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A role for cyclic AMP in expression of developmentally regulated genes in *Dictyostelium discoideum*

(slime molds/two-dimensional gels/*in vitro* translation)

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ABSTRACT **Starved cells of *Dictyostelium discoideum* begin to synthesize a new class of developmentally regulated proteins at about 13 hr of the 24-hr developmental program, concomitant with the formation of tips on the tight cell aggregates [Alton, T. H. & Lodish, H. F. (1977) *Dev. Biol.* 60, 180–206]. Continued synthesis of these proteins is normally dependent upon the integrity of the multicellular aggregates, because cells that have been disaggregated at 13 hr and shaken in suspension for 5 hr do not make these proteins. We show here that addition of 20 μM cyclic AMP to suspension cultures of disaggregated 13-hr cells caused synthesis of most of these late proteins to be maintained. Translation in an *in vitro* wheat germ system of total cellular RNA isolated from these cyclic AMP-stimulated suspension cells, or from normal aggregates, generated several proteins that were not encoded by the RNA isolated from equivalent suspension cells which had not been treated with cyclic AMP or from preaggregation cells. We conclude that cyclic AMP has a direct role in maintaining the synthesis of aggregation-dependent *Dictyostelium* proteins and in maintaining the level of the corresponding mRNAs.**

The cellular slime mold *Dictyostelium discoideum* presents an ideal eukaryotic system in which to study the control of developmentally regulated genes (1, 2). Growing cells exist as unicellular amoebae in the presence of a complete nutrient medium. When amoebae are starved and plated at an air–liquid interface, a well-defined developmental program ensues in which cells secrete 3′,5′-cyclic AMP (cAMP) (3) in a pulsatile manner (4–6), stream toward aggregation centers, and form multicellular aggregates that differentiate into stalk and spore tissues (1, 2). Some genes are expressed only during discrete stages of this developmental cycle: if cells are labeled with 15- to 30-min pulses of [^{35}S]methionine at various intervals throughout the 24-hr developmental program, and the protein products are separated on O'Farrell two-dimensional gels (7), about 100 of the 400 observable spots change substantially in relative intensity at some period of development (8). The most pronounced alteration occurs around 10–13 hr in the developmental cycle, at the time when tight cell–cell aggregates have formed and tips begin to appear at the top of the mounds: approximately 40 proteins that were made at low levels or not at all in 3-hr cells are induced to high levels of synthesis. The continued pronounced synthesis of these developmentally controlled proteins is dependent upon the maintainance of cell contact, because mounds that are disaggregated selectively arrest or attentuate the synthesis of these late species (9). In this paper, we present evidence that cAMP is directly involved in controlling the expression of this class of genes.

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MATERIALS AND METHODS

Conditions for axenic growth of strain AX-3 of *D. discoideum*, plating for development, labeling with [^{35}S]methionine, preparation of protein samples for electrophoresis on two-dimensional gels, fluorography, isolation of total cellular RNA, and *in vitro* translation in wheat germ extracts were all as described (8). Due to a change in the batch of Ampholines used in isoelectric focusing, our two-dimensional gel patterns do not correspond to those previously published (8); however, pH measurements made by slicing gels and eluting into boiled distilled water indicate that our present gradients are linear from pH 4.8 to 8.2.

Autoradiograms were made by exposing a gel containing 10^6 cpm of trichloroacetic acid-precipitable ^{35}S for 24 hr; gels containing less ^{35}S were exposed for proportionally longer periods of time. Hence our autoradiograms represent the relative, but not absolute, rate of synthesis of protein species.

Assays for cAMP were performed essentially by the method of Gilman (10) as detailed in the Calbiochem cAMP assay kit (catalog no. 869071). The absence of interfering activities was demonstrated by digesting samples with 0.02 unit of beef heart phosphodiesterase (Sigma) in 100 μl of 1 mM imidazole acetate/1 mM $MgSO_4$, pH 7.5, for 1 hr at 30°C. For measurement of extracellular cAMP, cells were removed by centrifugation, and the supernatant was made 1 M in perchloric acid and then neutralized with K_2CO_3 (11).

