The role of diffusible molecules in regulating the cellular differentiation of Dictyostelium discoideum

JEFFREY G. WILLIAMS
Imperial Cancer Research Fund, Clare Hall Laboratories, Blanche Lane, South Mimms, Potters Bar, Herts EN6 3LD, UK

(1) Summary

A central problem in developmental biology is to understand how morphogenetic fields are created and how they act to direct regionalized cellular differentiation. This goal is being pursued in organisms as diverse as moulds, worms, flies, frogs and mice. Each organism has evolved its own solution to the challenge of multicellularity but there appear to be common underlying principles and, once pattern formation is fully understood in any system, some general truths seem certain to be revealed. As a non-obligate metazoan, Dictyostelium discoideum has proven a particularly tractable system in which to identify and characterize cellular morphogens. Cyclic AMP and ammonia stimulate prespore cell differentiation and ammonia plays an additional role in repressing terminal cellular differentiation. Differentiation Inducing Factor (DIF) acts to direct prestalk cell differentiation and adenosine may play a synergistic role in repressing prespore cell differentiation. This review summarizes the evidence for these interactions and describes a number of models which show how this small repertoire of diffusible molecules, acting in concert, may direct the formation of a differentiated structure.

Key words: Dictyostelium discoideum, prestalk differentiation, diffusible molecules, cyclic AMP, prespore differentiation, ammonia, adenosine, DIF, pattern formation.

(2) The organism and its development

Dictyostelium discoideum, the most commonly studied cellular slime mould, exists during the vegetative phase of its life cycle as solitary amoebae, migrating through the surface layer of soil and forest litter. It is a haploid organism, with seven chromosomes, to which approximately 100 loci have been assigned using parasexual genetics (reviewed by Newell, 1982a). Because growth and differentiation can be observed in clonal populations of cells, and because development is facultative, developmental mutants are readily isolated. These have proved of considerable value in understanding Dictyostelium pattern formation and, with the recent development of DNA-mediated transformation (Nellen et al. 1984) and homologous gene disruption techniques (Lozanne & Spudich, 1987), the power of this approach in identifying specific genes involved in morphogenesis will be greatly enhanced.

When the bacterial food source is exhausted the amoebae enter the developmental pathway to form a mass of spores supported by a stalk (Fig. 1). The spores are ellipsoidal cells, resistant to environmental stress and the stalk is composed of highly vacuolated, cellulose-ensheathed dead cells. There is some continued cell division during development but this is not obligatory. Approximately 80 % of the cells that enter development form spores and the remainder form stalk cells. Aggregation of amoebae occurs in response to pulsatile emissions of cAMP from a signalling centre which arises, apparently at random, and entrains up to 10^5 amoebae. This is dependent upon the local cell density and development is highly regulative, over a very wide range of cell number (Bonner & Slifkin, 1949; Stenhouse & Williams, 1977). Cells respond to receipt of a cAMP stimulus by migrating towards the signalling centre and by synthesizing and releasing cAMP to relay the chemotactic signal through the aggregation territory. At the end of aggregation, a tip is formed at the top of the cell mound, and this elongates to form a structure known as the first finger (Fig. 1). By this stage in development, the aggregate has become a discrete entity, surrounded by an extracellular matrix composed of protein and cellulose, with all the cells...
behaving cooperatively to facilitate efficient spore formation. On the forest floor, the first finger will normally be formed below the surface and, under the conditions of light, humidity and ionic strength found there, the first finger collapses onto the substratum to form a migratory slug (Fig. 2). The slug is exquisitely responsive to light and temperature gradients and these sensitivities direct it to the surface where it culminates to form a mature fruit. Under laboratory conditions, the migratory slug phase can be eschewed and the whole process of development then takes 24–28 h.

The anterior one fifth of the slug is composed of cells which will, under normal conditions, form stalk cells. The posterior four fifths is predominantly composed of prespore cells but approximately 10–15 % of cells in this region display the morphological and biochemical characteristics of prestalk cells and hence are termed anterior-like cells (Sternfeld & David, 1981, 1982; Devine & Loomis, 1985). At culmination, the slug sits on end and backward migration of prestalk cells through the prespore zone, coupled with the swelling that accompanies their differentiation to form stalk cells, acts to lift the spore mass off the substratum. The migratory slug is of great value because it provides a ready source of cells fated to one or other of the two differentiation pathways, prespore and prestalk cells being readily separable by centrifugation in percoll gradients (Tsang & Bradbury, 1981; Ratner & Borth, 1983). It is approximately 1 mm in length and sufficiently robust to tolerate microdissection and transplantation. These have shown the slug to have some of the characteristics of the vertebrate embryo.

If the slug is bisected into a presumptive prestalk and prespore zone then each segment will, after a period of migration, regulate to form a correctly proportioned culminant (Raper, 1949). If, however,
the separated segments are forced to culminate immediately, the prestalk zone forms a culminant with a disproportionately large stalk while the prespore zone forms a more normally proportioned culminant (Raper, 1940; Sampson, 1976). This is probably a consequence of the presence of anterior-like cells in the prespore region, which rapidly migrate to the cut edge of the slug and reconstitute a prestalk zone (Sternfeld & David, 1981, 1982). These experiments show that cells are not irreversibly committed to their fate at the slug stage and the behaviour of anterior-like cells in isolated prespore segments suggests that cell sorting may play a role in the primary differentiation of prestalk and prespore cells.

