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Detection and developmental regulation of the mRNA for the regulatory subunit of the cAMP-dependent protein kinase of *D. discoideum* by cell-free translation

Jean de Gunzburg, Jakob Franke[1], Richard H.Kessin[1] and Michel Véron

Unité de Biochimie Cellulaire, Institut Pasteur, 28 rue du Dr Roux, 75724 Paris Cedex 15, France, and [1]Department of Anatomy and Cell Biology, College of Physicians and Surgeons, Columbia University, 630 West 168th Street, New York, NY 10032, USA

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cAMP is an important effector of the development of *Dictyostelium discoideum* amoebae and could exert its effects on gene expression through the cytosolic cAMP-dependent protein kinase (cAK). Antibodies, specific for the regulatory subunit (R) of the cAK, were used to investigate the developmental regulation of the corresponding mRNA (R-mRNA) by *in vitro* translation and immunoprecipitation. Under such conditions, a single polypeptide of the same mol. wt. as R (42 kd) is detected, showing that the protein is not synthesized as a large precursor. The level of the R-mRNA, which is low in vegetative cells, increases 10- to 20-fold during the first hours of development. Its expression is stimulated by the treatment of AX3 cells with cAMP either added to a concentration of 1 mM or given as 0.1 μM pulses every 5 min, whereas such treatments have little or no effect in cells of strain AX2. The R-mRNA remains highly expressed (0.01–0.03% of translatable mRNA) throughout post-aggregative development; it is not affected by mechanical disaggregation of the multicellular organism. The parallel developmental time courses of the translatable R-mRNA and the R protein produced *in vivo* suggest that the expression of this polypeptide is regulated at the level of mRNA synthesis.

Key words: Dictyostelium/cAMP/cAMP-dependent protein kinase/development

Introduction

The developmental cycle of *Dictyostelium discoideum* cells, initiated by starvation, leads a homogeneous population of isolated cells to form a multicellular fruiting body composed of a stalk supporting a mass of spores (for reviews, see Loomis, 1982). Adenosine 3′:5′cyclic monophosphate (cAMP) plays a crucial role throughout development. As an extracellular chemoattractant, it is periodically secreted and relayed from cell to cell in order to drive the aggregation of the isolated amoebae (Darmon and Brachet, 1978). cAMP is also an important effector of gene expression. During aggregation, the pulsatile or continuous addition of cAMP either represses or stimulates the expression of a number of genes including those coding for discoidin I (Williams *et al.*, 1980), a protein homologous to the oncogene product *ras* (Reymond *et al.*, 1984), the extracellular cyclic nucleotide phosphodiesterase and its inhibitor glycoprotein (Mullens *et al.*, 1984; Rossier *et al.*, 1983), and the cysteine proteinase 1 (Williams *et al.*, 1985). cAMP has also been shown to induce the expression of most pre-stalk- and pre-spore-specific genes (Mehdy *et al.*, 1983; Chisholm *et al.*, 1984) as well as to maintain the transcription and stability of the corresponding mRNAs following mechanical disaggregation of the multicellular slugs (Landfear and Lodish, 1980; Mangiarotti *et al.*, 1983; Chisholm *et al.*, 1984). Under conditions where they are unable to aggregate and form fruiting bodies, cells can be induced to differentiate into pre-spore as well as stalk cells in the presence of high levels of cAMP (Kay *et al.*, 1978; Kay and Jermyn, 1983).