RESULTS

Dictyostelium cells of strain AX-3 which are transferred to pH 6.5 starvation buffer (8) containing 7 mM 2-(*N*-morpholino)-ethanesulfonic acid, 20 mM KCl, 5 mM $MgSO_4$, and 0.3 mg of streptomycin per ml (hereafter referred to as MES-PDF) and plated on filter pads saturated with MES-PDF aggregate and form mature fruiting bodies after 24–26 hr at 22°C; formation of tight cell aggregates with tips generally occurs between 10 and 13 hr. Cells that are pulse labeled with [^{35}S]methionine for 20 min at 3 hr of development give rise to several hundred spots on fluorograms of two-dimensional O'Farrell gels (Fig. 1*A*); these spots presumably represent the most abundantly synthesized proteins that focus within the pH and size range of the gel. As reported previously (8), cells labeled at 13 hr—i.e., after tip formation—make almost all of the proteins characteristic of 3-hr cells plus, reproducibly, 38 species that are either new (spots 1–13, 15, 18, 22–25, 28, 30–32, 35, and 36 in Fig. 1*B*) or substantially enhanced (spots 14, 16, 17, 19–21, 26, 27, 29, 33, 34, 37, and 38 in Fig. 1*B*) compared to 3-hr cells. Continued synthesis of most of these proteins requires intact cell–cell contacts (9), because aggregates that are washed off filters,

Abbreviation: cAMP, 3′,5′-cyclic AMP.

