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A gene expressed in undifferentiated vegetative *Dictyostelium* is repressed by developmental pulses of cAMP and reinduced during dedifferentiation

(gene regulation/differentiation/*Dictyostelium discoideum*/adenylate cyclase)

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ABSTRACT **We describe the gene *M4-1*, whose unique pattern of developmental expression will allow us to study the molecular mechanisms controlling expression in undifferentiated cells in addition to repression in response to cAMP during development and reinduction during dedifferentiation. *M4-1* is a *Dictyostelium* gene expressed in the undifferentiated cell. We have shown that *M4-1* continues to be expressed very early during the developmental cycle but is repressed at a later stage of development, at a time coincident with the establishment of oscillations in the cAMP pool. Studies on the expression of the *M4-1* gene in shaking culture, under conditions that mimic early development, have established that pulsatile stimulation of cells with cAMP is sufficient to repress *M4-1* expression. Consistent with this, cells that are exposed to high levels of cAMP are unable to respond normally to cAMP oscillations and continue to express *M4-1* at vegetative levels. These data indicate that low-level oscillations of cAMP are required for the repression of *M4-1* expression rather than the continuous high levels of cAMP responsible for the regulation of a different class of *Dictyostelium* genes. We suggest that cAMP may mediate developmental expression of the *Dictyostelium* genome by different mechanisms. We also show that cell–cell interaction, a developmental event that occurs subsequent to the cAMP pulse, does not normally influence the regulation of *M4-1*. Finally, we have shown that when cAMP-pulsed cells are induced to dedifferentiate, M4-1 RNA sequences rapidly reappear in nuclei and cytoplasm, suggesting that regulation of *M4-1* expression is primarily mediated at the level of transcription.**

Dictyostelium discoideum grows as undifferentiated ameboid single cells. Development can be initiated by plating the cells on filter pads in a low-salt buffer in the absence of a food source. Synchronous development then proceeds at the air–water interface; gradients of cAMP are established and amebae move toward regions with high cAMP concentrations (1, 2). Stimulation of amebae by extracellular cAMP results in a rise in intracellular cAMP concentrations. cAMP is then secreted, thus, amplifying and propagating the original cAMP concentration wave (3–5). After relaying the cAMP signal, cells become transiently refractory to further stimulation (6, 7). During this period of adaptation or desensitization, extracellular cAMP concentrations decrease and cells recover their sensitivity to extracellular cAMP stimulation. This adaptation/deadaptation cycle repeats itself with cAMP pulses initiating from aggregation centers with a periodicity of ≈6 min. The result is the assemblage of groups of $\approx 10^5$ cells into multicellular aggregates. Individual aggregates then develop into mature fruiting bodies consisting of predominantly spore and stalk cells. The addition of high (mM) concentrations of cAMP early in development inhibits aggregation and continued development (8).

Specific changes in the expression of the *Dictyostelium* genome correlate closely with different stages of the developmental cycle. Many of the early developmental changes can be observed in cells in shaking culture. We report here studies on the regulation of a gene (*M4-1*) that is expressed in undifferentiated (vegetative) cells but not in cells in the latter part of the developmental cycle. Cells incubated in shaking culture in the presence or absence of continuous high concentrations of cAMP, conditions that do not promote differentiation, express this gene at vegetative levels. In contrast, expression is repressed by pulses of a low concentration of cAMP, a condition that mimics early developmental events and leads to an increase in intracellular cAMP levels. By exposing cAMP-pulsed cells to growth medium, *M4-1* expression can rapidly be reinduced. The rapid rise in the level of nuclear RNA derived from this gene suggests strongly that the observed changes in the expression of *M4-1* during differentiation and dedifferentiation are mediated at the level of transcription. Possible mechanisms mediating the regulation of *Dictyostelium* gene expression by cAMP will be discussed.

MATERIALS AND METHODS

Growth and Manipulation of Cells. Wild-type cells were used for all developmental studies on solid substrata. Axenic (Ax-3) cells were used for differentiation in shaking culture. Vegetative cells were grown to 2×10^6 cells per ml and resuspended at 1×10^6 cells per ml in the low-salt buffer PDF (9). cAMP was added as described in the text. Aggregated cells were physically disrupted in PDF buffer containing 5 mM EDTA (10).