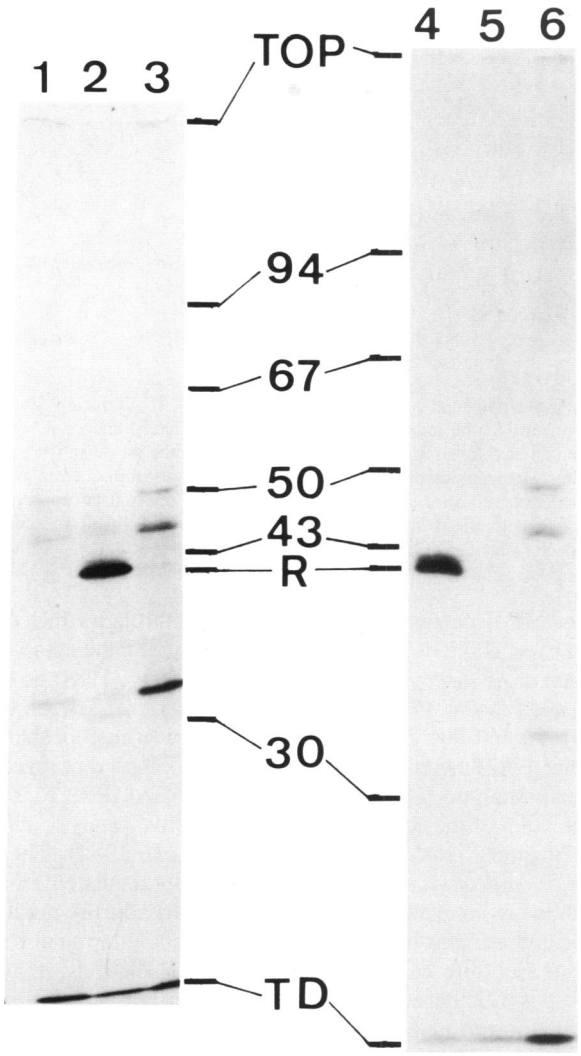

Fig. 1. Immunoprecipitation of *in vitro* synthesized regulatory subunit. 26 μg RNA from 4 h starved AX2 cells was translated *in vitro* in a total volume of 108 μl as described in Materials and methods. After pre-clearing, the supernatant was immunoprecipitated with: 12 μg pre-immune IgG **(lane 1)**; 12 μg anti-regulatory subunit IgG **(lane 2)**; 12 μg anti-regulatory subunit IgG that had been pre-incubated for 1 h at 4°C with 1 μg purified regulatory subunit **(lane 3)**. In a further experiment, the supernatants from these immunoprecipitations **(lanes 1,2,3)** were subjected to a second immunoprecipitation with 12 μg anti-regulatory subunit IgG **(lanes 4,5,6**, respectively).