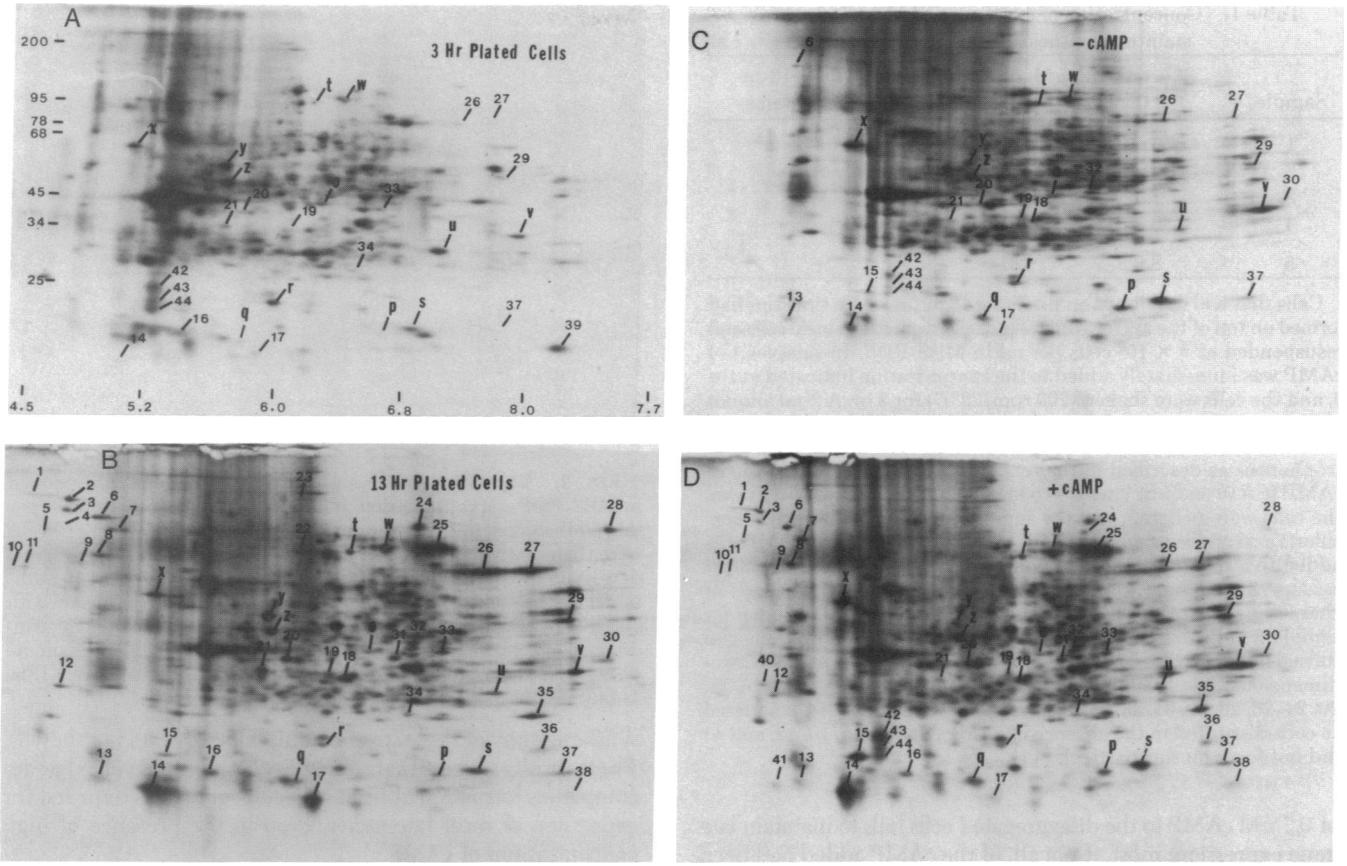

FIG. 1. (*A* and *B*) About 2.5×10^7 cells of *D. discoideum* developing on Whatman filters saturated with MES-PDF buffer were pulse labeled with 225 μCi (1 Ci = 3.7×10^{10} becquerels) of [^{35}S]methionine for 20 min and lysed in sample storage buffer (8), and the products were separated on O'Farrell two-dimensional gels (7). An approximate calibration of the pH gradient of the isoelectric focusing gel is shown on the horizontal axis of *A*, and a molecular weight $\times 10^{-3}$ calibration appears on the vertical scale. The pH gradient decays in the basic region of the isoelectric focusing gel, where the pH actually drops from 8.2 to 7.7. In all figures, numbers refer to polypeptides whose rate of synthesis changes during development or in the presence of cAMP; letters *o* through *z* are reference spots to facilitate comparison of different gels. (*A*) Three-hour plated cells. (*B*) Thirteen-hour plated cells. (*C*) At 13 hr, cells were washed off of the filters into cold MES-PDF containing 10 mM EDTA and disaggregated by repeated vigorous pipetting. This suspension culture, at a density of 5×10^6 cells per ml, was shaken at 200 rpm and 22°C for 5 hr, and then a 2.0-ml aliquot was labeled with 450 μCi of [^{35}S]methionine for 30 min. The cell pellet was prepared for electrophoresis as above. (*D*) Cells were treated as for *C* except that cAMP was added to a concentration of 1 mM at 0, 2, and 4 hr after disaggregation. Samples assayed at 2, 4 (just before addition of the second and third dose of cAMP), and 5 hr indicated that no significant degradation of the cAMP had occurred. Microscopic examination after 5 hr of shaking in suspension revealed that amoebae in both *C* and *D* had been completely dispersed into single cells, with a few aggregates of 2–10 cells. Numbers refer to spots that are either new or substantially enhanced in either 13-hr or cAMP-treated cells.

dispersed by vigorous pipetting, and shaken in suspension for 5 hr either no longer synthesize these species or make them at greatly reduced rates (Fig. 1*C*); the protein profile reverts to one very similar to that for 3-hr cells. However, if 1 mM cAMP is added to the suspension cells at 0, 2, and 4 hr after disaggregation, the synthesis of most of the late (13-hr specific) proteins continues (Fig. 1*D*).

Of the 38 proteins synthesized by 13-hr cells that are made in reduced levels or not at all by 3-hr cells, 32 (spots 1–3, 5–16, 18, 20, 24–38) continue to be made at high levels by suspension cells in the presence of cAMP. Most of these are not made or are made at reduced rates by disaggregated cells in the absence of cAMP, but four of the 13-hr-specific proteins (spots 13, 29, 32, 37) do continue to be made at high levels by suspension cells in both the presence and absence of cAMP; possibly these represent translation products whose messages degrade slowly when cells are dispersed. There are six polypeptides (spots 4, 17, 19, 21–23) whose synthesis stops or decreases upon disaggregation but that are not maintained by cAMP. Finally, there are five proteins (spots 40–44) whose synthesis is apparently maintained by cAMP but that are not induced at 13 hr during normal development; note especially the triplet numbered 42, 43, and 44 (Fig. 1*D*) whose synthesis is turned off by 13 hr of normal development but is reinduced by cAMP. Spot 39 is made only by 3-hr cells. Letters *o* through *z* indicate constant spots that are included to facilitate comparison of different gels.*

Microscopic examination shows that over 90% of the suspension cultures, with or without cAMP, are single cells even at the end of the 5-hr incubation. Furthermore, these resuspended cells are still developmentally competent: when cells that had been shaken in suspension for 5 hr (with or without cAMP) were plated, they formed loose aggregates immediately, developed tips within 2–3 hr, and culminated normally to form mature fruiting bodies.

