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Aberrant pattern formation in myosin heavy chain mutants of *Dictyostelium*

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SUMMARY

In mutant *Dictyostelium* strains that fail to accumulate the myosin heavy chain (MHC A), development is relatively normal up to the tight aggregate stage but is arrested prior to formation of the apical tip (DeLozanne and Spudich 1987, Knecht and Loomis, 1987). We show that in aggregates formed by such MHC A deficient (MHC A–) strains the proportions of pstA and pstB cells, the two prestalk cell types, and of prespore cells are similar to those found during normal development but their distribution is radically different. During the initial stages of normal slug formation, pstA cells move to the tip, pstB cells accumulate in the base and prespore cells occupy the remainder of the aggregate. In the aggregates initially formed by MHC A– mutants pstA cells are present in a central core, pstB cells are present in the cortex and prespore cells lie sandwiched between them. Eventually, cells within the cortex differentiate into mature stalk cells but spores are never formed. Mixing experiments, in which MHC A– cells are allowed to co-aggregate with an excess of normal cells, show that MHC A– prestalk cells enter the aggregate relatively normally but are unable to enter the slug tip or to migrate into the stalk at culmination and that MHC A– prespore cells accumulate in the lower part of the spore head during culmination. Thus MHC A– cells appear to be able to move within the multicellular aggregate but are incapable of participating in normal morphogenesis.

The structures formed by MHC A– cells are very similar to those of the agglomerates that form when wild-type cells are developed in roller-tube culture, conditions that result in loss of the polarity imparted by the presence of an air-water interface. We propose formation of such a structure by MHC A– cells to be a default response, caused by their inability to undertake the shape changes and intercalatory cell movements that are necessary to form and extend the tip.

Key words: MHC A–, pattern formation, pstA and pstB cells

INTRODUCTION

Non-muscle myosin II has been identified in eukaryotic organisms as widely diverged as *Acanthamoeba* and *Drosophila* (Warrick et al., 1986; Hammer et al., 1987; Watts et al., 1987; Ketchum et al., 1990) and is implicated in cellular processes requiring the generation of force. These include cytokinesis and changes in cell shape and motility (Spudich and Spudich, 1982; Yumura et al., 1984; Knecht and Loomis, 1987; DeLozanne and Spudich, 1987; Fukui et al., 1990; Eliott et al., 1993). Myosin II exists in vivo as a hexamer, composed of two heavy chains complexed with two regulatory and two essential light chains. Inactivation of the *Dictyostelium* myosin heavy chain gene (*mhcA*) by gene disruption (DeLozanne and Spudich, 1987), or inhibition of its expression by production of an antisense mRNA transcript (Knecht and Loomis, 1987), blocks cytokinesis but cell movement and chemotaxis are only partially perturbed (Wessels et al., 1988; Eliott et al., 1993). Antisense inhibition of the expression of the *Dictyostelium* myosin essential light chain produces similar defects in cytokinesis and chemotaxis to those produced by inactivation of the *mhcA* gene (Pollenz et al., 1992).

There is evidence that myosin II may also play a role in the morphogenetic events that shape multicellular organisms. *Drosophila* myosin II has been shown to be the product of the *zipper* gene which, when mutated, results in morphogenic defects and embryonic death (Young et al., 1993) while the embryonic lethal, *spaghetti-squash* mutation encodes the regulatory light chain of myosin II (Karess et al., 1992). A role in morphogenesis has also been demonstrated in *Dictyostelium*, where mutants that do not express the *mhcA* gene arrest midway through development (Knecht and Loomis, 1987; DeLozanne and Spudich, 1987; Manstein et al., 1989; Eliott et al., 1993).

Dictyostelium development is initiated when the bacterial food supply is exhausted and cells aggregate into hemispherical mounds in response to cAMP pulses. Cells at the apex of the mound then form into an apical papilla, or tip, which elongates and transforms the aggregate into a cylindrical structure known as the first finger. Environmental conditions determine whether the first finger completes development on the spot or collapses onto the substratum to migrate as a slug. In the slug the MHC A protein is selectively localised to the cortical cytoplasm of prestalk cells while it is uniformly dis-

tributed in the cytoplasm of cells in the prespore region (Elliot et al., 1991; Yumura et al., 1992) These observations suggest that myosin II may be involved in slug movement and this idea fits rather well with the inability of MHC A– cells to form a tip, since slug movement and tip extension seem likely to be related processes. At culmination the slug transforms into a fruiting body, composed of a droplet of viable spore cells supported by a slender column of vacuolated stalk cells.

The prestalk cell population is heterogeneous with respect to the expression of the *ecmA* and *ecmB* genes. These genes are induced by DIF, the stalk-specific morphogen, and encode the EcmA and EcmB extracellular matrix proteins (McRobbie et al., 1988). Prestalk A (pstA) cells express the *ecmA* gene and pstB cells express the *ecmB* gene (Jermyn et al., 1987; Willams et al., 1989a; Jermyn and Williams, 1991). At culmination, pstA cells in the tip of the slug activate expression of the *ecmB* gene as they enter the stalk tube, the cylindrical matrix that surrounds the stalk cells. These cells co-express the *ecmA* and *ecmB* genes and are therefore termed pstAB cells (Gaskell et al., 1992). The pstAB population appears to be committed to stalk cell differentiation (Jermyn and Williams, 1991).

Although the role of cell movement during culmination is readily apparent, because the prestalk cells must move down through the underlying prespore mass to form the supporting stalk, the involvement of directed cell movement during tip formation has been a matter of debate (reviewed by Williams 1988). It is now generally accepted, however, that primary morphogenesis involves sorting of prestalk from prespore cells (Takeuchi et al., 1977; Gomer, et al., 1986; Williams, et al., 1989a; Esch and Firtel, 1991). When they first appear, pstA cells, pstB cells and prespore cells are scattered throughout the aggregate (Williams et al., 1989a,b; Esch and Firtel, 1991; D. Traynor, T. Abe and J. G. Williams, unpublished results). Subsequently, during slug formation, pstA cells migrate to the apex, pstB cells accumulate in the base and prespore cells occupy the region between the pstA and B cells. The upward movement of pstA cells during tip formation appears to be mediated by chemotaxis to cAMP signals emanating from the apex of the mound (Durston and Vork, 1979; Bonner, 1949; Rubin and Robertson, 1975; Rubin, 1976; Maeda, 1977, Traynor et al., 1992). When the apical tip forms it is primarily composed of pstA cells but, after it has elongated, a core of pstAB cells appears within it (Fig. 1). This lies at the position where the stalk will form during culmination.

The physical processes of tip formation and elongation require cell rearrangements, to convert the hemispherical mound into a cylindrical first finger. These processes are in some respects similar to the cellular movements that occur during gastrulation in higher embryos. Many *Dictyostelium* mutants, including MHC A– strains, appear to be incapable of tip formation but the precise nature of the defect remains obscure. Although MHC A– cells block in development as mounds, they show normal expression of prespore-specific genes (Knecht and Loomis, 1988). When mixed with wild-type cells, MHC A– cells are capable of forming spores but they are excluded from the tip of the migratory slug and of pre-culminates (Knecht and Loomis, 1988; Eliott et al., 1993).

