Gene expression in *Dictyostelium discoideum*: mutually antagonistic roles of cyclic-AMP and ammonia

By R. R. KAY

*From the Imperial Cancer Research Fund, London*

**SUMMARY**

Cyclic-AMP and ammonia have been previously identified as extracellular signals during *Dictyostelium* development. Both are important in controlling morphological movements and cyclic-AMP also in inducing gene expression. The work in this paper is concerned with their effects on developmental gene expression. Cyclic-AMP was found to act as an inducer during the aggregative (as exemplified by phosphodiesterase) and the post-aggregative (glycogen phosphorylase, UDP-galactose polysaccharide transferase, prespore vacuoles and stalk cells) phases of gene expression. Ammonia inhibited the appearance of each of the above markers and antagonized the inductive effects of cyclic-AMP on them. This inhibition by ammonia of cyclic-AMP inducible gene expression may involve a step linking elevated intracellular cyclic-AMP levels to gene activation.

It has been suggested that the specification of cells within the aggregate into the stalk and spore pathways of differentiation might be controlled by cyclic-AMP and ammonia. In this model for pattern formation cyclic-AMP would induce stalk cell differentiation and ammonia spore formation. The present results argue against this idea since cyclic-AMP induces and ammonia inhibits differentiation along both pathways. The function of these agents may rather be to coordinate the rates of biochemical differentiation of individual cells and link them to the overall morphological changes occurring during development.

**INTRODUCTION**

Upon starvation the initially isolated cells of the slime mould *Dictyostelium discoideum* embark on their developmental programme. They first aggregate to form multicellular structures, which after a further series of morphological movements, give rise to a fruit consisting of stalk and spore cells. Accompanying and in some sense coupled to these morphological movements there is an elaborate programme of developmental gene expression (Loomis, 1975). This results both in the appearance of particular markers during development and in the generation of two distinct cell types by its completion. Control of development is exercised by a number of cell interactions as well as by processes internal to each cell. Two substances, cyclic-AMP and ammonia (or ammonium ions),

1 Author’s address: Imperial Cancer Research Fund, Burtonhole Lane, London NW7 1AD, U.K.
have so far been unambiguously identified as extracellular signals during development of the organism (Konijn, van de Meene, Bonner & Barkley, 1967; Newell, Telser & Sussman, 1969; Schindler & Sussman, 1977a; Thadani, Pan & Bonner, 1977). Both were initially implicated as mediators of some of the morphological movements: cyclic-AMP as the chemotactic factor in aggregation, ammonia as the agent influencing territory size in aggregation and as the metabolite whose accumulation induces the transformation of an aggregate into a migrating slug.

More recent work has revealed a nice economy in the effects of chemotactic cyclic-AMP signalling; not only does it bring about cellular aggregation but, by causing an increase in intracellular cyclic-AMP concentrations, it also stimulates aggregative gene expression (Darmon, Brachet & Pereira da Silva, 1975; Gerish, Fromm, Huesgen & Wick, 1975). By treating cells with high external cyclic-AMP concentrations (1–5 mm, which like signalling, produce an increase in the intracellular concentration; Sampson, Town & Gross, 1978), we now have some evidence that cyclic-AMP also drives gene expression at times after aggregation (Town & Gross, 1978).

By analogy with the dual role of cyclic-AMP in controlling both morphological movements and gene expression, ammonia too might be expected to have important effects on gene expression. The work to be described here was stimulated by this idea and by the suggestion of Sussman & Schindler (1978) that cyclic-AMP and ammonia might have antagonistic and pathway-specific effects on gene expression. According to this hypothesis cyclic-AMP would act as a specific inducer of gene expression in the stalk cell pathway of differentiation and ammonia in the spore. These ideas have been explored here by examining the effects of cyclic-AMP and ammonia on suitable aggregative and post-aggregative markers. The results show that cyclic-AMP acts as an inducer of both aggregative and post-aggregative development and that ammonia acts on the same markers as an inhibitor and antagonist of the inductions by cyclic-AMP. However, neither agent shows any obvious pathway specificity.

