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A Pharmacologically Distinct Cyclic AMP Receptor Is Responsible for the Regulation of gp80 Expression in *Dictyostelium discoideum*

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The EDTA-resistant cell-cell adhesion expressed at the aggregation stage of *Dictyostelium discoideum* is mediated by a cell surface glycoprotein of M_r 80,000 (gp80). The expression of gp80 is developmentally regulated by cyclic AMP (cAMP). In vitro nuclear run-on experiments show that transcription of the gp80 gene is initiated soon after the onset of development. The basal level of gp80 transcription is significantly augmented by exogenous cAMP pulses. Interestingly, in analog studies, 2′-deoxy-cAMP, 8-bromo-cAMP, and N^6-monobutyryl-cAMP are all capable of inducing a rapid accumulation of gp80 mRNA, suggesting the presence of a unique cAMP receptor that responds equally well to these analogs. To determine whether intracellular cAMP plays a role in the regulation of gp80 expression, caffeine was used to block cAMP-induced receptor-mediated adenylate cyclase activation. Expression of gp80 mRNA was blocked in caffeine-treated cells but could be substantially restored by treatment with exogenous cAMP pulses, suggesting that adenylate cyclase activation is not required. gp80 expression was also examined in the signal transduction mutants *synag 7* and *frigid A*. In both mutants, gp80 was expressed at the basal level. Pulses of cAMP as well as 2′-deoxy-cAMP and N^6-monobutyryl-cAMP were capable of restoring the normal level of gp80 expression in *synag 7* cells. These results, taken together, indicate bimodal regulation of gp80 expression during development and the involvement of a novel cAMP receptor in the transmembrane signalling pathway that regulates gp80 gene expression.

The cellular slime mold *Dictyostelium discoideum* provides a unique eucaryotic model for the analysis of cyclic AMP (cAMP) regulation of gene expression. cAMP plays a central role in the coordination of the developmental program of this organism. In addition to its putative function as a second messenger, cAMP serves as a primary messenger mediating intercellular communication in this organism. Exogenous cAMP regulates the chemotactic apparatus as well as the activity of various developmental genes (4, 5, 11, 14).

Upon the depletion of their food source, *D. discoideum* cells differentiate into aggregation-competent cells, eventually culminating in the formation of mature fruiting bodies. At 5 to 6 h of development, cells undergo chemotactic migration towards areas of higher cAMP concentration and EDTA-resistant cell-cell binding sites begin to appear on the cell surface. Mutual cohesiveness of cells increases, enabling them to form multicellular aggregates. A cell surface glycoprotein of M_r 80,000 (gp80) has been found to mediate the EDTA-resistant cell-cell adhesion at the aggregation stage, and it also serves as a developmental marker (29, 35, 41, 42). Artificial pulsing of cells with low concentrations of cAMP at the early stages of development can amplify the in vivo cAMP signalling system during aggregation and accelerate the developmental program (12, 22). The expression of proteins characteristic of aggregation-stage cells, such as adenylate cyclase (23), cAMP receptors (3, 4), and cAMP-dependent protein kinase (7, 30), is stimulated.

The cell adhesion molecule gp80 is also under cAMP regulation. Under normal conditions, gp80 becomes detectable after 6 h of development. Then it rapidly accumulates between 6 and 10 h, with 90% of the glycoprotein located on the cell surface (38, 43, 51). When exogenous cAMP pulses were administered to the cells, a precocious and enhanced induction of gp80 expression was stimulated. This was preceded by an equally rapid accumulation of gp80 transcripts (43). In this report, we present evidence that the basal level of gp80 transcription is initiated during early development and does not require cAMP signalling. The transcription rate of the gp80 gene is enhanced by exogenous cAMP pulses. Pharmacological studies indicate that a novel surface cAMP receptor, which is sensitive to pulses of low concentrations of cAMP, 2′-deoxy-cAMP, 8-bromo-cAMP, and N^6-monobutyryl-cAMP, is responsible for gp80 regulation. This pharmacological specificity is also confirmed in the signal transduction mutant *synag 7*.

MATERIALS AND METHODS

Materials. cAMP, 2′-deoxy-cAMP, 8-bromo-cAMP, N^6,*O*-2′-dibutyryl-cAMP, N^6-monobutyryl-cAMP, cGMP, and 5′-AMP were all purchased from Sigma Chemical Co. (St. Louis, Mo.). Caffeine was obtained from Sigma, and α-amanitin was purchased from Boehringer Mannheim (Dorval, Quebec, Canada).

Cell strain and culture conditions. The wild-type strain NC4 was grown on agar plates in association with *Klebsiella aerogenes* as the food source (44). Cells were grown to a density of 1×10^8 to 2×10^8 cells per 100-mm plate and collected for experiments. Bacteria were removed by differential centrifugation. Cell pellets were suspended at 10^7 cells per ml in 17 mM Na_2-potassium phosphate buffer (pH 6.4), and cultures were shaken at 180 rpm for development. cAMP pulses were added to a final concentration of 2×10^{-8} M at 7-min intervals. The mutant strains *synag 7* and *frigid A* HC85 were kindly provided by M. B. Coukell (York University, Toronto, Canada) and were cultured in the same way as NC4 cells.

Cell cohesion assay. Intercellular EDTA-resistant cohesiveness was monitored by a modification (28) of the roller

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tube assay of Gerisch (10). Cells were dissociated mechanically and suspended at 2.5×10^6 cells per ml in 17 mM phosphate buffer, pH 6.4, containing 10 mM EDTA to block the EDTA-sensitive binding sites. Samples of 0.2 to 0.3 ml were rotated vertically at 180 rpm on a platform shaker. Cell counts were performed with a hemacytometer after 30 min. Both singlets and doublets were scored as unaggregated cells.

Polyacrylamide gel electrophoresis and immunoblotting. Sodium dodecyl sulfate (SDS)-polyacrylamide slab gels were prepared by the method of Laemmli (27). Gel electrophoresis was performed with a mini-gel apparatus at a constant current of 20 mA. Total cellular extracts were solubilized and reduced in 7 M urea–2% SDS–4% β-mercaptoethanol by boiling for 5 min. Approximately 10 μg of protein was loaded in each lane. Protein molecular size markers used were myosin (200 kilodaltons [kDa]), phosphorylase b (97 kDa), bovine serum albumin (68 kDa), ovalbumin (42 kDa), and α-chymotrypsinogen (25 kDa).