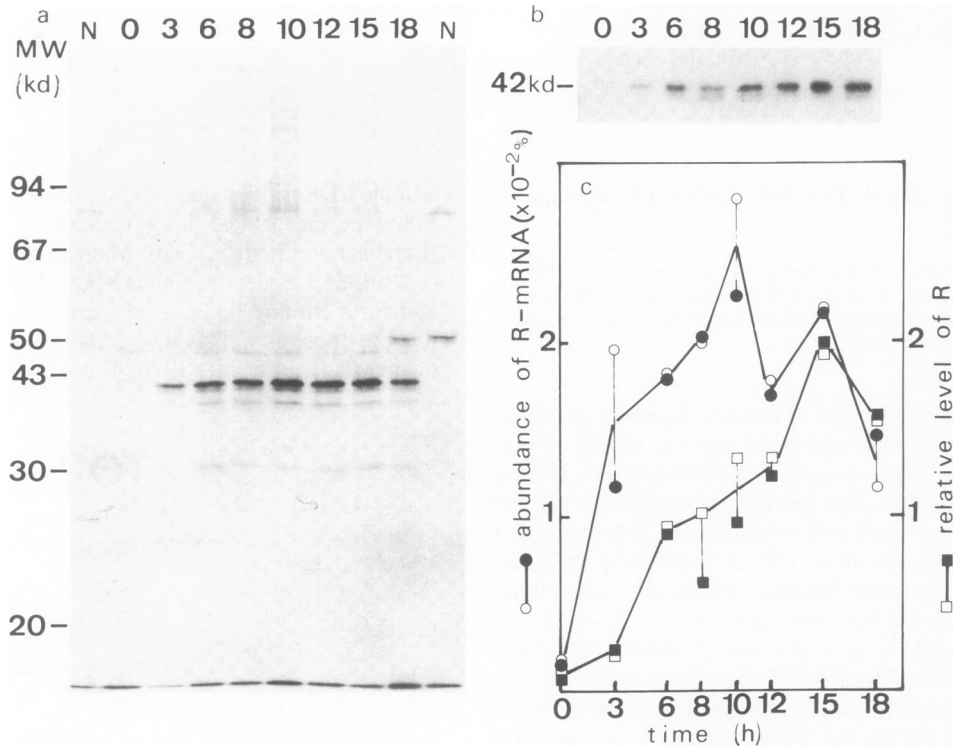

Fig. 2. Developmental regulation of R and its mRNA. Axenically grown AX2 cells were washed free of nutrient medium and plated on Whatman filters for development. At the indicated times, cells were collected and crude cellular extracts as well as total RNA were prepared. **(a)** *In vitro* translation of total RNA and immunoprecipitation by non-immune serum **(lanes N)** or anti-R antibodies **(lanes 0 – 18)**. The numbers above each lane indicate the time of development in hours. The samples precipitated with non-immune serum correspond to RNA from 6 h cells (far left) and 18 h cells (far right). **(b)** Immunoblot of crude cellular extracts. Lanes are numbered as in **a**. The relevant portion only of the blot is shown. **(c)** Quantitation of translatable R-mRNA and R protein. The level of R synthesized *in vitro* was measured by counting the band corresponding to R after excision from the gel shown in **panel a** (see Materials and methods for details). A similar curve was obtained by scanning the fluorogram. The relative level of R (expressed in arbitrary units) was measured by scanning the autoradiogram shown in **panel b**. Open and closed symbols correspond to two independent experiments.

A cAMP-dependent protein kinase (cAK) through which cAMP could exert some of its effects has been found in the cytoplasmic fraction of *D. discoideum* cells (Leichtling *et al.*, 1981; de Gunzburg and Véron, 1982; Rutherford *et al.*, 1982; Schoen *et al.*, 1984). As in other eukaryotic cells (reviewed in Flockhart and Corbin, 1982) the inactive holoenzyme is composed of regulatory (R) and catalytic (C) subunits; binding of cAMP to R induces dissociation of the complex and releases active catalytic subunits (de Gunzburg *et al.*, 1984; Majerfeld *et al.*, 1984). The cAK of *D. discoideum* is strongly developmentally regulated; its level, which is low in growing cells, increases early during pre-aggregation and remains high until culmination of the terminal fruiting body (Leichtling *et al.*, 1984; Part *et al.*, 1985).

We describe here the use of *in vitro* translation and immunoprecipitation to investigate the developmental regulation of the translatable mRNA coding for the regulatory subunit (R-mRNA) of the cAK from *D. discoideum* cells.

Results

Detection of the in vitro translation products

Total RNA was extracted from *D. discoideum* cells of the axenic strain AX2 and translated *in vitro* in a rabbit reticulocyte lysate using [^{35}S]methionine. The products were immunoprecipitated either with pre-immune antibodies, or with IgG specific to the regulatory subunit of the cAMP-dependent protein kinase of *D. discoideum* as described in Materials and methods. Figure 1 shows that when immune serum was used (lane 2), a strong band corresponding to a mol. wt. of 42 000 was detected after polyacrylamide gel electrophoresis in the presence of SDS (SDS-PAGE) and fluorography. This polypeptide was not detected with pre-immune IgG (lane 1) or when the antibodies had been adsorbed with purified regulatory subunit prior to immunoprecipitation (lane 3). Therefore this product represents the regulatory subunit of the cAMP-dependent protein kinase. The fact that the polypeptide purified from cellular extracts and the protein immunoprecipitated following *in vitro* translation have the same mol. wt. of 42 000 shows that R is not synthesized as a larger precursor. Minor bands of lower mol. wt. were also immunoprecipitated by anti-R antibodies in some experiments (see Figure 2); they either represented degradation products due to proteases, or translation products of truncated R-mRNAs.

