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Review

Regulation of cell differentiation and pattern formation in *Dictyostelium* development

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ABSTRACT Free-living cells of *Dictyostelium discoideum* aggregate to form a slug-shaped cell mass and differentiate into prestalk and prespore cells. The differentiation of prespore cells is characterized by expression of *Dp87* gene, the earliest event of prespore differentiation. It encodes a protein which first appears in ER of aggregating cells in a precursor form, is then translocated to prespore vacuoles and modified to a mature form and finally exocytosed to constitute the sorus matrix. The transcription of *Dp87* is regulated by the *cis*-acting region consisting of positive, prespore-specific, negative, non-prespore-specific and positive, cell-type-non-specific elements. Cells expressing *Dp87* appear at random in early aggregation streams and centers and then sort out to the posterior part of the slug. Intercellular signals required for prestalk and prespore differentiation were investigated by incubation at a low cell density of disaggregated cells. cAMP is inhibitory at the first and second stages of prespore differentiation, while it is required at the third stage. The stalk differentiation is divided into four stages: cAMP is required at the second stage and differentiation inducing factor (DIF) at the third stage, where a low molecular weight secretory substance is also required. At the third stage, cAMP inhibits both *ecmA* and *ecmB* expression, while 8-Br-cAMP specifically induces *ecmB* and maturation of prestalk to stalk cells. The relationship between the differentiation tendency of preaggregative cells and the cell-cycle phase at the initiation of development was studied by the use of cells synchronized for growth by a temperature-shift method. Cells starved at the mid-late G2 phase develop rapidly and initiate aggregation, while those starved just before mitosis aggregate more slowly. When aggregation centers form tips, however, the latter cells sort out to the tip portion and finally to the anterior prestalk region of a slug, while the former cells lag behind to occupy the posterior prespore region.

KEY WORDS: *cell differentiation, pattern formation, gene expression, cell cycle, Dictyostelium*

Introduction

Free-living cells of the cellular slime mold, *Dictyostelium discoideum* grow and multiply during the vegetative growth period. When the cells are depleted from the nutrient, they initiate development and subsequently aggregate at common collecting points through chemotactic movement towards cAMP signal produced by neighboring cells (Devreotes, 1982). A tissue formed at the center of aggregation containing as many as 100,000 cells first assumes the shape of a mound, but soon elongates and transforms into a slug shape. The slug eventually culminates and forms a fruiting body consisting of a cellular stalk and a spore head.

Within the slug, differentiation between the anterior and posterior parts has long been recognized: the anterior cells are predetermined to become the stalk cells of a fruiting body at their final state of differentiation, while the posterior cells will become the spore cells. Moreover, the proportion of the anterior prestalk cells to the posterior prespore cells remains constant irrespective of the size of a slug. Predetermination of the prestalk and prespore cells, however, is not fixed: when the prestalk or the prespore fragment is isolated from a slug, conversion of cell-type between prestalk and prespore cells occurs in each isolate without cell division, so that isolates containing normally proportioned prestalk and prespore cells are obtained within a matter of several hours.

These features of development make *Dictyostelium* an ideal organism to study the regulatory mechanism of cell differentiation and pattern formation (Takeuchi, 1991). In the present paper, we shall review our recent studies on (1) temporal and regional expression of a prespore-specific gene with its implication in pattern formation, (2) cell-cell interactions in regulation of prespore

Abbreviations used in this paper: 8-bromo cAMP, 8-bromoadenosine-3',5'-cyclic monophosphate; cAMP, adenosine-3',5'-cyclic monophosphate; CAT, chloramphenicol acety l transferase; ER, endoplasmic reticulum; X-gal, 5-bromo-4-chloro-3-indolyl-ß-D-galactoside.

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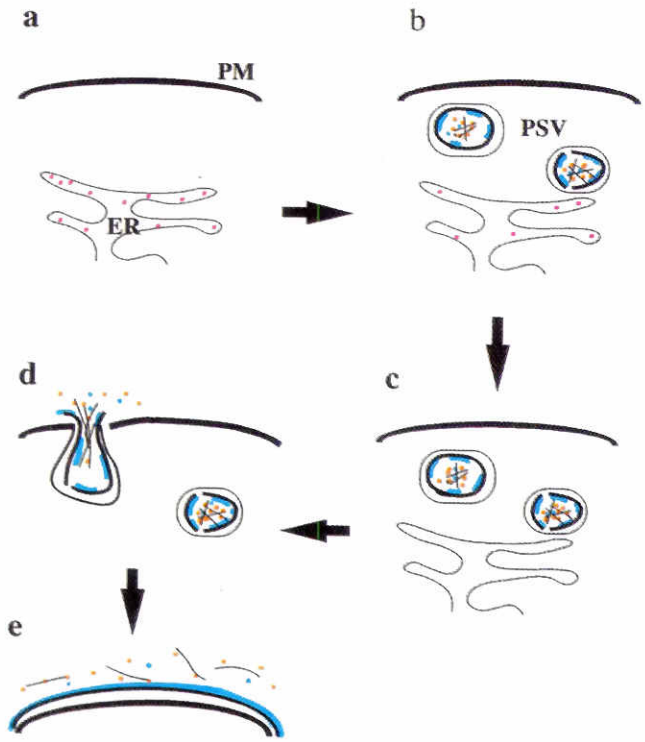

Fig. 1. Intracellular localization of the sorus matrix and the spore coat proteins in developing *D. discoideum* cells. (a) *The 81 kDa protein (pink) of Dp87 gene product (the sorus matrix protein) is synthesized in the ER of prespore I cells at the early aggregation stage. PM, plasma membrane.* **(b)** *The 83 kDa protein (orange) of Dp87 is concentrated in the fibrous matrix of PSVs of prespore II cells at the tipped aggregate stage. Spore coat proteins (blue) are located in both fibrous matrix and surrounding membranous structure. The 81 kDa protein is still present in the ER.* **(c)** *The 81 kDa protein is no longer detectable at the migrating slug stage.* **(d)** *At the culmination stage, PSVs are fused with the plasma membrane and the spore coat protein mainly covers the surface of spores, while the 83 kDa protein is exocytosed.* **(e)** *The spore coat proteins cover mature spores, while the 83 kDa protein is located in the intercellular space and constitutes the sorus matrix.*

and prestalk differentiation, and (3) dependence of pattern formation on the cell-cycle phase variation.

