Transcriptional Transitions during *Dictyostelium* Spore Germination†

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Many protozoa form spores in response to adversity; therefore, spore germination is a key process in their life cycle. *Dictyostelium discoideum* sporulates in response to starvation following a developmental program. Germination is characterized by two visible changes, spore swelling and the emergence of amoebae from the spore capsule. Several studies have indicated that an additional process termed spore activation is also required, but the physiological changes that characterize the three phases are largely uncharacterized. We used microarrays to monitor global transcriptional transitions as a surrogate measure of the physiological changes that occur during germination. Using two independent methods to induce germination, we identified changes in mRNA levels that characterized the germination process rather than changes that resulted from the induction method. We found that germination is characterized by three transitions. The first transition occurs during activation, while the spores appear dormant, the largest transition occurs when swelling begins, and the third transition occurs when emergence begins. These findings indicate that activation and swelling are not passive occurrences, such as dilution of inhibitors or spore rehydration, but are active processes that are accompanied by dramatic events in mRNA degradation and de novo transcription. These findings confirm and extend earlier reports that genes such as celA are regulated during spore germination. We also found by mutation analysis that the unconventional myosin gene *myoI*, which is induced during early germination, plays roles in the maintenance of dormancy and in spore swelling. This finding suggests that some of the observed transcriptional changes are required for spore germination.

The formation of asexual spores is a common protozoan response to adverse conditions and is a major factor in the dispersal of pathogenic protozoa. The process of spore germination is required to restore the growth of the unicellular organism upon a return to favorable conditions. Several common regulatory pathways in spore germination have been recognized, but the main difficulty is in distinguishing between mechanisms that regulate germination per se and mechanisms that regulate the subsequent growth processes (31, 43).

Protozoan organisms lend themselves to analysis by functional genomics methods, but only a few genomics-scale studies of spore germination in these organisms have been performed. For example, a comprehensive study of mutations that inhibit postgermination growth in *Saccharomyces cerevisiae* revealed the involvement of recombination and chromosome segregation genes as well as several putative germination-specific factors; that study also showed a limited correlation (about 16%) between germination-specific gene expression and gene function (13).

Spore germination in the social soil amoeba *Dictyostelium discoideum* is a regulated process (9). Spores differentiate in response to starvation after a 24-h developmental program that involves aggregation, differentiation into two cell types, and fruiting body formation (29). The fruiting body contains a cellular stalk that carries a ball of spores (sorus). The spores remain dormant in the sorus due to the action of the germination inhibitor discadenine and due to the presence of high salt concentrations in the sorus (1, 8). Upon dispersal, the spores may germinate if the inhibitor is diluted or inactivated. Efficient and synchronous germination can be induced in the laboratory by means of a short heat shock or by incubation with dimethyl sulfoxide (DMSO). Spores may also become competent for germination after prolonged incubation in the sorus in a process known as autoactivation (12). Microscopic examination reveals two morphological transitions during germination. First, the spores increase in size and lose their birefringence (swelling). Next, the spore cases break and the amoebae emerge (9). The process is dependent on mRNA synthesis, since transcription inhibitors inhibit germination (14, 20). Several examples of specific gene regulation during spore germination have been reported, although there is no evidence that this expression is necessary for the process. Two putative cellulase genes, *celA* and *celB*, and the *gerD* gene of the 270 gene family are expressed after 1.5 to 3 h of DMSO-induced germination (18). The *gerA*, *gerB*, and *gerC* genes of the 109 gene family are also regulated during the early stages of spore germination (15). Vegetative genes of the V and H gene families are expressed during the final stages of heat shock-induced spore germination (37). More recent work has shown that spore germination is accompanied by changes in the actin

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cytoskeleton as well (33). These findings indicate that spore germination is an active and regulated process.