Protein samples were transferred electrophoretically onto nitrocellulose filters, which were blocked with 5% (wt/vol) skim milk in phosphate-buffered saline for 1 h and then incubated with the anti-gp80 monoclonal antibody 8OL5C4 (42) at 4°C overnight. Filters were washed three times with 5% skim milk, 10 min each, followed by incubation with alkaline phosphatase-conjugated goat anti-mouse immunoglobulin G antibody for 1 h at room temperature. Filters were then rinsed with two changes of skim milk, 10 min each, once with TTBS (20 mM Tris hydrochloride [Tris-HCl, pH 7.5], 0.5 M NaCl, 0.05% Tween 20) for 15 min, and then with two changes of TBS (20 mM Tris-HCl [pH 7.5], containing 0.5 M NaCl), 10 min each. Color development was carried out with Nitro Blue Tetrazolium and 5-bromo-4-chloro-3-indolylphosphate as substrates.

Protein determination. Protein determination was carried out with the bicinchoninic acid protein assay kit (Pierce Chemical Co., Rockford, Ill.), with crystalline bovine serum albumin as the standard.

Isolation of RNA. Total cellular RNA was isolated by the method of Chirgwin et al. (2). From 2×10^8 to 5×10^8 cells were collected and solubilized in a guanidium thiocyanate solution (5 M guanidium thiocyanate, 0.5% sodium lauryl sarcosine, 25 mM sodium citrate, 0.7% β-mercaptoethanol, 0.1% Antifoam A [pH 7.0]). The lysate was placed on top of a 4-ml 5.7 M CsCl cushion and centriguged at 24,000 rpm for 16 h at 20°C in an SW40 rotor. The RNA pellet was solubilized and extracted once with chloroform-isoamyl alcohol (4:1, vol/vol) and then precipitated with ethanol. Purified RNA samples were dissolved in sterile water which had been treated with 1% diethylpyrocarbonate and then stored at −70°C.

RNA blot. RNA samples were size-fractionated in a 1% agarose gel containing 6.7% formaldehyde. Gel electrophoresis was performed with 20 mM MOPS (3-[*N*-morpholino] propanesulfonic acid) in the gel running buffer (pH 7.0) at 100 V for 4 h. RNA samples were capillary-transferred from the agarose gel onto a nylon membrane (Hybond-N; Amersham) with 20× SSC (3 M NaCl, 0.3 M sodium citrate [pH 7.0]). RNA was cross-linked to the membrane by UV irradiation for 5 to 15 min, followed by 1 h of baking at 80°C. Prehybridization was carried out at 42°C for 6 h in a solution containing 50% deionized formamide, 5× Denhardt solution (0.1% Ficoll, 0.1% polyvinylpyrroline, 0.1% bovine serum albumin), 6× SSPE (1.1 M NaCl, 0.6 mM disodium EDTA, 60 mM sodium phosphate [pH 7.7]), 0.1% SDS, and 100 μg of denatured herring sperm DNA per ml. Hybridization was carried out at 42°C for 12 to 48 h in the same buffer containing a ^{32}P-labeled DNA probe (10^6 cpm/ml; specific activity, 2×10^8 cpm/μg). The DNA probe was labeled by using a random-primed DNA labeling kit (Boehringer Mannheim). The filters were then washed with two changes of 2× SSC at room temperature, 15 min each, followed by two changes of 0.1% SDS–2× SSC at 65°C, 15 min each, and then two changes of 0.1% SDS–0.1× SSC at 65°C, 15 min each. The filters were partially air-dried, and autoradiography was carried out with X-ray films. The probe was routinely stripped off the filter by incubation in 5 mM Tris (pH 8.0)–2 mM disodium EDTA–0.1× Denhardt solution at 65°C for 2 to 4 h. The filters were then used for rehybridization with an actin probe to ascertain that similar amounts of RNA were applied to all the lanes.

For slot blots, RNA samples were denatured in 14% formaldehyde–7.5× SSC at 65°C for 15 min before loading onto the filter. Prehybridization and hybridization conditions were essentially the same as above. In general, two to three different RNA concentrations were used, and quantitation was carried out by densitometric scanning of the autoradiograms.

Isolation of nuclei. Nuclei were prepared by the method of Nellen et al. (36). Lysis buffer 1 (50 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, pH 7.5], 40 mM $MgCl_2$, 20 mM KCl, 0.15 mM spermidine, 5% sucrose, 14 mM β-mercaptoethanol, 0.2 mM phenylmethylsulfonyl fluoride) was prepared and stored at 4°C. NC4 cells (3×10^8 cells) at different developmental stages were collected by centrifugation at 600 × *g* and washed with 17 mM phosphate buffer (pH 6.4). The cell pellet was suspended in 3 ml of lysis buffer 2 (lysis buffer 1 with 10% Percoll). All subsequent procedures were performed at 4°C. Nonidet P-40 (30 μl) was added to the cell suspension and mixed well by vortexing gently for 10 s. Nuclei were pelleted at 3,000 × *g* for 5 min at 4°C. The pellet was suspended again in 3 ml of lysis buffer 2, and intact cells were spun down at 150 × *g* for 5 min. The supernatant was centrifuged at 3,000 × *g* for 5 min to yield a nuclei pellet. It was then washed once in 1.5 ml of lysis buffer 1. The final nuclei pellet was suspended in 300 μl of storage buffer (40 mM Tris [pH 8.0], 10 mM $MgCl_2$, 1 mM EDTA, 50% glycerol, 14 mM β-mercaptoethanol), frozen in a dry ice-ethanol bath, and stored at −70°C.

Nuclear run-on assay. The standard reaction mixture (100 μl) contained 40 mM Tris (pH 8.0), 250 mM KCl, 10 mM $MgCl_2$, 5% glycerol, 0.1 mM dithiothreitol, 200 μM each ATP, GTP, and CTP, 0.4 U of RNasin per μl, 100 μCi of [α-^{32}P]UTP (600 Ci/mmol, 10 mCi/ml), and 20 μl of thawed nuclei (from 2×10^7 cells). For nuclei isolated from 8-h cells, the in vitro transcription reaction was performed in the presence or absence of α-amanitin (10 μg/ml). The reaction mixtures were incubated at 23°C for 30 min, and the reactions were terminated by addition of a stop solution containing 100 mM Tris-HCl (pH 8.0), 20 mM EDTA, and 2% SDS. The mixtures were extracted once with an equal volume of phenol-chloroform (1:1, vol/vol). ^{32}P-labeled nuclear run-on RNAs were separated from unincorporated [^{32}P]UTP by Sephadex G50 column chromatography. After ethanol precipitation, the RNA pellet was solubilized in TES solution (10 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid [pH 7.4], 10 mM EDTA, 0.2% SDS).

