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Cell patterning in *Dictyostelium*

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We summarize studies on stalk and spore cell formation in *D. discoideum* cell monolayers, aimed at revealing factors involved in controlling the prestalk:prespore pattern in this organism. We propose that there are no cell interactions dependent on cell contact *per se*. Formation of mature stalk cells from isolated amoebae incubated in a buffered salts medium requires only cyclic AMP and a lipid-like factor (DIF) released by cells developing at high density. In addition, a variety of sporogenous mutants can form spores rapidly and efficiently when incubated at low density in tissue culture dishes containing a similar cyclic AMP and salts medium. In some cases spore formation is improved by the addition of one or other of a variety of protective agents such as bovine serum albumin. Wild-type amoebae at low density form prespore cells under the same conditions.

We present some evidence that DIF is the activator of prestalk cell formation in a two-component patterning mechanism of the kind proposed by Wolpert *et al.* (*Symp. Soc. exp. Biol.* **25**, 391–415 (1971)) and Gierer & Meinhardt (*Kybernetik* **12**, 30–39 (1972)). We also provide data indicating that the role of inhibitor is played by ammonia, an idea first mooted by Sussman & Schindler (*Differentiation* **10**, 1–5 (1978)).

Introduction

A few hours after the onset of starvation, amoebae of the cellular slime mould *D. discoidium* aggregate by chemotaxis to cyclic AMP to form a number of hemispherical mounds of cells (Konijn *et al.* 1967; Loomis 1975). By about the twelfth hour of development (t_{12}) a papilla develops on the top of each aggregate and by extension of this papilla the aggregate is transformed into an elongated cylinder with a tapering anterior end, the tip. This standing slug, as it is called, may either construct a fruiting body on the spot, or it may collapse onto the solid stratum and migrate for some time before fruiting (Newell *et al.* 1969*b*). Each fruiting body consists of a mass of spore cells held aloft by a tapering cellulose-ensheathed stalk constructed of vacuolated stalk cells. The stalk cells are derived from the cells of the anterior third of the slug, while the spores derive from the posterior cells (Raper 1940). The two types of slug cell display a number of biochemical differences that anticipate their presumptive fates (Newell *et al.* 1969*a*; Alton & Brenner 1979; Tsang & Bradbury 1981). To date, the most useful means of distinguishing the cells has been the use of a fluorescent antibody that specifically stains prespore (and spore) cells (Takeuchi 1963). Formation of prespore cells, as defined by this and related techniques begins shortly before papilla formation (Hayashi & Takeuchi 1976; Forman & Garrod 1977; Müller & Hohl 1973). From the time of their earliest appearance prespore cells are absent from the anterior tip region, and the definitive prestalk–prespore pattern is fully developed by the standing slug stage (Takeuchi *et al.* 1978).

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Amputated anterior slug fragments produce 'stalky' fruiting bodies if induced to form fruits immediately (Raper 1940), while amputated posterior regions of older slugs form fruiting bodies with disproportionate numbers of spores (Sampson 1976). These results, and others (see Tsang & Bradbury 1981) indicate that anterior and posterior cells show some degree of commitment to their respective fates. On the other hand, the pattern is also capable of regulating since isolated anterior regions give rise to normally proportioned fruiting bodies if they are permitted to migrate for some time, and posterior fragments of young slugs form normal fruits even without migration (Raper 1940; Sampson 1976).