Isolation and Hybridization of RNA. Total, cytoplasmic, and nuclear poly(A)$^+$ RNAs were prepared by phenol extraction as described (11, 12). RNA was denatured in 50% formamide/6% formaldehyde and separated on denaturing formaldehyde/agarose gels by electrophoresis, blotted on nitrocellulose, and hybridized to specific probes (11–13).

Labeling of DNA *in Vitro*. Double-stranded DNA was labeled *in vitro* by nick-translation using DNA polymerase I and DNase I and single-stranded M13 probes were labeled using a hybridization probe primer and the Klenow fragment of DNA polymerase I (11–13).

cAMP Assay. Shaking cultures were assayed periodically for the accumulation of endogenous or exogenous cAMP by a radioimmunoassay using cAMP antiserum complex (14).

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Abbreviation: kb, kilobase(s).

RESULTS

Polarity of Transcription and Developmental Expression of *M4-1*. The *M4* region of the *Dictyostelium* genome is comprised of two developmentally regulated transcription units (Fig. 1). The single-copy *M4-1* gene encodes a 0.9-kb mRNA present at 0.1% of total poly(A)$^+$ RNA in vegetative cells (11). The second transcription unit *M4-4* is composed of repeat and single-copy DNA. M4-4 mRNA represents 0.01% of total mRNA in vegetative cells; however, the relative level of expression of this gene increases ≈5-fold as development proceeds (13). The organization and developmental expression of the *M4-4* gene have been described in detail (11–13). The close (≈2 kb) proximity of these two differentially regulated transcription units led us toward a closer examination of the *M4-1* gene.

As an initial step toward a complete analysis of the structure of the *M4-1* gene, we have determined its polarity of transcription. M4-1 sequences were subcloned in both orientations in M13 single-stranded cloning vectors. Strand-specific probes were hybridized to nuclear and cytoplasmic poly(A)$^+$ RNA (Fig. 2). Only one strand of the *M4-1* gene hybridized to RNA from either nuclei or cytoplasm. Thus, the *M4-1* gene is asymmetrically transcribed. Interestingly, its transcription is divergent from the *M4-4* gene (see Fig. 1); the 5′ upstream sequences of each gene are contiguous. The M4-1 nuclear RNA is 1 kb, 10% larger in size than the M4-1 0.9-kb cytoplasmic mRNA (11). Since nuclear poly(A) lengths are generally greater than their cytoplasmic equivalents (15) and since *Dictyostelium* introns and, hence, mRNA precursors are relatively small (12, 16), it is not possible to conclude that the M4-1 mRNA is synthesized as a larger molecular weight precursor.

The developmental expression of the *M4-1* gene was also monitored. Poly(A)$^+$ RNA was isolated from wild-type cells at various times during the *Dictyostelium* developmental cycle. Equal amounts of RNA were separated electrophoretically on an agarose gel, blotted onto nitrocellulose, and hybridized with a probe specific for the M4-1 mRNA. As seen in Fig. 3, M4-1 mRNA was present at similar levels in bacterially grown undifferentiated vegetative cells and cells early in development. By aggregation (10 hr), the level of M4-1 RNA decreased to undetectable levels (<1 mRNA per cell). M4-1 mRNA did not reappear throughout later development (20 hr).

cAMP Pulses Repress *M4-1* Expression. Since the levels of M4-1 RNA are similar in vegetative cells and cells that have developed for 5 hr, it seemed unlikely that the initiation of development *per se* was sufficient to depress the expression of the *M4-1* gene. Rather, the timing of *M4-1* repression would appear to correlate with the establishment of the cAMP signal-relay system during early development (3–5). We thus examined the expression of *M4-1* in cells in shaking cultures that did or did not exhibit cAMP signaling.