Table 1 demonstrates that addition of cAMP to a concentration of 7–20 μM is sufficient to maintain synthesis of these late proteins. By contrast, addition of an initial concentration

* Of the spots listed in Figs. 1–3, all except spots 1, 4, 5, 10, 11, and 13 showed the indicated changes on at least two independent sets of gels. These spots appear in peripheral regions of the gel and occasionally do not focus with particular sets of Ampholines.

Table 1. Concentration of exogenous cAMP required to maintain synthesis of late proteins

Sample	cAMP, μM $t = 0$	cAMP, μM $t = 5$ hr	Synthesis of 13-hr-specific proteins
1	670	840	+
2	95	58	+
3	7.3	0.1	±
4	0.7	<0.1	−
5	8.6	20	+

Cells that had developed on filters for 13 hr (at which time tips had formed on top of the aggregates) were disaggregated to single cells and resuspended at 5×10^6 cells per ml in MES-PDF. In samples 1–4 cAMP was immediately added to the concentration indicated at $t = 0$, and the cells were shaken (200 rpm, 22°C) for 5 hr. A 2-ml aliquot of each sample was then labeled with 450 μCi of [^{35}S]methionine for 30 min, and the sample was prepared for two-dimensional gel electrophoresis as described in the text. Immediately after addition of cAMP ($t = 0$) and 5 hr later ($t = 5$ hr) a 500-μl aliquot was withdrawn, the cells were pelleted, and the supernatant was made 1 M in perchloric acid and assayed for cAMP. In the case of sample 5, cAMP was added to a nominal concentration of 10 μM at 0, 2, and 4 hr after resuspension. The + symbol indicates the pattern of synthesis of characteristic late polypeptides shown in Fig. 1*D*. The ± symbol for sample 3 indicates that synthesis of many of the late proteins was turned on, but to a lesser degree than for samples 1, 2, and 5. A two-dimensional gel of sample 5 indicated that spots 2, 3, 6–9, 14–16, 18, 20, 24–26, 28, 30, 31, 33, 35, 38, and 42–44 were enhanced, compared to cells disrupted in the absence of cAMP. Spots 4, 5, 10–12, and 41 did not focus on this particular gel.

of 0.7 μM cAMP to the disaggregated cells fails to maintain late gene expression; most, if not all, of the cAMP added had been hydrolyzed at the end of 5 hr. Consequently, 20 μM represents the estimate for the minimum level of cAMP necessary to ensure continued expression of late genes. Finally, the response is specific for cAMP, because 1 mM 5′-AMP was completely ineffectual in maintaining synthesis of late proteins (data not shown).

Although cAMP can sustain the expression of late genes in cells that have already aggregated and have then been disrupted, addition of cAMP to aggregation-competent cells that have not actually aggregated does not turn on these late genes. Cells that have been shaken in starved suspension for 16 hr are considered aggregation competent, because they possess surface proteins required for cell–cell cohesion (12, 13), they contain the cAMP receptor and phosphodiesterase (14) required for cAMP signaling, and they aggregate rapidly if plated on a surface (15); these aggregates induce normal synthesis of the 13-hr-specific proteins (ref. 9; data not shown). Fig. 2 reveals that cells that were shaken for 16 hr in starved suspension and then resuspended in MES-PDF containing 1 mM cAMP did not synthesize any of these late polypeptides; an assay of the medium indicated that the cAMP had not been degraded during the 5-hr incubation. However, when these cAMP-treated suspension cells were plated, they aggregated and formed tips within 7 hr and developed normally to the culmination stage.

Amoebae that have been plated on filters for 8 hr are in the process of streaming together, and are in loose contact, but they have not yet formed tight cell–cell contacts, and they do not make any of the characteristic "late" proteins. When these 8-hr plated cells were dispersed and shaken in suspension cultures in the presence of 1 mM cAMP for 5 hr, only a small subclass of the cAMP-dependent proteins (specifically, numbers 1, 2, 6, 15, 16, 25, and 42–44) were expressed at the increased levels characteristic of 13-hr cells resuspended in cAMP after 13 hr of development; controls resuspended without cAMP did not