The supernatants from the first immunoprecipitation were subjected to a second immunoprecipitation using anti-R antibodies. When the first precipitation was performed with pre-immune serum (lane 4), the polypeptide of mol. wt. 42 000 was detected. In contrast, when the first reaction had been performed with anti-R antibodies (lane 5), no protein was detected by the second immunoprecipitation. This demonstrates that under the conditions used all of the regulatory subunit synthesized was immunoprecipitated. Therefore the intensity of the band on the fluorogram reflects the abundance of the R-mRNA in a given RNA sample.

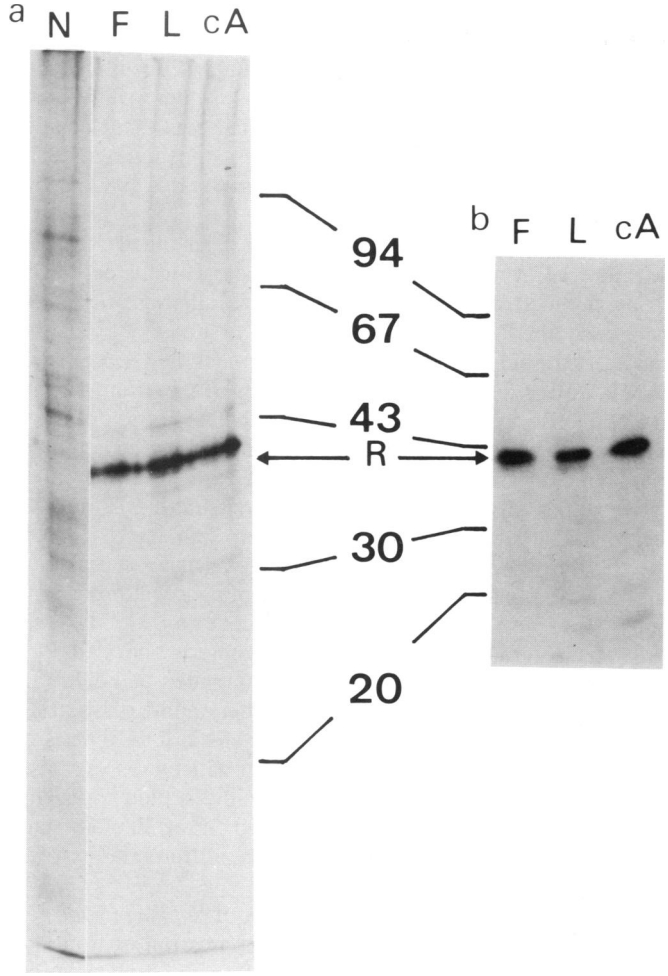

Fig. 3. Levels of R and its mRNA following mechanical dissociation of slugs. AX2 cells were allowed to develop on Millipore filters up to the slug stage (15 h). They were dissociated as described in Materials and methods and either replated on new filters (F), or resuspended in liquid in the absence (L) or presence (cA) of 100 μM cAMP. After 4 h, cells were harvested and crude cellular extracts as well as total RNA were prepared. **(a)** *In vitro* translation of total RNA and immunoprecipitation with non-immune serum (lane N) or anti-R antibodies (other lanes). **(b)** Immunoblot of crude cellular extracts. The mol. wt. (kd) of marker proteins is shown.