When *Dictyostelium* were differentiated in culture at a high shaking speed and low cell density, endogenous cAMP signaling was inhibited. Logarithmic-phase cells were collected from growth media, resuspended in low-salt buffer (PDF) at 1×10^6 cells per ml, and shaken at 200 rpm. We were able to confirm that under the conditions described, these cells were unable to generate an endogenous cAMP signal. Aliquots were assayed for endogenous cAMP levels every minute during the expected periods of maximal signaling. In addition, there was no accumulation of extracellular cAMP levels during the entire culture period of 10 hr. When cAMP was added to 25 nM at 6-min intervals to cultures shaking

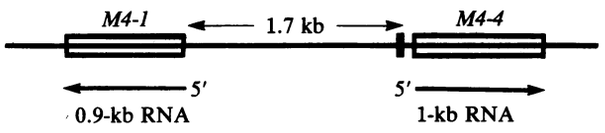

FIG. 1. The *M4* region in the *Dictyostelium* genome contains two transcription units. *M4-1* encodes a 0.9-kilobase (kb) mRNA and *M4-4* encodes a 1-kb mRNA. The directions of transcription are indicated as is the approximate distance between 5′-transcription initiation sites. The black box in *M4-4* indicates the location of a repetitive sequence found in association with certain developmentally regulated genes.

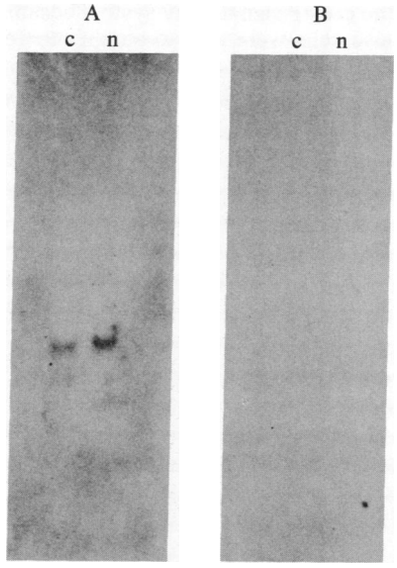

FIG. 2. Determination of orientation of the *M4-1* transcription unit. Strand-specific probes were constructed by using restriction fragments of *M4-1* cloned in both orientations in M13 vectors. Strand-specific probes were hybridized to RNA blots of cytoplasmic (c) and nuclear (n) poly(A)$^+$ RNA. The direction of transcription of *M4-4* has been determined (see Fig. 1). (*A*) Orientation represents hybridization to the strand opposite that of M4-4 mRNA. (*B*) Orientation represents hybridization to the same strand that hybridizes to M4-4 mRNA.

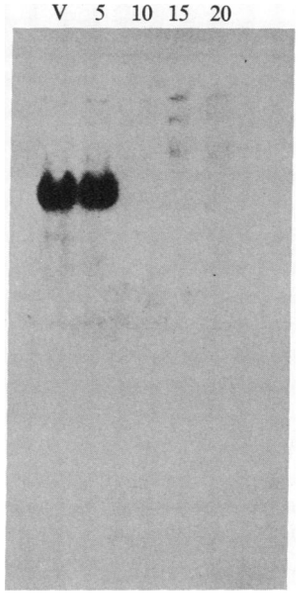

FIG. 3. Developmental expression of *M4-1*. A probe specific to *M4-1* was hybridized to RNA blots containing equal amounts of poly(A)$^+$ RNA from vegetative (V) cells and cells developed for 5, 10, 15, and 20 hr.

identically, the cAMP signal-relay system became expressed normally; these cells were able to amplify the exogenously supplied cAMP pulse. As a control, we observed normal endogenous cAMP signaling in the absence of an exogenously supplied cAMP pulse in cells shaken at a slow speed (90 rpm) at a high density (2×10^7 cells per ml).