Hybridization was performed with RNA samples containing equal amounts of radioactivity. DNA (10 μg) from pBR322, plasmids containing cloned gp80 cDNA, or actin cDNA was immobilized on a nitrocellulose filter in triplicate with a slot-blot apparatus. DNA-RNA hybridization was

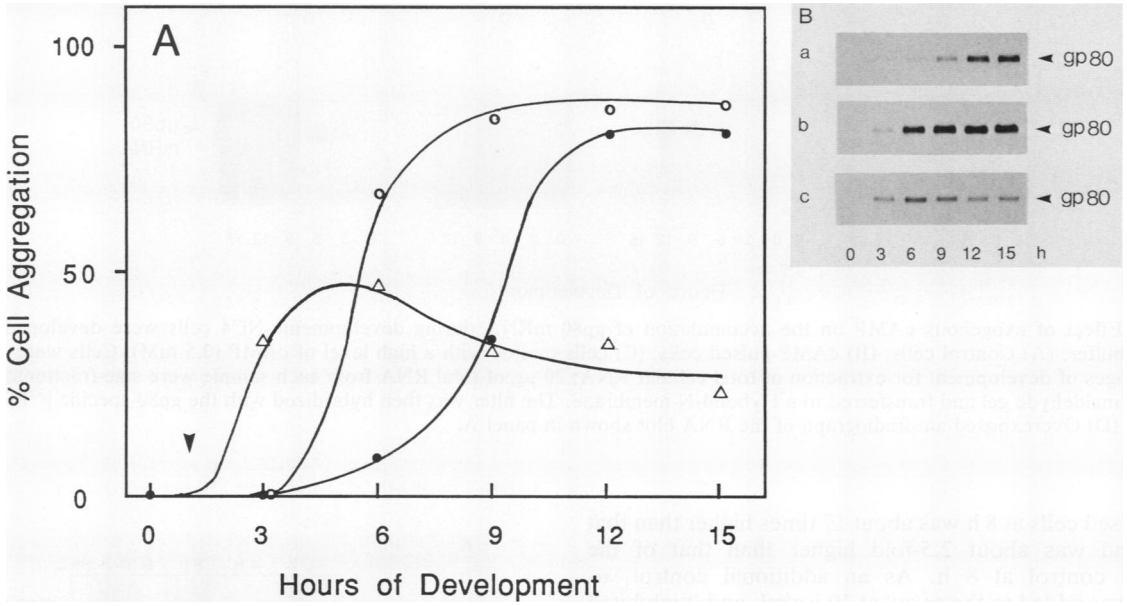

FIG. 1. Effect of cAMP on gp80 expression and EDTA-resistant cell-cell adhesion during development. NC4 cells were collected at the mid-exponential growth phase, washed free of bacteria, and suspended in 17 mM phosphate buffer (pH 6.4) at a density of 10^7 cells per ml for development. Cells were divided into portions and shaken at 180 rpm. Samples tested included the control (a, ●), cells pulsed with cAMP at 7-min intervals to give a final concentration of 20 nM (b, ○), and cells treated with a moderately high level of cAMP, where cAMP was added to a final concentration of 0.5 mM at 1 h of development and supplemented with 0.1 mM cAMP at hourly intervals (c, △). Cell samples were collected at the indicated times of development for the cell cohesion assay (A) and protein blot analysis (B).

carried out as described above. The filters were then air-dried and exposed to X-ray films. Quantitation of the relative rate of transcription of gp80 gene was performed by densitometric scanning of the autoradiograms.

RESULTS

Modulation of gp80 expression by extracellular cAMP. To investigate the mechanism by which cAMP regulates gp80 expression, the kinetics of gp80 expression under different developmental conditions were first examined. Cells were developed in shaken suspension, and a sample was treated with low-level cAMP pulses at 7-min intervals, with each pulse giving a final concentration of 20 nM cAMP. Another sample was treated with a constant level of a moderately high concentration of cAMP which was added to a final concentration of 0.5 mM at 1 h of development and supplemented with 0.1 mM at hourly intervals. These two modes of cAMP addition generated very different patterns of gp80 expression (Fig. 1). A high level of exogenous cAMP caused premature expression of gp80, similar to cAMP pulsing, and it also appeared to be more effective in inducing gp80 expression in the initial stages of development. The EDTA-resistant cell-cell adhesion also became clearly detectable at 3 h in cells treated with 0.5 mM cAMP (Fig. 1A). However, after the initial period of 3 to 4 h, the level of gp80 molecules in this sample was significantly lower than in the control or cells receiving cAMP pulses. At 12 h, the level was about 50% of that in the control and 13% of that in the cAMP-pulsed cells. The extent of cell reassociation also decreased slightly from the peak of 44% at 6 h. On the other hand, control cells began to express EDTA-resistant cell-cell adhesion at 6 h, which peaked at 12 h. cAMP-pulsed cells attained the peak of cell cohesion at least 3 h earlier than the control, confirming our earlier observation (43).

RNA blots were carried out to determine whether the expression of gp80 mRNA paralleled the expression of the EDTA-resistant cell-binding sites (Fig. 2). In control cells, gp80 mRNA accumulated gradually at first, peaking quickly between 9 and 12 h, and then decreased thereafter. At 6 h, cAMP-pulsed cells had already accumulated a significant level of gp80 mRNA, comparable to the peak level of control cells. The mRNA level continued to rise to maximal expression at 9 h and was significantly decreased at 15 h of development. Cells exposed to a high level of exogenous cAMP expressed a substantial level of gp80 mRNA even at 3 h. The amount was about sevenfold higher than that of cAMP-pulsed cells at the same stage, as evidenced by slot-blotting results (data not shown). However, these cells failed to accumulate a higher level of gp80 mRNA at later stages.

Since gp80 was detected occasionally in control cells at 3 h of development (Fig. 1B), it was of interest to determine whether gp80 gene transcription began before the cells began to generate their own cAMP relay system. An overexposure of the RNA blot shown in Fig. 2A revealed that a low level of gp80 mRNA was present as early as 3 h under normal conditions of development (Fig. 2D). This suggests that transcription of the gp80 gene is turned on early in development by a factor other than cAMP.