**MATERIALS AND METHODS**

*Cell growth and differentiation*

*Dictyostelium discoideum*, strains Ax2 (ATCC 24397) and HM1 (an acriflavine-resistant derivative of V12M2) were used. Ax2 cells were grown in axenic medium with glucose at 22 °C and with shaking as described by Watts & Ashworth (1970). They grew with a doubling time of 8–10 h and before they had reached a density of 5 × 10⁶ cell/ml they were harvested by centrifugation at 200 g for 3 mins. After two washes in KK₂ (KK₂ is 16-6 mM-KH₂PO₄, 3-8 mM-K₂HPO₄, 2 mM-MgSO₄ pH ~6-2) the pellet was resuspended in 10 or 20 mM-K₂HPO₄/KH₂PO₄, 2 mM-MgSO₄ pH 7-5 and adjusted to 10⁷ cell/ml. The cell suspensions were shaken at 125 rev./min in siliconized flasks of five to ten times
the sample volume at 7-5 °C or 22 °C. At various times samples were withdrawn and the cells pelleted for 20 sec in a microfuge and either stained immediately for prespore vacuoles or stored in duplicate at -20 °C for enzyme assay. The convention \( t_0, t_1, t_2, \ldots t_n \) is used here to indicate the number of hours for which the developing cells have been starved.

HM1 cells were grown on SM plates in association with *Klebsiella aerogenes* (Gross, Peacey & Trevan, 1976) and prepared for stalk cell induction as described previously (Kay, Garrod & Tilly, 1978). Briefly, cells were harvested and washed, spread on 1.5% agar containing 5% Bonners salts (100% is 0.6 g/l NaCl, 0.75 g/l KCl, 0.3 g/l CaCl₂), 200 μg/ml streptomycin, 10 mM Tris-HCl pH 7.5, with the indicated additions and then overlaid with washed cellophane held in Perspex stretchers. The plates were incubated at 22 °C in a moist atmosphere and scored by phase-contrast microscopy for stalk cells at various times.

**Assays**

Cell pellets frozen at -20 °C were thawed and lysed in 50 mM tris-HCl, 0.15% cemulsol pH 7.5 for phosphodiesterase assay or in 100 mM tricine-HCl, 20% (v/v) glycerol, 0.15% cemulsol pH 7.5 for the assay of glycogen phosphorylase and UDP-galactose polysaccharide transferase. Phosphodiesterase was assayed as described by Henderson (1975) and glycogen phosphorylase as described by Town & Gross (1978). UDP-galactose-polysaccharide transferase was measured by a modification of the procedure of Sussman & Osborn (1964). Acceptor for the assay was prepared from slugs of a culmination-defective mutant of *Dictyostelium mucoroides* (LS-2 obtained from I. Takeuchi), essentially as described by Sussman & Osborn (1964) except that the total material precipitated by 65% ethanol was used as acceptor, rather than the cut precipitated between 35% and 65% ethanol as previously. The assay consisted of 0.02 mM [³H]UDP-galactose (~10,000 cpm per assay, from Amersham), 54 mM dimethylglutarate, 14 mM-KCl, 1.8 mM-MgCl₂, 0.55 mg of acceptor plus enzyme protein in a total volume of 62.5 μl. After 4 h of incubation at 0 °C a 50 μl aliquot was removed from each assay tube and spotted onto a 2.5 cm diameter Whatman no. 1 filter. Filters were batch washed four times with ice-cold 55% ethanol, then once with 100% ethanol and after drying counted in toluene plus fluoros.

Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951). Prespore cells were stained with the specific antibody obtained from Drs D. Garrod and D. Forman which was raised in rabbit against *Dictyostelium mucoroides* spores and absorbed with vegetative *Dictyostelium discoideum* cells. Cell pellets were treated with pronase/BAL (Hayashi & Takeuchi, 1976) for 15 min and passed through a 27G syringe needle twice to disaggregate them to single cells. These were pelleted by centrifugation for 5 sec in a microfuge, resuspended in 60% methanol and 5 μl portions air dried onto coverslips.
To each spot 15 μl methanol was added and they were again air dried before staining for 20 min with 20 μl of anti-spore serum. After three washes with KK₂0·8 % NaCl the cells were stained with a ‘sandwich layer’ of fluorescent goat-anti-rabbit antibody (Miles-Yeda) for 15 min. Finally after a further three washes the coverslips were mounted in glycerol and fluorescence examined with a Zeiss III photomicroscope equipped for epi-fluorescence. Cells having three or more distinct cytoplasmic fluorescent spots were scored as positive. No attempt was made to quantify the intensity of staining of individual positive cells at different times of development although it appeared generally to change in the same way as the proportion of such cells in the population.