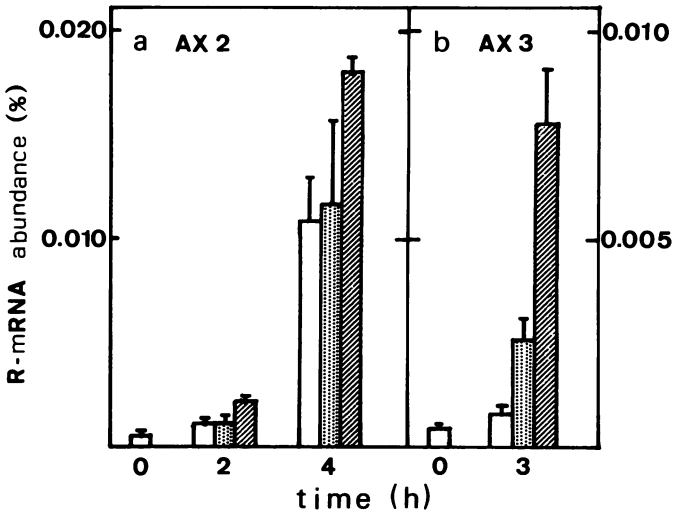

Fig. 4. Induction of the R-mRNA by cAMP. Axenically growing AX2 or AX3 cells were starved in liquid suspension and treated in the following way: open bars: no treatment; dotted bars: 1 mM cAMP was added to the suspension at the onset of starvation; hatched bars: cells were pulsed with 0.1 μM cAMP starting 30 min after the onset of starvation (see Materials and methods). Samples were withdrawn at the indicated times, total RNA was extracted and translated *in vitro*. The products were immunoprecipitated and the amount of R synthesized was quantitated as described in Materials and methods. **Panel a:** RNA from two different batches of AX2 cells were each translated *in vitro* in two independent experiments. Mean values ± s.e.d. are shown. **Panel b:** the same experiments as in **panel a** were performed with RNA from AX3 cells.

Developmental regulation of R and its mRNA

As shown in Figure 2 (panels a and c), the level of the R-mRNA was very low in vegetative cells, and increased 10- to 20-fold during the first 3 h of development. As development continued through aggregation to the multicellular stage, the level of the R-mRNA further increased to reach a maximum at the time of tight contact formation and morphogenesis of the tip (10 h of starvation). It remained high throughout differentiation of pre-stalk and pre-spore cells and exhibited a moderate decline upon culmination of the fruiting bodies. When the cells were starved in liquid suspension, a major increase also occurred between 2 and 4 h after nutrient deprivation (see below Figure 4).

The developmental time course of the R-mRNA was compared with variations in the level of the R protein produced *in vivo* by the same cells, as estimated by Western blotting (Figure 2b). Quantitation of the relative amounts of R and its mRNA (Figure 2c) shows that they exhibited roughly parallel time courses, with a lag of a few hours between the appearance of the R-mRNA and the accumulation of R protein. It is worth noting that the relative abundance of the R-mRNA (0.01 − 0.03% of translatable RNA) is similar to that of the R protein in cellular extracts (estimated by the enrichment factor necessary to purify the protein to homogeneity). All of these results strongly suggest that the amount of R present in the cells is directly dependent on the level of its mRNA.

Since the synthesis and stability of a number of mRNAs expressed at the multicellular stage of development are dependent on continued cell-cell contact or the presence of cAMP, it was of interest to determine whether this was the case for R. Figure 3 shows that when slugs were mechanically dissociated and the cells maintained single in a rapidly shaken suspension for 4 h, the respective levels of R and its mRNA did not diminish as compared with control cells allowed to re-aggregate on new filters. Moreover, the addition of 100 μM cAMP to the suspension had no effect. Thus R and its mRNA appear to remain metabolically stable during mechanical dissociation of slugs, which distinguishes this mRNA from the majority of the mRNAs expressed after aggregation.

