Genome-Wide Expression Analyses of Gene Regulation during Early Development of *Dictyostelium discoideum*

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Using genome-wide microarrays, we recognized 172 genes that are highly expressed at one stage or another during multicellular development of *Dictyostelium discoideum*. When developed in shaken suspension, 125 of these genes were expressed if the cells were treated with cyclic AMP (cAMP) pulses at 6-min intervals between 2 and 6 h of development followed by high levels of exogenous cAMP. In the absence of cAMP treatment, only three genes, *carA*, *gbaB*, and *pdsA*, were consistently expressed. Surprisingly, 14 other genes were induced by cAMP treatment of mutant cells lacking the activatable adenyl cyclase, ACA. However, these genes were not cAMP induced if both of the developmental adenyl cyclases, ACA and ACR, were disrupted, showing that they depend on an internal source of cAMP. Constitutive activity of the cAMP-dependent protein kinase PKA was found to bypass the requirement of these genes for adenyl cyclase and cAMP pulses, demonstrating the critical role of PKA in transducing the cAMP signal to early gene expression. In the absence of constitutive PKA activity, expression of later genes was strictly dependent on ACA in pulsed cells.

Multicellular organisms develop through a series of stages during which the patterns of gene expression are regulated to alter the properties of the cells and generate distinct cell types. These processes can be clearly recognized during development of the social amoeba *Dictyostelium* even though multicellularity is achieved by aggregation of vegetative cells to give the multicellular forms (5, 15, 19). During the first 10 h following the initiation of development, previously solitary amoebae acquire the ability to signal each other by secreting cyclic AMP (cAMP) and respond chemotactically to form mounds containing up to 10^5 cells. Definition of the genetic program that underlies these and subsequent developmental changes can benefit from genome-wide expression analyses with wild-type and mutant strains. Characterization of a large number of developmentally regulated cDNAs by the Japanese EST (expressed sequence tag) Project (28) has made it possible to recognize patterns of temporal and cell-type-specific expression of many developmentally regulated genes by use of microarrays (14, 25, 38). Microarrays not only expand the number of genes analyzed but also allow direct, quantitative comparisons between different genes.

A considerable number of genes are known to be expressed at high rates soon after the initiation of development (17, 20, 23, 26, 27, 29, 30). These include the genes for the cAMP receptor (*carA*), for the Go subunit that couples the receptor to adenyl cyclase (*gpaB*), for the activatable adenyl cyclase (*acaA*), and for the secreted and internal cAMP phosphodiesterases (*pdsA* and *regA*) (3, 11, 29, 34). The products of these genes function together with preexisting proteins to generate an oscillatory network that relays extracellular cAMP signals and regulates the levels of cAMP both inside and outside the cells (12, 18, 21). As the cells aggregate in chemotactic fields, they stick to each other in an EDTA-resistant manner as the consequence of expressing the genes, *csaA* and *lagC*, that encode the surface proteins gp80 and gp150, respectively (13, 40).

It has been known for some time that cells will express many developmental genes while suspended in buffer if they are given pulses of 30 nM cAMP at 6-min periods for several hours followed by high concentrations of cAMP to mimic conditions encountered on solid supports (17, 23, 26, 27, 30). Under these conditions, the cells are spatially homogeneous and can be expected to express developmental genes more synchronously than when developing on solid supports where there are local differences in cell density. Moreover, the effects of cAMP pulses on gene expression can be directly addressed. We compared the expression profiles in cells developing on solid supports with those in cells incubated in suspension with or without the addition of cAMP pulses. We found that most of the genes that are strongly expressed during the aggregation and mound stages of development are normally expressed in cells incubated in buffer and given cAMP pulses.

Three genes, *car1*, *gbaB*, and *pdsA*, have been shown to be expressed in suspension-developed cells even in the absence of cAMP signaling (10, 17, 23, 26, 27). Their products allow cells to respond to cAMP and regulate its exogenous levels. Surprisingly, these were the only cAMP pulse-independent genes recognized out of 6,345 targets carried on the microarrays.