Effect of cAMP pulses on the rate of gp80 transcription. To examine the effect of cAMP pulses on the transcription of the gp80 gene, in vitro nuclear run-on experiments were performed. Cells were developed in liquid suspension with or without cAMP pulses. Nuclei were isolated at 0, 4, and 8 h of development for the in vitro transcription assay. As shown in Fig. 3, there was no gp80 gene activity at 0 h, although it was initiated soon after the onset of development. The rate of gp80 transcription was highly augmented by exogenous cAMP pulses, and the effect was detectable as early as 4 h of development. The transcription rate in

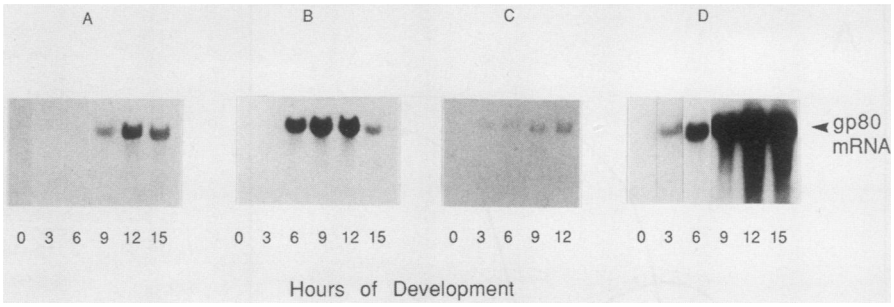

FIG. 2. Effect of exogenous cAMP on the accumulation of gp80 mRNA during development. NC4 cells were developed in 17 mM phosphate buffer. (A) Control cells; (B) cAMP-pulsed cells; (C) cells treated with a high level of cAMP (0.5 mM). Cells were collected at different stages of development for extraction of total cellular RNA; 20 μg of total RNA from each sample were size-fractionated on a 1% agarose–formaldehyde gel and transferred to a Hybond-N membrane. The filter was then hybridized with the gp80-specific [^{32}P]Ddgp80c-19 probe (51). (D) Overexposed autoradiograph of the RNA blot shown in panel A.

cAMP-pulsed cells at 8 h was about 17 times higher than that at 4 h and was about 2.5-fold higher than that of the nonpulsed control at 8 h. As an additional control, α-amanitin was added to the assay at 10 μg/ml, and it inhibited the transcription reactions in both nonpulsed and cAMP-pulsed cells (Fig. 3A), indicating that the observed differences were due to a change in the transcriptional rate by RNA polymerase II.

Effect of cAMP analogs on gp80 expression. The finding that maximal induction of gp80 protein and mRNA accumulation was brought about by periodic exogenous cAMP pulses suggests the participation of cell surface receptors in a transmembrane signal transduction pathway. To distinguish between the involvement of membrane receptors and the involvement of cAMP-dependent protein kinase in signal transduction, we examined the potency of a number of cAMP analogs in inducing gp80 mRNA accumulation. Cells were developed in liquid suspension and pulsed with various cAMP analogs. Total cellular RNA was purified from cells at 4 and 8 h of development. 2′-deoxy-cAMP, which binds to the surface receptor with high affinity (39, 50), stimulated the accumulation of gp80 mRNA (Fig. 4, lanes 4 and 12), consistent with the notion of receptor involvement. Quite unexpectedly, pulses of a low concentration of 8-bromo-cAMP and N^6-monobutyryl-cAMP, which are known to have higher affinity for the cytosolic kinase but poor affinity for the cAMP chemotactic receptor (8, 25), were able to induce gp80 expression to a level comparable to that of cAMP. cGMP, 5′-AMP, and N^6, *O*-2′-dibutyryl-cAMP did not have any inductive effect, and they exhibited inhibitory effects on gp80 expression.

Cell samples that were treated with the different cAMP analogs for 7 h were subjected to the cell cohesion assay. The cell-binding data correlated well with the RNA blot results. Cells pulsed with cAMP, 2′-deoxy-cAMP, 8-bromo-cAMP, and N^6-monobutyryl-cAMP were all able to achieve >85% cell reassociation, while the control sample showed only 40% aggregated cells.

The dose-response curves for cAMP, 2′-deoxy-cAMP, 8-bromo-cAMP, and N^6-monobutyryl-cAMP were also determined. Cells treated with pulses of different concentrations of cAMP or its analogs were collected at 8 h of development, and total RNA was extracted for slot blot analysis. Blots were hybridized with both gp80 and actin cDNA probes, and quantitation was carried out by densitometric tracings. Figure 5 shows that all three analogs behaved like agonists and stimulated the expression of gp80

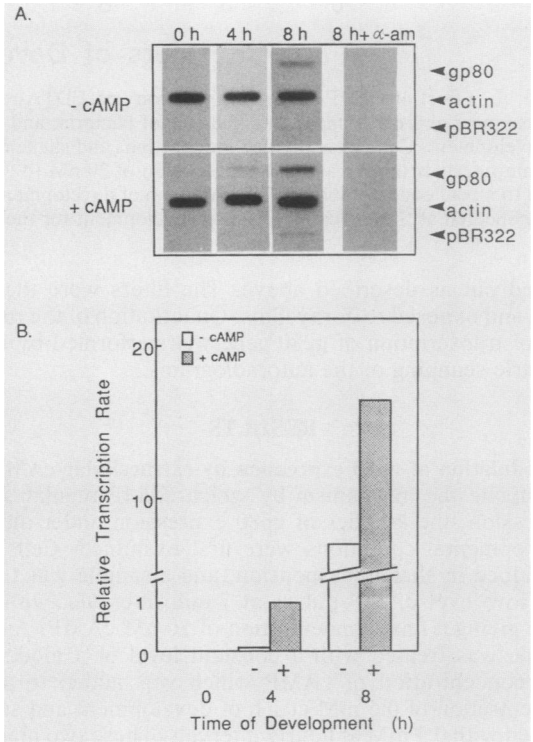

FIG. 3. Effect of exogenous cAMP pulses on the rate of gp80 transcription. NC4 cells were collected at exponential growth phase, washed free of bacteria, and suspended in 17 mM phosphate buffer (pH 6.5) at a density of 10^7 cells per ml for development, with or without exogenous cAMP pulsing. (A) In vitro nuclear run-on assays, primed with [α-^{32}P]UTP, were performed with nuclei isolated from 0-h, 4-h, and 8-h cells. The labeled transcripts were purified and hybridized with equal amounts of radioactivity to the cloned gp80 cDNA, actin cDNA, or pBR322 DNA immobilized on the nitrocellular filter. Each slot contained 10 μg of one of the plasmid DNAs. The in vitro transcription reactions for nuclei isolated from 8-h cells were also performed in the presence of 10 μg of α-amanitin (α-am) per ml. (B) Relative rates of gp80 gene transcription in cells developed in the presence (+) or absence (−) of cAMP pulsing were estimated by densitometric scanning of the autoradiograms. Hybridization of pBR322 DNA was subtracted from the corresponding samples to yield specific hybridization signals. The values represent results of a typical experiment. The experiment was repeated two times with two different nuclei preparations, and similar results were obtained.