It is generally agreed that cell sorting plays some part in pattern formation. Cells from the anterior and posterior of slugs sort out if grafted to heterologous positions in a slug (Bonner 1952; Yamamoto 1977) or when dissociated into single cells and mixed together (Tasaka & Takeuchi 1979; Sternfeld & David 1981; Takeuchi 1969; Matsukama & Durston 1979). Furthermore there is ample evidence for differences between vegetative cell populations that markedly affect their likelihood of ending up as stalk cells or spores in aggregates formed from appropriate cell mixtures (Bonner *et al.* 1971; Takeuchi 1969; Leach *et al.* 1973). However, the fact that the fate of a cell population depends upon which other cells it is mixed with (Leach *et al.* 1973), as well as the phenomenon of regulation of slug fragments already discussed, indicates that sorting out of 'pre-determined' cells is not by itself sufficient to account for the pattern of cell differentiation (see MacWilliams & Bonner 1979). Thus although there exist a small number of prestalk-like cells within the prespore region of slugs, and these migrate forward during regeneration of rear fragments (Durston & Vork 1979; Sternfeld & David 1981), the proportion of prestalk cells has been shown to increase from about 10% to 25–30% (Sakai 1973). The conclusion that there is respecification of cell type, presumably in response to changes in the levels of extracellular signal molecules, is even clearer in the case of anterior fragments since no prespore cells are present initially in such fragments (Sakai 1973). There is, however, no rigorous evidence as to whether cells change their differentiated type at random locations within regulating fragments and then sort out appropriately, or whether respecification is restricted to the vicinity of the amputation, as would be expected on various morphogenetic gradient models (Wolpert *et al.* 1971; Gierer & Meinhardt 1972).

In vitro studies relating to pattern formation

We shall now turn to the results of *in vitro* studies relating to the mechanism of pattern formation in *Dictyostelium*. The term '*in vitro*' in this context refers to any of a wide variety of experimental conditions which have in common only the fact that normal morphogenesis is prevented. These conditions range from situations where cells are plated on a solid substratum at such low density that they are unable to aggregate, to situations where dense suspensions are agitated at very low shearing forces so that large agglomerates, almost equivalent to normal slugs, are formed.

The role of cyclic AMP

The evidence for a role of cyclic AMP in early gene expression is well known (see Newell 1978) and will not be considered here. We, and others, have presented evidence that cyclic AMP is required for the synthesis of gene products accumulating later in development (Town & Gross 1978; Takemoto *et al.* 1978; Landfear & Lodish 1980), and some authors have

suggested that cyclic AMP may be involved specifically in the stalk pathway and hence could play a direct role in the patterning mechanism (McMahon 1973; Pan *et al.* 1974; Sussman & Schindler 1978; Brown & Rutherford 1980). However, *in vitro* observations do not support this view since there is good evidence that cyclic AMP is required for the formation of prespore vacuoles, prespore-specific enzymes and, indeed, mature spores (Town *et al.* 1976; Kay *et al.* 1978; Feit *et al.* 1978; Kay 1979; Coukell & Chan 1980; Ishida 1981).

The role of cell contact

Although only one model has implicated cell contact directly in the mechanism of pattern formation (McMahon 1973), many workers have concluded that cell contact plays an essential role in developmental gene expression. That there is a requirement for continued integrity of aggregates is not surprising; the problem is to analyse the nature of the interactions occurring within such aggregates. It is reasonably clear that part, at least, of the failure of dissociated cells to continue the accumulation of a variety of developmental products can be overcome by exposing them to high levels of cyclic AMP (Town & Gross 1978; Takemoto *et al.* 1978; Landfear & Lodish 1980), and this result hints at the continued existence within normal aggregates of some kind of signalling mechanism involving cyclic AMP (see Durston & Vork 1979).

The existence of additional contact-dependent interactions would be indicated by the demonstration of developmental competences that are not restored to dissociated cells by the addition of cyclic AMP or other soluble components. In our studies of the differentiation of cells in monolayers on agar (Town *et al.* 1976; Town & Stanford 1979) we showed that amoebae incubated at low density in the presence of cyclic AMP will develop into stalk cells if provided with a small molecular mass lipid-like factor (DIF), which is liberated into the extracellular medium during normal development (see below). Since stalk cells contain complex developmentally regulated carbohydrates (Loomis 1975), and depend on the products of many developmental genes for their formation (Town *et al.* 1976) it is evident that substantial developmental gene expression can occur in the absence of cell contact. Unfortunately it is technically difficult to determine which particular gene products are formed under these low density conditions, but we are attempting to do so.

