levels of transcription of α-mannosidase were observed by 4 h of development (12 to 15 times higher than those seen during vegetative growth on bacteria) and began to decline by 8 h (Fig. 7B). Thus, we conclude that the regulation of transcription is the major mechanism accounting for the observed increase in levels of α-mannosidase mRNA.

**Expression of α-mannosidase in a developmental mutant.** Although the α-mannosidase gene is induced in the same manner as other prestarvation response genes, its expression also appears to be linked to the further progression of the developmental program as evidenced by its inability to be expressed in mutants blocked very early in development (9, 20, 22). The aggregation-negative mutant HMW 404 is deficient in the synthesis of α-mannosidase following starvation and does not contain functional α-mannosidase mRNA as determined by in vitro translation assays (20). The lack of functional mRNA could be due to either improper transcription or splicing of the mRNA or to a level of transcription too low to be detected by in vitro translation assays. Therefore, we examined the levels of α-mannosidase mRNA in this mutant under developmental conditions by using Northern blot analysis. Figure 8 shows that unlike the parental strain AX3, the mutant was unable to accumulate any detectable α-mannosidase mRNA following starvation.

**FIG. 7.** α-Mannosidase is regulated at the transcriptional level. AX3 cells were prepared as described in the legend to Fig. 1 and resuspended in either starvation buffer (SUSPENSION) or axenic growth medium (TM). At the indicated times, cells were harvested and nuclei were isolated and used in nuclear run-on assays as described in Materials and Methods. Labeled RNA from these assays was hybridized to linearized plasmids that had been slot blotted to filters. (A) Results of nuclear run-on assays when radiolabeled RNA was hybridized to plasmids containing actin genomic sequences (ACTIN), α-mannosidase cDNA sequences (pM4), or vector sequences alone (VECTOR). (B) Fold increases in transcription of α-mannosidase when hybridization signals for α-mannosidase were normalized to signals obtained for actin and then compared with the normalized signal for 0 h, which was assigned a value of 1.

**FIG. 8.** α-Mannosidase mRNA does not accumulate in a developmental mutant. AX3 (wild type) and HMW 404 (developmental mutant) cells were prepared as described in the legend to Fig. 1 and were plated for development on filters. Cells were harvested at the indicated times, and RNA was extracted and subjected to Northern blot analysis as described in the legend to Fig. 1.
DISCUSSION

In this report, we describe the developmental regulation of the gene coding for the lysosomal enzyme α-mannosidase-1. We have demonstrated that the increase in enzyme-specific activity of α-mannosidase during development in *D. discoideum* is due to increased transcription of this gene, which results in at least a 20- to 30-fold increase in levels of α-mannosidase mRNA. In cells shifted from low-density growth to high-density starvation conditions, the increase in transcription occurred within 1 h, reached a maximum by 4 h of development, and began to decline by 14 to 22 h. The key factor regulating transcription of this gene proved to be high cell density. Starvation, while possibly involved, was not required for induction. This pattern of regulation identifies α-mannosidase as a member of the prestarvation response class of genes (36).

Some genes expressed as part of the prestarvation response are also regulated by cAMP pulses in later development (35). However, levels of α-mannosidase enzyme activity and mRNA in cells exposed to cAMP pulses or continuously high levels of cAMP were essentially the same as in control cells. Thus, cAMP appears not to play a secondary role in regulating α-mannosidase gene expression.

The possibility that cell-cell contacts might affect α-mannosidase gene expression was also examined by using EDTA to interfere with contacts early in development. EDTA had little effect on the accumulation of α-mannosidase mRNA, indicating that expression of this gene does not require cell-cell contacts. However, α-mannosidase enzyme activity did not accumulate in EDTA-treated cells. Further examination indicated that EDTA led to the accumulation of α-mannosidase precursor, which was detected in the medium. Cells treated with chloroquine or certain protease inhibitors also accumulate precursor polypeptide that is secreted into the medium (5, 37). These agents may all act in similar manners to affect the processing of the α-mannosidase enzyme. EDTA is commonly used to investigate the effects of cell-cell contacts on gene expression in developing cells. Our results suggest that EDTA should be used in such studies with caution, since it may affect protein processing, transport, and localization.