**MATERIALS AND METHODS**

**Growth, development, and preparation of RNA.** Strains AX4 (wild type), GP6 (*acaA^−*), TL149 (*acaA^−* *acaC^−*), and TL130 (*acaA^−* *acaC^−* *pkaC^−*) were grown axenically in HL5 medium and collected while in exponential growth (2, 29, 37). Strain GP6 was generated by homologous recombination in the *acaA* locus of AX3-derived strain H110 and was the kind gift of Peter Devreotes. Strain TL149 was the kind gift of Fredrick Soderbom, who used homologous recombination to delete *acaA* from strain GP6. Strain AK631 (*acaA^−* *pkaC^−*) was provided by Adam Kuspa.

For development on solid supports, cells were collected, washed, and deposited on nitrocellulose filters on buffer-saturated pads and allowed to develop...
synchronously at 22°C (37). Cells were scraped off the filters at 2-h intervals into deionized water, pelleted, and dissolved in Trizol reagent (Gibco/BRL), and total RNA was prepared.

For development in suspension, exponentially growing cells were collected, washed, and resuspended in 100 ml of 20 mM Na+ K+ phosphate buffer (pH 6.5) at a concentration of 10^7 cells/ml and rapidly shaken at 125 rpm on a rotary shaker. To simulate normal signaling, cells were given pulses of 30 nM cAMP at 6-min periods from 2 to 6 h followed by addition of 300 µM cAMP at 2-h intervals (30). At each time point, 5 × 10^7 cells were collected, pelleted, and dissolved in Trizol reagent (Gibco/BRL) for preparation of RNA.

**Preparation of microarrays.** Corning slides were microarrayed with 6,345 cDNA and genomic targets by using a Molecular Dynamics GenII robot in the BioGEM facility as previously described (14). Inserts from 5,655 cDNA were generously provided by the Japanese EST Project (28). A list of the genes is available online (http://www.biology.ucsd.edu/loomis-cgi/microarray/paper2.html).

All genes referred to in this study were sequence verified.

**Expression analyses.** Probes were prepared from total RNA collected at 2-h intervals as well as from time-averaged reference RNA as previously described (14). Superscript II DNA polymerase (Invitrogen, Carlsbad, Calif.) was used to incorporate either Cy5- or Cy3-conjugated dCTP (Amersham, St. Louis, Mo.) into DNA. Following incubation at 42°C for 3 h, unincorporated dyes were removed using Microcon-30 columns (Millipore, Burlington, Mass.) with three washes with 450 µl of Tris-EDTA buffer before drying and resuspension in 5% SSC (1 × SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.3% sodium dodecyl sulfate–25% formamide. Labeled probes were mixed and hybridized at 42°C to the microarrays for 6 to 12 h. Dyes for the sample and reference probes were interchanged in different experiments. Protocols are available online (http://www.biology.ucsd.edu/loomis-cgi/microarray/index.html).

Probed microarrays were analyzed in an Axon Genepix 4000B scanner, and the measurements were processed with the associated software. Total Cy5 signal was normalized to total Cy3 signal after background subtraction to allow independent slides to be compared. The ratios of Cy3 to Cy5 for individual genes were then calculated. Each sample was hybridized to two or more microarrays. Each developmental time course was repeated at least twice. Mean values were used for subsequent analyses and are available online (http://www.biology.ucsd.edu/loomis-cgi/microarray/paper2.html). Values were normalized to 1 at the start of each experiment.

**Statistical analyses.** The median and standard deviation of the time course values for each target were analyzed by a curve-fitting algorithm to extract the times of onset and cessation of transcription (31). The goodness of fit was measured by the statistic $s = \frac{1}{2E(t) - A(t)}$, where $E$ is the ratio of the background-adjusted fluorescence of the time sample (Cy3) to that of the reference sample (Cy5) normalized so that the mean expression level of each gene throughout development is unity and $A$ is the abundance of the gene transcript at time $t$. Since the denominator of the statistic is a measure of temporal variation found experimentally, $s$ is a measure of how much of that variation can be accounted for by the kinetic equation. The statistical significance ($P$ value) of the $s$ statistic for each gene was calculated by permuting the expression pattern 1,000 times and calculating the fraction of permutations whose $s$ statistic is less than that of the experimental pattern. Genes with good statistics that showed an increase of at least fivefold during development were K-means clustered into five groups that were consistently color coded. Averages of the clusters were normalized to the time of initiation of development ($t = 0$).