We have used *ecmA* and *ecmB* prestalk-specific markers to show that prestalk cell differentiation also occurs normally in MHC A– aggregates. The fact that prestalk cell differentiation

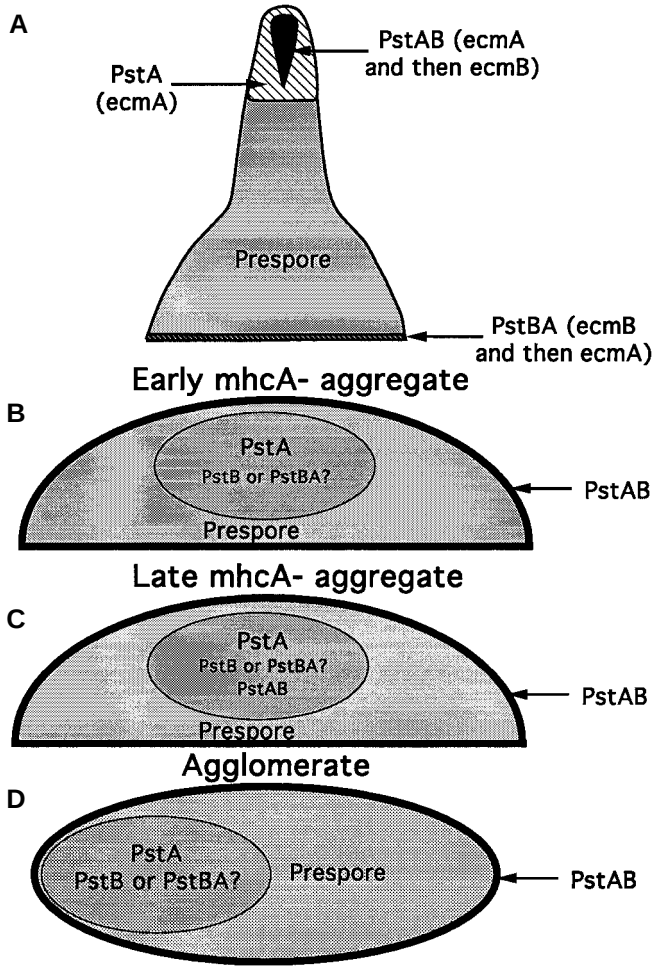

Fig. 1. A summary of the prestalk-prespore pattern observed during development. (A) By the first finger stage of normal development in wild-type strains the prestalk-prespore pattern is established. PstA cells express the *ecmA* gene and form the apical tip whereas pstAB cell express both the *ecmA* and *ecmB* genes and are found in a core within the tip. PstAB cells express the ST:lacZ marker. Cells at the base also co-express the *ecmA* and *ecmB* genes but here the order of gene expression is most probably reversed, so we term them pstBA cells. PstBA cells do not express the ST:lacZ marker. Prespore cells occupy the region between the tip and the base. (B) In MHC A– aggregates at 21 hours of development the pstA cells form a core within the aggregate. A number of cells within the core express the *ecmB* gene but the ST:lacZ construct is not expressed in the core. We can therefore be confident that they are not pstAB cells but they could be either pstB cells or pstBA cells. The periphery of the aggregate consists of pstAB cells some of which are vacuolated stalk cells. The remainder of the aggregate is composed of prespore cells. (C) MHC A– aggregates at 36 hours of development are very similar to aggregates at 21 hours of development, except that the ST:lacZ construct is expressed within the core. Thus at least some of the *ecmB*-expressing cells must be pstAB cells. (D) Wild-type cells developed in submerged conditions *in* vitro contain a core that is mainly composed of *ecmA*- and *ecmB*-expressing cells. There are virtually no pstAB cells but, again, the *ecmB*-expressing cells could be pstB cells or could be pstBA cells. Prespore cells occupy the intervening region of the aggregate. In the *mhcA* null aggregate and the agglomerate the size of the lettering gives some indication of the relative abundance of the different cell types.

is normal but no tip is formed suggests that the defect in morphogenesis is due to a defect in prestalk cell behaviour. Thus we have gone on to determine whether this defect prevents prestalk cells from moving to the apex of the mound or whether it prevents the subsequent cell movements and shape changes that are necessary for extrusion of the tip. We show that in MHC A− aggregates, prestalk and prespore cells do sort out from one another, albeit to form an aberrant structure, suggesting that their major defect is in tip extrusion. However, we confirm that MHC A− cells are excluded from the tip when synergised with normal cells, supporting the notion that they are also defective in their ability to move through the multicellular aggregate.

MATERIALS AND METHODS

Strains and culture conditions

Ax2 and Ax3 cells and their derived transformants were grown and maintained in axenic medium at 22°C (Watts and Ashworth, 1970). The thymidine auxotroph JH10 (kindly provided by J. Hadwiger and R. Firtel) was grown in HL-5 supplemented with 100 μg/ml thymidine or in association with *Klebsiella aerogenes* on SM-agar plates (Hadwiger and Firtel, 1992). The *mhcA* null mutant (kindly provided by K. Ruppel and J. Spudich) was created by rescuing JH10 with the pTZD1-Thy disruption vector containing the *thy1* gene and selecting for thymidine prototrophy (Dynes and Firtel, 1989). This plasmid is identical to pΔMHC-A5, except that the neomycin phosphotransferase (*neo*) gene is replaced with *thy1* (Manstein et al., 1989).

Cells were harvested and washed in KK_2 (16.5 mM KH_2PO_4, 3.8 mM K_2HPO_4, pH 6.2) by centrifugation before being plated for development at a density of $1\text{-}3\times10^6$ cells/cm^2 in KK_2 on 1.5% non-nutrient agar. For transfer experiments the MHC A− cells were developed on cellophane at a density of 4×10^6/cm^2. After 16-18 hours pieces of the cellophane containing approximately 50 aggregates were transferred to buffered (20 mM Na_2HPO_4, KH_2PO_4, pH7.0) 1.5% non-nutrient agar with various additions. After 12-15 hours incubation the aggregates were fixed and stained for β-galactosidase activity and the number of pstA cores within each aggregate determined by bright-field microscopy.

Ax3 and transformant MHC A− strains were mixed 70%:30% respectively, for synergy experiments. A total of $1\text{-}2\times10^7$ cells were plated for development and stained for β-galactosidase activity as described above.

In vitro development

Vegetative Ax2 transformant cells were harvested and washed twice in Bonner's Salt Solution (BSS, 10 mM NaCl, 10 mM KCl, 2.9 mM $CaCl_2$; Bonner, 1949) and then resuspended at $2\text{-}5\times10^6$ cells/ml in BSS. Agglomerates were formed by placing 3 ml of the cell suspension into 19 mm glass tubes, flushing them with oxygen and rolling them at 28 revs/minute (Tasaka and Takeuchi, 1981). Tight agglomerates formed after approximately 20 hours under these conditions and by 36 hours they displayed a refractive halo that indicated they had acquired an enveloping layer of slime sheath material.