Regulation of expression of a prespore-specific gene

The differentiation of prespore and prestalk cells is characterized by expression of cell-type-specific genes. Prespore-specific cDNAs and genes have been isolated by differential screening or immunological methods. For example, *D19* which encodes a membrane-associated protein named PsA was isolated by Early *et al.* (1988) and those of spore coat proteins, *SP96, SP70* and *SP60* were isolated by Dowds and Loomis (1984) and Tasaka *et al.* (1990) Recently, we have isolated a different type of prespore-specific gene named *Dp87* (Ozaki *et al.*, 1988). The expression pattern of this gene analyzed by Northern blot hybridization showed that the mRNA first appeared when cells formed early loose mounds, at a stage earlier than any other prespore-specific genes were expressed (Ozaki *et al.*, 1993). The mRNA ceased to be synthesized at the late culmination stage. This suggests that the mechanism of regulation of *Dp87* gene expression is different from the others.

Characteristics of Dp87 gene product

Dp87 mRNA contains one long open reading frame of 555 amino acids (Ozaki *et al.*, 1993). The peptide contains a leader sequence for secretion at the N-terminal and some N-glycosylation sites. It showed a low but significant homology to the spore coat proteins of *SP96* and *SP70*, but not to that of *SP60* (Fosnough and Loomis, 1989, 1991; Haberstroh and Firtel, 1990; Tasaka *et al.*, 1990). A polyclonal antibody was obtained against bacterially synthesized *Dp87* peptide, and the gene product was analyzed by the use of this antibody and those produced against spore coat proteins (Nakao *et al.*, 1994). The results are summarized in Fig. 1, which schematically illustrates intracellular localization of *Dp87* gene product and spore coat proteins.

By Western blotting with *Dp87* antibody, 81 kDa protein was first identified at the loose mound stage. Immunocytological and immunoelectronmicroscopical studies with the antibody showed that the protein was bound to ER (Fig. 1a). When the cell mound formed a tip, 83 kDa protein was detectable in addition to 81 kDa by Western blotting, and the positive immunostains were found not only in ER but also in prespore vacuoles (PSVs), which began to form at this stage of development (Fig. 1b). Within PSVs, the peptide identified by *Dp87* antibody is localized only in the inner fibrous matrix. In contrast, the spore coat proteins are located in both the inner fibrous matrix and surrounding membranous material of PSVs, but not in ER. At the migrating slug stage, 81 kDa peptide disappears gradually from ER, while 83 kDa peptide in PSVs is increased in amount (Fig. 1c). This suggests that *Dp87* gene product is first modified to 81 kDa within ER soon after it is translated, then translocated into PSVs when they are formed and instantly modified again to become 83 kDa. This suggests that there are two steps in prespore differentiation distinguishable by the process of modification of prespore-specific *Dp87* gene product: the product is localized in ER in step 1 (prespore I) cells and translocated to PSVs in step 2 (prespore II) cells.

At the culminating stage, the mRNA of *Dp87* disappears, but 83 kDa protein remains in PSVs. At the early stage of spore formation, 83 kDa protein is exocytosed out of prespore cells into the interspore space, with some attached to the surface of stalk tube and spore coat (Fig. 1d). In mature fruiting bodies, the protein is mainly deposited in the sorus matrix, with some attached to the stalk tube (Fig. 1e).

The function of *Dp87* protein is not clear, but its distribution pattern during development suggests at least two possibilities: it may be necessary for formation of PSV or for preservation of spores in mature fruiting bodies. It is probable that 81 kDa protein is a precursor component of PSVs, which after modification to 83 kDa constitutes the core of PSVs to assemble other spore coat proteins around it. It is also possible that the sorus matrix protein plays some roles for spore maturation or spore germination. It was shown that *Dp87* gene-disruptants were normal in development and formation of intact spores (Nakao *et al.*, 1994), but this is probably due to the fact that some other components might compensate for the *Dp87* gene product.

Regulation of expression of Dp87 gene

It has been shown by Southern blot hybridization that *Dp87* gene is unique (Ozaki *et al.*, 1993). The genomic fragments containing it have been isolated and sequenced. The gene in-

cludes one short intron near the end of the leader sequence at the N-terminal of *Dp87* peptide. The upstream region is extremely enriched with AT that is common among genes in *D. discoideum*. It has been shown by run-on assays that this gene is mainly regulated at the transcriptional level, like those of spore coat proteins such as *SP96* (Morio *et al.*, 1991).

To identify the regulatory mechanism of transcription of this gene, a part of the upstream region was conjugated with a reporter gene of CAT or ß-galactosidase (ß-gal) and the expression of the reporter genes was analyzed by the enzyme activity or staining with X-gal (Ozaki *et al.*, 1993; Morio *et al.*, 1994). The results are summarized in Fig. 2. It was found that the upstream region between -666 and +9 was sufficient for the transcription of this gene. This region can be separated into 4 parts depending on their functions. The first part consists of sequences between -666 to -158 and is related to prespore-specific transcription. This region is subdivided into at least 4 sub-regions, A (-666 to -432), B (-431 to -350), C (-349 to -232) and D (-231 to -158). Deletion experiments of these sub-regions showed that deletion of any single region did not affect prespore-specificity of the transcription, but that simultaneous deletion of regions C and D abolished prespore-specific transcription. This suggests that the complete set of 4 regions is not required for prespore-specific transcription, but that either region C or D is necessary.