**RESULTS**

Expression profiles during multicellular morphogenesis and in suspension. To characterize a large proportion of the genome, we arrayed 5,655 targets from cDNAs provided by the Japanese EST Project together with 690 targets previously chosen from specific genes (14, 28). Exponentially growing cells of wild-type strain AX4 were developed on filter supports, and samples were collected every 2 h. Fluorescently labeled copies were generated from RNA of each time sample and compared to labeled copies generated from time-averaged reference RNA prepared by pooling samples from different stages in development as previously described (14). Signals at 5,279 of the 6,345 targets either decreased or failed to increase significantly during development. These were mostly vegetative genes that are shut off during development. Values for each individual gene throughout development are available in supplemental materials found online (http://www.biology.ucsd.edu/loomis-cgi/microarray/paper2.html).

We have focused on genes that give at least a fivefold-higher signal at one stage or another during filter development of wild-type cells. Many of these genes are represented by several targets giving good reproducibility. A nonredundant set of 172 developmental genes clustered into five groups on the basis of their temporal profiles (Fig. 1A.). A cluster of 24 genes started to accumulate immediately after the initiation of development, another cluster of 42 genes started to be expressed at 4 h, another with 23 genes accumulated after 6 h, and two clusters started to accumulate at 8 h. Expression profiles for genes in...
the first four clusters were similar in cells incubated in buffer to which cAMP pulses were added for 6 h followed by high levels of cAMP (Fig. 1B). Many of the genes in the fifth cluster that were expressed after 8 h of development failed to be expressed in cells treated with cAMP in suspension, suggesting that they require intercellular signals in addition to cAMP.

In all, we found 125 targets that gave robust developmental signals in cells developed in suspension and treated with cAMP. In the absence of cAMP treatment, very few genes were expressed (Fig. 1C).

**Expression profiles of early genes.** The expression profiles for 18 genes (Table 1) that started to accumulate within the first 2 h of development in suspension are shown in Fig. 2. Except for *carA*, which encodes the high-affinity cAMP receptor, most of these genes were expressed at reduced or insignificant levels unless the cells were treated with cAMP pulses (Fig. 2A and B). When developed in suspension, the cells were at sufficiently high density that accumulation of cAMP could result in autonomous signaling after a few hours (12). Therefore, we analyzed a mutant strain, GP6, lacking the cAMP activatable adenyl cyclase, ACA.

In the absence of cAMP treatment, only four genes, *carA*, *gpaB*, *pdsA*, and *cbpB*, were expressed in cells lacking ACA (Fig. 2C). Three of these genes, *carA*, *gpaB*, and *pdsA*, have been previously shown to be expressed in cells developed in suspension in the absence of cAMP and at higher levels when the cells were treated with cAMP pulses (10, 17, 23, 26, 27). *cbpB* is unlikely to be a pulse-independent gene since it did not significantly accumulate in unpulsed wild-type cells (Fig. 2A).

Treatment of *acaA*− null cells with pulses of cAMP resulted in expression of all of the early pulse-dependent genes, except for *acaA*, which is mutated in these cells (Fig. 2D). cAMP induction of these genes is somewhat surprising since the signal transduction pathway leading from exogenous cAMP to alteration in the transcriptional pattern is thought to be mediated by PKA following stimulation by a rise in internal cAMP (4, 21, 32, 39). Yet these *acaA*− cells lack the major source of internal cAMP, the adenyl cyclase ACA that is coupled to the cAMP receptor.

Genes that were expressed later in wild-type cells while suspended in buffer and treated with cAMP pulses failed to be expressed in pulsed *acaA*− cells (Fig. 3). Expression of these late genes appears to depend on activation of ACA to generate high levels of cAMP within the cells.

**Role of the minor adenyl cyclase ACR.** The induction of early genes by cAMP in cells lacking ACA suggests that other signal transduction pathways may be stimulated upon ligand binding to CAR1. One such pathway involves a MAP kinase cascade that leads to the rapid activation of the MAP kinase ERK2 (24, 33). Mutant strains lacking ERK2 fail to accumulate cAMP and so are unable to aggregate. However, this phenotype can be suppressed by mutations in the gene encoding the internal cAMP phosphodiesterase, RegA (22; A. Kuspa, personal communication). These cells accumulate high levels of cAMP and are able to form aggregates by accretion. Thus, it appears that when ERK2 is activated by external cAMP binding to CAR1, RegA is inhibited and cAMP can accumulate to levels that activate PKA. However, there must be a source of cAMP other than ACA since at least 14 genes are activated by exogenous cAMP pulses in *acaA*− null cells.