We were, thus, interested in determining the levels of M4-1 mRNA in these fast-shaking cultures at low cell densities in the presence or absence of exogenous cAMP. One culture received pulses of cAMP to 25 nM at 6-min intervals. Again, samples were taken for assay to confirm that the cAMP concentration was not cumulative and that, indeed, these conditions were mimicking the cAMP pulses observed in normal development. A second culture was immediately adjusted to 500 μM cAMP but additional cAMP was provided to increase the final cAMP concentration by 100 μM every 60 min. These conditions are sufficient to inhibit development and the establishment of endogenous cAMP signaling (8). A third culture was not treated with cAMP. None of the three cultures exhibited agglomerate formation observable by light microscopy. Poly(A)$^+$ RNA was isolated from the vegetative cells and from the three shaking suspension cultures at incubation times of 2.5, 5, 7.5, and 10 hr. RNA blots were prepared and hybridized with an *M4-1* probe (Fig. 4). Cells incubated with high concentrations of cAMP as well as those incubated without cAMP continued to express the *M4-1* gene at levels similar to that of vegetative cells. Little or no change in expression was seen in cells pulsed with cAMP for 5 hr or less. By 7.5 hr, in the pulsed cells, the level of the M4-1 RNA had declined and by 10 hr it had decreased by a factor of >10 relative to that of vegetative cells. The timing of M4-1 mRNA decay in these cultures paralleled that observed for normal development and reflects the kinetics of appearance of oscillations in the intracellular cAMP pool. It should be noted that although the cAMP pulse in suspension culture was initiated at the onset of starvation, cells in suspension establish a cAMP signal-relay system at a time during differentiation that is coincident with that of normally developing cells. Thus, pulses of cAMP appear sufficient to elicit the repression of *M4-1* expression.

***M4-1* Is Induced During Dedifferentiation.** Developing cells will rapidly dedifferentiate if reexposed to fresh growth medium (17). Complete dedifferentiation yields vegetative cells that should express *M4-1* at normal growth levels. Vegetative cells were pulsed with cAMP in PDF buffer to depress *M4-1* expression, washed from this differentiation medium, and then resuspended in fresh medium. Poly(A)$^+$ RNA was purified after 2, 4, 7, and 10 hr of refeeding. Within 2 hr, the M4-1 mRNA began to reaccumulate and it reached vegetative levels within 4 hr (Fig. 5).

In a similar experiment we examined nuclear and cytoplasmic levels of M4-1 poly(A)$^+$ RNA from vegetative cells, cAMP-pulsed cells, and cells refed for 45 and 90 min (Fig. 6). In vegetative cells the relative abundance of the M4-1 sequences in the cytoplasm was slightly greater than in the nucleus. After pulsing with cAMP, the levels of M4-1 sequences in nuclear and cytoplasmic poly(A)$^+$ RNAs were reduced by a factor of >10 (see Fig. 6). Reexposing the cells to growth medium for 45 min resulted in a reappearance of M4-1 RNA in the nucleus and the cytoplasm. After 90 min the relative nuclear and cytoplasmic levels of M4-1 RNA increased further. The changes in the relative nuclear RNA levels suggest strongly that the *M4-1* gene is actively transcribed in dedifferentiated cells and that developmental cAMP pulses effect repression of *M4-1* gene activity.

Effect of Interrupting Cell–Cell Interaction on *M4-1* Expression. Several laboratories have shown that if aggregated cells are dissociated, the pattern of expression of many developmentally regulated genes is altered (see refs. 18 and 19). Some late genes expressed specifically in aggregates are deactivated, whereas some genes normally expressed earlier in development are reactivated. The addition of cAMP to dissociated aggregates often allows cells to maintain the aggregation-specific expression state of some of these developmentally regulated genes. It was of interest to determine if *M4-1* could be reactivated by dissociation of aggregates in the absence (or presence) of cAMP.

Wild-type cells were allowed to develop for 15 hr on filters and, consistent with our previous observations, vegetative cells were shown to possess M4-1 RNA sequences, whereas these sequences were not detectable in aggregates (Fig. 7). When the 15-hr aggregates were dissociated and then incu-