The tip of the migrating slug has some of the classical properties of an embryonic organizer. If a tip is transplanted to the posterior of another slug, it directs formation of a secondary axis and the slug splits into two (Raper, 1940; Rubin & Robertson, 1975). This behaviour, which is termed tip dominance, is a graded response. The ability of the transplanted tip to compete with the primary tip is a function of distance along the anterior–posterior axis of the slug (Durston, 1976; MacWilliams, 1982; Lokeshwar & Nanjundiah, 1985).

These phenomena, coupled with the precisely orchestrated cell behaviour displayed during slug migration and culmination, point to the continued action of diffusible signals in the aggregate. Four such molecules have been identified, cAMP, DIF, adenosine and ammonia.

(3) Cyclic AMP and prespore gene expression

While it is difficult to analyse oscillatory signalling in a cell mass, such as the slug, there is persuasive evidence (reviewed by Schaap, 1986) that emissions of cAMP, propagated from the tip, direct cell movement in the slug and are responsible for tip activation; the ability of a transplanted tip to establish a secondary slug. There is also now considerable evidence to suggest a primary role for cAMP in inducing prespore cell differentiation. Two general approaches have been used to show that cAMP acts to induce, or to maintain, the expression of prespore markers.

(A) Regulation of prespore cell number in intact slugs or large cell aggregates

Prespore cells can readily be identified by their characteristic morphology and by reliable enzymatic, immunological and mRNA markers. If intact slugs are allowed to migrate on agar containing 1 mM-cAMP, then the proportion of prespore cells increases by 5% (Schaap & Wang, 1986). The major effects of cAMP are to reduce the size of two non-prespore-staining zones, one normally found adjacent to the substratum and the other at the extreme posterior of the slug. This latter 'rearguard' zone contains prestalk cells which differentiate to form the basal disc, the structure that attaches the mature culminant to the substratum. Thus, in the intact slug, cAMP appears to induce the conversion of prestalk to prespore cells. It can also be shown to prevent the conversion of prespore to prestalk cells in a regulation experiment. When gradient-purified prespore cells are gently agitated in a simple salt solution, they form large aggregates in which approximately one-half of the prespore cells lose prespore markers and acquire some of the characteristic features of prestalk cells (Weijer & Durston, 1985). This transition is blocked by high concentrations of cAMP and, conversely, if endogenously produced cAMP is degraded, by the addition of cAMP phosphodiesterase, the rate of loss of prespore markers is accelerated (Weijer & Durston, 1985).

(B) Analysis of the role of exogenous cAMP on prespore cell differentiation under conditions that suppress endogenous cAMP production

In large aggregates, and in the intact slug, endogenous cAMP signalling makes it very difficult to analyse the direct, inductive, effect of cAMP on prespore cell differentiation. Hence an alternative approach has been to disrupt cAMP signalling in some way, and to determine the effect of exogenous cAMP. Cyclic AMP signalling is an autocatalytic process and some aspect of the process appears to be repressed in agglomerates of postaggregative amoebae. Thus cells shaken in suspension have been shown to express prespore markers much more rapidly in the presence of cAMP (Kay, 1979). In a more recent study, in which cells were rendered aggregation competent on agar and shaken in suspension, prespore-specific gene expression was found to be totally dependent upon the presence of exogenous cAMP (Schaap & Van Driel, 1985). A similar requirement has been demonstrated using a hypertonc incubation medium, which represses cAMP signalling (Oyama et al. 1983; Oyama & Blumberg, 1986a; Okamoto, 1986), and by using a mutant defective in cAMP signalling (Schaap et al. 1987).

When migrating slugs are disaggregated, and the cells rapidly shaken, the rate of transcription of many genes is reduced, the half life of the cognate mRNA sequences is greatly reduced and they quickly disappear (Chung et al. 1981; Mangiarotti et al. 1981, 1983; Landfear et al. 1982). Several of these genes have been shown to encode prespore-enriched mRNA sequences (Barklis & Lodish, 1983) and, for many of them, the presence of exogenous cAMP acts to maintain the rate of transcription and stabilize their
cognate mRNA sequences (Barklis & Lodish, 1983; Mehdy et al. 1983; Mangiarotti et al. 1983; Oyama & Blumberg, 1986a). Thus, under appropriate conditions, cAMP can be shown both to induce and maintain prespore gene expression.

A role of cAMP in prespore cell differentiation can also be demonstrated using isolated cells harvested from growth and incubated at very low density (Bonner, 1970; Town et al. 1976). This has proven to be an extremely useful assay for the effects of potential biological effectors of differentiation because cells are effectively removed from the influence of endogenously produced factors. Isolated amoebae of the strain V12M2 incubated at densities above \(10^3\) cells cm\(^{-2}\) differentiate to form prespore cells and stalk cells (Town et al. 1976; Kay et al. 1978). Sporogenous mutants of V12M2 are able to form mature spores under these conditions (Town et al. 1976; Kay et al. 1978). At very low cell densities, sporogenous mutants form spores with high efficiency but stalk cell differentiation does not occur (Kay, 1982a). Under these conditions cAMP is the only requirement for spore formation (Kay, 1982b). Thus, for sporogenous mutants, an alternative inducer produced endogenously would have to be active at an infinitesimally low concentration. This conclusion may not, however, apply to the development of wild-type cells because the sporogenous mutant could be a 'bypass' mutant (Rossier et al. 1980), which is able to dispense with some factor normally required. This same assay system also yielded the first evidence for a specific inducer of stalk cell differentiation.