The situation regarding requirements for the spore pathway is more uncertain. Formation of prespore cells (cells containing prespore vacuoles) did not occur in cells incubated on agar at low density in the presence of cyclic AMP but did occur in the same cells at high density. Furthermore, it was found that the density requirement for prespore cell formation could not be overcome by DIF or any other factor capable of crossing a cellophane barrier (Kay *et al.* 1978). A comparable result was obtained with a set of sporogenous mutants. These are derivatives of the parent strain which, unlike the latter, are able to form mature spores, not just prespore cells, when incubated as dense monolayers with cyclic AMP (Town *et al.* 1976). As expected, none of these derivatives formed spores efficiently at low density even when separated from a dense layer of helper cells by a sheet of cellophane. We concluded from these results that expression of genes essential for the spore pathway of development required some kind of 'short-range' or 'cell-contact' interaction (Kay *et al.* 1978, 1979).

Subsequent experiments have led us to modify this view. Using cells incubated on the surface of bacteriological plastic petri dishes under a modified salts medium we were able to obtain spore differentiation at lower densities than previously. Time-lapse filming at such densities

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showed that amoebae can differentiate into spores without making contact with any other cells (Kay & Trevan 1981). Since spore formation did not occur at very low densities under these conditions it appeared that there was a requirement for some diffusible (and presumably large molecular mass) substances, and indeed conditioned medium was found to stimulate spore differentiation at very low density (Kay, unpublished). However, the finding that a variety of proteins, e.g. lysozyme and bovine serum albumin (BSA), or even low levels of the detergent Tween 80, could substitute for conditioned medium provoked the suspicion that the isolated cells were merely somewhat unhealthy, and that spore formation did not require a specific 'factor'. Indeed it has now been possible to obtain efficient and quite rapid spore differentiation

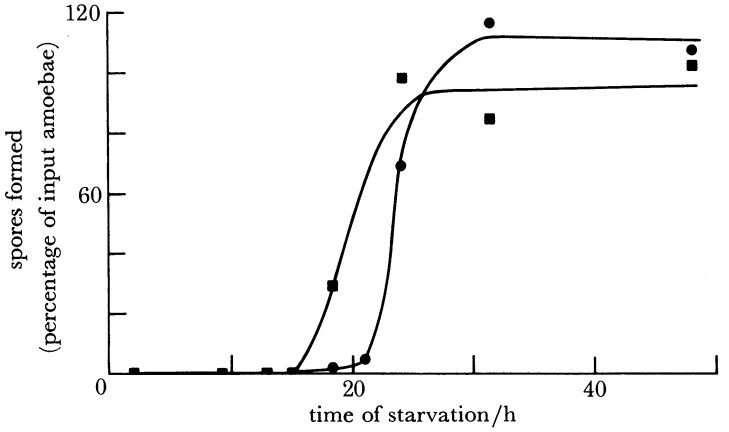

FIGURE 1. Amoebae can differentiate into spores at comparable rates at high cell density or when isolated at low density. Amoebae of the sporogenous strain HM29 were grown on SM agar in association with *Klebsiella aerogenes*. To initiate development they were freed of bacteria by repeated centrifugations, as described previously (Kay & Trevan 1981). Cells were plated at the stated densities in 5 cm diameter Sterilin tissue culture dishes containing 2 ml of a medium consisting of 10 mM 2-(*N*-morpholino)ethanesulphonic acid (MES), 20 mM NaCl, 20 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM cyclic AMP, 200 μg/ml streptomycin sulphate, 15 μg/ml tetracycline, 20 μg/ml bovine serum albumin, pH 6.2. After various periods of starvation the detergent Cemulsol was mixed into the medium to a final concentration of 0.3% to lyse the amoebae. The number of detergent-resistant spores per unit area of the plate was then determined by phase contrast microscopy. In the figure this is expressed as a percentage of the amoebae originally plated. In normal development of strain HM29 we have found that spores differentiate at a similar time to that found at high density *in vitro* (not shown). ■, High cell density (2.5×10^5/cm^2); ●, low cell density (2.5×10^2/cm^2).

in a variety of sporogenous derivatives of strain V12M2 at very low density (figure 1) simply by substituting tissue culture for bacteriological dishes, and adding Mg^{2+} and BSA to the salts medium. Even the mutant fruity-17 of strain NC4, for which a strong contact requirement has been claimed (Wilcox & Sussman 1978), gives some 20% spores when plated at low density in this medium (Kay, unpublished).