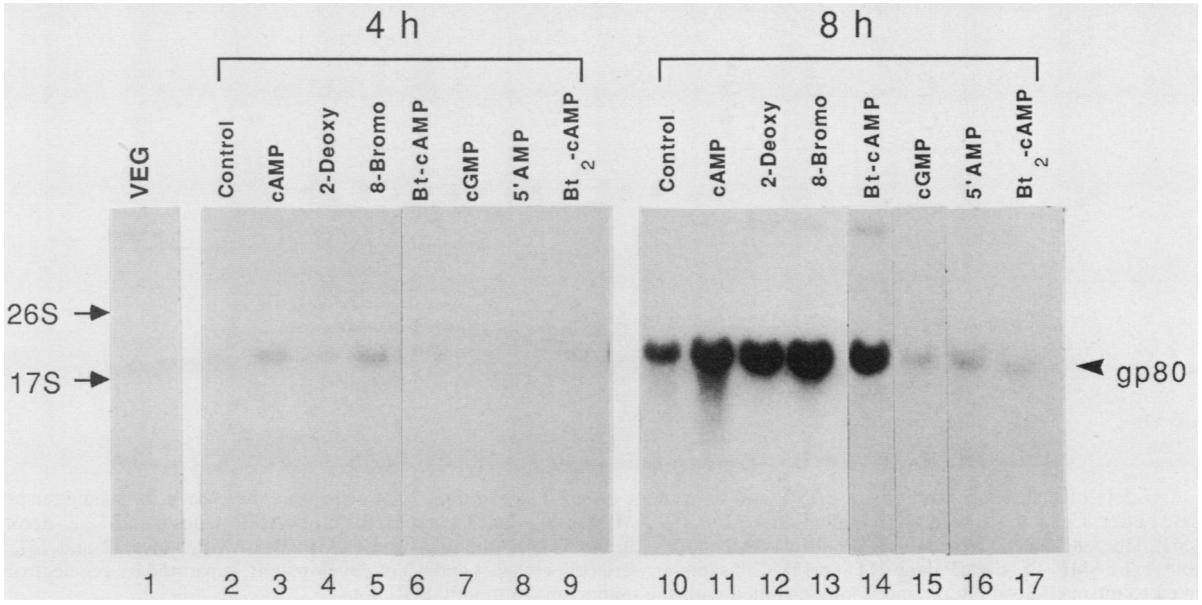

FIG. 4. Effect of cAMP analogs on gp80 mRNA levels. Cells were developed in 17 mM phosphate buffer, and cAMP or analogs were pulse-delivered to the cells at 2×10^{-8} M at 7-min intervals, starting at 1 h of development. Total cellular RNA was isolated at 0, 4, and 8 h, gel-fractionated, transferred to Hybond-N membrane, and hybridized with a ^{32}P-labeled gp80 cDNA probe. Lane 1, Vegetative cells; lanes 2 and 10, cells without cAMP treatment. The analogs used were 2'-deoxy-cAMP (2-deoxy), 8-bromo-cAMP (8-bromo), N^6-monobutyryl-cAMP (Bt-cAMP), cGMP, 5'-AMP, and N^6, *O*-2'-dibutyryl-cAMP (Bt_2-cAMP).

mRNA. cAMP and 2'-deoxy-cAMP showed a 50% effective concentration (i.e., concentration that increased gp80 expression by 50% of the amount over the control) of about 1 nM, and saturation was achieved at 5 nM. On the other hand, the 50% effective concentration for both 8-bromo-cAMP and N^6-monobutyryl-cAMP was about 6 nM, and saturation was reached at 10 nM. The levels of gp80 mRNA declined slightly at 10 nM cAMP and at concentrations of 30 nM and above for the three analogs.

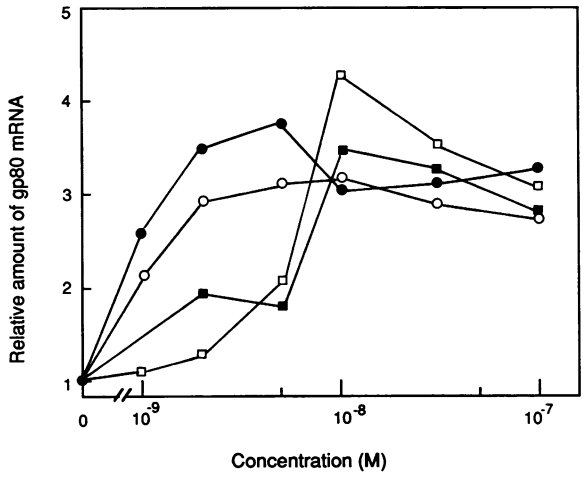

FIG. 5. Effect of different concentrations of cAMP and analogs on gp80 expression. Beginning at 2 h of development, cells were treated with pulses of cAMP or an analog at 7-min intervals to give final concentrations varying between 10^{-9} and 10^{-7} M. Samples were collected at 8 h, total RNA was extracted, and slot blot analysis was performed. RNA was loaded at 5 and 10 μg in duplicate and hybridized with ^{32}P-labeled gp80 or actin probe. Autoradiograms were quantitated by densitometric scanning, and values for gp80 mRNA were corrected for the amount of actin mRNA detected in the same sample. The amounts of gp80 mRNA were normalized against the amount expressed in the 8-h nonpulsed sample. Dose-response curves were determined for cAMP (●), 2'-deoxy-cAMP (○), 8-bromo-cAMP (□), and N^6-monobutyryl-cAMP (■).