Analysis of gene expression with microarrays is a means of monitoring genome-scale changes in gene expression. Early microarray studies raised hopes that process-specific gene regulation would reveal which genes regulate the process, but this idea appears to be largely inapplicable. Methodical studies of the yeast *S. cerevisiae* have shown that the correlation between gene expression and gene function is low, about 4%, meaning that one cannot hope to describe the specific function of a gene in a given process based on its regulation alone (17, 44). Modern microarray studies use the genome-scale gene expression profile as a surrogate measure for overall cell physiology (3, 21). Clustering of multiple genes having a common annotation is used to predict the participation of a genetic pathway in a process instead of the analysis of individual genes (45). For *Dictyostelium*, microarray analysis of global changes in gene expression have been used to describe physiological transitions during growth and development (41) and during dedifferentiation (23) as well as for the analysis of mutant cell physiology (19, 22, 24, 25). It has also been shown that combining cluster analysis and gene annotation can predict gene function. For example, clustering and annotation analyses showed that the *Dictyostelium dhkA* gene belongs to a group of signal transduction genes specifically regulated during dedifferentiation. This finding prompted a mutational analysis that demonstrated the direct involvement of dhkA in the process (23).

In the present study, we determined the transcriptional profiles of germinating spores by using an expression microarray. We found that the germination process is characterized by three major transitions in the mRNA content of the spores. The first transition occurs during the first 2 h after induction, the second and largest transition occurs 1 h later, during swelling, and the third major transition occurs during emergence. We also found that developmental mRNAs are degraded in the beginning of the process and that vegetative genes are induced immediately thereafter, during swelling. A germination-specific group of genes revealed possible functions in signal transduction, mRNA metabolism, and protein dephosphorylation, implicating these genes in the regulation or in the execution of the germination program. Mutational examination revealed that the unconventional gene myoI, which is regulated during spore germination, plays an important role in the process.

**MATERIALS AND METHODS**

**Strains and spore production.** *D. discoideum* AX4 cells (27) were maintained on SM nutrient agar plates in association with *Klebsiella aerogenes* (38). For spore collection, AX4 spores were mixed with a suspension of *K. aerogenes* in water and spread on nutrient agar plates made with SM2 (27.8 mM glucose, 5 g of Bacto Peptone [Difco]/liter, 0.5 g of Bacto Yeast Extract [Difco]/liter, 4.0 mM magnesium sulfate, 17.4 mM potassium phosphate [pH 6.2]). Spores were collected by passage of wet microscope slides over the tops of the sori 2 days after fruiting bodies appeared. Spores were resuspended and washed twice with KP buffer (10 mM potassium phosphate [pH 6.5]).

Mutant strains were generated by random restriction enzyme-mediated integration (REMI) (28) at the *Dictyostelium* Functional Genomics Project at Baylor College of Medicine (A. Kuspa and G. Shaulsky, unpublished data), at which the mutated genes also were cloned and sequenced.

**Spore germination.** For heat activation, spores were resuspended in KP buffer in glass test tubes and incubated in a heated water bath at 42°C for 30 min. The samples were removed from the water bath and adjusted to a density of 5 × 10⁷ spores ml⁻¹ with KP buffer at room temperature (time, 0 h). For dimethyl sulfoxide (DMSO) activation, spores were resuspended in 20% (vol/vol) DMSO in KP buffer in glass test tubes and incubated at room temperature for 1 h with occasional agitation. The spores were pelleted by centrifugation, the DMSO solution was aspirated, and the spores were resuspended with a rapid stream of KP buffer (time, 0 h). The final spore density was adjusted to 5 × 10⁷ spores ml⁻¹ in KP buffer.

Following activation, spores were shaken at 100 rpm in glass flasks at 22°C. Samples were collected at 1-h intervals, and differential counting of unswollen spores, swollen spores, and emerged amoebae was done by using a Zeiss phase-contrast microscope.

**Autocytosis.** Cells were developed as described above, and spores were allowed to mature in fruiting bodies for several days. Spores were removed from fruiting bodies by passage of dry microscopic slides over the tops of the sori. Spores at the edges of the microscope slides were transferred to 5 ml of KP buffer with or without 200 mM sucrose. In this assay, the spores were not washed by centrifugation to prevent the loss of dead and lysed spores. Instead, spore density was adjusted to 10⁶ ml⁻¹ to prevent spore-to-spore signaling (7). Spore suspensions (4 ml) were plated in 10-cm glass test tubes with microstirrer bars (1 by 3 mm) on a magnetic stirrer and stirred at the lowest speed required to keep the spores in suspension. Samples were removed, mixed with an equal volume of neutral red (1:10,000 [wt/vol] in water) on glass microscope slides, and incubated for 1 min. Bright-field microscopy (>400) was used to distinguish among viable dormant spores (unstained), viable swelling spores (weak punctate staining), or dead spores (strong red staining of the entire cytoplasm). Four hundred cells were counted at each time point. In some experiments, spore suspension samples of 0.4 ml were plated on 2% nonnutrient agar (Difco) containing KP buffer with or without 1% Bacto Peptone to determine spore viability and the ability to spontaneously germinate. All experiments were repeated at least three times.