Effect of cAMP on the R-mRNA level

We have examined the expression of the R-mRNA during the first hours of development by starving cells of strains AX2 and AX3 (another axenic strain independently isolated from the same parent as AX2) in liquid suspension. In both cases, a sharp increase in the level of R-mRNA was observed in the first 3 − 4 h of starvation (Figure 4). That the appearance of this mRNA is not an effect of cell density is demonstrated by the fact that in stationary phase cells, grown to the density at which they were starved in suspension, the level of the R-mRNA was even lower than in exponentially growing cells (not shown).

We investigated the effect of treatment of cells with cAMP on the expression of the R-mRNA during the first hours of starvation. In the case of strain AX2 (Figure 4a), the addition of

1 mM cAMP to the suspension at the onset of starvation had no reproducible effect; when cAMP was added in the form of 0.1 μM pulses every 5 min (which mimics the natural pulsatile signalling), a slight induction effect was found. In other experiments, 1 mM cAMP was added to the cell suspension at various times and the level of the R-mRNA was measured 1, 2 and 3 h later; addition of cAMP 1 h after the onset of starvation had no effect, whereas its addition after 5 h of starvation increased 1.5- to 2-fold the level of the R-mRNA (data not shown).

In contrast, treatment of AX3 cells with 1 mM cAMP at the onset of starvation stimulated 3- to 5-fold the expression of the R-mRNA measured after 3 h (Figure 4b). When, instead, cells were pulsed with cAMP, the induction of the R-mRNA was even greater (9- to 12-fold); when such a treatment was continued until 6 h of starvation, the induction effect persisted, although to a lesser extent (~5-fold). Estimation of the amount of R protein in those cells by Western blotting showed that its level was also increased in response to cAMP treatment; the extent of stimulation was similar to that of the R-mRNA with a lag of 1–3 h (data not shown).

Discussion

Upon precipitation of *in vitro* translation products of *D. discoideum* RNA with IgG specific for R, a single band corresponding to a mol. wt. of 42 000 is detected; that it indeed represents R has been demonstrated by several lines of evidence (see Results). Since the mol. wts. of R either purified from *D. discoideum* cells or synthesized *in vitro* from the RNA are the same, the protein is not synthesized *in vivo* in the form of a larger precursor that could resemble the regulatory subunits of other species. Indeed, regulatory subunits type I and II of cAMP-dependent protein kinases from mammalian origin (for a review, see Flockhart and Corbin, 1982) as well as those from lower eukaryotes such as yeast (Hixson and Krebs, 1980), *Mucor rouxii* (Moreno and Passeron, 1980), *Blastocladiella emersonii* (Brochetto-Bragga *et al.*, 1982) or *Neurospora crassa* (Trevillian and Pall, 1982) have substantially higher mol. wts. determined on SDS-gels (47 000–62 000) than the homologous protein from *D. discoideum*. The reason for these differences is probably due to the fact that these proteins carry a dimerization domain and (except for yeast) a second cAMP-binding site in addition to the cAMP-binding site and the domain of interaction with the catalytic subunit also present in the protein from *D. discoideum*. The fact that *Dictyostelium* R subunit has always been isolated as a monomer (Leichtling *et al.*, 1981; de Gunzburg and Véron, 1982) is in agreement with the model, as is the dimeric structure of the holoenzyme that we have reported (de Gunzburg *et al.*, 1984).

Thus the regulatory subunit from the cAK of *D. discoideum* represents the simplest known form of cAK regulatory subunits containing only one cAMP-binding domain and the domain of interaction with the catalytic subunit. It may well be an ancestral form of the molecule since, on the basis of rRNA sequences, it has been proposed that *Dictyostelium* belongs to the most ancient known eukaryotic branch of the evolutionary tree and diverged earlier than yeasts from the path leading to mammals (Olson and Sogin, 1982; McCarroll *et al.*, 1983).