Regions A, B and D respectively contain one or two of the CA-Box (ACACCCA/T) which is found in the *cis* regions of genes for spore coat proteins, *SP96* and *SP70* and in the cAMP responsible elements of *SP60* and *UDPGP1* genes (Pavlovic *et al.*, 1989; Haberstroh and Firtel, 1990; Fosnough and Loomis, 1991, 1993; Haberstroh *et al.*, 1991; Tasaka, 1991; Tasaka *et al.*, 1992). Region C contains the CAE-like sequence (CACACA) which was identified as the cAMP responsible element of *SP60* gene (Haberstroh *et al.*, 1991). Though there are four CA boxes in regions A, B, and D and a CAE-like box in region C, the deletion of any one or two of these boxes did not affect the normal staining pattern of transformant slugs with X-gal, the results being inconsistent with those of *SP60* (Haberstroh *et al.*, 1991).

The gel retardation analyses showed that nuclear proteins isolated from slug cells, but not from vegetative cells, made a sequence-specific binding with DNA of region B. Competition assays revealed that regions A and D and the *cis*-region of *SP96* gene formed similar complexes (Tasaka, 1991; Ozaki *et al.*, 1993). When region B was separated into two parts, each containing one CA-box, both fragments competed for binding. This suggests that multiple CA-boxes may bind with the same nuclear protein and that the binding between the CA-box and the protein must be important for positive regulation of prespore-specific transcription.

The second part of the regulatory region is region E (-157 to -94), a negatively-acting element. It suppresses the transcription of the gene at the vegetative growth phase and in prestalk cells. The third part is region F (-93 to -63). It is a general positive regulatory region in both vegetative and slug cells and increases the rate of transcription. The last part is region G (-63 to +9) which contains the putative TATA box and the start site of transcription. Region E failed to confer negative regulation to a heterogeneous promoter, suggesting that its proper function may require the presence of region G. These three parts, especially the existence of a negative regulatory region (E), are unique as compared to the other prespore-specific genes. This may be related to the fact that transcription of this gene precedes that of the other prespore-specific genes.

The transcription of *Dp87* gene was inhibited by exogenous

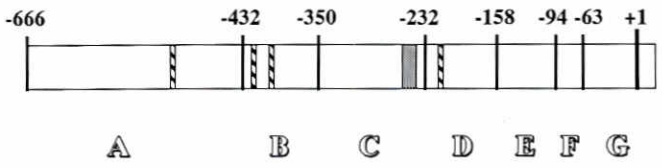

Fig. 2. The *cis*-acting elements of *Dp87* gene. *Regions A, B, C and D are prespore-specific, positive regulatory regions. Either region C or D is essential for prespore-specific transcription. Regions E and F are the negative and the positive regulatory regions, respectively. Region G is the basic promoter region containing putative TATA box. +1 is the start site of transcription and the numbers indicate the positions from the transcription initiation site. Hatched box, CA-box; Dotted box, CAE-box.*

differentiation inducing factor (DIF) which induces prestalk cell differentiation (Morris *et al.*, 1987), as is the case with the other prespore-specific genes (Early and Williams, 1988; Ozaki *et al.*, 1993). By the use of disaggregated slug cells, it was shown that the transcription was stimulated by exogenous cAMP (Takemoto *et al.*, 1990). It is suggested that the cAMP signal is mediated by a change in intracellular Ca^{2+} concentration, because Ca^{2+} ionophore A23187 also induced the transcription in disaggregated slug cells without the addition of cAMP (data not shown). Recently, we showed that the addition of cAMP brought about an increase in intracellular free Ca^{2+} concentration in prespore cells, though much less than in prestalk cells (Saran *et al.*, 1994).

Prespore cell differentiation and pattern formation

After slug formation, differentiated prestalk and prespore cells occupy the anterior one fourth and the posterior three fourths of a slug, respectively. Two contrasting models have been proposed to explain how such a pattern of differentiation of the two cell types is achieved in a migrating slug. The first model claims that cells differentiate after formation of a slug according to their positions along the antero-posterior axis of the slug. The second model proposes that cells differentiate randomly into either prestalk cells or prespore cells before slug formation and that prestalk cells sort out to the front while prespore cells sort out to the rear during slug formation.

To identify the process of prespore differentiation during development, the upstream region (-666bp to +149bp) of *Dp87* gene was jointed to the reporter gene of ß-gal and the chimeric gene was introduced into *D. discoideum* cells (Ozaki *et al.*, 1993). Cloned transformants were grown independently, allowed to develop on filter papers, fixed at different developmental stages and stained histochemically with X-gal. The results obtained from different clones were basically the same. The process of prespore differentiation as revealed by *Dp87* gene expression is summarized schematically in Fig. 3.

Histochemically stained cells were first detected in an early aggregation field. Initially, the number of stained cells was low and most of them were dispersed in aggregation streams which radiate from a premature aggregation center containing more cells than other places, but some stained cells were found outside the streams (Fig. 3a). Then, stained cells increased in number dramatically, as aggregation proceeded, but they were randomly dispersed in loose aggregates (Fig. 3b,c). When a tip appeared on an aggregation center, stained cells were mostly observed in the middle of the aggregate, unstained cells occupying the tip and the basal part (Fig. 3d). The distribution pattern of stained and unstained