The results just discussed all refer to work with sporogenous mutants. It is possible that such mutants lack a requirement for a contact-dependent interaction in the spore pathway that is present in wild-type cells. Although we cannot exclude this possibility, we do not think that any such additional requirement of wild-type cells can be important in relation to pattern formation since the sporogenous mutant HM29, which shows no such requirement (figure 1), makes normally patterned slugs (Tsang & Bradbury 1981). Moreover, wild-type amoebae form prespore vacuoles with quite high efficiency at low density under our optimal conditions (Kay, unpublished). Although prespore cell formation is delayed compared with cells plated at high

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density, we are inclined to attribute this delay to residual unhealthiness of the low-density cells as well as to possibly poorer responsiveness of wild-type cells, compared with sporogenous derivatives, to sustained levels of cyclic AMP (Marin 1977).

Further, we have attempted to repeat experiments performed by other workers from which a contact requirement for developmental gene expression was inferred. In the two instances examined we can offer an alternative interpretation.

(*a*) Marin (1977) reported that cells of the axenic strain A3, when shaken rapidly in suspension, failed to differentiate to aggregation competence. Since in other experiments he had found that cyclic AMP did not accelerate development when the cells were on a solid surface in the same Ca^{2+}-containing medium, he concluded that some cell interaction other than cyclic AMP signalling was required for early developmental gene expression. Using the same medium and strain (kindly provided by Dr Marin and Dr Rothman) we have found that 1 mM cyclic AMP added to rapidly shaken suspensions at t_2 does in fact efficiently induce contact site formation as well as the appearance of other aggregative and post-aggregative products, but that addition at t_0 is much less effective (Peacey, Dhokia & Gross, unpublished). We believe therefore that the effect of rapid agitation is simply to interfere with cyclic AMP signalling, and that the failure of 1 mM cyclic AMP to correct this defect when added at t_0, as well as its failure to advance development under more nearly optimal conditions, is due to the well documented sensitivity of early cells of strain NC4 and its derivatives to high levels of cyclic AMP (Marin 1977; Marin & Rothman 1980; Rossier *et al.* 1978; Ishida 1981). Moreover, since Klein & Darmon (1976) have shown that A x 2 cells plated at $2 \times 10^3/cm^2$ develop aggregation competence in the presence of a supernatant factor subsequently identified as phosphodiesterase (Brachet *et al.* 1977) it seems very unlikely that any cell interactions other than those involving cyclic AMP relay and destruction are required for early development.

(*b*) Landfear & Lodish (1980) report that amoebae of *D. discoideum* strain A3 failed to make the characteristic set of post-aggregative polypeptides when they were shaken at fast speed for 16 h in buffer and then exposed to high levels of cyclic AMP. Cells permitted to develop normally on a solid surface for 13 h, then dissociated and treated as above did, however, make these polypeptides in response to cyclic AMP. The authors infer that prior cell contact is required for expression of late genes in dissociated cells. We have repeated these experiments using the enzymes glycogen phosphorylase and UDP-galactose polysaccharide transferase as indicators of post-aggregative gene expression, and find that cyclic AMP added at t_6 invariably induces extensive accumulation of the post-aggregative enzymes (see figure 2). However, if cyclic AMP addition is delayed until t_{16} it fails to induce the enzymes if the cells are shaking in phosphate buffer, and gives variable results in the MES-PDF used by Landfear & Lodish (Peacey, Dhokia & Gross, unpublished). The strong induction in MES-PDF at t_6 is particularly interesting since we have confirmed the observation of Landfear & Lodish that the amoebae remain throughout as single cells under these conditions. Moreover, they show no signs of accumulating contact sites A in this medium (figure 2). Thus rather than supporting the idea that cell contact is a prerequisite for later enzyme synthesis these results point quite strongly in the opposite direction!