(4) DIF and prestalk gene expression

Differentiation Inducing Factor (DIF) was detected as a diffusible factor which induces isolated cells, incubated at low density, to form stalk cells (Town et al. 1976). If the exudate from cells incubated at high density, under the conditions described above, is added to low density cells, a very high proportion are induced to form stalk cells (Town & Stanford, 1979; Gross et al. 1981; Kay & Jermyn, 1983). Using this as an assay, five peaks of activity were detected by HPLC, with the vast majority of the activity migrating in a single peak (Kay et al. 1983). This molecule, DIF1, has been purified (Kay et al. 1983) and its structure has been determined (Morris et al. 1987b). The DIF1 molecule is unusual among biologically active substances in containing chlorine atoms (Fig. 3). At least two of the other peaks of DIF activity observed on HPLC, and termed DIF2 and DIF3, are related molecules of unknown function (Morris et al. 1987a). DIF1 is active at nanomolar concentration in inducing stalk cell formation in vitro and several pieces of evidence indicate that it plays a central role in the prestalk-stalk pathway of differentiation in vivo.

(A) The accumulation of DIF is developmentally regulated and a mutant defective in its accumulation is blocked in prestalk cell differentiation

A small amount of DIF is produced early during development but the major rise in concentration occurs between the loose mound and tipped-aggregate stage of development (Brookman et al. 1982), the time when overt cellular differentiation first becomes apparent. The HM44 mutant, which accumulates only 1 or 2 % of the wild-type amount of DIF, forms only a low percentage of stalk cells when incubated at high density in the submerged culture assay (Kopachik et al. 1983). If purified DIF is added, then stalk cells are formed with greater than 90 % efficiency. Under normal conditions of development, the mutant becomes arrested at the loose mound stage and expresses only prespore markers (Kopachik et al. 1983).

(B) In the presence of DIF prespore cells are induced to form stalk cells

If sporogenous mutants are exposed to DIF, in the submerged assay, then they are prevented from forming spore cells and are induced to form stalk cells (Kay & Jermyn, 1983). Prespore cells, isolated from slugs are induced to redifferentiate into stalk cells in the presence of DIF (Kay & Jermyn, 1983). This property of DIF is conveniently studied using the HM44 mutant. In the standard assay for DIF induction of HM44 cells (Fig. 4), amoebae are incubated in the presence of cAMP for 10–12 h and then DIF is added. Morphologically identifiable stalk cells are formed within 6–8 h. After the preincubation in the presence of cAMP, the cells contain prespore-specific proteins (Kopachik et al. 1983) and prespore-specific mRNA sequences detectable with cloned cDNA probes (A. Early & J. G. Williams, unpublished manuscript). The addition of DIF suppresses the expression of these markers. The accumulation of D19, a prespore-specific mRNA sequence, is repressed at the transcriptional level (A. Early & J. G. Williams, unpublished manuscript). This effect of DIF seems to result from a direct antagonism with
cAMP, in its induction of prespore cell differentiation and DIF appears to exert its inhibitory action at some step in intracellular signal transduction (Wang et al. 1986).

(C) DIF induces the transcription of prestalk-specific genes

The HM44 mutant provides an ideal system in which to assess the effect of DIF on prestalk and stalk gene expression, because cellular differentiation is dependent upon its addition. In the presence of DIF, a prestalk-specific isozyme of acid phosphatase (Kopachik et al. 1983) and an unidentified prestalk protein detected by two-dimensional gel electrophoresis (Kopachick et al. 1985) are induced to accumulate. Two genes, directly inducible by DIF, have also been identified.

The pDd56 and pDd63 cDNA clones were isolated by differential screening of a cDNA clone bank prepared from HM44 cells induced to differentiate into stalk cells by the addition of DIF (Jermy et al. 1987). They have been shown respectively to encode the ST430 and ST310 proteins of Morrissey et al. (1984). In the DIF induction experiments, the times indicated are the number of hours after the addition of DIF, subsequent to a 10 h preincubation in the presence of cAMP [see Fig. 4]. The low level of expression of the pDd63 gene, seen at late times in the absence of added DIF, almost certainly results from the finite but low level of DIF production by the HM44 mutant (Williams et al. 1987). The stages represented during normal development are A, late aggregate; T, tipped aggregate; F, first finger/slug; H, Mexican hat; P, early culminant; C, mature culminant.

These mRNA sequences are both dependent upon DIF for their accumulation but they differ in their precise kinetics of appearance. The pDd56 mRNA displays sigmoidal kinetics of accumulation while the pDd63 mRNA accumulates with linear kinetics (Jermy et al. 1987; Fig. 5). Both genes are induced at the transcriptional level and expression of the pDd63 gene is detectable within 15 min of the addition of DIF (Williams et al. 1987; Ceccarelli et al. 1987;
(5) The role of ammonia