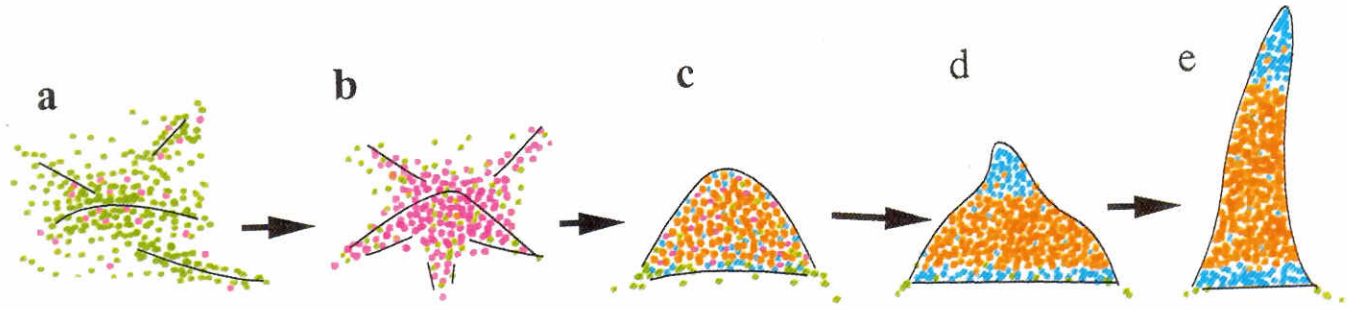

Fig. 3. A schematic representation showing the distribution pattern of differentiating prespore cells in aggregation streams and centers, as revealed by prespore-specific *Dp87* gene expression. *Green, pink, orange and blue cells represent undifferentiated, prespore I, prespore II and prestalk cells, respectively.*

cells was basically maintained at the standing (Fig. 3e) and migrating slug stages.

These observations appear interesting on two counts: the timing and location of prespore differentiation. The appearance of stained cells at the early aggregation stage indicates that *Dp87* gene transcription is the earliest event of prespore differentiation so far examined. It has been shown that the expression of prespore-specific spore coat genes and of prestalk-specific *ecmA* and *ecmB* genes occurs in loose cell mounds (Williams *et al.*, 1989; Harberstroh and Firtel, 1990), while *Dp87* gene expression was detected in cells in aggregation streams or even in isolated cells. This can be explained by the aforementioned fact that there are two steps in prespore differentiation. In prespore I cells, *Dp87* gene is transcribed and the product (81 kDa protein) is stored in ER, while in prespore II cells, not only *Dp87* gene but the other prespore-specific genes are transcribed and the products (83 kDa and spore coat proteins) are stored in PSVs.

The evidence that cells expressing *Dp87* appear randomly in the aggregation field indicates that prespore differentiation occurs independently of the positions in a cell mass and excludes the possibility that positional information plays any role in prespore cell differentiation. This supports the second model mentioned above and is consistent with the previous findings (Tasaka and Takeuchi, 1981; Takeuchi *et al.*, 1988; Williams *et al.*, 1989; Takeuchi, 1991). However, why only certain cells in a field differentiate into prespore cells, while leaving the other cells undifferentiated, remains to be solved. Some heterogeneity produced among growing cells, for example in the cell-cycle phase, or nutritious conditions might be involved. This will be in part discussed in the last chapter.

The fact that prespore I cells expressing *Dp87* gene were dispersed randomly in the aggregation stream indicates that there occurs no cell sorting between prespore I cells and undifferentiated cells during aggregation. In contrast, prespore II cells and prestalk cells which appear in late aggregation centers sort out each other to construct prestalk-prespore patterns in slugs. This is consistent with the previous finding that glucose-rich and glucose-poor cells aggregate together without cell sorting, but sort out each other during slug formation (Tasaka and Takeuchi, 1981).

Cell-cell interactions in prespore and prestalk/stalk differentiation

It has been shown that isolated, single cells of *D. discoideum* are unable to undergo differentiation and that single cells isolated from migrating slugs lose the differentiated characteristics once acquired in a short time (Takeuchi and Sakai, 1971). This implies that intercellular signals are essential for induction and maintenance of differentiation in this organism. In particular, the fact that the prespore/prestalk ratio is kept constant irrespective of the size of a slug cannot be explained without resorting to intercellular signals. We have analyzed what kind of intercellular signals are involved in the pathways of prespore and prestalk/stalk differentiation.

Intercellular signals in prespore differentiation

When vegetative cells are starved and incubated in a single cell state, no prespore differentiation occurs even in the presence of cAMP. However, if cells are allowed to develop up to the mound stage and then disaggregated, isolated cells differentiate into prespore cells if only cAMP (and albumin) was supplied (Yamada and Okamoto, 1990). This means that cells require some intercellular signals other than cAMP in the initial stages of development. By disaggregating cell masses at various stages of development and incubating disaggregated cells in a single state in the presence of various substances, we found that the pathway of prespore differentiation can be dissected into three distinct stages (Stages P1, P2 and P3) with respect to their requirements for differentiation (Fig. 4) (Yamada and Okamoto, 1990).

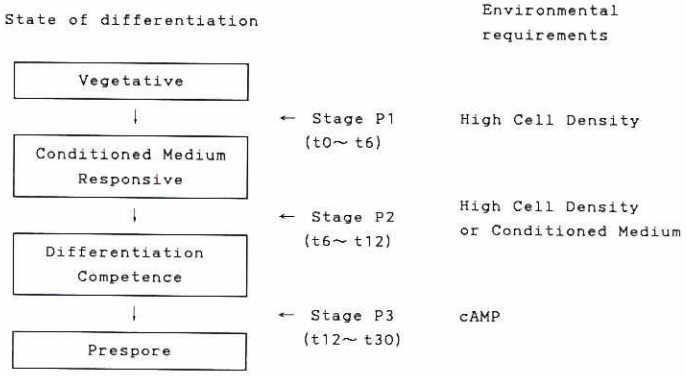

Fig. 4. The pathway of prespore differentiation dissected by variance in environmental requirement. *Approximate times for each stage are shown with cells developing in liquid culture (t indicates hours of starvation). For detailed explanation, see the text.*

During the first 5 h of starvation (Stage P1), cells must be incubated at a high cell density and any exogenous substances cannot replace this requirement. Biochemical events taking place in cells during this period have not been analyzed, but cells were shown to have acquired the responsiveness to a conditioned medium (see below) by the end of this stage. The presence of cAMP at this stage is rather inhibitory.