Differentiation-inducing factor (*DIF*)

DIF was originally detected as a substance or substances that can pass across a dialysis membrane from a dense to a sparse V12M2 cell monolayer and permit cells of the latter to differentiate into stalk cells in the presence of cyclic AMP (Town *et al.* 1976). DIF activity can

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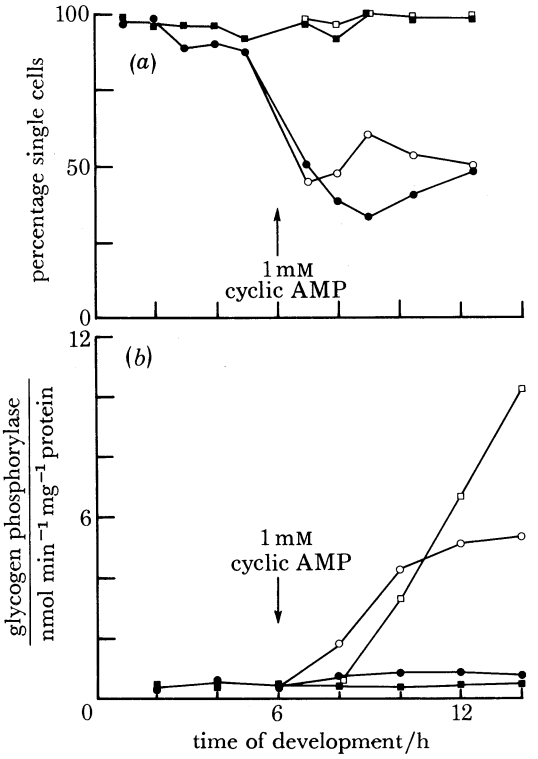

FIGURE 2. Induction of aggregative and post aggregative products by extracellular cyclic AMP, in fast shaken (250 rev/min) cells of strain A3, in MES-PDF (Landfear & Lodish 1980) and in KK_2 buffer (Sampson *et al.* 1978). Cells of strain A3 were grown axenically on an orbital shaker (180 rev/min) at 22 °C in axenic medium. The cells were harvested when the culture reached a density of approximately 2×10^6 cells/ml. Development was initiated by washing the amoebae free of nutrient and resuspending them in either MES-PDF or KK_2 buffer at 10^7 cells/ml. Samples for enzyme assay were collected by centrifugation and stored as cell pellets at −20 °C. Before assay, pellets were resuspended and lysed in 0.1 M Tricine buffer, pH 7.5, containing 20% glycerol and 0.15% Cemulsol. (*a*) EDTA-resistant contact formation was estimated as described by Sampson *et al.* (1978). (*b*) Glycogen phosphorylase, assayed as described by Town & Gross (1978). ■, MES-PDF; □, MES-PDF plus 1 mM cyclic AMP at t_6; ●, KK_2 buffer; ○, KK_2 buffer plus 1 mM cyclic AMP at t_6.

be found in dialysates of medium that has previously supported stalk cell differentiation (Town & Stanford 1979). It is readily extractable from this aqueous solution by petroleum ether, suggesting that it is a non-polar molecule. This view is supported by chromatography on silica gel where in different solvent systems it migrates with R_f's similar to those of neutral lipids (e.g. R_f in chloroform:methanol:water (14:6:1) is 0.76). The hydrophobic nature of DIF casts some doubt on the suggestion that it contains sialic acid and phosphate moieties, both of which would normally be expected to confer charge on it (Town & Stanford 1979). More recently, we have employed high-pressure liquid chromatography (h.p.l.c.) to purify the ether extracted material, with promising results: a single peak of DIF is reproducibly recovered (figure 3) (Kay, Jermyn & Coombs, unpublished). Although this material is still not pure, we have hopes that after a further h.p.l.c. step it will be. In any case this result suggests that DIF is a single molecular species (or a number of closely related species).

What is the role of DIF? The short answer is that we do not know. However, we are attracted by the idea that it functions as the activator of prestalk cell formation in a patterning mechanism of the kind proposed by Wolpert *et al.* (1971) and Gierer & Meinhardt (1972). If this were so,

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we would expect DIF to accumulate at the time when the pattern is established. This appears to be the case. Figure 4 presents the time course of accumulation of DIF and of two well characterized enzymes during normal development of cells of strain V12M2 on agar. Under these conditions cells complete aggregation at t_9 and tip formation occurs between t_9 and t_{12}. There is a brief period of slug migration around t_{18}, and fruiting body formation is complete by t_{24}. It can be seen that cell-associated DIF accumulates between t_9 and t_{12} with very much the same kinetics as does the prespore-specific enzyme UDP-galactose polysaccharide trans-