RESULTS

Effects of cyclic-AMP and NH₄Cl on cyclic-AMP phosphodiesterase

After about 4 h of starvation, cells plated on a surface start to aggregate and embark on the major phase of cyclic-AMP phosphodiesterase accumulation. Conveniently for the experiments described in this section phosphodiesterase (and other aggregative products, Town & Gross; 1978; Sampson et al., 1978) are also made with similar timing by cells starved in slowly shaken suspension. The time course of phosphodiesterase appearance in such conditions is shown in Fig. 1. It is apparent that accumulation of the enzyme is strongly inhibited not only by 20 mM-NH₄Cl, in agreement with Schindler & Sussman (1977b), but also somewhat surprisingly by 20 mM-KCl, added as an ionic strength control. In other experiments the inhibition by HN₄Cl was always severe, whereas that by KCl was often much less so.

The addition of 1 mM cyclic-AMP at t₂ resulted in an efficient induction of phosphodiesterase in the presence of KCl (Fig. 1) which was comparable to that obtained in its absence (Klein, 1975). However, consistently poor inductions were obtained in the presence of NH₄Cl. In the presence of either KCl or NH₄Cl the cyclic-AMP concentration required for a maximal induction of phosphodiesterase was found to be about 0·5 mM (results not shown). Since this is less than the 1 mM concentration used for the inductions shown in Fig. 1, it follows that NH₄Cl must act not only as an inhibitor of phosphodiesterase accumulation but also in such a way as to reduce the maximal level of induction which can be obtained with exogenous cyclic-AMP.

Effects of cyclic-AMP and NH₄Cl on post-aggregative functions

Cells starved in suspension will under the appropriate conditions continue development beyond the aggregative stage (Garrod & Forman, 1977; Sternfeld & Bonner, 1977; Takeuchi, Hayashi & Tasaka, 1977). Figure 2 shows such an experiment in which the cells start to accumulate the post-aggregative products glycogen phosphorylase, the major species of UDP-galactose polysaccharide transferase (‘transferase’) and prespore vacuoles (‘PSVs’) some considerable
Fig. 1. Ammonium chloride inhibits the accumulation of cell-associated phosphodiesterase by control and cyclic-AMP-treated cells. Ax2 cells were starved in slowly shaken suspension at a concentration of $10^7$/ml in 10 mM-KH$_2$PO$_4$, 2 mM-MgSO$_4$, pH 7.5. At $t_2$, as indicated by the arrow, the following additions were made to separate flasks: •, None; ■, +30 mM-KCl; ■, +30 mM-KCl + 1 mM cyclic-AMP (dotted line); ▲, +30 mM-NH$_4$Cl; ▲, +30 mM-NH$_4$Cl + 1 mM cyclic-AMP (dotted line).

time after the peak level of phosphodiesterase has been reached. The early peak of transferase (Fig. 2b) has not been previously observed but is regularly seen with Ax2 cells in suspension. It could be responsible for the synthesis of acceptor polysaccharide found by Sussman & Osborn (1964) in cells at 9 h of starvation, before the late enzyme has started to appear. It is apparent that the addition of 5 mM cyclic-AMP at $t_6$ or later results in a decline in phosphodiesterase levels (Town & Gross, 1978) but that glycogen phosphorylase, transferase and the proportion of cells staining for PSVs increase dramatically. In this and other experiments the prespore markers, transferase (Newell, Ellingson & Sussman, 1969) and PSVs, increased with similar kinetics whenever cyclic-AMP was added and with a delay that decreased from about 3 h when the addition was made at $t_6$, to 1 h when it was made at $t_{10}$. Glycogen phosphorylase, which is made by both prestalk and prespore cells (Rutherford & Harris, 1976), behaved slightly differently, in that at earlier times an induction was generally apparent about 1 h before the prespore cell markers. These results suggest that cyclic-AMP levels are rate-limiting for the accumulation of all three markers.
Fig. 2. Induction of post-aggregative functions and depression of phosphodiesterase levels by cyclic-AMP. Ax2 cells were starved in slowly shaken suspension at a concentration of $10^7$/ml in 20 mM-KH$_2$/K$_2$HPO$_4$, 2 mM-MgSO$_4$, pH 7.5. The numbered arrows indicate the time in hours at which 5 mM cyclic-AMP was added to a particular flask. The resulting changes in each of the markers assayed are shown by the numbered dotted lines, where the number again gives the time at which cyclic-AMP was added. Addition of tracer amounts of $[^3]$Hcyclic-AMP and separation of the products of cyclic-AMP hydrolysis showed that it took approximately 6 h in these conditions for the concentration of cyclic-AMP to be reduced to 1 mM.

throughout the period studied and hint that the prespore cell markers may be regulated differently from glycogen phosphorylase.