Further experiments were carried out to determine whether a constant high level of these three cAMP analogs would induce gp80 expression in a manner similar to that of cAMP. Cells developed in liquid suspension were treated with 0.5 mM of the analogs at 1 h, supplemented with 0.1 mM at subsequent hourly intervals. Cellular extracts from two stages of development were subjected to immunoblot analysis (Fig. 6). The analogs that induced gp80 mRNA accumulation in the pulsing experiment (i.e., 2'-deoxy-cAMP, 8-bromo-cAMP, and N^6-monobutyryl-cAMP) also induced a precocious increase in gp80 expression at 4 h. However, the effect did not persist as in the case of cAMP, and the levels of expression were substantially lower by 8 h. The extent of EDTA-resistant cell cohesion was also monitored for these analogs, and the results were similar to those obtained for cells treated with a constant high level of cAMP (data not shown).

gp80 expression in the absence of adenylate cyclase activation. If 8-bromo-cAMP and N^6-monobutyryl-cAMP pulses exerted their effects on gp80 expression by entering the cells and interacting with a cytosolic target protein(s), activation of adenylate cyclase would be required in the signal transduction pathway. To resolve the question of whether intracellular cAMP has the role of a second messenger in the enhancement of gp80 expression, cells were treated with caffeine to block the adenylate cyclase activity. Caffeine has been found to block the cAMP-induced receptor-mediated adenylate cyclase activation with 50% inhibition at about 150 μM (1). Cells were developed in shaking culture in the presence of 3 mM caffeine with or without cAMP pulses, and RNA was isolated for hybridization studies (Fig. 7). At 8 h of development, gp80 mRNA was expressed at a very low level

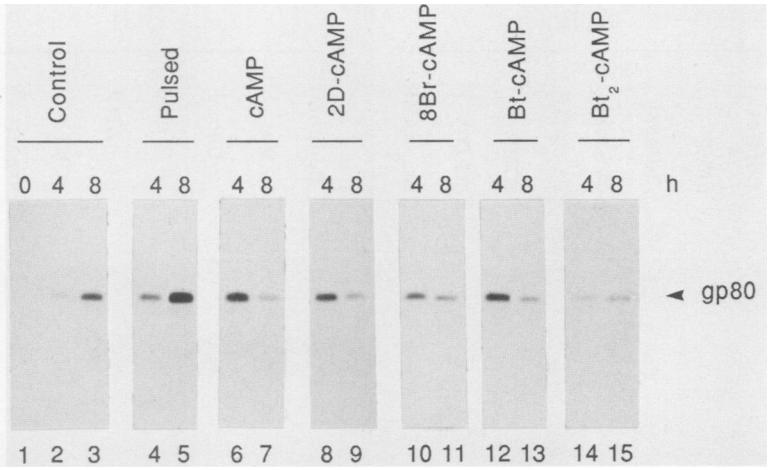

FIG. 6. Effect of high concentrations of cAMP and its analogs on gp80 expression. NC4 cells were developed in shaken suspension in phosphate buffer. Cells were given no treatment (lanes 1 to 3), cAMP pulsing (lanes 4 and 5), 0.5 mM cAMP (lanes 6 and 7), 2′-deoxy-cAMP (2D-cAMP, lanes 8 and 9), 8-bromo-cAMP (8Br-cAMP, lanes 10 and 11), N^6-monobutyryl-cAMP (Bt-cAMP, lanes 12 and 13), and N^6, *O*-2′-dibutyryl-cAMP (Bt_2-cAMP, lanes 14 and 15). Cell samples were collected at 4 and 8 h of development, separated by gel electrophoresis, transferred to nitrocellulose filters, and immunostained with the monoclonal antibody 8OL5C4.

in caffeine-treated cells, and it was only detectable when the autoradiogram was overexposed. However, when cAMP pulses were used together with caffeine, the expression of gp80 mRNA was restored to a level comparable to that observed in 8-h-pulsed cells without caffeine treatment. The result suggests that extracellular cAMP, and not intracellular cAMP, is important in the regulation of gp80 transcription.

Effect of cAMP and analogs on gp80 expression in mutant strains. *synag 7* cells are defective in receptor-mediated and GTP-stimulated adenylate cyclase activation, while retaining the expression of normal surface cAMP receptors (46). The effect of cAMP pulses on gp80 expression in *synag 7* cells was examined. While gp80 mRNA was barely detectable in the mutant cells, exogenous cAMP pulses were able to restore the level close to that in cAMP-pulsed wild-type cells (Fig. 8).

The effect of cAMP analogs was tested in the mutant strains *synag 7* and *frigid A*. *frigid A* mutants express moderate levels of surface cAMP receptors. However, these mutants cannot relay the cAMP signal or respond to exogenous cAMP (6) because they lack a functional $G_\alpha 2$ protein (19, 33). Mutant cells were developed in the presence of pulses of cAMP or a cAMP analog. The expression of gp80 molecules in cell samples taken at 4 and 9 h of development was analyzed by immunoblotting. *synag 7* cells had a very low level of gp80 expression at 9 h (Fig. 9). However, treatment with cAMP, 2′-deoxy-cAMP, 8-bromo-cAMP, or N^6-monobutyryl-cAMP pulses was capable of reviving the expression of gp80 to a level comparable to that of cAMP-pulsed NC4 cells. A low basal level of gp80 expression was also evident in *frigid A* HC85 cells. In this case, neither

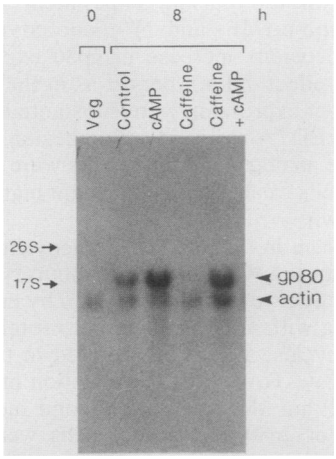

FIG. 7. Effect of caffeine on gp80 gene expression. NC4 cells were developed in shaken suspension at 10^7 cells per ml. Caffeine was added to give a final concentration of 3 mM at 1 h, with supplementary addition to 1 mM at 2-h intervals. In a separate sample, cells were treated with 3 mM caffeine plus cAMP pulses. Control samples with or without cAMP pulse treatment were also analyzed. Veg, Vegetative.

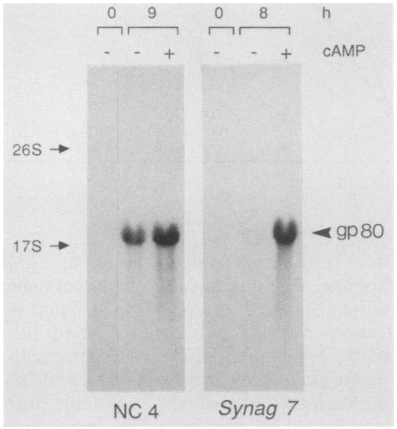

FIG. 8. gp80 gene expression in the mutant strain *synag 7*. NC4 and *synag 7* cells were developed in the presence or absence of exogenous cAMP pulses. Cells were collected at the indicated times of development for preparation of total cellular RNA for RNA blot analysis.