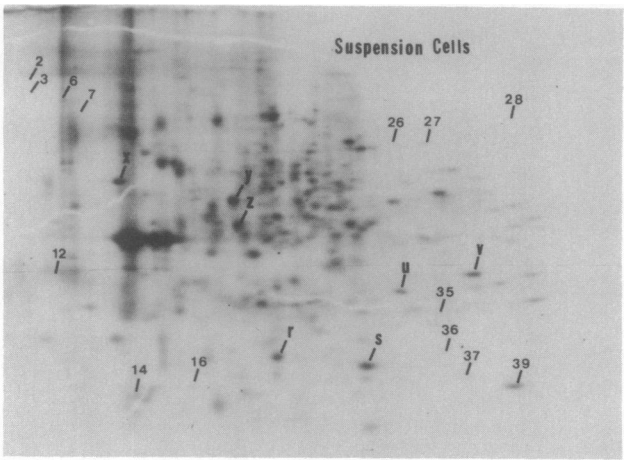

FIG. 2. Vegetative amoebae were washed twice and resuspended in MES-PDF starvation buffer at a concentration of 5×10^6 cells per ml and then shaken in suspension (200 rpm, 22°C) for 16 hr. The cells were then centrifuged, washed once, and resuspended at the original density in fresh MES-PDF containing 1 mM cAMP. After an additional 5 hr of shaking, a 2-ml aliquot was pulse labeled for 30 min with 450 μCi of [^{35}S]methionine, and the sample was prepared for two-dimensional gel electrophoresis. The numbers indicate approximate positions of some late proteins that are made by 13-hr plated cells (Fig. 1) but are not made by these starved suspension cells.

synthesize any of these species at high levels (data not shown). These results suggest that some developmental event that accompanies formation of tight cell–cell contacts is required for expression of most late genes, even in the presence of high concentrations of cAMP.

Previous results (8) indicate that the appearance of many of the 13-hr proteins coincides with the accumulation of messenger RNA that can program the synthesis of these same proteins in an *in vitro* wheat germ translation system. Comparison of Fig. 3 *A* and *B* indicates that eight species are new (spots 6, 7, and a) or enhanced (spots 14–16, b, and c) in the translation products from RNA isolated from 12-hr developing cells relative to the translation products of RNA from 3-hr cells. Five of these products (spots 6, 7, and 14–16) may be authentic developmentally regulated *Dictyostelium* proteins, because they comigrate on two-dimensional gels with authentic 13-hr-specific polypeptides. Fig. 3 *C* and *D* displays the translation products of RNA isolated from cells that were plated for 13 hr, disaggregated, and then shaken in suspension for 5 hr either with (Fig. 3*D*) or without (Fig. 3*C*) three additions of 1 mM cAMP. Three new polypeptides (spots 6, 7, and a) were present in the translation products of RNA from cAMP-treated cells that were not found in the translation product of RNA from cells incubated without cAMP; six more polypeptides (spots 14–16, 42–44) were enhanced in the translation products of RNA from cAMP-treated cells when compared to translation products of RNA from cells disaggregated without cAMP.†

In particular, the *in vitro* translation products of RNA from cAMP-treated cells (Fig. 3*D*) were identical to the *in vitro* translation products of RNA from normal 12-hr cells (Fig. 3*B*). Hence, at least for those polypeptides identified above, cAMP acts to maintain high levels of translatable mRNA encoding these proteins. For reasons discussed previously (8), not all authentic *Dictyostelium* proteins can be detected as products of a wheat germ translation reaction.

† There are several spots that appear, on this particular set of gels, to be selectively enhanced in translations of RNA from either 3-hr cells or aggregates dispersed in the absence of cAMP; however, these changes were not reproducible in a second set of gels.