During differentiation, a major fraction of cellular protein is degraded and a large amount of ammonia is produced. Migrating slugs can be induced to culminate by enzymatically depleting them of ammonia (Schindler & Sussman, 1977). A drop in ammonia concentration may, therefore, trigger fruiting during normal development. Ammonia may also be acting antagonistically to DIF earlier in development, in preventing the differentiation of prestalk into stalk cells, because stalk cells can be induced to differentiate within the slug by localized exposure to DIF and an enzyme cocktail that depletes ammonia (M. Wang and P. Schaap, personal communication). Ammonia has also been suggested to be important in facilitating prespore cell differentiation. Ammonia is required for spore cell formation in the differentiation of large clumps of cells in submerged culture (Sternfeld & David, 1979). When cells are rapidly shaken in a highly hypertonic medium, where cAMP relay is inhibited, ammonia is necessary for the optimal expression of several prespore genes (Oyama & Blumberg, 1986b). Further evidence for a role of ammonia in spore cell differentiation derives from experiments using a sporogenous mutant. If cells are incubated at high density in the submerged culture assay a mixture of stalk and spore cells is formed. In the presence of ammonium salts the proportion of cells differentiating into spores is increased (Gross et al. 1983). This has led to the suggestion that ammonia is an antagonist to DIF during primary cellular differentiation (Gross et al. 1983) and the observation that a mutant that is hypersensitive to the inhibitory effect of ammonia has a reduced prestalk zone (Newell & Ross, 1982; MacWilliams, 1982) is consistent with this idea.

(6) The role of adenosine in tip inhibition and the repression of prespore cell differentiation

Adenosine appears to function as an antagonist to cAMP in its role in mediating tip dominance and in its stimulatory effect on prespore cell differentiation.

In the migratory slug, the ability of the primary tip to repress secondary slug formation by a transplanted tip (tip inhibition) decreases along the axis of the slug, the prestalk zone being a region of high tip inhibition (Durston, 1976; MacWilliams, 1982). The size of the slug is a reflection of the balance between tip activation and tip inhibition; an overly large slug will break into two smaller slugs because of the formation of a secondary tip. Reduction of the adenosine concentration in the slug, by treatment with adenosine deaminase, reduces the size of the slug (Schaap & Wang, 1986). Conversely, exposure to a high external concentration of adenosine increases the average size of the slug twofold (Schaap & Wang, 1986). During early aggregation, exogenous adenosine acts to increase the average size of aggregation territories by reducing the number of signalling centres (Newell & Ross, 1982). At late stages during aggregation, adenosine acts as an inhibitor of the binding of cAMP to its cell surface receptor which reduces cAMP relay (Newell, 1982b; Van Haastert, 1983; Theibert & Devreotes, 1984; van Lookeren Campagne et al. 1986). These observations, taken together, suggest that adenosine is responsible for tip inhibition and that it functions by counteracting tip activation by cAMP.

In regulation experiments, using submerged aggregates of gradient-separated prespore cells, adenosine inhibits the stabilization of prespore markers by cAMP (Weijer & Durston, 1985). In the intact slug, reduction of the adenosine concentration, by enzymatic depletion, results in the appearance of prespore cells within the prestalk zone (Schaap & Wang, 1986). In the most extreme cases, this treatment produces a slug which stains uniformly with a prespore-specific antibody. The effect of exposure to a high adenosine concentration is much less dramatic, with no increase in the proportion of non-prespore cells. There is, however, a redistribution of non-prespore staining cells. In aggregation-competent cells, rendered dependent upon exogenous cAMP for the rapid induction of prespore-specific markers, adenosine acts as a potent competitive inhibitor (Schaap & Wang, 1986). Presumably, it again exerts its effect by reducing cAMP binding to the cell surface receptor.
The stimulation of prespore cell differentiation by other nonstructurally identified factors

In addition to these four substances, of known structure, a number of other biological activities have been detected, using various assays, but no corresponding specific factor has as yet been structurally identified. When incubated in submerged culture at low density, wild-type cells will form spores only in the presence of conditioned medium from high-density cells (Kay, 1982). Exposure to a low molecular weight factor, termed CMF, is necessary for Ax3 cells incubated at low density to express a number of markers, including several prespore-specific mRNA sequences (Mehdy & Firtel, 1985). Other, apparently distinct, factors, termed SPIF (Wilkinson et al. 1985) and PIF (Kumagai & Okamoto, 1986), which stimulate prespore cell differentiation, have also been detected and partially purified. Again, the ability of sporogenous mutants to differentiate into spores in the absence of any factor other than cAMP suggests that, at least in this mutant, the requirement for additional stimuli can be bypassed. Also, there is no evidence for the rapid induction of prespore gene expression by any of these factors. They may, therefore, act as permissive factors, facilitating cellular differentiation but not directly inductive. The observation that CMF stimulates the expression of non-prespore mRNA sequence is consistent with this suggestion (Mehdy & Firtel, 1985).

Cell-type-specific markers and the timing of cellular differentiation

In order to assess the role of these various molecules in pattern formation, it is necessary to know when prestalk and prespore cell differentiation occur. This apparently simple question has led to considerable controversy and confusion and hence is worth considering in some detail.