The presence of the protein synthesis inhibitor cycloheximide substantially reduced the induction of α-mannosidase mRNA in NC4 cells. When low-density NC4 cells were incubated overnight in a bacterial suspension prepared in conditioned buffer plus cycloheximide, the cells accumulated only trace amounts of α-mannosidase mRNA. Because high levels of PSF were present in the conditioned buffer, this result indicates that synthesis of some other component(s) of the signal transduction pathway is needed for maximum α-mannosidase induction. The trace response that did occur may be the result of a low level of protein synthesis (about 5% of control levels) that persisted in the presence of cycloheximide. Similar results have been obtained for other prestarvation response genes (35).

In a separate experiment, Ax3 cells were starved at high density in the presence and absence of cycloheximide. Under these conditions, the level of induction of α-mannosidase mRNA in cycloheximide-treated cells approached 50% of control levels. It remains to be determined whether the higher level of induction seen in this experiment was a function of the strain (Ax3 versus NC4) or of the difference in conditions (starvation versus growth). One possibility is that starvation induces α-mannosidase transcription through a pathway that is independent of PSF and does not require protein synthesis (existing data show that starvation is not necessary for α-mannosidase induction but do not address the question of whether it is sufficient). A second possibility is that growing Ax3 cells (but not NC4 cells) contain low levels of all components required for PSF-mediated induction of α-mannosidase mRNA. These possibilities can be distinguished experimentally, and the appropriate studies are in progress.

An interesting observation in the present study was that bacterially grown Ax3 cells, washed free of bacteria and resuspended in axenic broth at high density, produced higher levels of α-mannosidase mRNA than cells resuspended in starvation buffer. We do not yet know whether this super-induction is confined to axenic strains. Studies of the discoidin I gene family, another member of the prestarvation response class, have shown that discoidin I is regulated by cell density in both wild-type and axenic strains but is additionally induced in axenic strains by axenic growth conditions. Classical genetic analysis demonstrated that the accumulation of high levels of discoidin I protein during axenic growth was the property of a mutation, daxA, present on linkage group II in Ax3, and that this effect was separable from the prestarvation response (36). In Ax2, another axenic strain, all three members of the discoidin I gene family were found to be induced during development, but only two were induced in axenic growth (8). Deletion analysis of promoter elements for the discoidin I gene in Ax2 identified two cis-acting sequence elements, of which one enhanced expression during axenic growth and the other enhanced expression during development (44). Therefore, α-mannosidase may also be subject to dual regulatory mechanisms.

It has long been recognized that high cell density as well as starvation is required for the normal progression of development in *D. discoideum* (12, 13, 17, 29, 31). The prestarvation response, which takes place during late-exponential growth, encompasses what has been traditionally regarded as the earliest stage of development (7, 35, 36). It provides a mechanism for determining whether conditions are appropriate (high cell density and low food supply) for the initiation of development. Further progression of development requires starvation and possibly additional factors produced by starving cells. It remains to be seen whether PSF-mediated gene induction is required for later development. This can be addressed by inactivation of PSF or identification of strains that do not produce PSF.

There are several aggregation-negative mutants of Ax3 that fail to accumulate α-mannosidase enzyme activity during development even though they carry a normal structural gene for this enzyme (9, 20, 22). One such strain, HMW 404, was examined in this study. Mutant cells fail to synthesize any detectable levels of α-mannosidase mRNA under high-density starvation conditions. This mutant may be a good candidate for a strain that is deficient in some element of the prestarvation response pathway. If this is the case, it could be used to help define the PSF regulatory pathway and elucidate the relationship between the prestarvation response and later developmental events.

Future studies will include the isolation of α-mannosidase genomic clones and a detailed analysis of the upstream sequence elements contained in the promoter of this gene. The analysis will involve the identification of both *cis*-acting sequence elements and *trans*-acting factors involved in the induction of this gene.
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