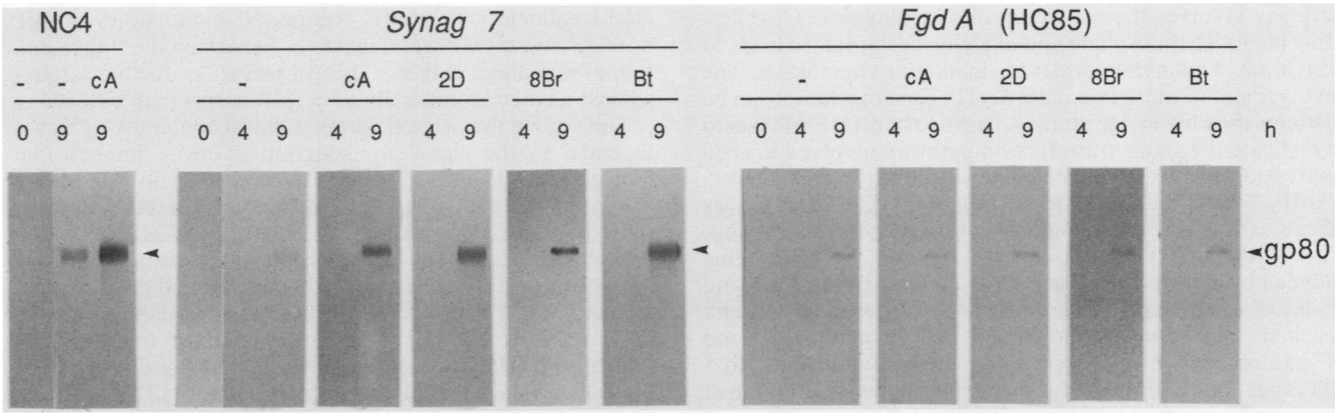

FIG. 9. Immunoblot showing the effect of cAMP analogs on the developmental expression of gp80 in mutant strains *synag 7* and *frigid A*. Wild-type NC4 cells and the mutant strains *synag 7* and *frigid A* HC85 were developed in 17 mM phosphate buffer (pH 6.4). Cells were pulsed with cAMP (cA), 2'-deoxy-cAMP (2D), 8-bromo-cAMP (8Br), or N^6-monobutyryl-cAMP (Bt) to give a final concentration of 20 nM at 7-min intervals. Cell samples were collected at the indicated times of development for immunoblotting analysis.

cAMP nor any analog was capable of stimulating gp80 expression above the basal level (Fig. 9).

The levels of gp80 expression in these two mutant strains closely correlated with the extents of EDTA-resistant cell-cell adhesion. EDTA-resistant cell reassociation was absent in both nonpulsed *synag 7* and *frigid A* HC85 cells (Fig. 10). However, *synag 7* cells achieved about 70% aggregation upon stimulation by cAMP pulses. Similar to cAMP, 2'-deoxy-cAMP, 8-bromo-cAMP, and N^6-monobutyryl-cAMP were able to induce *synag 7* cells to express EDTA-resistant cell aggregation. In the case of HC85 cells, neither cAMP nor its analogs had any effect on cell-cell adhesion. HC85 cells were unable to undergo EDTA-resistant cell aggregation in all instances. On the other hand, both mutant strains expressed EDTA-sensitive cell-binding sites and were able to undergo reaggregation in the absence of EDTA.

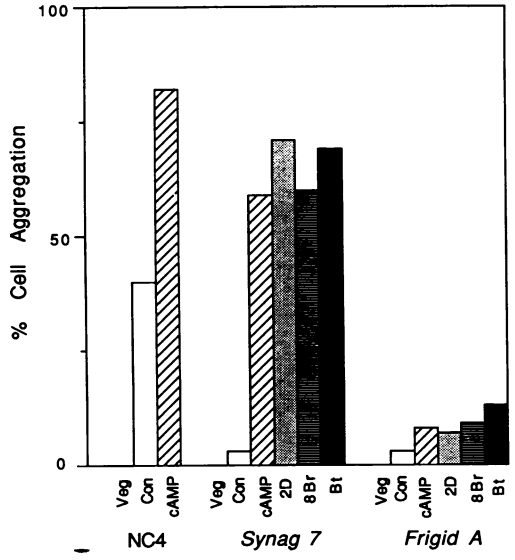

FIG. 10. Effects of cAMP analogs on the EDTA-resistant cell-binding sites in the signal transduction mutants. Wild-type NC4 cells and the two mutants *synag 7* and *frigid A* HC85 were developed in liquid cultures. Cells were given pulses of cAMP, 2'-deoxy-cAMP (2D), 8-bromo-cAMP (8Br), or N^6-monobutyryl-cAMP (Bt). Cell cohesion assays for vegetative (Veg) and 9-h cells were performed. Results represent the average from two independent experiments, with each done in triplicate.

DISCUSSION

Results from these studies indicate that gp80 expression is under bimodal regulation during development. In the initial phase, gp80 transcription is triggered soon after the beginning of the developmental cycle. gp80 transcripts are maintained at a relatively low level during this period. Although the amount of gp80 protein expressed is very low, it is still detectable in the signal transduction mutants *synag 7* and *frigid A* strain HC85. A similar observation has been made for *synag 7* recently (32). These results indicate that the initiation of gp80 transcription is dependent on a signal other than cAMP. The mechanism is, however, unknown, and it may involve secreted protein factors, such as the conditioned-medium factor (14, 32, 34).

Full induction of gp80 expression requires exogenous cAMP signals in pulsatile form, suggesting that cell surface cAMP receptors, which oscillate between excitable and adapted states (25, 26), are involved in the augmentation of gp80 expression in the second phase of regulation. The results of our nuclear run-on experiment indicate that cAMP pulses lead to an increase in the rate of gp80 transcription. At 8 h of development, the transcription rate of gp80 in cells receiving exogenous cAMP pulses is about threefold higher than in the control.