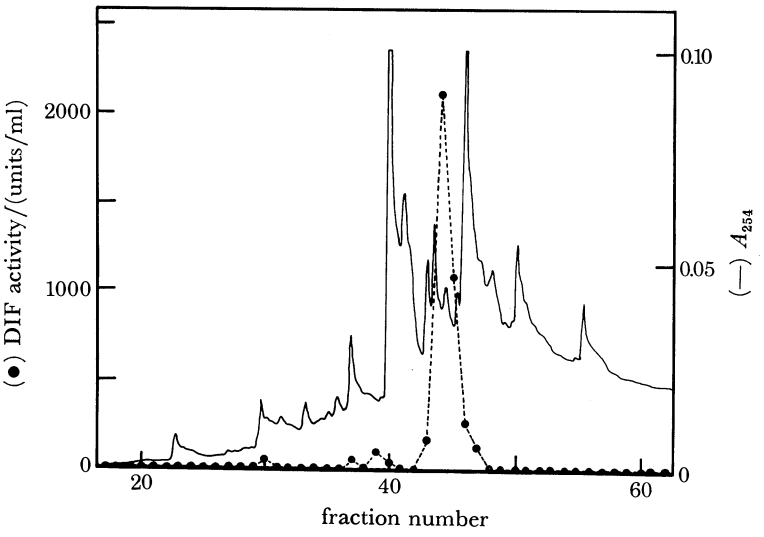

FIGURE 3. Purification of DIF by high-pressure liquid chromatography. DIF was obtained by dialysis from the medium in which cyclic AMP had induced the differentiation of a dense layer of amoebae into stalk cells (Town & Stanford 1979). This medium was concentrated 50–100-fold by rotary evaporation and the DIF extracted by shaking with petroleum ether. It was then dried down, redissolved in ethanol and loaded onto a C_{18} ODS reverse-phase column. The column was eluted with a gradient of 15–100 % ethanol in water and fractions collected. DIF activity in these fractions was assayed as follows: up to 10 µl of sample was added to 2 ml of 10 mM MES, 2 mM NaCl, 10 mM KCl, 1 mM $CaCl_2$, 1 mM cyclic AMP, 5 µg/ml BHT (2,6-di-*t*-butyl-4-methylphenol), 200 µg/ml streptomycin sulphate, 15 µg/ml tetracycline, pH 6.2, in a 5 cm diameter Sterilin tissue culture dish. Washed vegetative cells of strain V12M2 (Town & Stanford 1979) were included at a density of 750/cm² and allowed to differentiate at 22 °C. After 2 or 3 days, amoebae and stalk cells were scored by phase contrast microscopy. In the absence of added DIF on average fewer than 1 % of the cells were stalk cells; a unit of DIF is defined as giving a 1 % increase in stalk cells above this background level.

ferase, and thereafter remains approximately constant in level during slug migration and fruiting (Brookman, unpublished). In other experiments it has been found that DIF accumulates in the extracellular medium with similar kinetics (Town & Stanford, unpublished).

The idea that DIF is the activator of prestalk cell formation has several additional consequences. First, it predicts that DIF levels should be higher in the anterior, prestalk, zone of slugs than in the posterior, prespore, zone. Secondly, DIF should be required for the formation of prestalk cells, not just for the production of mature stalk cells, which is all we have shown directly as yet. Prestalk cell markers, which should permit us to test this expectation, are now available (Alton & Brenner 1979; West & McMahon 1979; Morrissey *et al.* 1981). Thirdly, DIF should not be required for the spore pathway, and should actually manifest itself as an 'inhibitor' of this pathway. We have already described conditions in which isolated wild type and sporogenous amoebae will form prespore vacuoles and mature spores respectively (see