**Extraction and preparation of RNA for microarray analysis.** To ensure efficient extraction of RNA, spores (5 × 10⁷) were collected by centrifugation, frozen rapidly in liquid nitrogen, and ground with a mortar and pestle until all of the spores appeared to be broken upon microscopic examination (32). The ground-up spores were resuspended in 1 ml of Trizol reagent (Life Technologies), and RNA was extracted according to the manufacturer’s recommended protocol.

Gene expression analysis was performed as described previously (41). Briefly, purified total RNA was reverse transcribed with Cy3-conjugated homopolymers of 18 deoxythymidine (dT18) as primers. A reference sample made from a pool of equal portions of RNA samples prepared from six developmental stages (0, 3, 6, 12, 17, and 24 h) was reverse transcribed with Cy3-conjugated (dT18) oligonucleotides as primers. This reference sample was used in all of the experiments as well as in previous work and thus allowed comparison among the different germination experiments and between germination and development. The experimental samples and the reference sample were mixed and hybridized to a microarray containing 7,744 hybridization targets (41).

The analysis of DMSO-induced spores was done in three independent biological replications, and the analysis of heat shock-induced spores was done in two independent biological replications. Each RNA sample (except for one of the DMSO-induced samples) was hybridized two independent times to a microarray on which each of the 7,744 targets was printed in duplicate (i.e., each measurement was repeated eight times). All of the data presented are averages of these multiple replications.

**Microarray data analysis.** Data normalization was performed as described previously (23, 41) with the following modifications. The normalized single-chip data sets were scaled to have a variance of 1 and combined into two multiple-experiment data sets according to the treatment (DMSO or heat shock induction). The cross-time mean gene expression value during germination was subtracted from the gene expression values separately for each combined data set. The data sets were then combined so that for each gene, the expression value was a vector of length 14 (two treatments at seven time points each).

To identify genes with invariant expression patterns in both treatments, we applied an analysis of variance to each vector. Smooth curves were fitted to these vectors by using linear regression. The polynomial bases of the smooth curves were of degree 5 and were generated by using the “poly(5)” function in the R statistical software (version 1.6.1). Briefly, for the polynomial used here (αx⁵ + βx⁴ + δx³ + γx² + χx + j), the function finds orthogonal polynomials (by generating values corresponding to Xᵢ, Xᵢ², . . . , Xᵢ₅) in a way that facilitates the subsequent calculation of the coefficients (α, β, . . . , j) by regression. Coefficients for the smooth curves and F statistics for the fit were recorded. A total of 90 genes were filtered out based on F statistics (P < 0.05). This procedure ensures that only genes whose expression is significantly regulated during germination are
considered and that the regulation is due to the process of germination and not to the method of induction. The regulation of the remaining genes is documented in the supplemental material.

The smooth-curve coefficients of these 1,397 genes were further analyzed with the K-means algorithm to obtain gene clusters. Briefly, K-means is an algorithm that optimizes the grouping of objects (gene trajectories in this case) into a predetermined number of groups (K). We tried different numbers of clusters (K ranging from 2 to 10) and chose 4 as the proper number of clusters based on the slope changes of the within-cluster sum-of-squares errors. The data set was repeatedly clustered into four clusters, and optimized cluster centers were chosen, resulting in four groups containing 360, 396, 357, and 284 genes. The genes within each cluster were ordered based on the correlation between their coefficients and the group center (median).

To determine the statistical significance of each cluster, we performed a simulation in 2,000 iterations. The data at each time point were permuted to generate simulated data sets, and the filtering and clustering procedure was performed as described for the real data. The mean correlation coefficients between the smooth-curve coefficients and the cluster centers were calculated. The statistical significance of the clusters in the real data was estimated from the mean correlation coefficient distribution; the P values were <10^-7 for the group containing 360 genes, <10^-4 for the group containing 396 genes, <0.04 for the group containing 357 genes, and <0.15 for the group containing 284 genes. The last group was excluded from further analyses.