An unambiguous marker of a particular cell type should not, by definition, be expressed in any other lineage and should, if it is to be useful in determining the time of differentiation, be detectable as early as possible in its ontogeny. Prespore cells are easily distinguishable morphologically. They contain prespore vesicles which, at culmination, fuse with the spore cell wall (Hohl & Hamamoto, 1969; Ikeda & Takeuchi, 1971; Devine et al. 1983). The enzyme UDP-galactose polyaccharide transferase is highly enriched in prespore over prestalk cells (Newell et al. 1971) and two-dimensional gel electrophoresis reveals many other proteins, which are not expressed prior to prespore differentiation and which are not expressed in prestalk cells (Ratner & Borth, 1983; Morrissey et al. 1984). It also indicates there to be considerable overlap in the genes expressed in prespore and mature spore cells. Many cDNA clones have been isolated that derive from mRNA sequences that are highly enriched in, and possibly totally specific to, prespore cells (Barklis & Lodish, 1983; Mehdy et al. 1983; Dowds & Loomis, 1984). Finally, there are reliable polyclonal (Takeuchi, 1963) and monoclonal (Krefft et al. 1985) antibodies that can be used to stain individual prespore cells. When these various markers are used to analyse the timing of cellular differentiation there is a high degree of concordance. The first prespore-specific markers appear late in aggregation, or just prior to, the time of tip formation ( Muller & Hohl, 1973; Hayashi & Takeuchi, 1976; Forman & Garrod, 1977a; Tasaka et al. 1983; Morrissey et al. 1984; Dowds & Loomis, 1984; Krefft et al. 1984; Williams et al. 1987). The situation on the stalk cell pathway of differentiation has, however, been much more confused because of the lack of comparable markers.

Prestalk cells are morphologically very similar to cells at the late aggregation stage of development (Schaap, 1983) and also share many of their biochemical characteristics. Thus, they possess relatively high levels of cell-surface cAMP phosphodiesterase (Tsang & Bradbury, 1981; Mee et al. 1985; Otte et al. 1986) and they are more highly responsive to cAMP than prespore cells (Matsukuma & Durston, 1979; Mee et al. 1985). Very few of the genes expressed in mature stalk cells are also expressed in prestalk cells (Ratner & Borth, 1983; Morrissey et al. 1984), again suggesting that prestalk cells are arrested in development in a form little different from that of cells late in aggregation. Prestalk cells do, however, differ from both late aggregation and prespore cells in the pattern of carbohydrate modification of several cytoplasmic enzymes (Knecht et al. 1984; Loomis & Kuspa, 1984) and an isozyme of acid phosphatase, which is modified in the prestalk-specific manner, has proven to be a useful marker (Oohata, 1983). Using this criterion, prestalk cell differentiation has been suggested to occur several hours later than prespore cell differentiation, the isozyme first becoming detectable at, or just after, the tipped aggregate stage of development (Oohata, 1983).

However, a quite opposite conclusion was drawn from the analysis of two monoclonal antibodies that react with epitopes expressed at a high relative level in prestalk cells (Tasaka et al. 1983) and from cDNA clones derived from mRNA sequences which are similarly enriched (Barklis & Lodish, 1983; Mehdy et al. 1983). These markers are first expressed during cellular aggregation, well in advance of prespore markers, with several of the mRNA sequences being expressed in vegetative cells (Mehdy et al. 1983;
Chisholm et al. 1984; Oyama & Blumberg, 1986a). However, these cDNA clones have now been shown to hybridize to mRNA sequences which are expressed in both prespore and prestalk cells (Jermyn et al. 1987). This fact, coupled with their expression prior to overt cell differentiation, strongly suggests that they are first expressed in precursors of both cell types and are then lost at a somewhat higher rate from prespore cells. This suggestion is supported by the observation that several monoclonal antibodies of this class stain a majority of cells during aggregation (Tasaka et al. 1983; Barclay & Smith, 1986).

The pDd63 and pDd56 mRNA sequences differ radically from previously described prestalk mRNA markers. Within the limits of currently available gradient purification techniques, they appear to be totally prestalk-specific (Jermyn et al. 1987). They are present in both prespore and stalk cells, giving confidence that their expression is diagnostic of the prespore-prespore cell lineage. They also differ from previously described markers in having a ‘slug-specific’ function as extracellular matrix proteins, presumably involved in maintaining the integrity of the slug (S. J. McRobbie, K. A. Jermyn, K. Duffy, K. Blight & J. G. Williams, unpublished manuscript), rather than in some aspect of earlier development, such as cellular aggregation. Finally, they are dependent for their expression upon, and rapidly induced by, DIF, a known stalk cell inducer. The two mRNA sequences are first detectable at the tipped aggregate stage of development (Williams et al. 1987; Jermyn et al. 1987), confirming the original observation using the acid phosphatase isozyme (Ohohata, 1983). Their expression at this time also helps to invalidate a potential criticism of the prestalk isozyme, for which there is some indirect evidence, that its appearance is a late event which is effectively uncoupled from primary prestalk cell differentiation (Weijer & Durston, 1985).

(9) Interactions between cAMP, DIF, ammonia and adenosine during normal development

These biological effectors have so far been considered in isolation and the properties ascribed to them largely derive from studies of individual cells or artificial aggregates. The speculative scheme presented in Fig. 6 suggests how they might interact to direct cellular differentiation during normal development. A central premise is that prespore differentiation precedes prestalk differentiation. It is also assumed that cells destined to become prestalk cells do not first express prespore markers and then transdifferentiate into the prestalk cell pathway. The critical steps in differentiation in this scheme are as follows.

(A) The induction of prespore cell differentiation

Both cAMP and ammonia appear to play a role in prespore cell differentiation and other factors such as CMF, SPIF or PIF may also be necessary. The evidence clearly supports the notion that prespore gene expression is induced by cAMP. However, cAMP also acts earlier during development to induce the expression of many non-cell-type-specific genes (reviewed by Williams et al. 1986; Gerisch, 1987). The cAMP responsiveness of the definitive prestalk genes pDd63 and pDd56 is not known but cAMP is known to be required for cells to become DIF-responsive (Sobolewski et al. 1983). Cyclic AMP cannot, therefore, be considered a truly cell-type-specific inducer in the way that DIF is specific for prestalk cell differentiation.