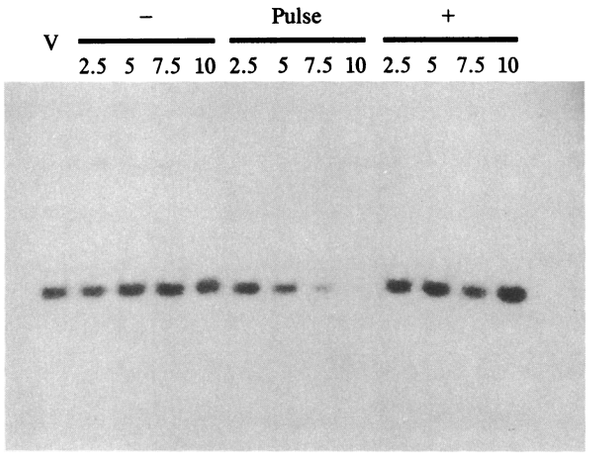

FIG. 4. Expression of *M4-1* in shaking cultures in the presence or absence of exogenous cAMP. Vegetative (V) cells were washed and resuspended in PDF buffer. One culture (with cAMP, +) was adjusted to 500 μM cAMP and received cAMP to an additional 100 μM every 60 min. One culture (pulse) received pulses of cAMP to 25 nM every 6 min. One culture (without cAMP, –) did not receive any exogenous cAMP. Poly(A)$^+$ RNA was isolated from vegetative cells and cells incubated in shaking culture for 2.5, 5, 7.5, and 10 hr. RNA blots were prepared and hybridized to an *M4-1* probe.

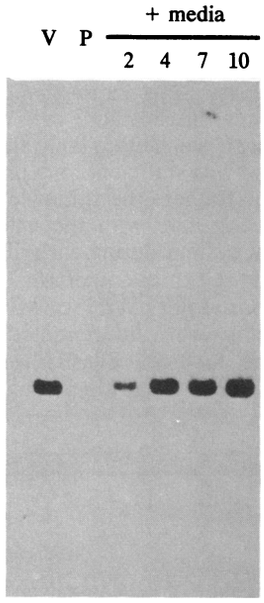

FIG. 5. Expression of *M4-1* during dedifferentiation. Vegetative (V) cells were resuspended in PDF buffer, pulsed with cAMP (P), and subsequently incubated in fresh medium (+ media). RNA blots were prepared by using poly(A)$^+$ RNA isolated at the times indicated (in hours) and hybridized to an *M4-1* probe.

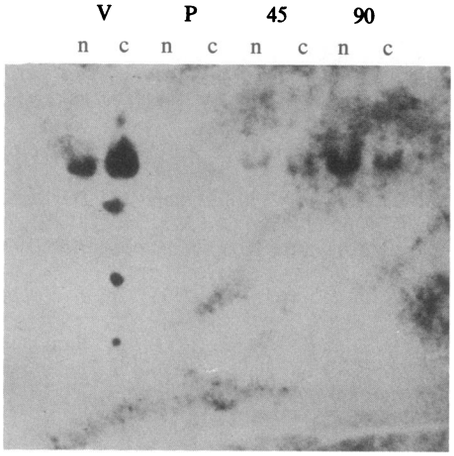

FIG. 6. M4-1 RNA levels in nuclei and cytoplasm of vegetative (V) cells, cAMP-pulsed (P) cells, and cells refed for 45 and 90 min. An *M4-1* probe was hybridized to RNA blots containing equal amounts of poly(A)$^+$ RNA isolated from nuclei (n) or cytoplasm (c) of indicated cell preparations.

bated for 4 hr in the presence or absence of cAMP, no reaccumulation of the M4-1 RNA occurred (Fig. 7).

DISCUSSION

M4-1 is one of the first genes identified that is expressed in undifferentiated cells but repressed during development. More specifically, it is negatively modulated by the cAMP pulses that occur during early *Dictyostelium* development. Exposing developed cells to fresh growth medium promotes dedifferentiation and induces a rapid reaccumulation of M4-1 RNA sequences. Results comparing the relative nuclear abundance of M4-1 RNA sequences among vegetative, developing, cAMP-pulsed, and dedifferentiating cells strongly suggest a transcriptionally mediated control of *M4-1*. If *M4-1* transcription has ceased by 5 hr, the decay of M4-1 mRNA

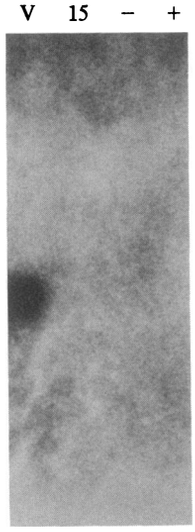

FIG. 7. Effect of dissociation of aggregated cells on *M4-1* expression. Vegetative (V) cells were plated for 15 hr to form tight cell aggregates. Aggregates were disrupted and incubated for 4 hr in fast-shaking cultures as single cells with and without continuous exposure to high levels of cAMP. Poly(A)$^+$ RNA was isolated from vegetative cells, 15-hr developed cells, disaggregated cells without cAMP (−), and disaggregated cells exposed to cAMP (+) and hybridized on RNA blots to an *M4-1* probe.

during development would suggest an mRNA turnover of 2 hr.