The level of R present in the cells is strongly developmentally regulated (Leichtling *et al.*, 1984; Part *et al.*, 1985) and it has been shown that these rises correspond to *de novo* protein synthesis (Leichtling *et al.*, 1984). This report shows that the mRNA coding for R is also highly developmentally regulated, exhibiting a 10- to 20-fold increase at the onset of pulsatile signalling; it reaches a maximum at the time when pre-stalk and pre-spore cells differentiate and remains high until morphogenesis of the final fruiting body. The parallel increases in the levels of the R-mRNA and the amount of R *de novo* synthesized and accumulated in the cells make it unlikely that this species is under translational control but rather suggest a transcriptional control mechanism as is the case for most genes whose expression is induced during the development of *D. discoideum* (Williams *et al.*, 1980; Lodish *et al.*, 1982).

Similarly to proteins involved in cAMP metabolism (Klein and Darmon, 1977) as well as the mRNA coding for the extracellular cyclic nucleotide phosphodiesterase (Mullens *et al.*, 1984), the level of the R-mRNA rises during the first hours of starvation and its expression is induced by the treatment of AX3 cells with cAMP. In the case of the closely related AX2 strain, such an induction effect of cAMP treatment on the expression of the R-mRNA was much less obvious for reasons that we do not understand. In contrast to most of the proteins expressed early in development, the level of R and its mRNA remains high throughout the multicellular stages of development. The mRNA species specific for post-aggregative differentiation usually appear much later than the R-mRNA, i.e. at the end of aggregation or at the time of establishment of tight cell-cell contacts (Mehdy *et al.*, 1983; Chisholm *et al.*, 1984). Their synthesis and stability in the slug have been shown to require continued cell-cell interactions or the presence of cAMP (Landfear and Lodish, 1980; Lodish *et al.*, 1982), whereas the level of the R-mRNA is unaffected by mechanical disruption of the multicellular organism. Therefore, the mRNA coding for R exhibits a unique developmental regulation and it is highly expressed during all of the stages at which cAMP exerts its effects on the differentiation of *D. discoideum* cells.

There is ample evidence that treatment of *D. discoideum* cells with cAMP specifically promotes the expression of a number of genes (Williams *et al.*, 1980; Landfear and Lodish, 1980; Mehdy *et al.*, 1983; Chisholm *et al.*, 1984) and the presence of a cAK makes it possible that cAMP plays a role as a second messenger during *D. discoideum* development. However, there are other candidates for this role including cGMP and Ca^{2+} (Mato *et al.*, 1977; Wurster *et al.*, 1977; Lappano and Coukell, 1982; Europe-Finner and Newell, 1984), and using a morphogenetic mutant incapable of relaying cAMP signals, Wurster and Bumann (1981) have suggested that transient elevations in the intracellular cAMP concentration are not necessary for differentiation to aggregation competence. Directed evidence for the involvement of cAMP as a second messenger will only be obtained through selective means to modulate the intracellular levels of cAMP or of cAMP-dependent phosphorylations, such as the transformation of *D. discoideum* cells with sense or antisense sequences coding for the cAMP-phosphodiesterase or either subunit of the cAK.

Materials and methods

Strains, conditions for growth and differentiation

D. discoideum amoebae of the axenic strains AX2 and AX3 were grown at 22°C in HL 5 broth (Watts and Ashworth, 1970) to a density of $4-7 \times 10^6$ cells/ml. They were collected by centrifugation and washed twice in 20 mM K/Na_2-phosphate buffer pH 6.2 containing 0.5 mM $CaCl_2$ (buffer A). For development in liquid suspension, cells were resuspended in buffer A at a density of 2×10^7 cells/ml and shaken (120 r.p.m.) at 22°C. When indicated, cells were supplemented with 1 mM cAMP at the onset of starvation (90% of which was degraded after 3 h). In certain cases, cells were pulsed with 0.1 μM cAMP every 5 min starting 30 min after nutrient deprivation. For development on filters, cells were resuspended in 40 mM K/Na_2-phosphate buffer pH 6.2 containing 20 mM KCl, 2.5 mM $MgCl_2$ and 0.5 mM $CaCl_2$ (buffer B) and plated at a density of 3×10^6 cells/cm^2 either on a Whatman 50 filter resting over two Whatman 3 pads, or on a Millipore HABP filter resting over two Schleicher & Schüll no. 8 pads.