Stage P2 lasts for the next few hours and advancement of this stage is also inhibited by exogenous cAMP (Yamada and Okamoto, 1992). During this period, however, cells are converted to become cAMP-requiring. Although incubation at a high cell density is again needed at this stage, a conditioned medium prepared from shake cultures at a high cell density replaces this requirement and even an isolated single cell can proceed to develop with it. A signal substance contained in the conditioned medium, termed DCF (differentiation competence factor), is neither cAMP, ammonia nor methionine. DCF is sensitive to heat, acid and alkaline, and quite unstable, probably due to proteolytic degradation, and this makes its purification highly difficult.

After cells acquire the responsiveness to cAMP at Stage P2, prespore-specific antigen is ready to be synthesized in the final phase (Stage P3), where only cAMP and albumin are required, as described earlier (the latter substance is probably needed for protection of cells). To summarize, various types of signals are involved in each stage and concerted action of these signals promotes cells to proceed from one stage to the next. It should be noted that cAMP is the only kind of signal that has been identified, but many other unidentified signals are also involved in prespore differentiation.

Intercellular signals in prestalk/stalk differentiation

One of the most characteristic properties of prestalk/stalk differentiation is the requirement of, besides cAMP, a small signal molecule, termed DIF, which is secreted by developing cells (Town *et al.*, 1976). DIF-1, the most abundant species of DIF, which is now identified as 1-(3,5-dichloro-2,6-dihydroxy-4-methoxyphenyl)-1-hexanone (Morris *et al.*, 1987), was shown to act later than cAMP (Sobolewski *et al.*, 1983) to specifically induce expression of prestalk genes (Jermyn *et al.*, 1987).

By applying the same method as was used to dissect the prespore pathway, we also examined the pathway of stalk differentiation to determine signals involved in the process (Yamada and Okamoto, 1994) (Fig. 5). To assess the expression of stalk-specific genes in individual cells, the expression of *LacZ* gene ligated to the promoter regions of prestalk-specific *ecmA* and *ecmB* genes (Jermyn *et al.*, 1989) was histochemically visualized.

For the first 5 h of starvation (Stage T1), cells acquire responsiveness to cAMP when incubated at a high cell density. If incubated at a low cell density, subsequent incubation at a high cell density cannot restore the cells to express the prestalk genes. Signal substances involved at this stage have not been clarified.

The next 5 h (Stage T2) is the period during which cells acquire responsiveness to DIF-1 when incubated with cAMP at a high cell density. The requirement for a high cell density is not replaceable with conditioned medium, cell lysates, cell membrane fractions, albumin or 8-bromo-cAMP. It is worth noting that cAMP is necessary for the stalk pathway at this stage, although this is inhibitory for prespore differentiation in the same period (Yamada and Okamoto, 1992; see also the preceding section). This implies that completely distinct processes are involved in these two pathways at such an early stage of development.

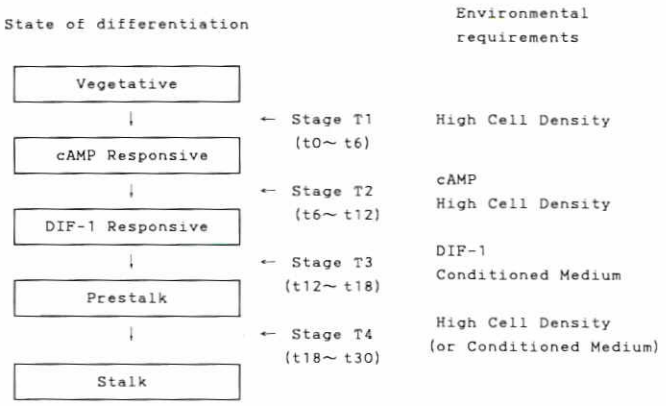

Fig. 5. The pathway of prestalk/stalk differentiation dissected by variance in environmental requirement. *Approximate times for each stage are shown with cells developing in liquid culture (t indicates hours of starvation). For detailed explanation, see the text.*

During the third stage (Stage T3), cells express stalk-specific genes in the presence of DIF-1, at a high cell density. At this stage, however, if conditioned medium prepared from a high cell density culture is supplied together with DIF-1, even cells plated at a low density can express these genes. This indicates that the requirement for a high cell density is simply due to the accumulation of some secreted factors in the intercellular space. We can thus conclude that both a yet unidentified signal substance(s) and DIF-1 are required for prestalk gene expression at this stage.

Although both *ecmA* and *ecmB* are prestalk/stalk-specific genes, the expression of these genes is dissimilarly controlled both spatially and temporally. In a slug, *ecmA* is expressed roughly in the whole anterior region, while *ecmB*-expressing cells are restricted to the central cone-like region in the anterior part (Jermyn *et al.*, 1989). This poses a question as to what signals regulate such dissimilar expression of the two genes. cAMP was proposed to be responsible for this, since in shake cultures of a high cell density, only *ecmB* expression was inhibited by this nucleotide (Berks and Kay, 1990). However, in the case of cells plated at a low density, we found that cAMP inhibited both *ecmA* and *ecmB* expression to the same extent (a half maximum inhibition was obtained by 5 μM cAMP). We therefore conclude that cAMP itself has no differential effects on the expression of the two genes and that there must be some other signals for such dissimilar expression. The apparently dissimilar effect of cAMP as observed in a high-density culture might be due to formation of cell clumps, where *ecmA*- and *ecmB*-expressing cells would have sorted out and hence been exposed to different concentrations of cAMP.