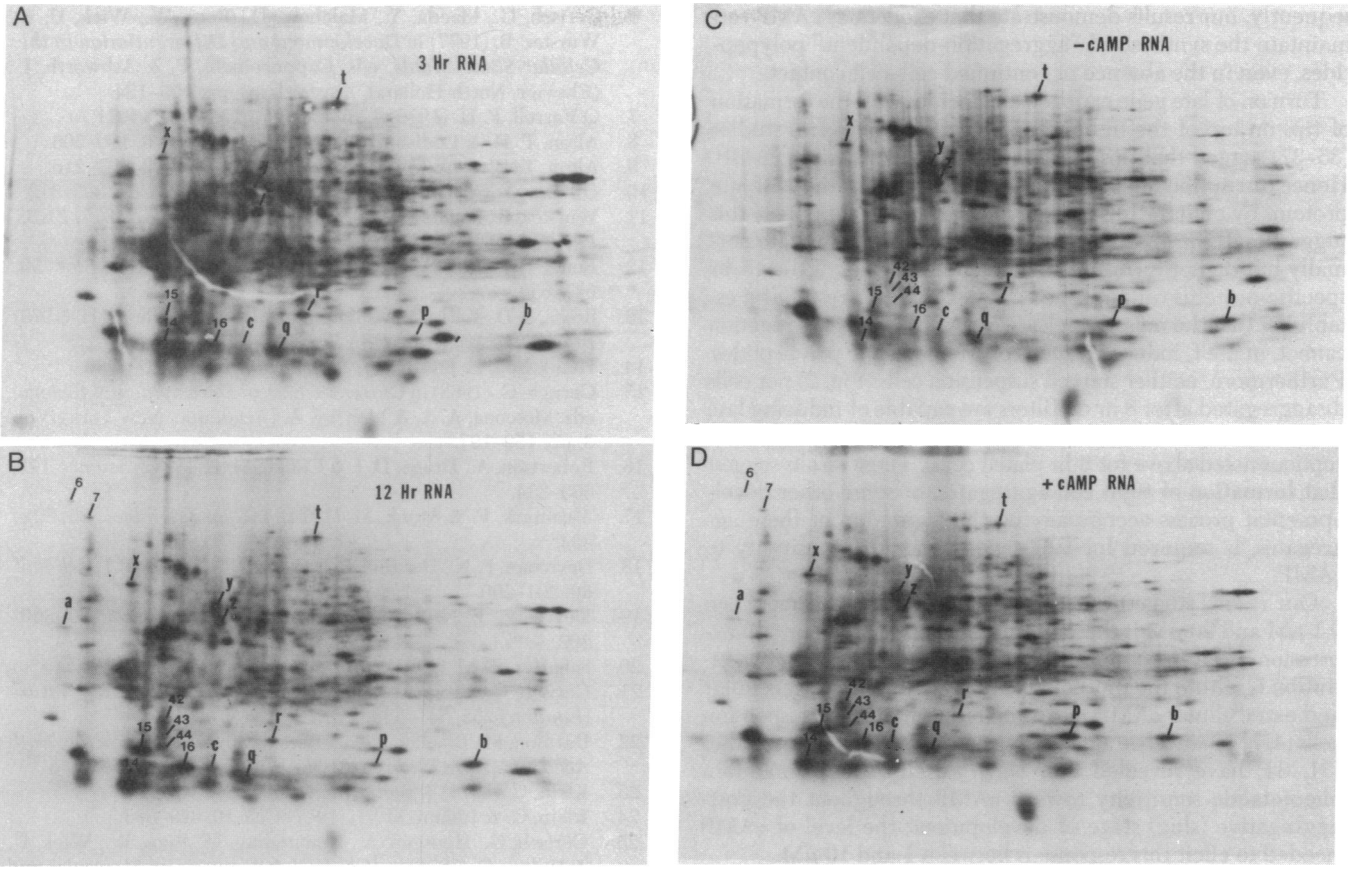

FIG. 3. *In vitro* translation products of wheat germ reactions primed with total cellular RNA were resolved on two-dimensional gels. RNA was isolated from: (*A*) 3-hr plated cells, (*B*) 12-hr plated cells, (*C*) 12-hr plated cells, disaggregated and shaken in suspension (5×10^6 cells per ml) for 5 hr, (*D*) suspension cells as in *C* except that cAMP was added to a concentration of 1 mM at 0, 2, and 4 hr after disaggregation. Spots scored with either numbers or the letters *a*, *b*, and *c* are those that are either new or substantially enhanced in translation products of RNA isolated from 12-hr or cAMP-treated cells. Numbers designate authentic *Dictyostelium* proteins that are induced during development or by addition of cAMP. Letters *a*, *b*, and *c* indicate spots that are found only in the translation products of RNA isolated from 12-hr or cAMP-treated cells and are not synthesized in that form by the intact cells.