The situation with ammonia is in some ways the reverse of that with cAMP. There is no evidence for
an absolute requirement for ammonia for prespore cell differentiation nor is there evidence for the induction, from a low background, of prespore-specific genes. There is, however, both biochemical evidence for the stimulation of prespore cell differentiation and genetic evidence for the repression of prestalk cell differentiation. It may be, therefore, that ammonia is truly directive in stimulating prespore cell differentiation and suppressing prestalk cell differentiation, but further evidence using definitive prespore and prestalk markers is required to establish this unambiguously.

(B) Repression of prespore cell differentiation in a proportion of cells
This is presumably the result of the action of a specific repressor. Both adenosine and DIF are competent to suppress prespore cell differentiation in regulation experiments and it is difficult at present to decide whether one, both or neither of these factors fulfil this function during primary differentiation. Adenosine is an attractive candidate because of its likely higher concentration in the prestalk zone (Armant & Ruth erford, 1979; Tsang & Bradbury, 1981) and because enzymatic reduction of adenosine levels within the slug leads to prespore differentiation in the prestalk zone (Schaap & Wang, 1986). However, exposure to high adenosine levels does not lead to an increase in the total proportion of prestalk tissue in the slug (Schaap & Wang, 1986) while the analogous experiment using DIF results in a large reduction in the proportion of prespore staining cells (D. Traynor and R. Kay, personal communication). This difference need not necessarily indicate a more important role for DIF. It might simply be a consequence of differences in the spatial distribution, or mechanism of action, of exogenous adenosine and DIF. Given that adenosine and DIF act at different levels in the cAMP signal–transduction pathway (Schaap & Wang, 1986; Wang et al. 1986), one attractive idea is that they might function synergistically to repress prespore gene expression.

(C) Induction of prestalk cell differentiation in the subpopulation of cells that fail to express prespore markers
This supposition is based upon the relative times of appearance of definitive prespore and prestalk markers and it assumes that the non-prespore-staining cells, detected at the tight aggregate stage (Kreft et al. 1984), are the direct precursors of the cells that express prestalk markers at the tipped aggregate stage. Independent of the validity of these assumptions, the rapid transcriptional induction by DIF of prestalk genes, such as that encoding the ST430 protein, provides convincing evidence that DIF acts at, or around, the tipped aggregate stage to induce prestalk cell differentiation.

(D) Terminal differentiation at culmination triggered by a drop in ammonia concentration
The evidence that ammonia is antagonistic to DIF in preventing stalk cell differentiation has been discussed. There is also evidence for a similar antagonism between cAMP and DIF. After an initial period where cAMP is required (Sobolewski et al. 1983), it becomes totally inhibitory to stalk cell induction in isolated cells of the strain NC4 and reduces the rate of stalk cell induction by DIF in V12M2 cells (M. M. Berks and R. R. Kay, personal communication). The fact that, at this late stage in development, both ammonia and cAMP can both be shown to be antagonistic to DIF may indicate that they also function in this way during primary cellular differentiation. Again, however, definitive proof of this is as yet lacking and cAMP is known to be required for cells to become DIF-responsive. Possibly, therefore, cAMP fulfils different functions at different stages in development.

If the overall scheme is correct then Dictyostelium differentiation can be viewed as a cAMP driven differentiation down the spore cell pathway with ammonia acting to facilitate prespore cell differentiation. Cyclic AMP may also be necessary for prestalk cell differentiation but it later acts in conjunction with ammonia to repress terminal stalk cell differentiation. DIF, possibly acting in conjunction with adenosine, acts to divert a proportion of the cells into the stalk cell pathway. A central problem is now to determine how these molecules act to produce regionalized cellular differentiation.

In the differentiation of Dictyostelium, only two cell types are produced and their direct precursors are arranged in a linear array along the longitudinal axis of the slug. It has, therefore, proved an attractive system in which to formulate and test models for pattern formation. However, this apparent simplicity belies a potentially complex set of cellular interactions and the search for a relevant model awaits the resolution of a central question – where does primary cellular differentiation occur? There are two opposed viewpoints – that position determines fate or that fate determines position.

(10) The spatial distribution of prestalk and prespore cell precursors and of the morphogenetic signals that induce their differentiation
Agglomerates of cells in a uniform aqueous environment form differentiated structures containing both prespore and prestalk cells (Sternfeld & Bonner,
1977; Forman & Garrod, 1977b; Tasaka & Takeuchi, 1981). Thus cellular differentiation is not dependent upon the positional signals that normally orient the aggregate. As amoebae enter development they are heterogeneous in metabolic state, cell size and stage in the cell cycle. By varying the composition of the nutrient broth in which the cells are grown, or by purifying cells at different stages in the cell cycle, it is possible to generate populations of cells that selectively sort to different parts of the slug or that will develop to give a skewed prestalk-to-prespore, or stalk-to-spore, ratio (Leach et al. 1973; Tasaka & Takeuchi, 1981; McDonald & Durston, 1984; Weijer et al. 1984; Blaschke et al. 1986). It seems plausible to suggest, therefore, that cells entering development are weakly predetermined. This does not, however, preclude the possibility that cells sort to specific regions of the aggregate where they differentiate in response to a spatially localized signal.