It has been suggested that *ecmB* expression requires activation of protein kinase A (Harwood *et al.*, 1992). Therefore, we examined the effect of 8-bromo-cAMP, a cell-permeable activating agent for protein kinase A, on the prestalk gene expression with Stage T3 cells plated at a low density, and found that this agent, together with DIF-1, greatly stimulated *ecmB* expression without the conditioned medium. Since this was the case with single cells, there should be no other intercellular signals required. Hence, we conclude that activation of protein kinase A and the induction of a signal by DIF-1 are necessary and sufficient conditions for *ecmB* expression. This further suggests that the conditioned medium contains a

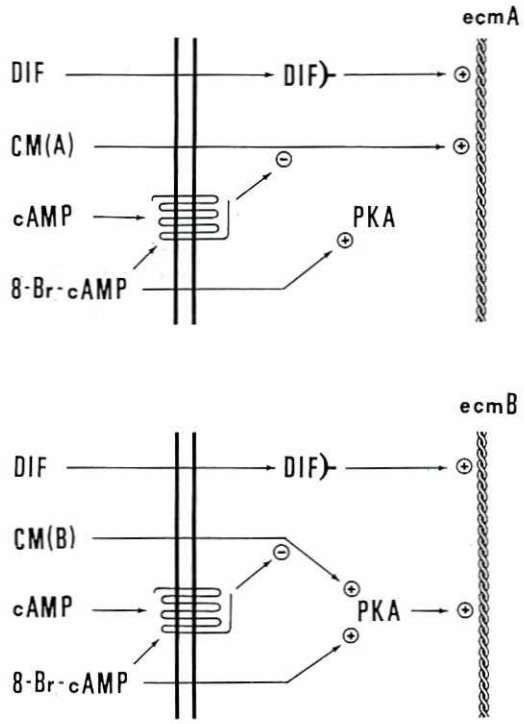

Fig. 6. Hypothetical schemes for actions of intercellular signals in induction of prestalk specific genes, *ecmA* and *ecmB*. *DIF penetrates into the cell and binds with its intracellular receptors to exert its action. Conditioned medium (CM) is assumed to contain two active components, A and B, which activate ecmA and ecmB genes, respectively. For activation of ecmA or ecmB, both DIF and the CM components are required. Binding of cAMP with the surface receptors inhibits expression of either gene probably by blocking the signalling pathways of the CM components. CM(B) appears to activate protein kinase A (PKA), as its effect is replaceable with 8-Br-cAMP. This is not the case with CM(A) (see the text).*

substance that leads to activation of protein kinase A. Preliminary experiments indicate that such substances are of low molecular masses, distinct from DCF and consist of multiple components separable by HPLC chromatography. In contrast to *ecmB* expression, *ecmA* expression is not induced but rather inhibited by 8-bromo-cAMP. This inhibition is probably caused by its binding to the surface receptors for cAMP (Fig. 6) (remember that cAMP inhibits the gene expression), since the concentration of the analogue was high enough (10 mM) to allow such heterologous binding. In the case of *ecmB* expression, such an inhibitory effect is bypassed, probably because protein kinase A is located downstream of the inhibition site of cAMP (Fig. 6). By contrast, activation of protein kinase A is perhaps not involved in *ecmA* expression.

The last stage (Stage T4) of stalk differentiation is the maturation process of prestalk cells. For the analysis of this step, prestalk cells were separated from normally developed slugs by a Percoll density-gradient centrifugation and the degree of stalk maturation was microscopically monitored (Kubohara *et al.*, 1993). When isolated prestalk cells were incubated in a single cell state, no stalk maturation occurred. The addition of DIF-1 to these cells caused only a fraction to mature. Further addition of adenosine or dimethyloxazolidine-dione or depletion of ammonia failed to raise the efficiency of maturation. However, we found that 8-bromo-cAMP induced stalk maturation at a high efficiency (~90%). The efficiency was essentially unaffected even when the cell density was greatly reduced. Therefore, the involvement of other intercellular signals is very unlikely and activation of protein kinase A would be the only requirement for the conversion of a prestalk cell to a stalk cell.

It should be pointed out that Maeda (1988) and Kay (1989) have previously shown that spore maturation is also induced by 8-bromo-cAMP. Taking these together, it appears that once prespore and prestalk cells are differentiated, the final process of maturation is promoted by activation of protein kinase A in either pathway. Since 8-bromo-cAMP is not a naturally occurring substance, there must be a natural signal substance which causes an elevation of intracellular cAMP. In the stalk pathway, a substance contained in the conditioned medium is a good candidate. In fact, the conditioned medium causes cells in the stalk pathway to proceed through not only Stage T3 but also Stage T4 to mature stalk cells (Yamada, personal communication), suggesting that the factor raises intracellular cAMP level in both stages through a yet unclarified mechanism.

In conclusion, we have shown that a number of different signals are involved in the development of this organism. Some are stage-specific, pathway-specific, or even gene-specific. Although cAMP is one of the most frequently used signals, we showed that it is stimulative in one stage, but highly inhibitory in the other stages in either differentiation pathway. Therefore, when we consider the mechanism of action of a signal, we should specify the stage in which it works. Dissection of the developmental pathway is thus important for such a study.

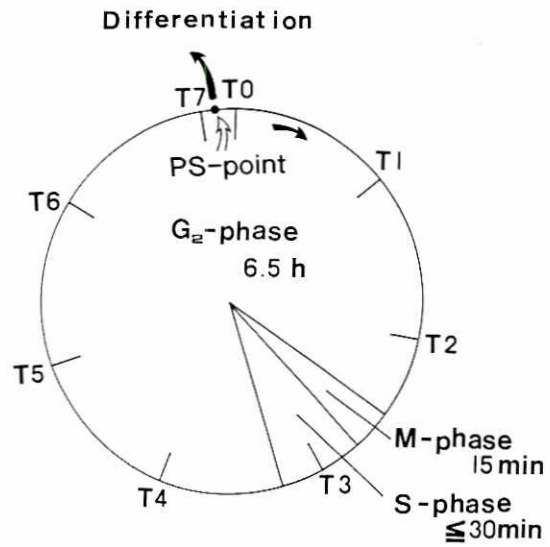

Fig. 7. The cell cycle of a growing *D. discoideum* Ax-2 cell and its relation to differentiation after starvation. *Ax-2 cells may progress through the cell cycle to a particular point (putative shift point; PS-point), irrespective of the presence or absence of nutrients, and enter the differentiation phase from this point under conditions of nutritional deprivation (Maeda et al., 1989). The PS-point is interposed near the cell-cycle position of T7. T0, T1 and T7 indicate 0 h, 1 h, and 7 h after the temperature shift from 11.5°C to 22.0°C for cell synchrony, respectively.*