Plasmid constructs and transformation of *Dictyostelium*

Ax3 cells were transformed with a mixture of 8 μg of plasmid pA6mhcA and 4 μg of cell type-specific β-galactosidase fusion genes using the calcium-precipitation method described in Harwood et al. (1992), except that the glycerol shock was reduced to 2 minutes. The pA6mhcA construct (a kind gift of David Knecht and William Loomis) contains a portion of the *mhcA* gene in the antisense orientation relative to the *Dictyostelium* actin 6 promoter (Knecht and Loomis, 1987). The *mhcA* null mutant strain was transformed with prestalk-specific β-galactosidase fusion constructs (Dingermann et al., 1989) containing the *neo* resistance gene as a selectable marker. These vectors were made by sub-cloning a 2.19 kb *Bgl*II fragment encompassing the *ecmA* promoter and the first four codons of the translated sequence, or a 1.97 kb *Bgl*II fragment containing the *ecmB* promoter and the first three codons, into the *Bgl*II site within the polylinker of pDdGal-17 (Harwood and Drury, 1990; Jermyn and Williams, 1991). This yielded the ecmA:lacZ and ecmB:lacZ constructs. The stalk tube-specific *lacZ* fusion construct (ST:lacZ) contains sequences from –877 to +112 of the *ecmB* promoter and is equivalent to construct A in fig. 1 of Ceccarelli et al. (1991), except that the *lacZ* vector is pDdGal-17 instead of pDDlac-1. A prespore-specific construct was generated by sub-cloning the *pspA* (formerly D19) promoter as a 1.2 kb *Bam*HI-*Bgl*II fragment, generated by the polymerase chain reaction from the genomic clone (Early et al., 1988), into *Bam*HI-*Bgl*II restricted pDdGal-17. The *Bgl*II site was created within the fourth codon of the *pspA* gene.

Ax2 was transformed, as described above, with 5-10 μg of the cell type-specific β-galactosidase fusion constructs. In all cases transformants of every strains were selected and maintained in HL5 medium containing 20-40 μg/ml of the aminoglycoside G418 (Geneticin; GIBCO UK).

Histochemical and immunological staining

Cellular differentiation and migration was monitored in cells developing on 1.5% non-nutrient agar by analysis of β-galactosidase staining in whole mounts or sections. Aggregates were fixed and stained for β-galactosidase activity as described in Harwood et al. (1992). Stained aggregates were embedded in OCT compound (BDH,UK) and frozen longitudinal sections were cut to 8 μm thickness. The sections were collected onto slides subbed with 1-3% gelatin and mounted in gelvatol prior to microscopic analysis.

In order to quantitate different cell types, aggregates were dissociated in buffer (20 mM Na_2HPO_4, 20 mM NaH_2PO_4, 20 mM EDTA, pH 7.0) at $1\text{-}2\times10^6$ cells/ml by trituration. The cell suspension was fixed and washed by centrifugation, before being resuspended at $0.5\text{-}1\times10^7$ cells/ml in staining solution (Harwood et al., 1992). After 4-8 hours incubation at 37°C, 5 μl of the cell suspension was spotted onto poly-L-lysine (80 mg/ml in 10 mM Tris-HCl, pH 8.0) subbed slides and the number of stained cells determined using bright-field microscopy. Prespore cells were detected by indirect immunofluorescence using the PsA-specific monoclonal antibody MUD1 as described by Early et al. (1988).

RESULTS

Morphological characteristics of the aggregates formed by MHC A− strains

We analysed both a gene disruptant (DeLozanne and Spudich, 1987; K. Ruppel and J. Spudich, personal communication) and a strain containing a fragment of the *mhcA* gene in an antisense orientation under the control of the actin 6 promoter (Knecht and Loomis, 1987). Development in both strains is extremely asynchronous, with aggregation streams still present after 30 hours of incubation. The mounds that formed appeared flattened in comparison to wild-type mounds. There are many loosely associated, apparently undifferentiated cells surrounding a surrounding layer of flattened and tightly adherent cells, which marks the outermost extent of cellular differentiation within the aggregate. We find the phenotype of strains that lack the MHC A protein as a result of gene disruption to be subtly different from that of antisense strains. Although both are retarded in development compared to the wild type and show

the basic structure described above, the antisense strain forms looser mounds than the *mhcA* gene disruptant strain. Despite this slight difference in gross morphology, both the gene disruptant and the antisense mutant have the same pattern of cellular differentiation, as assayed using reporter gene constructs. The majority of data presented below is derived from analysis of the *mhcA* gene disruptant.

Prestalk and prespore cell differentiation are not inhibited in MHC A– cells

Although inactivation of the *mhcA* gene blocks normal morphogenesis, it does not alter the timing and level of expression of several prespore-specific genes (Knecht and Loomis, 1988). We confirmed that differentiation of prespore and prestalk cells ocurs normally in MHC A– cells using *lacZ* fusion genes, containing the promoters of the *ecmA* gene and the *ecmB* gene, and a prespore-specific monoclonal antibody. The numbers of pstA, pstB and prespore cells were determined in *mhcA* null structures disaggregated at various times after the initiation of development. A few *ecmA*-, *ecmB*- and *pspA*-expressing cells were present after 9 hours of development, when only loose mounds with ill-defined aggregation streams had formed (data not shown). The proportion of expressing cells rose very rapidly thereafter until, by 15 hours of development, the numbers of pstA, pstB and prespore cells had reached a plateau (Fig. 2). These plateau levels are similar to those present at the slug stage in parental Ax3 cells (Traynor et al., 1992).

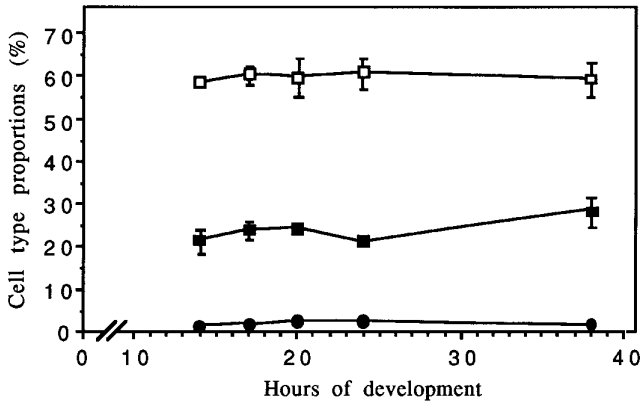

Fig. 2. Cell type proportions in the *mhcA* null strain. The proportions of cells expressing the ecmA:lacZ (■—■) and ecmB:lacZ (●—●) fusion genes together with the MUD-1 antigen (□—□) were determined in *mhcA* null aggregates dissociated at various times of development.