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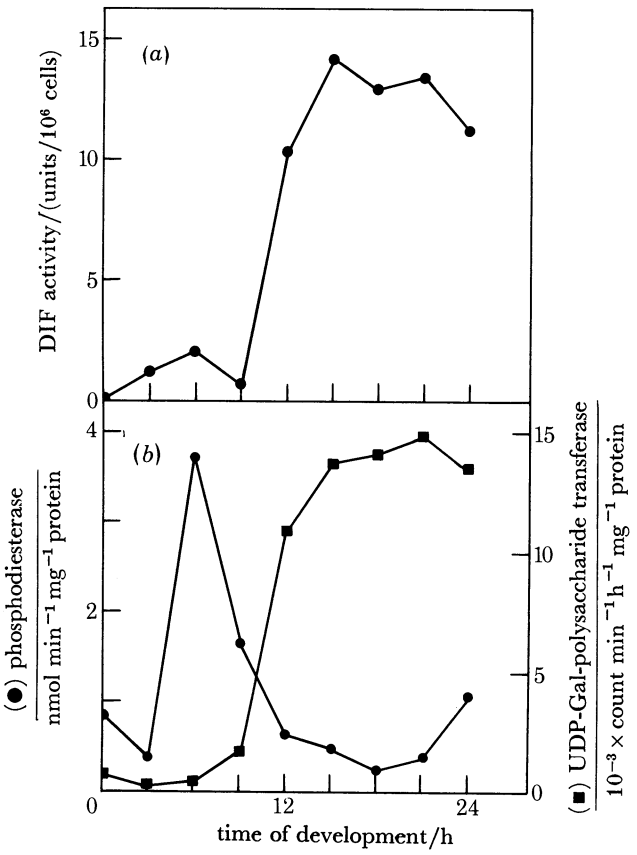

FIGURE 4. (*a*) Regulation of cellular levels of DIF activity during development. Cells of strain V12M2 were grown and harvested as described by Kay & Trevan (1981). For development, cells were spread at a density of $3 \times 10^6/cm^2$ on 80 ml of 1.5% agar (Oxoid L28) containing 100% Bonners salts (10 mM NaCl, 10 mM KCl, 2 mM $CaCl_2$) with 5 mM MES, pH 6.2, in 14 cm Sterilin petri plates and placed in a humid atmosphere at 22 °C. Approximately 1.3×10^9 cells were harvested at each time point and frozen at −20 °C.

Extraction of DIF from cells. DIF was extracted from thawed cells by the lipid extraction procedure of Bligh & Dyer (1959). BHT (5 μg/ml) was routinely added to the solvents during extraction. The chloroform layer containing the DIF was concentrated by rotary evaporation to a small volume and the DIF taken up in ethanol. BHT was then added to make a final concentration of 1 mg/ml and the extracts stored at −20 °C.

Assay for DIF activity. Volumes ranging from 0.2 to 5 μl of the extracts from each developmental time point were assayed for the ability to induce stalk cell differentiation in isolated cells as described in the legend to figure 3.

(*b*) Enzyme activities were determined on cell pellets (about 10^7 cells) stored frozen at −20 °C. Before assay the pellets were thawed and lysed as described by Kay (1979). Cyclic AMP phosphodiesterase was assayed as described by Henderson (1975). UDP-galactose polysaccharide transferase was measured as described by Kay (1979). Protein was determined by the method of Lowry *et al.* (1951).

legend to figure 1). DIF is not required. In fact we have found recently that the addition of small amounts of DIF-containing extract inhibits both prespore and spore cell formation under these conditions (Kay & Jermyn, unpublished). We have not yet tested purified DIF preparations. This result provides tentative support for the idea that DIF is involved in cell patterning rather than just maturation of stalk cells.

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Towards a unitary model of pattern formation in *Dictyostelium*

When cell aggregates exceed a certain critical size, two or more roughly equally spaced tips are formed, each one generating a separate finger, and ultimately a distinct fruiting body (Hohl & Raper 1964). It seems attractive to consider each tip as equivalent to a prestalk zone and to view the subdivision of larger aggregates into separate territories as merely a slightly different expression of the same equilibrium between activator and inhibitor that is responsible for patterning each territory into prestalk and prespore regions. If this unitary view is correct, factors affecting the stalk:spore ratio should also influence critical aggregate size. This appears to be true for the 'petite' mutant P4 (Hohl & Raper 1964; Forman & Garrod 1977; Kopachik 1980) as well as for another 'stalky' mutant, Brown E9, isolated by Dr H. MacWilliams (Bradbury, unpublished). Temperature changes also seem to affect the two processes co-ordinately. Thus lowering the temperature has the effect of lowering critical mass (Peacock & Soll 1978) and it also results in stalkier slugs and fruits (Bonner & Slifkin 1949; Forman & Garrod 1977). We note also that Durston (1976) could account for the results of his experiments on inhibition of tip formation by means of precisely the same kinds of two-variable models that are used to generate the prestalk:prespore pattern.