To annotate the putative functions of selected genes, we used gene ontology (GO), which is a controlled vocabulary for describing gene function (5). Nearly full-length cDNA sequences of each microarray target were matched to the sequences in the GO database (http://www.godatabase.org/dev/database). GO identifiers for each array target were determined based on the BLASTX score and the GO evidence code of the homologous genes (see supplemental material for details). A recursive traversal of the GO directed acyclic graphs provided the GO identifiers that are overrepresented in the gene list compared to the entire array. A detailed explanation of the method and a simple example are provided in the supplemental material. Lists of genes with significantly high representation were identified by comparing the number of genes having a common GO category in the experimental group to the total number of genes having that category on the entire array. The data are presented graphically, where bar lengths represent the ratio (fold enrichment) of the list frequency (number of genes in the list/total number of all genes at the GO level) to the array group frequency (number of genes with a specific GO annotation on the array/total number of all array genes at a particular GO level). The x-axis is the scale for that ratio.

Developmental gene expression data were reanalyzed from previously published experiments (41).

## RESULTS

### Morphological transitions during spore germination.

Spore germination is accompanied by two morphological transitions, swelling and emergence. Before germination, the dormant spores appear oval and bright under phase-contrast microscopy. The first morphological transition occurs 1 to 3 h after induction, as the spores swell and lose their birefringence. In the second transition, the spore cases break and the amoebae emerge. We used two different methods to induce spore germination, DMSO treatment and heat shock (6, 10). In the first hour after DMSO treatment, 30% of the spores were swollen, and swelling peaked at 55% after 2 h. Amoebae emergence began after 2 h (6%) and continued throughout the duration of the experiment (Fig. 1A). Heat shock-induced germination was more synchronous and efficient, but the transitions were delayed by 1 h compared to those seen with the DMSO treatment. It took 3 h for about 97% of the spores to swell, about 70% of the amoebae emerged by 4 h, and the process was complete after 6 h (Fig. 1B).

### Transcriptional transitions during spore germination.

Spore morphology during germination indicates that the process consists of at least two stages, swelling and emergence. Previous experiments indicated that these two stages must be the result of more complicated physiological processes, but the details of the processes are largely unknown (9). We wanted to find how many physiological transitions occur during the process and how they are related to the morphological transitions. Microarray profiling of gene expression is an established method for distinguishing among physiological states caused by mutations, growth conditions, and drugs (3, 21). It is also applicable to the analysis of Dictyostelium growth and development (19, 24, 41). We therefore used this tool to characterize the process of spore germination.

We first collected RNA from germinating spores at 1-h intervals and analyzed gene expression with a microarray representing most of the expressed genes in Dictyostelium (41). We then calculated the relative level of expression of each gene across the seven experimental time points and used all of the values to determine the dissimilarity among the seven RNA samples. The dendrograms in Fig. 2A show the results. In the dendrograms, each leaf represents a time point in germination, from 0 to 6 h, and the height of each join is directly proportional to the dissimilarity between the leaves or the clades (group of leaves) that it connects.

For DMSO induction, the greatest dissimilarity was observed between the 0- to 1-h clade and the 2- to 6-h clade, suggesting that the time between 1 and 2 h of germination is accompanied by the greatest change in cellular mRNA content. For heat shock induction, the greatest dissimilarity was...
observed between the 0- to 2-h clade and the 3- to 6-h clade, suggesting that the time between 2 and 3 h of germination is accompanied by the greatest change in cellular mRNA content. These major shifts (2 to 3 h with heat shock induction and 1 to 2 h with DMSO induction) (Fig. 2A) occur at the time of maximal spore swelling after the respective induction (Fig. 1), showing a correlation between the physiological and the morphological observations.

The results shown in Fig. 2A were nearly identical for the two induction methods, indicating that the process of germination was robust and that the observed gene expression changes were due mainly to the process of germination rather than to the method of induction. We therefore averaged the two data sets and plotted the dissimilarity between the RNA samples as described above (Fig. 2B). Again, the largest dissimilarity in the data was observed during the swelling process, between the 0- to 2-h clade and the 3- to 6-h clade (Fig. 2B), indicating that swelling is accompanied by the largest physiological transition. The second most prominent change was observed between the 0- to 1-h clade and the branch at 2 h (Fig. 2B). This change occurs at the onset of swelling, indicating the existence of a preswelling transition in mRNA content. The third largest change was observed between the 3- to 5-h clade and the 6-h branch (Fig. 2B), indicating the existence of yet another physiological change, during the emergence of amoebae from the spore cases. These results indicate that there are three major physiological transitions during spore germination: one that follows induction, one during spore swelling, and one during the emergence of amoebae from the spore cases.