All filters were saturated with buffer B; they were incubated at 22°C under incandescent light. Under those conditions, cells had aggregated by 8 h and tips were formed by 10–12 h. At 15 h, all organisms were at the slug stage; they were culminating at 18 h.

For experiments dealing with dissociated slugs, cells were recovered from filters after 15 h of differentiation (slug stage). Aggregates were dissociated by vigorous pipetting through a glass thin-tipped pipette; cells were then either replated on fresh Millipore filters, or diluted to a density of 5×10^6 cells/ml in buffer B in the presence or absence of 100 μM cAMP and shaken for 4 h at 250 r.p.m.

Preparation of crude cellular extracts for immunoblotting

At the appropriate times of development, 2×10^8 cells were collected, washed twice in buffer A and resuspended in 1 ml of buffer A. A 0.1 ml aliquot was saved for the determination of protein content (Bradford, 1976). The remainder was diluted 2-fold with SDS sample buffer, heated at 100°C for 2 min, and stored at −20°C. The presence of R was estimated by Western blotting using anti-R antibodies as previously described (Part *et al.*, 1985). Upon scanning of the autoradiogram, the area of the peak was proportional to the amount of protein loaded on the gel for one given sample.

Extraction and translation of RNA

2×10^8 cells were harvested at the indicated times of development and washed twice in buffer A; they were stored as frozen pellets at −80°C. Total cellular RNA was extracted according to Alton and Lodish (1977) and translated in a nuclease-treated rabbit reticulocyte extract prepared as described by Pelham and Jackson (1976). Conditions for *in vitro* translation using [^{35}S]methionine (1500 Ci/mmol, Amersham International plc) and determination of efficiency of incorporation were as described by Rossier *et al.* (1983).

Immunoprecipitation following in vitro translation

In order to compare samples containing equal amounts of translatable mRNA, aliquots of the translation mixtures representing the same quantity of TCA-precipitable radioactivity (usually $1-3 \times 10^6$ c.p.m.) were withdrawn for immunoprecipitation and adjusted to the volume of the largest aliquot by the addition of buffer C (50 mM Tris-HCl pH 7.4, 0.15 M NaCl, 5 mM EDTA, 0.02% NaN_3, 1% Nonidet P-40, 1% sodium deoxycholate, 0.2% SDS, 0.5% bovine serum albumin). They were diluted by the addition of eight volumes of cold buffer C and pre-cleared using one volume of a 10% (w/v) suspension of fixed *Staphylococcus aureus* cells (Pansorbin, Calbiochem). The supernatants were reacted overnight at 4°C with 12 μg of IgG purified from pre-immune or immune serum through two successive precipitations with ammonium sulfate (35% saturation).

Immune complexes were recovered with 10 μl Pansorbin, washed and prepared for analysis on 10% SDS-polyacrylamide gels as previously described (Mullens *et al.*, 1984). Supernatants from the first immune precipitation were subjected to a second precipitation with immune serum in order to ascertain that the reaction with the antibodies was complete. Gels were subjected to fluorography using Amplify (Amersham International plc) and exposed to pre-flashed Kodak XAR-5 film at −80°C. For quantitation of the amount of immune-precipitated regulatory subunit, the relevant areas of the gels were excised after fluorography and counted in a liquid scintillation counter using 4 ml of OCS (Amersham International plc). The number of c.p.m. precipitated with non-immune serum was subtracted from that precipitated with immune serum. The abundance of the translatable R-mRNA was calculated as the percentage of radioactivity in R over total [^{35}S]methionine incorporated into TCA-precipitable material.

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