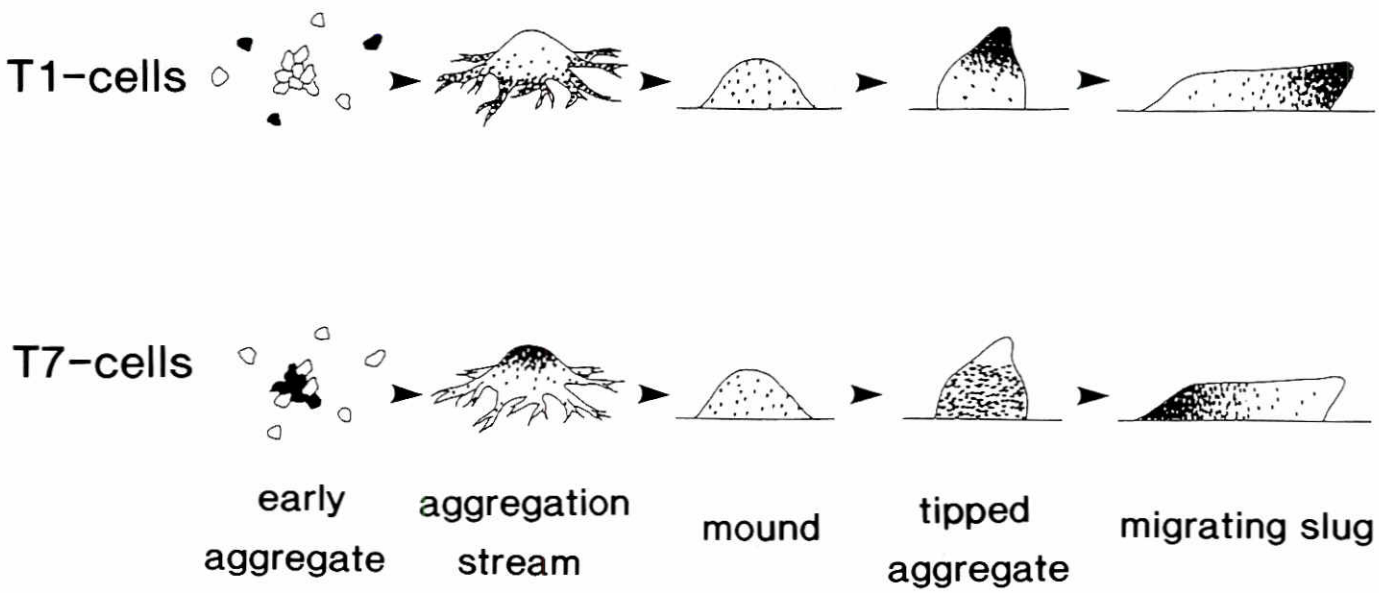

Fig. 8. A schematic representation showing the sorting behaviors of T1 and T7 cells during development, when mixed with non-synchronized cells. *The respective synchronized cells are shown as black ones. It is noteworthy that the spatial exchange between T1 and T7 cells occurs sometime between the aggregation stream and tipped aggregate stages.*

Another important aspect in discussions of signal requirements is that there is a large variation in the degree of requirement among different strains of *D. discoideum*. The strain NC4 and sporogenous mutants are probably two extremes. In our study, we mostly used NC4, which shows the highest requirement.

Cell-cycle dependent formation of differentiation pattern

Recently, evidence has been accumulated indicating that the tendency of preaggregative cells to become either prestalk or prespore cells is correlated with the cell-cycle phase when the cells are starved to initiate development (Zada-Hames and Ashworth, 1978b; McDonald and Durston, 1984; Weijer *et al.*, 1984b; Sharpe and Watts, 1985; Maeda, 1986, 1993; Ohmori and Maeda, 1987; Wang *et al.*, 1988; Maeda *et al.*, 1989; Zimmerman and Weijer, 1993; Araki *et al.*, 1994). We will describe the cell-cycle phase of this organism and its implications in development, especially formation of the differentiation pattern.

Synchronous growth and the cell cycle

For precise analysis of the cell cycle and related phenomena, good methods are required for inducing synchronous growth of cells. Axenic cells have been exclusively used for this kind of study. In the culture system of an axenic strain, Ax-2, three main methods have been used for cell synchrony: 1) a mitosis wash-off procedure (McDonald and Durston, 1984), 2) a stationary-phase release method in which stationary-phase cells are released (diluted) to fresh growth medium (Zada-Hames and Ashworth, 1978a; Weijer *et al.*, 1984a), and 3) a temperature-shift method in which exponentially growing cells at 22.0°C are shifted to a low temperature of 11.5°C, shaken for 20.0 h and then re-shifted to 22.0°C (Maeda, 1986). We found that both the wash-off procedure and the stationary-phase release method were either ineffective or resulted in only partial synchrony. In contrast, the temperature-shift method gives excellent synchronous growth of Ax-2 cells (Maeda, 1986).

The axenically growing cells show a doubling time of about 7.2 h at 22°C. There is little or no G1-phase and a short S-phase lasting 30 min or less, while the G2-phase lasts about 6.5 h (Weijer *et al.*, 1984a; Ohmori and Maeda, 1987) (Fig. 7). In the population of Ax-2 cells synchronized by the temperature shift method, cell doubling occurs over about a 2-h period after a lag phase of about 1 h.