We also observed that the dendrograms shown in Fig. 2B represented the temporal relationships between the samples (i.e., time point 3 is adjacent to time points 2 and 4, and so forth). This finding is highly significant because we did not use the temporal information in the dissimilarity calculations and in rendering the dendrograms. Therefore, it indicates that the transcriptional profile faithfully represents the temporal progression of the spores through the physiological process of germination.

Coordinated regulation of gene expression during spore germination. In order to identify genes that are coordinately regulated during germination, we sought genes that exhibited a time-dependent pattern that was highly similar between the heat-shocked samples and the DMSO-treated samples. Using an analysis of variance, we identified 1,397 such genes. We then subjected the genes to a cluster analysis that sorted them into four groups based on all gene expression values at each time point. For data collected from germinating spores after DMSO induction (left panel), the two most dissimilar groups are shown in green (0 to 1 h) and magenta (2 to 6 h), and the 2-h sample (blue) is an outgroup of the clade shown in magenta. For data collected from germinating spores after heat shock induction (right panel), the two most dissimilar groups are shown in cyan (0 to 2 h) and red (3 to 6 h), and the 2-h sample (blue) is an outgroup of the clade shown in green. The scale on the left indicates dissimilarity in arbitrary units. (B) The dissimilarity was calculated from the averaged data from the two treatments. The two most dissimilar groups are shown in cyan (0 to 2 h) and red (3 to 6 h). The 2-h sample (blue) is an outgroup of the clade shown in cyan and was different from the 0- to 1-h samples (green).

The genes in group II exhibited an inverse pattern relative to the group I genes (Fig. 3A, group II, DM and HS). Transcripts of the 396 genes in group II began to accumulate after 1 to 2 h in the DMSO-induced spores and after 2 to 3 h in the heat shock-induced spores (Fig. 3A, group II, DM and HS, respectively). These mRNAs continued to accumulate throughout the process of germination (Fig. 3B, group II). Examination of these genes in the developmental process showed that their mRNAs were expressed during the vegetative stage (0 h) and during the first 6 to 8 h of development, after which the mRNA levels were greatly reduced (Fig. 3A, Devel.). These findings
are consistent with the morphological observation that the emerging amoebae in the germination experiment were quite similar to the vegetative amoebae before development. Therefore, the regulation of the group II genes may be an indicator for the physiological process that prepares the germinating spores for subsequent growth.

Group III mRNAs were not present at high levels in dormant spores, but their expression was induced after 1 h of germination and peaked after 3 h (Fig. 3A, group III, DM and HS, and Fig. 3B, group III). Following the peak, the abundance of the 357 group III mRNAs was reduced to an average level throughout the process of germination. Examination of the group III transcripts during development revealed that the expression of many of these genes was induced immediately after starvation (2 h), decreased during the processes of aggregation and cell type divergence, and increased again toward the end of development, at the time of spore encapsulation (Fig. 3A, group III, Devel.). This pattern was observed before for spore- and stalk-enriched genes during development (41).

These genes may be involved in regulating or executing the germination program.

One of the genes on the microarray, celA, was previously described as a germination-specific gene that is induced at 1.5 to 3 h of germination after DMSO induction (15). In our microarray experiment, celA expression peaked at 2 h of germination after DMSO induction (Fig. 3B, DM), thus validating our microarray findings. In the heat shock-induced spores, celA expression was also regulated but peaked at 4 h of germination (Fig. 3B, HS). celA (and other genes of its type) was not included in group III because it does not fulfill the requirement of nearly identical trajectories with both methods of induction. These results indicate that our analysis may have underestimated the number of genes regulated during spore germination.

The data presented in Fig. 3 indicate that the process of germination is accompanied by vast changes in mRNA abundance and that mRNA accumulation begins immediately after induction, when the spores still appear dormant. The large
transitions in mRNA abundance in the spores (Fig. 2) are due to both increases and decreases in mRNA abundance, suggesting that both synthesis and degradation of mRNA are involved.