At early stages of formation of MHC A– aggregates pstA cells are present in a central core and pstB cells occupy the periphery

During normal development pstA cells differentiate at apparently random positions and then move to the apex of the aggregate (Williams et al., 1989a; Traynor et al., 1992). In

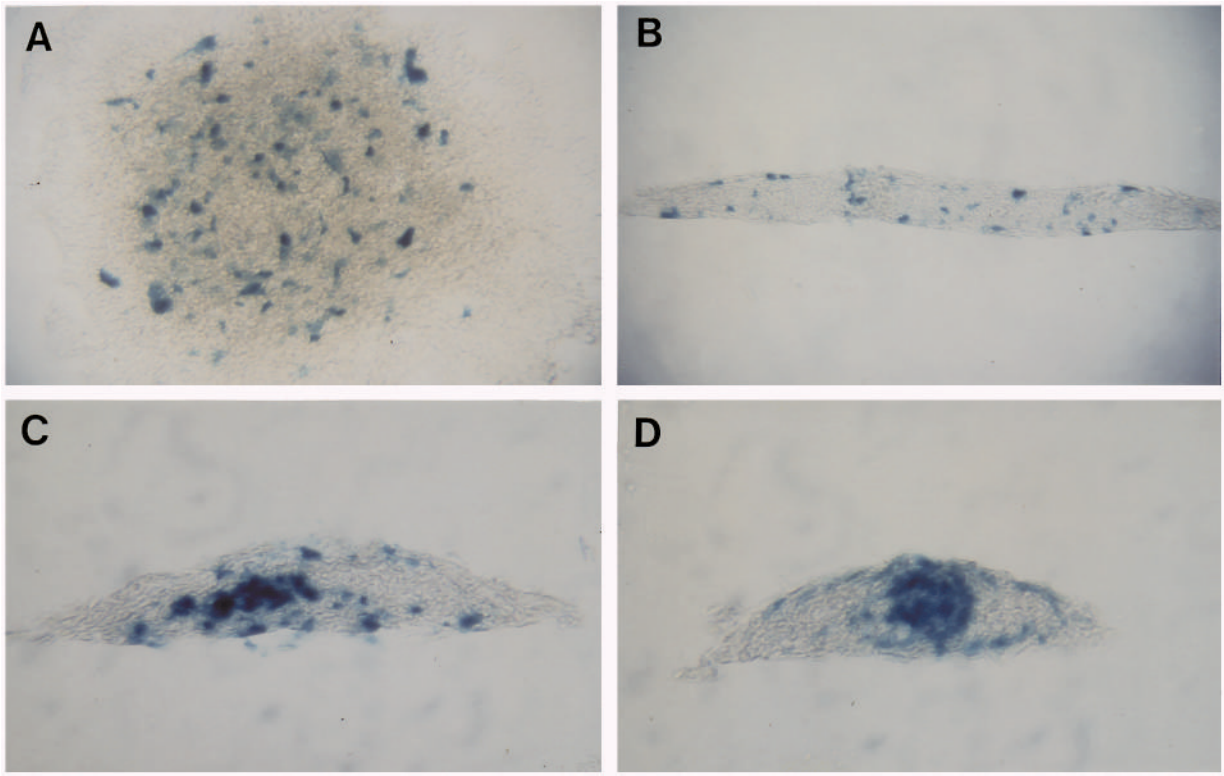

Fig. 3. PstA cell differentiation and movement within MHC A– aggregates. (A) A whole mount of a representative *mhcA* null aggregate transformed with ecmA:lacZ and stained for β-galactosidase activity after 12 hours. Longitudinal sections through the middle of similar *mhcA* null transformant aggregates stained for β-galactosidase activity after (B) 12 hours, (C) 15 hours, and (D) 18 hours of development. The whole mount is viewed from the top whereas all sections are orientated with the apex of the aggregate towards the top of the page. Magnification: in A and B, 130×; C and D, 320×.

MHC A– strains *ecmA*-expressing cells also differentiate at random (Fig. 3A,B) but they do not become localised to the apex of the aggregate. Rather, they accumulate in the interior of the aggregate to form a core (Fig 3C,D). This forms within 15 hours of the initiation of development (Fig. 3C) and larger aggregates contain multiple cores (data not shown). In normal development, pstB cells first appear at apparently random positions within the aggregate and subsequently accumulate at the base (Williams et al., 1989a,b; D. Traynor and J. G. Williams, unpublished data). Whole mounts of MHC A– aggregates, fixed early in development, show scattered *ecmB*-expressing cells (data not shown) but, after sectioning, the number of stained cells is too low to determine whether they occur at random positions throughout the aggregate. After 15 hours of development the majority of pstB cells are present in the periphery of the aggregate (Fig. 4A,B). Thus pstA cells initially accumulate in the core while pstB cells accumulate in the cortex.

At intermediate stages in the development of MHC A– aggregates there are *ecmA*-expressing cells in the cortex and *ecmB*-expressing cells in the core but co-expressing, pstAB, cells are found only in the cortex

By 18 hours of development there are also *ecmA*-expressing cells in the cortex. (Fig. 3D). Three pieces of evidence indicate that at least some of these express both the *ecmA* and the *ecmB* gene (Fig. 1). Firstly, there are *ecmB*-expressing cells in the cortex by 15 hours (Fig. 3B). Secondly, many of the *ecmA*- and *ecmB*-expressing cells in the cortex are vacuolated by this time and, during normal development, mature stalk cells inevitably derive from pstAB cells (i.e. cells that express both the *ecmA* and *ecmB* genes). During culmination, the *ecmB* gene is activated both in pstA cells, as they enter the mouth of the stalk tube, and in anterior-like cells (ALC), as they sort to surround the spore head (Jermyn and Williams, 1991). The ST:lacZ construct contains a sub-fragment of the *ecmB* promoter that directs expression in cells within the stalk tube but not within ALC (Ceccarelli et al., 1991). Thus pstAB cells can be directly identified using ST:lacZ as a marker.

Scattered cells within the cortex express the ST:lacZ construct by 15 hours of development (data not shown) and by 22 hours many of the cortical cells are expressing the gene (Fig. 5A). This suggests very strongly that these are pstAB cells rather than pstBA cells (i.e. cells that first express the *ecmB* gene and then express the *ecmA* gene, the behaviour shown by the *ecmB*-expressing cells that migrate to the base during the first finger stage), because pstBA cells do not express ST:lacZ (Ceccarelli et al., 1991). The apparently contradictory fact, that *ecmA* expression is detected a little later than *ecmB* expression, could reflect slight diffences in timing of development between transformant strains or could result from the ecmB:lacZ construct being present at a higher copy number than ecmA:lacZ (and therefore having a higher level of expression).

The core of pstA cells forms between 15 and 18 hours of development and from approximately 21 hours onwards some of the cells within the core express the ecmB:lacZ fusion gene (Fig. 4C). The virtual absence of ST:lacZ expression within the core at 22 hours (Fig. 5A) indicates that they are not pstAB cells. It is, however, possible that they are cells, which first expressed the *ecmB* gene and subsequently activated expression of the *ecmA* gene, i.e. that they are pstBA cells. Thus the core at this time could be a mixture of pstA and pstB cells, it could contain pstA and pstBA cells or it could contain all three cell types (Fig. 1).