In subsequent experiments testing the effects of NH$_4$Cl and cyclic-AMP on post-aggregative development a protocol more convenient than that of Fig. 2 was used. For the first 15–18 h of starvation the cells were shaken at 7.5 °C and
Fig. 3. Ammonium chloride inhibits the accumulation of post-aggregative products by control and cyclic-AMP-treated cells. Ax2 cells were starved in slowly shaken suspension at a concentration of 10⁷/ml in 20 mM-KH₂PO₄, 2 mM-MgSO₄, pH 7.5 at 7.5 °C overnight. At the start of the experiment the flasks were transferred to 22 °C and the following additions made immediately: ●, None; ■, +5 mM cyclic-AMP; ▲, +30 mM-NH₄Cl; ▼, +30 mM-NH₄Cl+5 mM cyclic-AMP.

then transferred to 22 °C at the start of the experiment. Development proceeded at a reduced rate at the lower temperature, so that the cells generally had not initiated the synthesis of glycogen phosphorylase at the time of transfer but did so shortly thereafter. Figure 3 demonstrates the induction of glycogen phosphorylase, transferase and PSVs by cyclic-AMP in these conditions and shows that 30 mM-NH₄Cl added at the time of transfer totally inhibits the appearance
Fig. 4. Ammonium chloride inhibits cyclic-AMP-induced stalk cell formation. Washed cells of strain HM1 were spread on agar containing 5% Bonner's salts, 200 μg/ml streptomycin, 5 mM cyclic-AMP, 10 mM tris pH 7.5 plus the indicated additions and overlayed with cellophane. Stalk cells were scored by phase contrast microscopy. ●, No addition; ■, +20 mM-NH₄Cl; ▲, +30 mM-NH₄Cl; ▼, +40 mM-NH₄Cl.

of glycogen phosphorylase and PSVs. It also inhibits the main rise in transferase activity but seems to somewhat prolong the early period of accumulation, which is already under way at the time of transfer of the cells to 22 °C. In other experiments 20 mM-NH₄Cl was almost as effective and 10 mM half as effective as 30 mM-NH₄Cl in inhibiting glycogen phosphorylase and PSV accumulation. However, unlike the situation with phosphodiesterase, 20-40 mM-KCl had little effect. It is also apparent from Fig. 3 that NH₄Cl strongly antagonizes the inductive effects of cyclic-AMP. Again the concentration of cyclic-AMP used is at least as great as that required for maximal induction of glycogen phosphorylase and PSVs, so it appears that NH₄Cl acts in a way so as to reduce the maximal level of induction obtainable.

The results presented so far indicate that NH₄Cl acts as an inhibitor of differentiation of cells at least along the spore pathway, for which transferase and PSVs are specific markers, and probably the stalk pathway too, since glycogen phosphorylase is inhibited and is normally present in both prestalk and prespore
Gene expression in Dictyostelium cells. Because of a lack of suitable specific markers for prestalk cells to assay in suspension the effect of NH$_4$Cl on differentiation along the stalk cell pathway was investigated in the 'cellophane-monolayer' system for stalk cell induction (Town, Gross & Kay, 1976; Kay et al. 1978). As shown previously, cells of strain HM1 (a V12M2 derivative) plated at high density on agar and overlayed with cellophane are efficiently induced to become stalk cells by cyclic-AMP but make none in its absence. NH$_4$Cl alone does not induce stalk cell differentiation and Fig. 4 shows that in fact it strongly inhibits stalk cell induction by cyclic-AMP. This is equally true whether the NH$_4$Cl is added at $t_0$ or at $t_{15}$ (not shown). As with other post-aggregative functions KCl, up to 40 mM, has little effect on stalk cell induction.