The responsiveness of genes under extracellular cAMP regulation can be divided into two categories, adaptive and nonadaptive (11, 20). Constant levels of cAMP effectively induce postaggregative late-gene products in a nonadaptive manner and are mediated by the cell surface cAMP receptor (13, 15, 39, 40). On the other hand, most early genes are under adaptive cAMP regulation, responding to cAMP pulses. The regulation of certain early genes, such as M3, D2, K5, and M4-1, is also mediated by a surface cAMP receptor-coupled mechanism (11, 21, 31–33). The basis of adaptation is probably at the cAMP receptor level, in the form of oscillation between the phosphorylated state and the dephosphorylated state (24–26).

Two different cAMP receptor-mediated signal transduction pathways have been postulated (9, 11, 17, 37). The first one involves cAMP-induced activation of adenylate cyclase in the establishment of the cAMP relay system. The second

pathway involves the activation of phospholipase C, generating inositol triphosphate and diacylglycerol. It also leads to activation of guanylate cyclase in mediating chemotaxis. The involvement of these two pathways in gene regulation can be distinguished by analog studies. Our use of cAMP analogs to investigate the signal transduction pathway involved in gp80 expression yielded unexpected results. Pulses of 2'-deoxy-cAMP, 8-bromo-cAMP, or N^6-monobutyryl-cAMP are as effective as cAMP in the induction of gp80 gene expression. This is the first instance of a *Dictyostelium* gene being induced by all three of these cAMP analogs. Both cAMP and 2'-deoxy-cAMP exhibited similar dose-response curves and reached saturation at about 5 nM. 8-Bromo-cAMP and N^6-monobutyryl-cAMP were fairly ineffective in the 1 to 5 nM range, but both caused a sharp rise in gp80 expression at between 5 and 10 nM (Fig. 5). The 50% effective concentration for these latter analogs was only sixfold higher than that of cAMP or 2'-deoxy-cAMP. The observations thus suggest that the cAMP receptor involved in gp80 gene expression is quite different from the well-characterized chemotactic receptor. Although 2'-deoxy-cAMP is known to have a high affinity for the cell surface chemotactic receptor, 8-bromo-cAMP and N^6-monobutyryl-cAMP bind with affinities several hundredfold lower than cAMP (39, 50).

An alternative interpretation of these results is that intracellular cAMP is involved in stimulating gp80 expression, since both 8-bromo-cAMP and N^6-monobutyryl cAMP are known to have high affinities for cytoplasmic protein kinase. 8-Bromo-cAMP binds the regulatory subunit of the cytosolic cAMP-dependent protein kinase with a 2.5-fold higher affinity than cAMP, whereas 2'-deoxy-cAMP binds with a 10^4-fold lower affinity (8, 15, 25, 47, 50). N^6-Monobutyryl-cAMP also has a high affinity for protein kinase (39). However, N^6, *O*-2'-dibutyryl-cAMP, which is a membrane-permeable protein kinase activator commonly used as a substitute for cAMP in cultured mammalian cells to elevate the intracellular cAMP level (16, 52), has no effect on gp80 gene expression. It is therefore unlikely that 8-bromo-cAMP and N^6-monobutyryl-cAMP act intracellularly as a second messenger. This conclusion is further supported by the caffeine experiment, which shows that adenylate cyclase activation is not essential in the signalling pathway, thus excluding a role for intracellular cAMP.

It is of interest that a high concentration of cAMP can stimulate a rapid transient expression of gp80 molecules but fails to induce maximal gp80 expression (Fig. 1 and 2). 2'-Deoxy-cAMP, 8-bromo-cAMP, and N^6-monobutyryl-cAMP show similar effects when added to cells in high doses (Fig. 6). This observation thus supports the notion that these three analogs exert their effects by binding to the same cell surface receptor.

Results from our studies on the mutant strains *synag 7* and *frigid A* HC85 are also consistent with the involvement of a unique surface cAMP receptor in gp80 regulation. The *synag 7* mutant is blocked specifically in the receptor-mediated activation of adenylate cyclase, failing to relay cAMP pulses (46). The *frigid A* mutant HC85 carries a deletion in the gene encoding $G_\alpha 2$-protein and is blocked in the receptor-mediated activation of both adenylate cyclase and the putative phosphatidylinositol bisphosphate cycle (19, 33). Despite the fact that exogenous cAMP pulsing cannot activate in vivo adenylate cyclase activity and cAMP synthesis in *synag 7* cells, it is capable of augmenting gp80 gene expression. Hence, adenylate cyclase activation cannot be involved in the transmembrane signalling pathway regulating gp80 transcription. On the other hand, HC85 cells remain "frigid" after treatment with cAMP. Neither N^6-monobutyryl-cAMP nor 8-bromo-cAMP was capable of bypassing the transmembrane signalling defect. This observation further argues against a role for intracellular cAMP in this pathway.

The finding that accumulation of intracellular cAMP is not essential in the signal transduction pathway implies that 8-bromo-cAMP and N^6-monobutyryl-cAMP do not act by activating the cytosolic cAMP-dependent protein kinase. It is remarkable that pulses of 2'-deoxy-cAMP and N^6-monobutyryl-cAMP at low concentrations are also capable of restoring EDTA-resistant cell cohesion and gp80 expression in *synag 7* cells. These results, taken together, indicate the involvement of a novel cAMP receptor in gp80 gene regulation. This receptor is pharmacologically distinct from the cell surface chemotactic cAMP receptor (18, 25, 45, 50). Since gp80 is not induced in HC85 cells, it is likely that this novel cAMP receptor is also coupled to a pathway dependent on a funtional $G_\alpha 2$ protein.

A similar stimulatory effect of 8-bromo-cAMP and N^6-monobutyryl-cAMP has recently been observed for the expression of the chemotactic cAMP receptor CAR-1 (unpublished data). This suggests that the same surface receptor may be responsible for the expression of a number of early genes.

The identity of this novel cAMP receptor is not clear at present. The cAMP-binding activity at the cell surface has been found to be heterogeneous (49). The chemotactic cAMP receptor has been identified by photoaffinity labeling (46). It is noteworthy that several minor bands, other than the major chemotactic cAMP receptor represented by a doublet of 40 and 43 kDa (40/43-kDa) have been reported to be labeled by the photoaffinity probe 8-N_3[^{32}P]cAMP (47). Recently, Tsang and Tasaka (48) have characterized a 57-kDa cAMP-binding protein, CABP-1. The properties of CABP-1 are clearly distinct from those of the 40/43-kDa chemotactic cAMP receptor. These surface receptor proteins might represent potential candidates responsible for gp80 expression. Further endeavors to identify and characterize this novel cAMP receptor should aid in delineating the signalling pathway involved in gp80 expression and in our understanding of the diverse effects of cAMP in *Dictyostelium* development.

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