

Establishment and maintenance of stable spatial patterns in *lacZ* fusion transformants of *Polysphondylium pallidum*

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Summary

Polysphondylium pallidum cells were transformed with a construct containing the *Dictyostelium discoideum* *ecmA* promoter fused to a *lacZ* reporter gene. Two stably transformed lines, one in which β -galactosidase (β -gal) is expressed in apical cells of the fruiting body (p63/2.1), and one in which it is expressed in basal cells (p63/D), have enabled us to infer how cells move during aggregation and culmination. Several types of cell movement proposed to occur during slime mold culmination, such as random cell mixing and global cell circulation, can be ruled out on the basis of our observations.

Cells of the two transformant lines express β -gal very early in development. In both cases, stained cells are randomly scattered in a starving population. By mid to late aggregation, characteristic spatial patterns emerge. Marked cells of p63/2.1 are found predominantly at tips

of tight aggregates; those of p63/D accumulate at the periphery. These patterns are conserved throughout culmination, showing that marked cells maintain their relative positions within the multicellular mass following aggregation.

Neither the apical nor the basal pattern appears to be regulated within the primary sorogen by de novo gene expression or by cell sorting as whorls are formed. However, marked cells within a whorl re-establish the original pattern in secondary sorogens. This must be achieved by cell migration, since β -gal is not re-expressed.

Key words: *Polysphondylium pallidum*, cellular slime molds, development, patterning, cell sorting, promoter-*lacZ* fusions.

Introduction

Development in the cellular slime molds provides an excellent opportunity to examine cell movement and differentiation in the absence of cell division. When a population of amoebae starve, they cease dividing and aggregate into mounds. These mounds elongate to form finger-like structures, which in many species then fall over onto the substratum and undergo a period of migration. During culmination, each multicellular mass (now called a sorogen) is led by the tip off the substratum as a central stalk differentiates. Ultimately, a mature fruiting body is formed, consisting of spore and stalk cells.

The slime mold genus *Polysphondylium* provides special opportunities for studying these developmental processes. During culmination of the sorogen, cell masses are released at regular intervals at the sorogen base (Harper, 1929; Spiegel and Cox, 1980). These cells differentiate into whorls of secondary (2°) fruiting bodies arrayed at right angles to the primary (1°), central, stalk (Cox et al., 1988). Fruiting bodies within each whorl are evenly spaced (Cox et al., 1988). The regular spacing of whorls along the stalk, and the even distribution of 2° fruiting bodies within whorls, lend a

special fascination to studies of morphogenesis in this organism.

Once a whorl of cells is pinched off from the base of a 1° sorogen, it forms a distinct population which no longer interacts with the rest of the developing sorogen. Thus the morphogenetic history of each sorogen is recorded unambiguously in the 2° fruiting bodies arranged linearly along the central stalk. By analyzing a fruiting body that contains several whorls, and the 2° sorocarps which derive from them, it is thus possible to study the fate of the cells that cooperated to form the primary sorogen.

In the studies reported here, we transformed *P. pallidum* with a vector carrying a *D. discoideum* prestalk promoter fused to β -gal (Jermyn and Williams, 1991). The clones that we obtained expressed β -gal stably and with several different spatial and temporal patterns. Here we report on the use of two of them to mark cell subpopulations and examine their spatial distribution during aggregation and culmination.

Materials and methods

Transformation

All manipulations were carried out at 21°C. Prior to

transformation, *P. pallidum* PN500 cells (Francis, 1975) were grown axenically in Bis-Tris HL-5 medium (Knecht et al., 1986). Cells were transformed essentially as described by Nellen et al. (1984), using 5–10 μg of the *D. discoideum* promoter fusion construct, p63 NeoGal (generously provided by A. Harwood and J. Williams; Jermyn and Williams, 1991). After incubation overnight, cells were plated in the presence of 200 $\mu\text{g ml}^{-1}$ G418 on lawns of *Escherichia coli* strain B/r-1, a neomycin-resistant derivative of strain B/r (provided by J. Hughes and D. Welker). This antibiotic concentration was necessary to prevent growth of untransformed *P. pallidum* cells. Transformants from well-separated plaques were re-cloned on bacterial streaks and propagated in the presence of 200 $\mu\text{g ml}^{-1}$ G418.