The cell cycle and differentiation pattern

When synchronized cell populations were harvested, washed and allowed to develop on non-nutrient agar at various phases of the cell cycle (referred to as Tt cells, t indicating hours after the temperature shift-up), they exhibited different developmental features. For example, cells starved just before mitosis (M-phase) such as T1 cells (cells harvested at 1 h after the temperature shift) preferentially sorted out to the anterior prestalk region of slugs, whereas cells starved at the mid-late G2-phase such as T7 cells (cells harvested at 7 h after the shift-up) sorted out to the posterior prespore region (Ohmori and Maeda, 1987; Maeda *et al.*, 1989; Araki *et al.*, 1994). When T1 cells were incubated for 6 h in the absence of nutrients, they showed developmental features similar to those of T7 cells. Cell doubling and subsequent DNA synthesis occur in T0.5 and T1 cells even in the absence of nutrients, but not in T3, T5, or T7 cells (Maeda *et al.*, 1989). These facts indicate that the cells progress through the cell cycle to a particular point (putative shift point, PS-point), irrespective of the presence or absence of nutrients, and enter the differentiation phase in case of nutritional deprivation, as schematically shown in Fig. 7. The developmental features of cells starved at different phases of the cell-cycle indicate that the PS point is located near the cell-cycle position of T7 cells (Ohmori and Maeda, 1987).

The time needed for cell aggregation and tip formation varies in a cell-cycle dependent manner: T7 cells show the most rapid development (Ohmori and Maeda, 1987). Furthermore, cells ac-

quire chemotactic ability towards cAMP and EDTA-resistant cohesiveness at different times of development depending on the cell cycle (Ohmori and Maeda, 1987); T7 cells acquire these properties earlier than T1 cells. This raised the possibility that T7 cells may function as autonomously signaling aggregation centers and chemotactically attract neighboring cells (Ohmori and Maeda, 1987).

This idea was supported by our recent finding (Araki *et al.*, 1994) that T7 cells transformed by a vector (pAct15-Gal) bearing bacterial ß-gal are predominantly located in the premature aggregation center when mix-cultured with non-synchronized (non-transformed) cells (Fig. 8). Moreover, the number of aggregates increases in proportion to that of externally added T7 cells (data not shown). In spite of the fact that T1 cells starved at the late G2-phase aggregate more slowly than T7 cells, they then sort out to the tip portion of tipped aggregates and finally to the anterior prestalk region of slugs. At the mound stage, both T1 and T7 cells exhibit temporarily uniform distribution throughout the cell mass; the former are in transit to the tip region, while the latter are to the basal region. Thus, the anterior T1/posterior T7 pattern is established during slug formation and mostly maintained at the later stages. The sorting behaviors of T1 and T7 cells are schematically shown in Fig. 8.

The precise mechanism of cell sorting is still unknown, but it is most likely that chemotaxis is closely involved in the process. Although aggregating cells are most active in chemotaxis, slug cells are still chemotactically sensitive to cAMP (Maeda, 1977) and the sensitivity is higher in the prestalk cells than in the prespore cells (Matsukuma and Durston, 1979; Mee *et al.*, 1986). It was previously shown that the chemotactic sensitivities of T1 and T7 cells are reversed around the aggregation stage (Ohmori and Maeda, 1987). This reversion might explain the fact that T1 cells and T7 cells interchange their relative positions in the cell mass between the aggregation stream and the tipped aggregate stages.

Some of our results described above are in disagreement with those obtained by the use of the stationary-phase release method. According to McDonald (1986), Ax-2 cells starved at the S-phase (2-3 h after the stationary release) display the initial sign of aggregation earlier than those starved at the late G2-phase. Wang *et al.* (1988) also reported that Ax-2 cells starved at the S- or the early G2-phase are the first to initiate aggregation centers, most active in chemotaxis and signal relay and eventually sort out to the anterior part of slugs. These observations are apparently in contrast to the sorting behavior of T7 cells starved at the mid-late G2-phase, especially in that they initiate center formation but eventually sort out to the posterior part of slugs. Although the reason for such a conflict is presently unknown, it is likely that the methods used for cell synchronization and the efficiency of cell synchrony have great influences on the sorting behavior of the cells.

There is another disagreement in the ratio of prestalk to prespore cells in migrating slugs derived from cells starved at different phases of the cell cycle. We found no differences in the ratio among slugs derived from T1, T3, T5 and T7 cells; in all cases, about 72% of slug cells are prespore cells, the same value as obtained with slugs derived from non-synchronized cells (Maeda *et al.*, 1989). This indicates that cell-type proportioning is strictly regulated in a cell mass, independently of cell-cycle phase. Alternatively, several workers (Weijer *et al.*, 1984b; Wang *et al.*, 1988) using the stationary-phase release method have reported that the ratio varies depending on the cell-cycle phase: S-phase cells form slugs with about 50% prespore cells, while late G2-phase cells form slugs with about 90% prespore cells. We reexamined these results using the same method for cell synchrony, but found no marked difference in the ratio of prespore cells during progression of the cell cycle (Maeda *et al.*, 1989).

Using cell-autonomous markers, Williams *et al.* (1989) traced the origins of two types of prestalk cells (Pst A and Pst B cells) during development. Pst A cells are dispersed at random throughout the mound when first detectable and then sort out to the apex during tip formation, whereas Pst B cells appear randomly in the mound and then sort out to the basal region during tip formation. As described in a preceding section, cells expressing prespore-specific Dp87 gene first appear at random in the aggregation stream as well as in the cell mound and then sort out to the central region of the tipped aggregate. It is of particular interest to find out whether the differentiation of these cells is somehow related to the cell-cycle phases. In this connection, it is noteworthy that the behaviors of T1 and T7 cells during aggregation appear similar to those of Pst A and prespore cells, respectively. To examine such a relationship, the use of double transformants bearing both a stable cell marker and cell-type-specific markers would be promising.

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