**Biological processes affected by spore germination.** Genes that may have important roles in biological processes can be found by annotation of gene groups discovered by microarray experiments (21, 45), despite the fact that there is little correlation between the function and the expression of individual genes (17, 44). Because the annotation of the *Dictyostelium* genome is incomplete, we compared the gene sequences to sequences of several well-annotated genomes. The germination-regulated genes were annotated by using the GO scheme (5) as described previously (23). First, the sequences of all of the genes in the three groups (Fig. 3) were compared to the sequences in the GO database, and the matches with the highest scores were recorded. Then, the GO identifiers pertaining to the *Dictyostelium* gene list were mapped to the GO data structure, and a tabulation of counts below the nodes in the GO tree was obtained as part of the tree-traversal process. Gene sublists with significantly high representation were identified by comparing the number of genes in the experimentally defined group having a common GO category (e.g., phospholipid metabolism gene in group I) to the total number of genes in that category on the entire array (e.g., all phospholipid metabolism genes on the array). The results are shown in Fig. 4, where the length of the bar represents the fold enrichment of genes in the list relative to their total number on the array. A detailed explanation is provided in the supplemental material.

Group I consists of genes that are induced during development and down-regulated during germination (Fig. 3). It is also the only group that contains genes with a “developmental process” annotation (Fig. 4, panel I), consistent with the expectation that one major event in germination is the erasure of the developmental state. The “physiological process” annotation of genes in group I reveals the down-regulation of purine biosynthesis as well as phospholipid, lipoprotein, and sulfur metabolism (Fig. 4, panel I). The “cellular process” annotation reveals the down-regulation of transport mechanisms and of cell wall organization genes (Fig. 4, panel I), consistent with the expectation that cell wall organization is required for spore production but not germination.

Group II consists of genes that are up-regulated toward the end of germination and down-regulated during multicellular development (Fig. 3). The physiological processes of the two largest subgroups in group II are annotated as biosynthesis and protein metabolism (Fig. 4, panel II). Mo-molybdopterin biosynthesis genes are also included, suggesting the possibility that dehydrogenase activity is required. The cellular process subgroup includes chromatin modulation and GTPase signal transduction genes (Fig. 4, panel II). The results shown in Fig. 4, panel II, indicate that germinating spores induce a wide range of cell growth genes, the same genes that are turned off during spore differentiation. It is important to note that the spores are germinated in the absence of nutrients, so that the induction of the cell growth genes is not likely to be a result of growth.

Group III genes are transiently up-regulated during early spore swelling (Fig. 3). Their main cellular process annotation indicates that intercellular signaling is involved (Fig. 4, panel III). The major physiological processes are annotated as mRNA metabolism and transcription as well as protein translation and modification; organic acid biosynthesis and disaccharide biosynthesis are also observed (Fig. 4, panel III). These results suggest that spores which seem to be dormant upon morphological examination (Fig. 1) are quite active physiologically after they are induced.

**Function of germination-regulated genes in spore germination.** Gene expression patterns may predict which genes participate in spore germination. To test whether the germination-regulated genes are necessary for germination, we tested the effects of mutations in some of them. The microarray target sequences of the genes found in group III were compared to a collection of 1,200 sequences of mutated genes from the Baylor College of Medicine *Dictyostelium* Functional Genomics Project. Mutant strains were generated by REMI mutagenesis, picking of several thousand random clones, plasmid rescue of the insertion sites, and sequencing of the mutated genes (28). Mutant cells were grown and developed, spores were collected, and their ability to germinate was tested. We found that spores of one strain, V10704, failed to germinate normally (data not shown). The gene mutated in V10704 is myoI, an unconventional myosin gene (40). The REMI insertion in strain V10704 occurred at the DpnII site starting at base 7090 of the sequence at GenBank accession number L35321. This location corresponds to the codon for amino acid 2237 of the 2,358 amino acids in the predicted MyoI protein sequence. We did not determine how the insertion might have affected protein activity or stability, but REMI insertions have been documented to cause null mutations even when they occur at the untranslated 3′ end of genes (34).