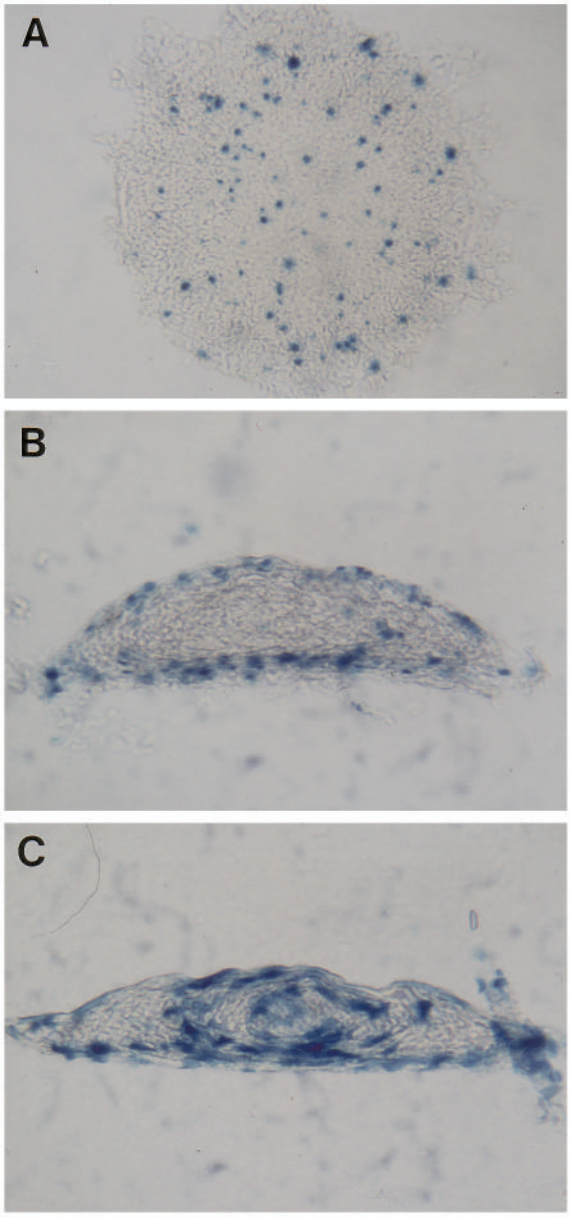

Fig. 4. PstB cell differentiation and movement within MHC A– aggregates. (A) A whole mount of a representative *mhcA* null aggregate transformed with ecmB:lacZ and stained for β-galactosidase activity after 15 hours. Longitudinal sections were cut through the middle of similar transformant aggregates stained for β-galactosidase activity. (B) *mhcA* null aggregate after 15 hours of development. (C) Ax2 transformed with the *mhcA* antisense construct pA6mhcA (which gives similar results to that obtained with the null mutant) after 21 hours of development. The whole-mount is viewed from the top whereas both sections are orientated with the apex of the aggregate towards the top of the page. Magnification in A, 130×; B and C, 200×.

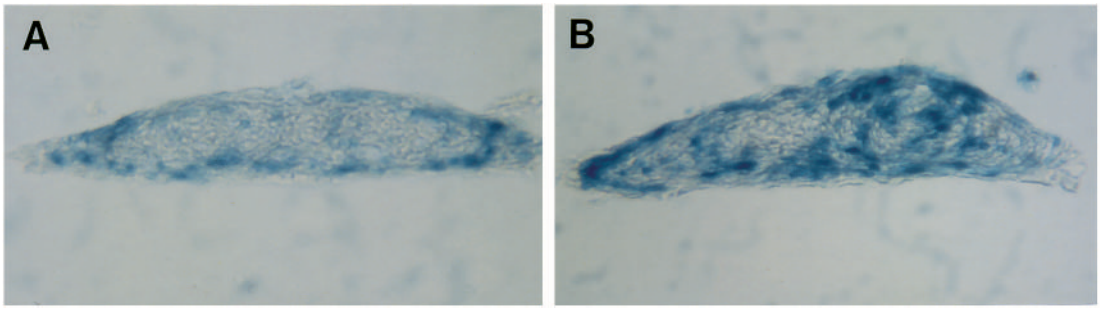

Fig. 5. Stalk cell-specific gene expression within MHC A– aggregates. Longitudinal sections were cut through the middle of representative aggregates the *mhcA* null strain transformed with ST:lacZ and stained for β-galactosidase activity after (A) 22 hours and (B) 36 hours of development. Both sections are orientated with the apex of the aggregate towards the top of the page. Magnification in A, 200×; B, 320×.

In terminally differentiated structures there are pstAB cells in the core

By 36 hours of development the ST:lacZ marker is also expressed in the central core (Fig. 5B), indicating the presence of pstAB cells (Fig. 1). In contrast to the pstAB cells in the cortex, the pstAB cells in the core of MHC A– aggregates are not vacuolated.

Prespore cell differentiation in MHC A– aggregates

Prespore cell differentiation, as defined by expression of the pspA–gal reporter gene, is first detectable at approximately the same time as prestalk cell differentiation. At 15 hours of development stained cells appear to be randomly distributed in the mhcA null mounds (Fig. 6A). Later, the prespore cell distribution is the complement of that observed for the prestalk cells, i.e. there are no stained cells in the periphery and in the core region of the mounds (Fig. 6B,C). Disaggregation of *mhcA* null mounds yields no morphologically identifiable mature spore cells and it was not possible to isolate detergent resistant cells (data not shown). This latter test is a reliable indicator of the presence of very small numbers of viable spores. Thus, although there is terminal differentiation along the stalk cell pathway, in those cells that form the periphery of the aggregate, differentiation along the spore cell pathway is arrested in MHC A– aggregates.

The core of pstA cells in MHC A– aggregates shows some of the characteristic properties of the apical tip.

Since at early times it is composed of pstA cells we reasoned that the core in the centre of MHC A– aggregates might display other properties of the tip and we tested this using adenosine and cAMP, substances which affect tip formation during normal development.

The number of tips formed by an aggregate seems to be controlled by interactions between cAMP and its breakdown product adenosine (Schaap and Wang, 1986). Exogenously added adenosine increases the amount of tissue controlled by a single tip and suppresses the formation of competing tips. Adenosine produces a similar effect on the number of pstA cores formed within mounds of the *mhcA* null strain. When mounds were transferred to agar containing 5′-AMP, before appearance of the pstA cores, the number of cores that subsequently formed was not significantly affected (Table 1). Transfer to concentrations of adenosine of 10^{-3} M and above decreased the number of pstA cores by 40-70% compared to mounds placed on buffered agar. The formation of cores was completely inhibited by 0.1-5 mM cAMP and transfer to these concentrations of cAMP also disrupted previously formed cores (data not shown). These effects of adenosine and cAMP on pstA core formation are very similar to those reported for the apical tip (Nestle and Sussman, 1972; George, 1977; Wang and Schaap, 1985; Schaap and Wang, 1986).

MHC A– cells are unable to enter the tip when synergised with normal cells and cells that differentiate as prespore cells also show an atypical distribution

Synergy experiments, wherein MHC A– cells are mixed with an excess of normal cells, provide the opportunity to observe the behaviour of MHC A– prestalk and prespore cells at stages beyond the tight aggregate stage; the stage at which they arrest when developing alone. Previous studies show that MHC A– cells form spores when mixed with wild-type strains but are excluded from the slug tip (Knecht and Loomis, 1988; Eliott et al., 1993). Here we analyse the individual behaviours of *ecmA*-, *ecmB*- and *pspA*-expressing cells when synergised with normal cells.

MHC A– strains, transformed with the ecmA:lacZ, ecmB:lacZ or psA:lacZ fusion genes, were mixed with an excess of Ax3 cells and allowed to develop on agar. Development appears normal up to the mound stage, the MHC A– cells differentiate into pstA, B and prespore cells, which lie scattered throughout the aggregate (Fig. 7). As development proceeds pstA, pstB and prespore MHC A– cells accumulate in the base of the aggregates and, by tip formation, the majority of MHC A– cells are located in the lower half of the aggregate. Presumably as a result of this asymmetric distribution, the basal half of the aggregate at the first finger stage is significantly thicker than when Ax3 cells are allowed to develop alone. As the MHC A– structure becomes a slug, the base, which contains most of the stained cells, is pinched off and remains on the substratum as a hemispherical mound that does not undergo further morphogenesis.