The most direct evidence for a totally deterministic mechanism of cellular differentiation comes from experiments where isolated cells were observed by time-lapse cinematography (Gomer & Firtel, 1987). Depending upon the stage of the cell cycle at which development was initiated, cells were found to express either a prestalk or a prespore marker. However, the prestalk marker used is not a definitive marker because the prestalk cathepsin mRNA is present in both prestalk and prespore cells (Pears et al. 1985). The sporadic expression of beejin, the prespore marker, might simply reflect intrinsic variability in the ability of cells, at different stages in the cell cycle, to proceed to the prespore stage of development as isolated cells. Such differences may be of importance and may help explain why populations, enriched in particular stages of the cell cycle, display distorted stalk-to-spore ratios. However, regulation experiments show that cells are not irreversibly committed to their fate and imply the existence of diffusible signals acting to control the proportion of prestalk and prespore cells. There is also suggestive evidence to indicate that these signals may be localized and that they may play a pivotal role in primary cellular differentiation.

One indirect but strong argument for some form of morphogenetic field comes from a consideration of other slime mould species. In some species, the slug is composed almost entirely of prespore cells and, at culmination, cells at the extreme anterior transdifferentiate into stalk cells (O'Day, 1979; Schaap et al. 1985). In other species, a stalk is formed continuously during slug migration but again this is formed by the differentiation of prespore cells at the prespore–stalk boundary (Gregg & Davis, 1982; Schaap et al. 1985). A similar phenomenon occurs late during the culmination of Dictyostelium discoideum, where a proportion of prespore cells at the prespore–prestalk boundary differentiates into stalk cells (Schaap, 1983). In these cases, the evidence strongly appears to favour the existence of a localized signal acting to cause cellular differentiation. The only fundamental difference between Dictyostelium discoideum and these other species is the existence of a large prestalk zone containing cells fated to stalk-cell differentiation. It seems somewhat implausible, therefore, that Dictyostelium discoideum evolved a radically new mechanism of pattern formation wherein cell sorting plays an indispensable role. There is also direct evidence that, for prespore cells at least, differentiation in D. discoideum occurs in a localized fashion. Prespore vesicles, and the antigens recognized by prespore-specific polyclonal and monoclonal antibodies, are restricted to cells in the more basal part of the aggregate and never appear in the tip (Muller & Hohl, 1973; Takeuchi, 1981; Kreft et al. 1985). There is, as yet, no equivalent study using a definitive prestalk marker, such as the ST430 protein, but clearly this will be of importance in helping to resolve this fundamental issue.

The different models make different predictions of the distribution of potential morphogens within the aggregate and these might therefore be expected to be a simple way of distinguishing them. Unfortunately, while such measurements have been made for some of these molecules, their interpretation is not at all straightforward. The total cAMP concentration is known to be about 50% higher in the prestalk zone (Brenner, 1977). However, the effects of cAMP on postaggregative gene expression are known to be mediated through the specific cell-surface cAMP receptor (Schaap & Van Driel, 1985; Oyama & Blumberg, 1986c; Haribabu & Dottin, 1986; Gomer et al. 1986). The relevant parameter is, therefore, the perceived extracellular cAMP concentration and this measurement is not technically feasible at present. The activity of DIF has been established, by slug cutting, to be at least twofold higher in the prespore than the prestalk zone (Brookman et al. 1987). A similar caveat again applies, because total-cell-associated DIF levels were measured and not all of the DIF may be biologically active (Neave et al. 1985). Based upon the levels of enzymes that act to generate adenosine (Armant et al. 1980; Tsang & Bradbury, 1981), it appears likely to be present at a higher concentration in the prestalk zone but this has yet to be demonstrated. The distribution of ammonia is also unknown.

This whole issue is central to understanding Dictyostelium pattern formation but it is a very difficult problem to resolve (for a more extensive review of...
the continuing debate see MacWilliams & David, 1984). One approach is to formulate models, based upon one or other mechanism, and then to determine how closely the models fit observed behaviour.

(11) Models of pattern formation

(A) Models based upon position-dependent differentiation
Several models of this form have been comprehensively reviewed elsewhere and only one of these, which satisfactorily explains both primary differentiation and proportion regulation and is consistent with other features of pattern formation in *D. discoideum*, will be presented here (MacWilliams & Bonner, 1979).

(i) The activator–inhibitor model
This is a specific formulation of the general class of reaction–diffusion models (Gierer & Meinhardt, 1972), which allows for proportion regulation. Two interacting molecules are invoked. An activator of cellular differentiation, which autocatalytically stimulates both its own production and that of an inhibitor. Since the inhibitor acts to repress the autocatalytic stimulation of activator production, the system is self-regulating. In order to account for regionalized differentiation, the activator is assumed to have a very short effective diffusion range while the inhibitor is supposed to have an effective range encompassing the entire differentiating structure. Thus the activated zone is the primary site of production of both the activator and the inhibitor. However, it is only in the active region that the amount of activator exceeds a threshold limit allowing cellular differentiation. In the formulation of this model described by MacWilliams & Bonner (1979), the activated zone is assumed to be the prestalk region and DIF has been proposed to be the activator, and ammonia the inhibitor, in such a model (Gross *et al.* 1981). In order to account for the observed retention of polarity in separated prestalk and prespore zones MacWilliams & Bonner (1979) further suggest the existence of a variable ‘basal’ level of production of activator in all cells entering development. Cells with the highest level of production are then proposed to sort to the front of the slug. This formulation is, therefore, a hybrid model but, once established, the pattern of cellular differentiation depends solely upon the existence of stable, regionally localized morphogenetic fields.