We hypothesized that the failure of V10704 spores to germinate was due to a direct role of myoI in germination. Alternatively, it could be due to an indirect consequence of a defective sporulation process in this mutant (i.e., a dependent-sequence effect). The timing of sporulation and the shape of the mutant spores were indistinguishable from those of the wild type (data not shown); therefore, we could not use those parameters to distinguish between the two possibilities. We therefore relied on the fact that spores of wild-type strain AX4 do not germinate spontaneously unless they are aged for 6 days in the sorus (26). Wild-type and *myoI* mutant spores were collected immediately after fruiting body formation and after 1, 3, 6, and 9 days of aging in the sorus at 22°C, and neutral red staining was used to identify spores that had autoactivated. The data in Fig. 5A show that both wild-type and mutant spores did not exhibit significant staining on days 0 and 1, indicating that the spores were intact and dormant. These findings argue against the dependent-sequence possibility and suggest that *myoI* has a direct role in germination. After 3, 6, and 9 days of aging in the sorus, 20, 33, and 67% of the mutant spores, respectively, were stained with the dye, whereas the fraction of stained wild-type spores never exceeded 10% (Fig. 5A). These findings suggest that the *myoI* mutant spores underwent abortive autoactivation while in the sorus.

To further characterize the abortive autoactivation phenotype, we collected 1-day-old spores of both strains, diluted them to remove the germination inhibitors, and incubated them in suspension with shaking. Under these conditions, the wild-type spores did not germinate or swell (Fig. 5B), but the
FIG. 4. Annotation of germination-regulated genes. Sequences of the genes from groups I to III (Fig. 3) were compared to several GO-annotated genomes, and the *Dictyostelium* genes with significant sequence similarity were assigned the respective GO annotations. The biological process annotations of significantly enriched gene groups (*P*/H ≤ 0.1) are shown. The GO tree level of the biological process annotation is indicated to the left of each bar (2 to 8). Numbers to the right of the bars indicate the number of genes in the group (List), the number of genes with that annotation on the entire array (Total), and the *P* value representing the statistical significance of the enrichment. The length of the bar represents the ratio of the list frequency in the data (number of genes in the list/total number of genes in the group at that particular GO level) and the group frequency in the entire array (number of genes with that GO annotation on the array/total number of genes on the array at that particular GO level). The *x* axis is the scale for the ratio. A detailed explanation of the method is provided in the supplemental material. In the connected bars, the lower bars are subgroups of the bars immediately above them, as indicated by the branching pattern. A branch shown by a broken lines indicates that the group did not show significant enrichment at an intermediate GO level. The bar color and pattern represent the group annotation at GO level 2: striped bars indicate the developmental process, black bars indicate the physiological process, and gray bars indicate the cellular process. Panels I, II, and III represent genes from groups I, II, and III, respectively, in Fig. 3.
occurs before and at the beginning of spore swelling. This swelling process but is dispensable for osmoregulation. maintaining dormancy in the sorus and for completion of the myoI complete failure to swell (Fig. 5B). This bated them in buffer containing 200 mM sucrose and found a were still sensitive to inhibition by high osmolarity, we incu-

spore germination (8, 42). To test whether the V10704 spores releasing amoebae (data not shown). (A) Fruiting bodies were aged for the indicated times (days). Spores were collected and stained with neutral red. The fraction of stained spores (percentage) is shown. (B) One-day-old spores (24 h after the completion of development) were collected and germinated in suspension with (solid symbols) and without (open symbols) 200 mM sucrose. Samples were removed at the indicated times (hours) and stained with neutral red to identify abortively swollen spores. The fraction of stained spores (percentage) is shown. The data are means and standard deviations from at least three independent experiments. Error bars smaller than the symbols are not shown.

mutant spores began to germinate and became swollen, as indicated by the steady increase in neutral red-stained spores (Fig. 5B). The deeply stained swollen spores failed to release viable amoebae during the 6 h of the experiment, and a few of the swollen spores were almost completely lysed by the end of the experiment. When these spores were plated on agar containing 1% Bacto Peptone, a small fraction of the unswollen spores released visible amoebae. The remaining 35% of the spores, which did not swell during the first 6 h of the experiment, were viable and capable of responding to heat shock. However, more than 95% of them entered the swelling stage of germination abortively (as indicated by a deep neutral red staining) and died without releasing amoebae (data not shown).