Chimeric slugs contain some MHC A– cells but again their behaviour differs from that of the Ax3 cells, in that they appear to be defective in their ability to move within the aggregate. Thus MHC A– cells expressing the ecmA:lacZ fusion gene are

entirely excluded from the slug tip, even after prolonged migration. There are very few cells expressing the ecmB:lacZ fusion gene within the slug and the core of pstAB cells in the slug tip is not visible. This cohort of cells is believed to be derived from a subset of the pstA cells that prematurely enter the stalk cell pathway of differentiation (Jermyn and Williams, 1991; Sternfeld, 1992). Given that there are no MHC A– cells that express the *ecmA* gene within the slug tip, the absence of a core of pstAB cells is therefore readily explicable.

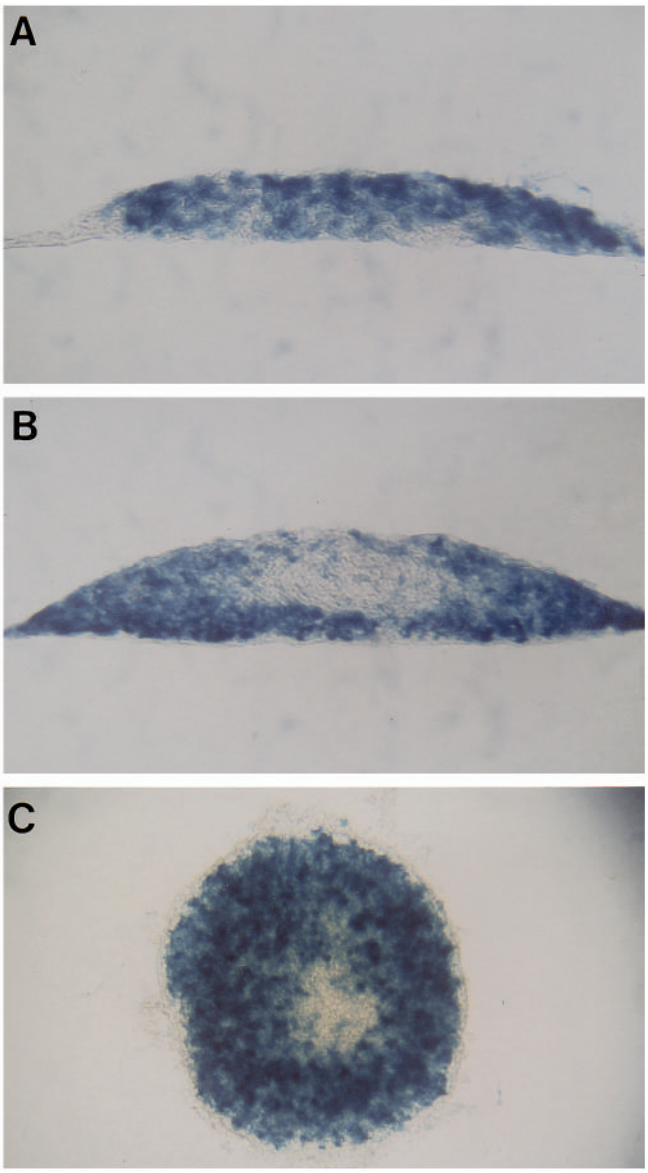

Fig. 6. Prespore cell differentiation within MHC A– aggregates. Longitudinal sections were cut through the middle of representative aggregates of the *mhcA* null strain transformed with the prespore-specific pspA:lacZ construct and stained for β-galactosidase activity after (A) 15 hours and (B) 21 hours of development. (C) A whole mount of an aggregate from the same pspA:lacZ strain and the time of development as in B. Both sections are orientated with the apex of the aggregate towards the top of the page but the whole mount is viewed from above. Magnification: in A and B, 200×; C, 130×.

The apparent inability of MHC A– cells to enter the tip also explains why there are no *ecmA*- or *ecmB*-expressing cells within the stalk at culmination. There is, however, no bar to *ecmB* expression because MHC A– cells expressing the ecmB:lacZ fusion gene accumulate at the prestalk-prespore boundary and at the base of the spore head during culmination (Fig. 7). This is exactly the pattern of gene expression and cellular localisation observed during the normal development of wild-type strains (Jermyn and WIlliams, 1991).

Those MHC A– cells that differentiate down the spore cell pathway of differentiation also display an asymmetric distribution but this only occurs during culmination, when they become selectively localised in the lower half of the emerging spore head (Fig. 7). In contrast to the MHC A– prestalk cells, this aberrant localisation does not interefere with their further differentiation because they go on to form mature spores (Knecht and Loomis, 1998).

MHC A– aggregates are very similar in structure to agglomerates observed in cells deprived of an air-water interface

The distribution of prestalk and prespore cells in MHC A– aggregates is very similar to that previously described for the agglomerates that form when cells are developed under submerged conditions (Sternfeld and Bonner, 1977; Garrod and Forman, 1977; Tasaka and Takeuchi, 1979, 1981; Takeuchi et al., 1988). In these previous studies prestalk cells were identified by their failure to express prespore-specific antigens, by their selective staining with vital dyes and by the morphological appearance of the cells. To confirm the apparent similarity with aggregates formed by MHC A– cells, agglomerates were analysed from Ax2 cells transformed with the same reporter constructs used to characterise the MHC A– aggregates.

With the ecmA:lacZ marker, scattered stained cells are observed 24 hours after cells were placed in the roller tube (not shown) and by 36 hours these pstA cells accumulate within the aggregate (Fig. 8A). At intermediate stages in the generation of agglomerates the prespore cells surround the prestalk cells (Tasaka and Takeuchi, 1979), but by the late stages shown here the prestalk and prespore cells come to lie side by side. There

Table 1. Effects of adenosine on the number of pstA cores formed within mhcA null aggregates

Addition	(M)	Number of cores per mound χ±s.e.m.	(*n*)	% Control
None		2.48±0.43	(170)	100
5′-AMP	5×10^{-3}	2.92±0.71	(70)	117
Adenosine	10^{-6}	2.72±0.20	(68)	110
	10^{-3}	1.44±0.07	(214)	58
	5×10^{-3}	0.65±0.04	(229)	26

The *mchA* null strain transformed with ecmA:lacZ was developed at a density slightly higher than normal to ensure the formation of large mounds which would contain multiple cores. Adenosine at a concentration of 5 mM had additional effects on the morphology of the aggregates and caused a number of them to partially disintegrate, which may explain why the pstA core failed to form in some mounds (on average <1 core was formed). The average number of pstA cores per aggregate is indicated together with number of aggregates scored (in brackets) over two independent experiments.