**DISCUSSION**

The effects of exogenous cyclic-AMP on *Dictyostelium discoideum* are potentially mediated by two routes. In the first, cyclic-AMP acts as an extracellular 'first messenger' and its effects are transmitted into the cell via the cell surface receptors. In the second, with high exogenous levels such as have been used in this work, cyclic-AMP leaks directly into the cell (Sampson et al. 1978) and by elevating the internal concentration can bring about the observed cellular responses. In this case cyclic-AMP would act as a 'second messenger' (Robison, Butcher & Sutherland, 1971). The induction of prespore cells (as marked by prespore vacuoles and UDP-galactose polysaccharide transferase) by cyclic-AMP reported here confirms the earlier results obtained in the cellophane-monolayer system for cell differentiation (Kay et al. 1978). In the present work cyclic-AMP has been added to cells after the end of the aggregative phase of development. It follows therefore that apart from its effect on aggregative gene expression, cyclic-AMP also acts as an inducer of prespore cell differentiation during post-aggregational development. A similar post-aggregative role for cyclic-AMP in the stalk cell pathway is indicated by the observation that in the cellophane-monolayer system post-aggregative cells do not differentiate to stalk cells unless cyclic-AMP is added (Kay, unpublished observation). Combining these results with others which show the induction by cyclic-AMP of two post-aggregational enzymes (Town & Gross, 1978) and possibly of prespore cell maturation (Feit, Fournier, Needleman & Underwood, 1978), it seems reasonable to propose that cyclic-AMP stimulates some developmental gene expression throughout post-aggregative development.

Ammonia (as NH$_4$Cl), in the physiological concentration range (Schindler & Sussman, 1977a) inhibits the appearance of each of the cyclic-AMP-inducible functions investigated in this work. The effect would appear to be specific for development, since growth of Ax2 cells in axenic medium at pH ~7.5 is, apart from an initial lag phase, unaffected by the same concentrations of NH$_4$Cl (Kay, unpublished observation). The inhibition by ammonia of cyclic-AMP-inducible gene expression is potentially explicable by the finding of Schindler &
Sussman (1979) that treatment of cells with ammonia results in a rapid decline in the intracellular cyclic-AMP concentration. However, the fact that in the presence of ammonia, the induction of phosphodiesterase and glycogen phosphorylase, by saturating levels of cyclic-AMP, is much less efficient than in its absence suggests that ammonia has an inhibitory effect additional to that on the endogenous cyclic-AMP concentration. This could involve a reduced uptake of exogenous cyclic-AMP into the cell or more likely the inhibition of some part of the process linking elevated intracellular cyclic-AMP levels to gene activation. The dual effects of ammonia suggested by these results and those of Schindler & Sussman (1979) would be unified if a single component was inhibited (such as a cyclic-AMP-dependent protein kinase, Sampson, 1977) which had a role both in raising cyclic-AMP levels and in coupling them to gene expression.

Since cyclic-AMP and ammonia affect gene expression at the time of development when prestalk and prespore cells arise within the aggregate, it is possible that these agents could act selectively to induce the differentiation of one cell type but not of the other. Sussman & Schindler (1978) have suggested a model for pattern formation in Dictyostelium that utilises this idea, in which cyclic-AMP would specifically direct cells along the stalk cell pathway and ammonia along the spore. Spatial gradients of cyclic-AMP and ammonia within the aggregate would then be responsible for the regional differentiation of the two cell types. The present results argue strongly against the part of this model concerned with cellular differentiation. Cyclic-AMP is found to induce differentiation along both the spore and stalk pathways, rather than just the stalk one, whilst ammonia inhibits differentiation in both pathways, rather than inducing it in the spore one. Further, since other distinct requirements have been shown for the induction of stalk and spore cells in the cellophane-monolayer system (Town et al. 1976; Kay et al. 1978; Kay, Town & Gross, 1979), it may be unnecessary to propose that levels of cyclic-AMP and ammonia control the specification of cells into the two pathways. Rather these agents, by acting on both pathways of differentiation, could serve to coordinate the rates of biochemical differentiation in the individual cells and couple these to aggregational movements and the subsequent morphological changes of the aggregate as development proceeds.

I should like to thank Julian Gross and my other colleagues for helpful discussions.

REFERENCES


Gene expression in Dictyostelium


*(Received 20 December 1978, revised 8 February 1979)*