Osmoregulation is one of the hallmarks of Dictyostelium spore germination (8, 42). To test whether the V10704 spores were still sensitive to inhibition by high osmolarity, we incubated them in buffer containing 200 mM sucrose and found a complete failure to swell (Fig. 5B). This finding suggests that the myoI mutation does not affect the osmosensory aspects of germination. Therefore, myoI appears to be important for maintaining dormancy in the sorus and for completion of the swelling process but is dispensable for osmoregulation.

DISCUSSION

The largest change in mRNA content during germination occurs before and at the beginning of spore swelling. This observation was made by using two very different methods of induction, DMSO and heat shock, indicating that the changes are related to the actual process of germination and not to technical effects. The changes include both an increase and a decrease in mRNA abundance, suggesting that both mRNA degradation and gene expression events must take place. These results were somewhat unexpected because the major morphological changes occur later, during spore swelling and during amoeba emergence; therefore, the initiation of spore swelling had been considered a less complex event (9, 11). Moreover, our results suggest that germination is accompanied by two other large transitions, one immediately after induction and the other during emergence. Therefore, the microarray data indicate that spore germination is an active and regulated process, even at the early stages of induction and swelling, and that there are physiological changes at the onset of germination that are not accompanied by obvious morphological events.

A causative relationship between transcription and germination has already been demonstrated. Transcription inhibitors inhibit spore germination, suggesting that de novo transcription is essential for germination (14, 20). The DNA-binding transcription inhibitor 4-nitroquinoline 1-oxide inhibits germination even when the spores are treated with the drug for a short time during activation (20). This finding implies that transcription is required very early in germination, but it was subject to alternative interpretations because 4-nitroquinoline 1-oxide forms stable DNA adducts that may have had a lasting effect that inhibited germination at a later stage. Our present results show that major transcriptional transitions do occur very early in germination, supporting the idea that transcription is indeed essential.

During development, vegetative amoebae differentiate into spores, and during germination, spores turn into amoebae. The expression of the germination-regulated genes during development is consistent with the reciprocal relationship between the processes. The patterns of expression of group I and group II genes during germination were almost the exact opposite of the pattern of expression during development. Group I genes are expressed at a low level in vegetative amoebae before development, induced to high levels of expression after cell type divergence and during spore maturation, and expressed at a lower level again during spore germination. This pattern indicates that the group I mRNAs are deposited in the spore during the process of encapsulation and are then degraded upon spore germination. The biological function annotation of group I genes supports this idea as well, because group I is the only one that contains genes with a developmental function annotation. Since the mRNAs of group I are deposited in the spore before germination, we propose that the major cause of the change in the level of these transcripts is mRNA degradation.

Group II mRNAs are highly abundant in vegetative cells and in early developing cells, before aggregation and cell type divergence. Their levels drop in differentiated cells so that their abundance in the spores is low. Upon swelling, group II genes are induced, and their levels continue to rise as the amoebae emerge from the spore cases. Because we germinated the spores in buffer and not in growth media, we propose that the
expression of group II genes anticipates the onset of growth but is not induced by growth. *Dictyostelium* development takes place under starvation conditions, while ribosomes and structural components are degraded for energy and metabolites (29). Therefore, spores are much smaller than vegetative cells, and the emerging amoebae must replenish their resources upon return to growth. Therefore, it is not surprising to find that group II genes have biological function annotations mainly in biosynthesis and protein metabolism.

While group I and group II genes suggest that germination is an exit from development and is followed by entry into growth, respectively, group III genes appear to be more specific to the process of germination itself. Group III contains many signal transduction genes that are induced during early germination, an interesting finding in the context of the regulation of germination. Previous studies showed that spore germination is regulated by two mechanisms (11). The germination inhibitor discadenine, a modified adenine nucleotide, is a potent inhibitor of spore germination (1). The discadenine signal is probably mediated by the histidine kinase DhkB and the downstream response regulator phosphodiesterase RegA (46). RegA regulates the activity of the cyclic AMP-dependent protein kinase A (PKA-C), and mutations in regA or in the genes that encode the regulatory and catalytic subunits of protein kinase A (pkaR and pkaC, respectively) have profound effects on germination efficiency (2, 4, 36). The main source of cyclic AMP in the germinating spores is the germination-specific adenylyl cyclase AcgA, and mutations in the corresponding gene, acgA, lead to unregulated germination. Another interesting aspect of AcgA is that the cyclase catalytic activity is phosphorylation is directly involved in spore germination (16, 33). It

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