There is a minor adenyl cyclase activity in vegetative cells that could be the source of cAMP (16, 35). To test the hypothesis that this adenyl cyclase is necessary for pulse induction when ACA is missing, we characterized suspension-developed cells of a strain in which both adenyl cyclase genes, *acaA* and *acrA*, are disrupted. As shown in Fig. 4, the early pulse-independent genes were expressed with or without cAMP pulses but none of the pulse-dependent genes were expressed at significant rates even when the double mutant cells were treated with cAMP. Thus, internal cAMP appears to be required for expression of these genes. Characterization of a strain in which only *acrA* is missing showed that either ACA or ACR is sufficient since all early genes were expressed in pulsed *acrA*− null cells (see the supplementary materials available online).

**Constitutive PKA activity bypasses the requirement for internal cAMP.** If increases in the internal levels of cAMP regulate gene expression by stimulating PKA activity, then constitutive PKA activity should be sufficient for full expression independently of cAMP synthesis. The catalytic subunit of PKA is inhibited when associated with its regulatory subunit but is activated when cAMP binds to the regulatory subunit. However, when the catalytic subunit outnumbers the regulatory subunit, some of the PKA activity would be expected to be cAMP independent and constitutive (1). We disrupted *acaA* in strain AK631, in which *acaA* is disrupted and actin 15/*pkaC* is carried in a multicopy vector. The resulting strain, TL130, was shown to have high levels of PKA activity that was independent of cAMP (2). Early genes, both pulse independent and pulse dependent, were fully expressed in both unpulsed and pulsed cells of this *acaA*− *pkaC*− strain that is unable to synthesize cAMP in response to exogenous cAMP pulses. As expected, *acaA* was not expressed since it is mutated in this
FIG. 2. Expression profiles of early genes. Wild-type AX4 cells (A and B) and acaA/H11002 null cells (C and D) were developed in suspension without addition of cAMP or in the presence of 30 nM cAMP between 2 and 6 h. Expression profiles were considered pulse independent (blue) if the target was not increased in the absence of cAMP pulses. Those that increased only in the presence of cAMP were considered pulse dependent (yellow). Genes that increased threefold or more in the absence of cAMP pulses or in cAMP-treated cells were considered to be pulse independent (Table 1). Values for each gene are available in the online supplemental materials. Bar graphs of the expression profiles here and in Fig. 4 represent the average change (n-fold).
strain. It appears that constitutive PKA activity is sufficient for expression of these genes.

**DISCUSSION**

Previous Northern analyses of *carA* and *gbaB* have indicated that these genes are expressed at basal levels in the absence of cAMP pulsing and at a higher rate when exposed to cAMP pulses (17, 23, 26, 27). Results from the microarray analyses confirmed these observations but showed that pulsing resulted only in a modest two- or threefold increase in accumulation of these mRNAs (Fig. 2 and 4). Even when one or both of the developmental adenyl cyclases were disrupted, the pulse-independent genes were expressed within the first few hours of development.

When development is induced by shifting the cells to a nonnutrient environment, *carA* and *gbaB* mRNAs start to accumulate immediately such that their products, the cAMP receptor CAR1 and the trimeric G protein subunit Go2 that couples it to activation of ACA, are rapidly synthesized. The cells then become responsive to cAMP and express these genes as well as pulse-dependent genes at high levels. The only other pulse-independent gene that was recognized on the microarrays, *pdsA*, encodes the extracellular cAMP phosphodiesterase. Previous studies have shown that transcription of *pdsA* is regulated by three independent cis-acting regions. The distal promoter is activated in the first few hours of development in a pulse-independent manner (10). Rapid synthesis of the phosphodiesterase allows the cells to keep the extracellular levels of cAMP within bounds as they start to produce cAMP pulses. Strains carrying null mutations in either *carA* or *gpaB* fail to generate cAMP pulses or show any signs of aggregation when developed on solid supports (29). Likewise, strains carrying...
null mutations in *pdsA* fail to show chemotactic movement (36).