DISCUSSION

cAMP plays a central role in the early stages of development in *D. discoideum*. This nucleotide is the chemotactic agent that directs aggregation of single amoebae into multicellular masses during the first 10 hr after initiation of starvation (3, 16). Certain cells first secrete cAMP in a pulsatile manner, inducing surrounding cells to migrate toward the original source of cAMP (17). The affected cells secrete cAMP in response to the original pulse, resulting in signal amplification and relay and giving rise to periodic outward propagating waves of cAMP that direct cell movement toward the origin (4–6, 17–20). Another role for cAMP early in development has been revealed by observations that either pulses or high continuous levels of exogenous cAMP induce prematurely the appearance of several developmentally regulated proteins (21–28). High concentrations of cAMP can also induce formation of stalk (29, 30) and spore (30) cells under conditions in which normal development is arrested. cAMP may also be involved in sorting cells into separate stalk and spore tissues later in the developmental program (31, 32). Pulses of exogenous cAMP activate the membrane-bound adenylate cyclase and increase the intracellular concentration of cAMP (26); both pulses and high continuous levels of exogenous cAMP may exert their influence on development by raising the intracellular cAMP concentration.

The most pronounced change in gene expression in developing amoebae occurs between 10 and 13 hr of development, when tight cell–cell contacts form and a large number of late proteins begin to be synthesized (8). These observations are further supported by the discovery that the population of messenger RNAs doubles in complexity (from about 4000 to 8000 different mRNA species) at this time in development (ref. 33; unpublished data). Apparently several thousand genes are activated at this time; the translation products of only a subpopulation of this group can be observed by our two-dimensional gel technique. Furthermore, synthesis of these late proteins is dependent upon continued cell–cell contact and is arrested by disruption of multi-cell aggregates. The principle result reported here is that many of these developmentally controlled proteins continue to be made at high levels even in disaggregated cells if exogenous cAMP is present. These observations suggest that cAMP plays a direct role in late gene expression in *Dictyostelium*.

Takemoto *et al.* (34) have also reported that addition of 1 mM cAMP to disaggregated slugs mimics the effect of continued cell–cell interactions by suppressing the accumulation of enzymes that are normally expressed early in development, synthesized at low levels in slugs, but reexpressed at high levels in disaggregated slugs in the absence of cAMP. Town and Gross (28) have observed that addition of 0.2 mM cAMP to fast-shaken starved suspension cells induces the accumulation of UDP-glucose pyrophosphorylase and glycogen phosphorylase, two developmentally controlled enzymes. However, their observations differ from ours, because their fast-shaken suspension cultures of strain V12 M2 exist mainly as small agglomerates at the time of enzyme induction, whereas our cultures are about 90% single cells, even after 5 hr of suspension in cAMP. Con-

sequently, our results demonstrate that exogenous cAMP can maintain the synthesis of "aggregation-dependent" polypeptides, even in the absence of continued cell–cell contact.

Turn on of late gene expression coincides with the formation of tips on top of the tight cell aggregates (8); several studies (35–37) suggest that tips are centers for the synthesis of cAMP. Hence disruption of aggregates may suppress synthesis of late proteins by obliterating the source of cAMP. In support of this suggestion is the observation that mutants that aggregate normally but do not form tips fail to synthesize any of the 13-hr specific proteins (unpublished data); this latter result also establishes that the pulsatile release of cAMP during aggregation cannot, in itself, induce synthesis of 13-hr-specific polypeptides. Furthermore, neither starved suspension cells (Fig. 2) nor cells disaggregated after 8 hr on filters are capable of inducing late genes in the presence of exogenous cAMP (with the few exceptions noted above for 8-hr plated cells). These results suggest that formation of tight cell aggregates, or some other developmental process accompanying the formation of these aggregates, is required for late genes to acquire sensitivity to cAMP.

Our results suggest that a concentration of cAMP between 0.1 μM and 20 μM is the minimum necessary to maintain expression of late proteins in disaggregated cells. This concentration is within the physiological range for both intracellular and extracellular cAMP measured either in starved suspension cells (4) or in amoebae aggregating on filters (38). Recent studies (31, 32) have revealed that *Dicytyostelium* cells maintain chemotactic sensitivity toward cAMP throughout the post-aggregative (slug) stage of development; the level of cAMP needed to elicit this response is between 1 and 10 μM.

The results of wheat germ translation reactions indicate that cAMP prolongs synthesis of late proteins in disaggregated cells by maintaining sufficiently high levels of the messenger RNAs encoding these species. Whether this effect manifests itself at the level of synthesis, processing, or stability of these messages remains to be determined. It also remains to be determined whether all 4000 genes whose expression is induced at 13 hr of development (ref. 33; unpublished data) continue to be expressed in disaggregated cells in the presence of cAMP.

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