(B) Models based upon position-independent differentiation
It is possible to conceive of primary differentiation, into prestalk and prespore cells, being entirely predetermined by some heterogeneity inherent in the cells as they enter development. However, it would then be necessary to postulate a totally distinct mechanism acting to regulate the proportion of prestalk to prespore cells in the slug and this is inherently unattractive. Three models, which simultaneously explain primary differentiation and cell-type regulation, have been proposed. They invoke the existence of homeostatic mechanisms to control the prestalk-to-prespore ratio coupled with cell sorting to generate pattern.

(i) Double-negative feedback acting on prestalk and prespore cells
In this model (Weijer & Durston, 1985; Fig. 7A), the regulation of cell type proportions is mediated by
diffusible molecules, produced by prestalk and prespore cells, which act to prevent their own formation from cells of the opposite type. There is assumed to be a random probability of conversion, with cell sorting acting to establish a spatial distribution of the two cell types during the aggregation stage. When the inhibitors start to take effect, an equilibrium is established, further interchange is prevented and cell sorting is no longer required to maintain the pattern. The model derives from the observed effects of cAMP and its breakdown products on the regulation of cell agglomerates (Weijer & Durston, 1985). Cyclic AMP is proposed to be the prespore-to-prestalk inhibitor and cAMP breakdown products, such as adenosine, the prestalk-to-prespore inhibitor.

(ii) Double-negative feedback acting on anterior-like cells
This conceptually similar model derives from an analysis of the ratios of prestalk, prespore and anterior-like cells in slugs formed by mixing mutant cells with altered prestalk-to-prespore ratios or by comparing wild-type cells grown in the presence or absence of glucose (MacWilliams et al. 1985; Blaschke et al. 1986; Fig. 7B). The model assumes that all conversions between prestalk and prespore cells occur via anterior-like cells and that negative feedback loops act to control these transitions. Again cAMP plays the role of inhibitor of prestalk cell formation, by preventing their formation from anterior-like cells, but DIF is proposed to act as the inhibitor of prespore cell formation. Adenosine, produced by anterior-like cells, is suggested to act as a homeostatic regulator of the formation of anterior-like cells from prestalk cells by antagonizing the inductive effect of cAMP.

(iii) The consumed substrate model
This is a reaction–diffusion scheme which does not involve an inhibitor but instead relies upon localized depletion of a specific substrate to create inhomogeneity. The substrate is consumed in the autocatalytic formation of an activator which directs cellular differentiation. In the specific model formulated for Dictyostelium, the activated region is assumed to be the presumptive prestalk cells creating around themselves a region of relatively high activator concentration, it becomes very difficult to distinguish this from a 'positional information' model. Distinction between position-dependent and position-independent differentiation will, therefore, require identification of the activator, determination of its initial site of formation and measurement of its diffusion range.

Perspectives
There now exist models for pattern formation in Dictyostelium that utilize known potential morphogens. Genes that respond to some of these agents have been isolated and these can be used to test some of the predictions of these models. They can also be used to delineate the sequence of intracellular events leading from receipt of a morphogenetic signal to activation or repression of specific gene expression. The biochemistry of cAMP signal generation and reception in Dictyostelium is very well understood (for recent reviews see Newell, 1986; McRobbie, 1986; Gerisch, 1987) and this provides a solid basis for determining how extracellular cAMP acts to regulate gene expression. A major aim now is to produce, for each candidate morphogen, an integrated description of its mode of action and role in pattern formation. It seems reasonable to expect that over the next few years Dictyostelium development will be understood at such a level.

I would like to thank Stuart McRobbie, Karen Duffy, Rob Kay, Julian Gross and Malcolm Maden for their constructive comments on this review.

References
Arment, D. R. & Rutherford, C. L. (1979). 5' AMP nucleotidase is localized in the area of cell–cell contact.


LOZANNE, B. D. & SPUDICH, J. A. (1987). Disruption of
Two feedback loops may regulate cell type proportions in
DICTYOSTELIUM. In Microbial Development (ed. R.
Harbor Press.

prestalk and prespore pattern in cellular slime moulds.
Differentiation 14, 1–22.

formation in DICTYOSTELIUM. In Microbial Development
Spring Harbor Press.

MANGIAROTTI, G., CECARELLI, A. & LODISH, H. F. (1983). Cyclic AMP stabilizes a class of
developmentally regulated DICTYOSTELIUM discoideum

disaggregated DICTYOSTELIUM discoideum cells. Dev.
Biol. 89, 82–91.


Microbiology 13, 335–375.

McRobbie, S. J., TILLY, R., BLEIGT, K., CECARELLI, A.


expression in DICTYOSTELIUM discoideum. Mol. cell. Biol. 5, 705–713.


MORRIS, H. R., MASENTO, M. S., TAYLOR, G. W.,
eclucidation of two Differentiation Inducing Factors
(DIF2 and DIF3) from DICTYOSTELIUM discoideum.
Biochemistry (in press).

MORRIS, H. R., TAYLOR, G. W., MASENTO, M. S.,


DICTYOSTELIUM discoideum: Temporal and spatial
distribution of prespore vacuoles. Differentiation 1,
267–276.

differentiation inducing factor and other lipids during the differentiation of *Dictyostelium discoideum*. *Biochem. cell. Biol.* **64**, 85–90.


(Accepted 23 February 1988)