A set of pulse-induced genes was coordinately expressed starting at 2 h of development such that their mRNAs reached high levels by 6 h of development. Most of them declined thereafter when the levels of exogenous cAMP were raised. These genes were induced by cAMP pulses even in cells lacking the cAMP-stimulated adenylyl cyclase, ACA (Fig. 2D). Later genes were not expressed in *acaA* null cells (Fig. 3), suggesting that they depend on the high internal levels that ACA can generate.

*acaA* is expressed rapidly in pulsed cells and leads to a high rate of cAMP synthesis during the aggregation stage. Another pulse-induced gene, *csaA*, has previously been shown to be cAMP regulated (9). It encodes the cell adhesion glycoprotein gp80. There are five pulse-induced genes that encode calcium binding proteins, *cbpA*, *cbpB*, *cbpD*, *cbpF*, and *cbpG*; *cbpB* becomes enriched in prestalk cells while *cbpD* becomes enriched in prespore cells (14). Although *cbpB* was expressed in unpulsed *acaA*− cells, it was not expressed in unpulsed wild-type AX4 or *acaA*− *acrA*− double mutants and so is unlikely to be a pulse-independent gene. Loss of *cbpA* results in aberrant aggregation, further indicating a role for calcium regulation in early development (7). SSL579 encodes a protein related to CbpB and other calcium binding proteins and has four EF-hand domains.

*dia-2* encodes a protein of unknown function which is essential for normal aggregation (6). *pgmA* encodes phosphoglucomutase, which plays a role in rapid breakdown of glycogen during early development (19). Three members of the *lagC* family are pulse-induced genes. The product of *lagC*, gp150, is a surface glycoprotein which makes cells mutually adhesive at the mound stage and is necessary for later gene expression (8, 40). *lagC2* encodes a protein which is 55% identical to LagC and may play a similar role. Two novel genes from the EST collection, SLD188 and SSG481, were found to be expressed in pulsed wild-type and *acaA*− null cells (Table 1).

The signal transduction pathway leading from CAR1 ligand binding to activation of these genes appears to require a source of internal cAMP to activate PKA. Only when both *acaA* and *acrA* were disrupted did cAMP pulses fail to induce these genes (Fig. 4). While the stimulation of ACA upon ligand binding to CAR1 is well defined, it has not previously been realized that ACR is able to generate sufficient cAMP for PKA activation in pulsed cells. A model accounting for the kinetic interactions of these components following a pulse of exogenous cAMP is given in Fig. 5. Ligand binding to CAR1 leads to GTP replacement of Ga2-bound GDP and dissociation of the trimeric G protein. In wild-type cells, the βγ subunits lead to activation of ACA while the Ga2 subunit leads to activation of the MAP kinase ERK2 (4). If ERK2 results in the inhibition of the internal phosphodiesterase, cAMP can build up and activate PKA. It has been proposed that PKA activity indirectly leads to inhibition of ERK2 and the reactivation of RegA (4, 18, 21). Even in the absence of ACA, this model can account for the activation of PKA in cells treated with exogenous pulses of cAMP.

On a longer time scale, expression of pulse-independent genes within the first few hours following initiation of development can be seen to prepare the cells for expression of pulse-dependent genes. Entrainment of pulse production among cells ensures that they will be coordinated for subsequent differentiation (12, 18). At least one pulse-dependent gene, *lagC*, is necessary for progression through subsequent developmental stages (8; our unpublished data). Strains carrying *lagC* null mutations form only loose aggregates and fail to express postaggregation marker genes even when treated with cAMP. Other pulse-dependent genes may also play critical roles in the temporal sequence of gene expression. Genome-wide expression analyses can uncover previously unsuspected genes within coordinately controlled groups and focus attention on their physiological roles. The newly recognized developmentally regulated genes behaved in a consistent manner in the different strains under various experimental conditions and provide robust markers for the early stages of development. It will be interesting to define later groups on the basis of genome-wide expression profiles in strains lacking specific gene products and determine the networks that interconnect them.

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