We have tentatively ascribed to DIF the role of activator. What about the inhibitor? If it exists, one would expect it to be present in the DIF preparations that we routinely make by collecting the medium under a layer of amoebae developing on a dialysis membrane. Because DIF is hydrophobic it can readily be removed from such preparations by adsorption to XAD-2 columns. We have sought to detect the inhibitor by adding aliquots of such DIF-free preparations to amoebae incubated in our standard stalk cell induction medium at pH 6.2, but no inhibitory effect could be detected (Gross, unpublished). This outcome prompted us to reconsider the suggestion concerning the role of ammonia put forward by Sussman & Schindler (1978), since at pH 6.2 very little of the ammonia present in our preparations would be free, most of it being present as NH_4^+.

Schindler & Sussman (1977) have demonstrated that the decision to form migratory slugs is dependent on high levels of free NH_3 and they proposed that ammonia also plays a role in cell patterning. Specifically they suggested that in the presence of high cyclic AMP concentrations cells accumulate stalk-specific materials, that low cyclic AMP concentrations favour spore formation, and that NH_3 interferes with cyclic AMP synthesis and hence favours spore formation. As mentioned earlier, present evidence does not support the idea that cyclic AMP concentrations control the pathway of differentiation. However, this does not eliminate the possibility that ammonia acts in some other way so as to favour the spore pathway. Kay (1979) did not detect a differential effect of ammonia, but for technical reasons the two pathways were not studied under strictly comparable conditions. For this reason, and also because our *in vitro* cell differentiation conditions have been improved, it seemed worthwhile to re-examine the question. We now consistently find strong inhibition of stalk cell formation at levels of NH_4Cl between 2.5 and 5 mM in Tris buffer, pH 7.5, much lower levels than previously found necessary (Kay 1979) and these NH_4Cl concentrations have little effect on the accumulation of prespore cells. Moreover, in preliminary experiments with sporogenous mutants (Gross & Peacey, unpublished) we have found that ammonia dramatically shifts the stalk:spore ratio in favour of spores (table 1). The effects of NH_4Cl are dependent on elevated pH in mutant HM18, though not so much in HM29, and are not mimicked by KCl in either strain. In addition, higher pH

TABLE 1. THE EFFECT OF AMMONIA ON STALK AND SPORE CELL FORMATION IN SPOROGENOUS STRAINS

sporogenous strain	pH	NH_4Cl mM	KCl mM	percentage spores	percentage stalk cells	percentage amoebae
HM18	7.5	0	—	21	62	17
		1	—	24	45	31
		2	—	42	31	27
		3	—	61	17	22
		4	—	56	12	32
		5	—	53	5	42
	7.5	—	0	27	56	17
		—	5	22	59	19
	6.2	0	—	12	75	13
		5	—	13	70	17
HM29	7.5	0	—	27	34	39
		1	—	24	26	50
		2	—	25	16	59
		3	—	37	4	59
		4	—	40	2	58
	7.5	—	0	22	34	44
		—	5	23	37	40
	6.2	0	—	13	76	11
		5	—	37	42	21

Washed amoebae were incubated for 3 days on Nunc tissue culture dishes containing 5 mM cyclic AMP, 1 mM $CaCl_2$, 2 mM NaCl, 10 mM KCl, 200 μg/ml streptomycin sulphate, 10 mM Tris, pH 7.5, or 10 mM MES, pH 6.2, with the additions as given in the table. Over 200 cells were scored in each case. Cell density was $10^5/cm^2$.

itself appears to reduce stalk formation and increase the yield of spores. It is interesting to note that the inhibitory effect of ammonia on DIF-dependent entry into the stalk pathway may account for the otherwise rather mysterious 'requirement' for NH_4Cl for spore formation reported by Sternfeld & David (1979) and Wilcox & Sussman (1978). Clarification of the details of the interactions between DIF and ammonia, and of how synthesis of these factors is in turn related to cyclic AMP levels, awaits further study.

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