It has been shown recently that *M4-4*, the gene adjacent to *M4-1* in the genome (see Fig. 1), was developmentally regulated in a manner different from that of *M4-1* (13); *M4-4* expression increases early in development, whereas *M4-1* is repressed. We also suggested that certain sequences responsible for the developmental increase in expression of *M4-4* were located 5′ to the site of transcription initiation. We have shown that the *M4-1* gene is transcribed divergently from *M4-4* (see Fig. 1). If 5′ control regions are associated with different modes of regulation, they should lie within 1.5 kb of each other in the genome. It may be likely that all of the regulatory regions of both genes would be present on the isolated 4.5-kb genomic clone and facilitate their analyses. In addition, their organizations are suggestive of individual gene regulation rather than control of large chromosomal regions.

Although the *M4-1* gene is, to our knowledge, the first vegetative gene isolated whose expression is negatively controlled during the described developmental stage, previous studies of proteins synthesized in vegetative and developing cells suggest that several other moderately expressed genes may show the same developmental kinetics of expression (20–22). Hybridization studies also indicate that there is a very limited class of vegetative genes that are repressed at this developmental stage (18, 19, 23). Identification and comparison of regulatory sequences within such a presumptive limited gene set may lead to a clearer understanding of the mechanisms involved in repressing genes during early development as well as mechanisms for reinducing them during dedifferentiation.

M4-1 repression can be observed in shaking culture under conditions in which endogenous oscillations of cAMP are absent but into which pulses of cAMP have been introduced exogenously. Since these cultures do not form agglomerates, cell contact does not appear to be an obligatory, associative event involved in *M4-1* repression. *M4-1* is repressed in the absence of and prior to aggregation. Aggregates dissociated in the presence of cAMP appear to maintain their developmental properties, whereas aggregates dissociated in the absence of cAMP regress to an earlier developmental stage equivalent to that of cAMP-pulsed cells. Dissociation of aggregates in the presence or absence of cAMP has no effect on *M4-1* expression, again consistent with *M4-1* repression occurring independently of cell contact. We have confirmed that genes putatively affected by cell contact (24) exhibit the expected differential expression in disaggregated cells shaken in the presence or absence of cAMP (10, 25, 26). It is likely that cAMP pulses are sufficient to elicit the repression of *M4-1* and that cell–cell contact is not required for this repression.

We have begun to examine the expression of *M4-1* in certain cell lines that do not exhibit normal developmental or erasure (dedifferentiation) properties. Cell lines that develop in the presence or absence of high concentrations of cAMP are of particular interest since they do not respond normally to cAMP pulses but will form small aggregates that develop into fruiting bodies (27). A dedifferentiation deficient cell line also exists, which abnormally retains its ability to reaggregate rapidly (28). Such studies may allow us to focus on regulatory events only apparent when normal patterns of development are disrupted.

The physiological effect of *M4-1* repression on developing *Dictyostelium* is not yet known. The M4-1 gene product may be essential for vegetative growth or necessary for early development; its protein product may have a negative effect on later developmental stages. If this is true, the M4-1 protein may be unstable during later development or become specifically sequestered or modified. The identification and subcellular localization of the protein encoded by *M4-1* in

conjunction with expression studies may direct us toward an understanding of the function of the M4-1 protein and eventually of other proteins regulated similarly during vegetative growth and development.

Finally, we are interested in the mechanism that mediates the regulation of *M4-1* expression by cAMP. The data presented here are most consistent with an intracellular effect of cAMP. It is interesting that at the same time of development that *M4-1* expression becomes repressed, there is an increase in activity of the cAMP-dependent protein kinase (29). It has been postulated that an increase in the intracellular cAMP pool during development eventually results in the phosphorylation of specific proteins that are responsible for altering the pattern of gene expression in *Dictyostelium*. It should now be possible to determine if genes regulated by cAMP signaling (e.g., *M4-1*) are controlled in a manner similar to or different from genes such as those preferentially expressed in prespore or prestalk cells, whose expressions are dependent upon continuous high levels of extracellular cAMP.

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