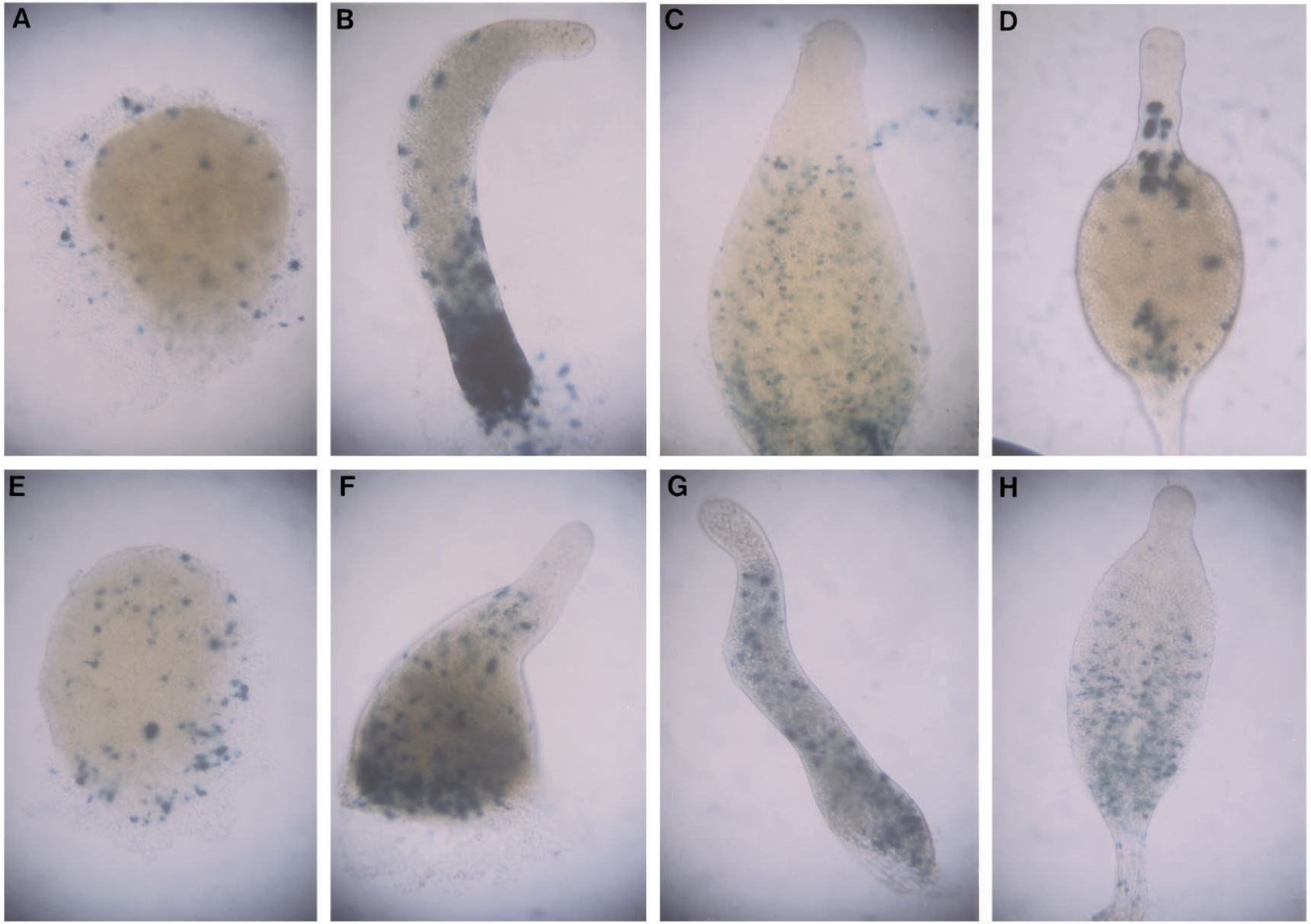

Fig 7. The differentiation and accumulation of MHC A– cells in a synergistic mixture with normal cells. Whole mounts of chimeric aggregates formed during the development of Ax2 mixed with the *mhcA* null mutant (70%:30% respectively) transformed with (A–C) ecmA:lacZ, (D) ecmB:lacZ and (E-H) pspA:lacZ. The aggregates were fixed then stained for β-galactosidase activity and are orientated with the tip upwards except A and E, which are viewed from above. The structures shown are (A,E) mounds, (B) first finger, (C, D,H) culminant (sorogen), (F) extended tipped mound and (G) slug. The magnification in all micrographs is 130× except in D, which is 240×.

is very weak cortical expression of the ecmA:lacZ fusion gene (Fig. 8A) and strong expression of both the ecmB:lacZ (Fig. 8B) and the ST:lacZ fusion genes. (Fig. 8C). The outer cells of agglomerates are vacuolated and are believed to be stalk cells (Garrod and Forman, 1977; Sternfeld and Bonner, 1977; Tasaka and Takeuchi, 1979). The pstAB cells in the cortical layer of the agglomerates are vacuolated showing them to be stalk cells (Fig. 8C; data not shown).

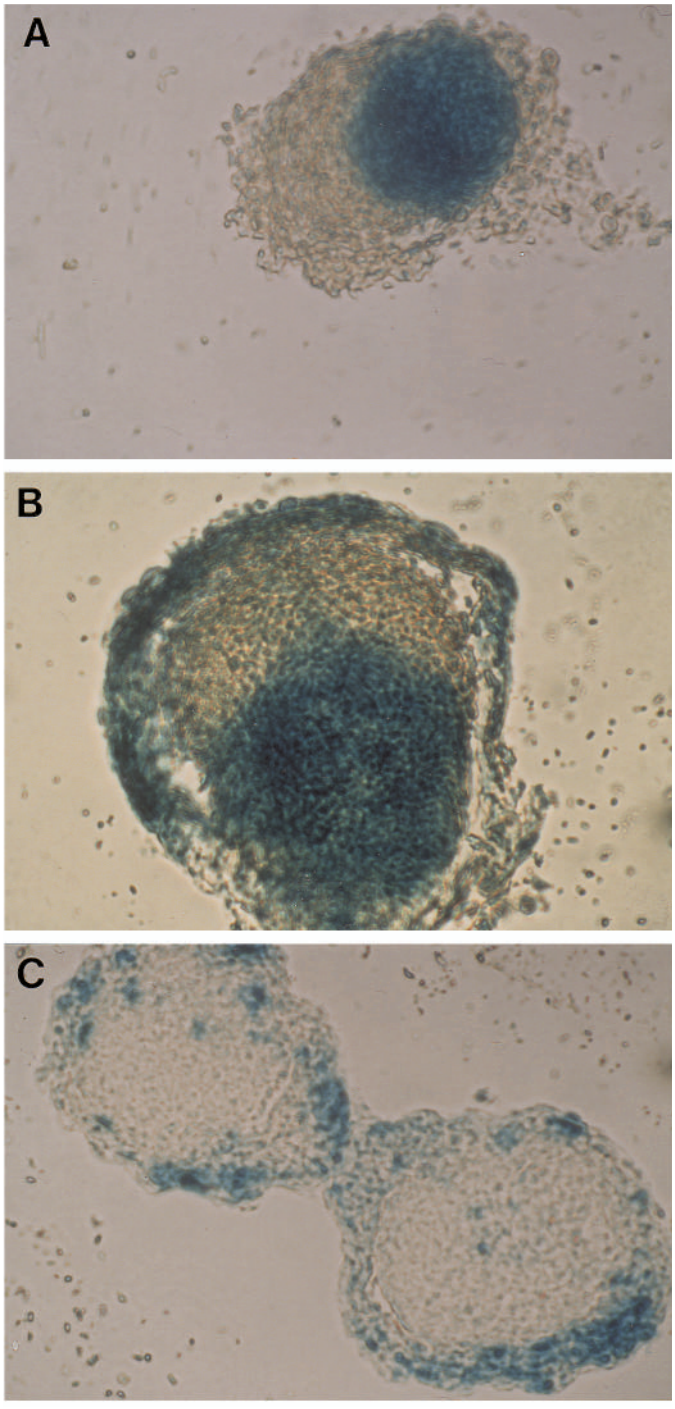

Fig. 8. Prestalk and stalk-specific gene expression within wild-type cell agglomerates formed in rolling culture. Longitudinal sections were cut through the middle of representative aggregates stained for β-galactosidase activity after 36 hours of development in rolling culture. Ax2 transformed with (A) ecmA:lacZ, (B) ecmB:lacZ and (C) ST:lacZ. The magnification in all micrographs is 320×.

This distribution of *ecmB*-expressing cells is very similar to that of the terminal structures formed by MHC A– aggregates (Fig. 1). The major difference between them is that fewer cells within the core of the submerged agglomerates express the ST:lacZ fusion gene (compare Figs 5B and 8C). Thus in the case of the MHC A– aggregates a higher fraction of cells in the core are pstAB cells than are pstB or pstBA cells.

DISCUSSION

We have analysed pattern formation in aggregates of MHC A– cells. Both the initial timing and extent of prestalk and prespore cell differentiation are comparable to those that occur during normal tip formation. Three prestalk cell types, pstA, pstB and pstAB cells, are all present; with pstA and pstB cells appearing first and pstAB cells only appearing at later times of development. Here the direct similarities end, because the differentiated cells in MHC A– aggregates become arranged in a manner quite unlike that of normal aggregates at the equivalent temporal stage (Fig. 1).

Despite this apparent dissimilarity, there may be regions of equivalence between the normal aggregate and the MHC A– aggregate; especially when early stages in their development are compared. During normal tip formation pstA cells migrate to the apex of the tight mound, pstB cells accumulate in the base and prespore cells occupy the intervening region (Williams et al., 1989a,b; Traynor, Abe and Williams, unpublished data; Fig. 1). During the early stages of formation of MHC A– aggregates pstA and B cells are also scattered. PstA cells then accumulate in the central core while pstB cells accumulate in the periphery of the aggregate. Whilst it is not possible to follow these processes directly, we believe that they probably move to these positions in cell sorting processes which mirror those occurring during normal development. If so, then the centre of the MHC A– aggregate must act as the equivalent of the apex of the normal mound, drawing in the pstA cells by chemotactic sorting to cAMP. Since pstB cells accumulate at the periphery of the MHC A– aggregate, the cortex would appear to be equivalent to the normal base. Later in the development of MHC A– aggregates this correlation seems to break down because cortical cells start to express ST:lacZ, a construct which, during normal development, is only expressed within the stalk tube.

The core of an MHC A– aggregate has some properties typical of the apical tip, in that the number of cores formed and their integrity are altered by exposure to exogenous adenosine and by cAMP (Nestle and Sussman 1972; George, 1977; Schaap and Wang, 1986). Subsequent to their movement to the core, a subset of the pstA cells activate expression of the *ecmB* gene and become pstAB cells but they do not become vacuolated. This is again very reminiscent of normal development. PstA cells migrate to the apex of the aggregate and then, during tip elongation, a core of pstA cells within the tip activate expression of the *ecmB* gene to become pstAB cells. These cells are also not vacuolated. During migration the slug occasionally sheds the core cells and, after deposition into the slime trail, they become vacuolated (Sternfeld, 1992). It would

appear, therefore, that conditions within the slug tip repress terminal stalk cell differentiation; even in cells that have made the first step along the stalk pathway by differentiating into pstAB cells, and that the same is true of the core of MHC A– aggregates. It may be that the eventual appearence of stalk cells within the cortex of MHC A– aggregates is a default response, caused by the bar to normal morphogenesis that is imposed by their inability to form a tip.

Our results suggest that MHC A– cells can move within the multicellular environment of the aggregate but they cannot undertake the coordinated cell shape changes and intercalatory cell movements necessary for tip formation and extension. The synergy experiments confirm previous observations, of a defect in cell movement in the late multicellular stages (Eliott et al., 1993), and further show that this defect affects both those MHC A– cells that differentiate as prestalk cells and those that differentiate as prespore cells.

Even though an apparently normal slug tip is formed in synergy experiments, the MHC A– cells that differentiate as prestalk cells are almost entirely excluded from entering it. Hence they are also excluded from the stalk tube at culmination and they remain arrested in their state of differentiation, as pstA or pstB cells. While the prespore cells can differentiate into spore cells (Knecht and Loomis, 1988), they become selectively localised in the lower half of the spore head, as it moves up the stalk at culmination, so they also appear to have a defect in cell movement.

The above interpretation of the behaviour of MHC A– cells is supported by the observation that the agglomerates which form when cells are allowed to develop in the absence of an air-water interface have a very similar structure to the MHC A– aggregates. They have an outer layer containing mature stalk cells and a core containing pstA cells and pstB cells, with the prespore cells initially sandwiched between them but later coming to lie adjacent to the prestalk cells (Tasaka and Takeuchi, 1979). If an agglomerate is placed on a solid substratum the core of prestalk cells form the apical tip of the resultant slug (Takeuchi et al., 1988).

It is because of these similarities in structure, especially the fact that the internal core of prestalk cells in both cases seems to correspond to the tip, that it seems reasonable to draw inferences about the formation of MHC A– aggregates using the agglomerate as a model. Since prestalk cells can be stained with neutral red, it is possible directly to observe their movement within the agglomerates. When slug cells are dissociated and allowed to form agglomerates the prestalk cells are initially scattered but soon migrate to the centre, in a periodic manner, to form the core (Takeuchi et al., 1988). This type of movement is strongly suggestive of chemotaxis to cAMP signals emanating from the centre of the agglomerate. If the parallel with agglomerates is correct this would suggest that a similar chemotactic process is occurring in MHC A– aggregates.

During normal development, tip formation always occurs at the apex of the mound. The characteristic organisation observed in cell agglomerates, formed by agitating cells in suspension, is believed to result from the effective removal of the air-water interface. Under these depolarizing conditions the centre of the aggregate seems to become the dominant signalling centre and the prestalk cells migrate to it. Thus the signalling centre appears to remain at the apex of the aggregate only when tip formation is possible. When tip formation at the apex of the aggregate is not possible, a pseudo-tip is formed within the centre of the agglomerate. We suggest that the same default pathway occurs in MHC A– cells as occurs in agglomerates; because tip formation is not possible the pstA cells accumulate in a central core. These observations seem likely to be relevant to other mutants that block at the tight aggregate stage of development. The apparent inability of cells that have cytoskeletal defects to undertake similar morphogenic movements may explain why removal of other cytoskeletal components, such as F-actin cross-linking proteins, results in arrest at this stage of development (Witke et al., 1992). It will be of interest to determine the structure of such aggregates.

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