Histochemical staining

Cells were grown axenically to prevent contaminating β -gal activity from *E. coli*. They were typically grown in 60 mm Petri dishes containing 3.5 ml of HL-5 (Watts and Ashworth, 1970) with 200 $\mu\text{g ml}^{-1}$ G418. They were harvested, centrifuged at 3000 *g* for 2 minutes, rinsed once in phosphate buffer (PB, 8.35 mM Na_2HPO_4 , 8.35 mM KH_2PO_4 , pH 6.2), and dispensed in monolayers upon 6 \times 6 mm squares of dialysis membrane placed on 2% agar plates (2 g agar in 100 ml distilled H_2O). At various developmental times, cells were fixed and stained with X-gal (5-bromo-4-chloro-3-indoyl- β -D-galactopyranoside) as described by Dingeramn et al. (1989). Triton X-100 was added to the fixing solution to 0.5% and, for transformant p63/D, the staining solution was diluted tenfold. Staining was usually apparent within 5 to 10 minutes at room temperature and was allowed to continue for 60 minutes or longer before mounting in 90% glycerol, 10% PB. Specimens were photographed on a Zeiss Photomicroscope III using Kodak Ektar 25 film.

Results

P. pallidum cells were transformed with the plasmid construct, p63 NeoGal. This construct contains the promoter of the *D. discoideum* prestalk gene, *ecmA* (Williams et al., 1987), fused to β -gal (Jermyn and Williams, 1991). It also contains the *neo* gene, which confers resistance to G418. A battery of β -gal-expressing transformants was isolated. Untransformed cells did not stain (data not shown). Each clone was assigned to one of several distinct categories on the basis of its characteristic staining pattern. For each clone, the pattern has proved to be stable and reproducible. Evidence from Southern blotting and PCR experiments shows that the transformants have integrated at different sites and in different genetic arrangements in the genome (C. D. Vocke, M. I. Carrin and E. C. Cox, unpublished data).

Two transformants from our collection exhibit the following staining patterns. p63/2.1 expresses β -gal predominantly in cells in the apical half of the 1° sorogen. In contrast, p63/D stains at the base of the 1° sorogen; additional β -gal-expressing cells are scattered randomly throughout the sorogen. These two clones were chosen to examine the origin and subsequent location of apical and basal cell populations during development.

Early cell patterns in an apically staining transformant, p63/2.1

Freely dividing amoebae begin to group together to form small mounds about 1 hour after starvation. At this time, p63/2.1 β -gal expression is very weak; no stained cells are detected after a 30 minute staining period (data not shown). However, when the staining reaction is allowed to proceed for 5 hours, a few faintly stained cells can be detected (Fig. 1A). By the time aggregation streams are readily apparent, following the loose mound stage, expression is stronger (detected within 10 minutes of staining) and is evident in a greater numbers of cells (Fig. 1B, C). Marked cells appear to be randomly scattered throughout the aggregate and within the incoming streams. As aggregation nears completion (Fig. 1D, E), most of the marked cells have moved to the center of the aggregate (note the peripheral arrangement of unstained cells in tight aggregates, Fig. 1E). As the late aggregate elongates into a finger, most of the marked cells are positioned in the apical half (Fig. 1F). Thus, a recognizable pattern of expression in the anterior has been established by late aggregation.

The p63/2.1 staining pattern is maintained throughout culmination

The youngest culminants are those that have recently risen off the substratum and have short stalks and no whorls. In these sorogens, expression is confined to a subset of cells positioned within the apical one-third to one-half of the multicellular body (Fig. 1G). β -gal activity also persists in the dead and highly vacuolated mature stalk cells. This result demonstrates that β -gal is stable in *P. pallidum*, as it is in comparable experiments in *D. discoideum* (Dingeramn et al., 1989; Haberstroh and Firtel, 1990; Esch and Firtel, 1991; Jermyn and Williams, 1991; Harwood et al., 1991; Ceccarelli et al., 1991) and many other organisms (Bassford et al., 1978; Rose et al., 1981; Guarente and Ptashne, 1981; Lis et al., 1983; Fire, 1986; Goring et al., 1987; Teeri et al., 1989).

The distribution of stained stalk cells appears to be random regardless of stalk length (see stalks in Fig. 1 micrographs; other unpublished observations). This randomness is probably determined by the order of deposition of cells at the tip into the stalk tube. Since only a subset of cells in the apical region of p63/2.1 express β -gal, stained cells are intermingled with unstained cells as the stalk forms. By this criterion, β -gal-expressing cells represent about 20% of the cells in the apical section of the 1° sorogen.

The expression pattern established by late aggregation is maintained in older culminants. The first whorl mass to be pinched from the base of the ascending 1° sorogen contains few, if any, marked cells, and the boundary between the expressing and the non-expressing zones, though not absolute, is maintained (Fig. 1H). In culminants with multiple whorls, the oldest, most basal, whorls are largely unmarked, whereas whorls that pinch off above the original apical/basal boundary contain marked cells in the same proportion as the

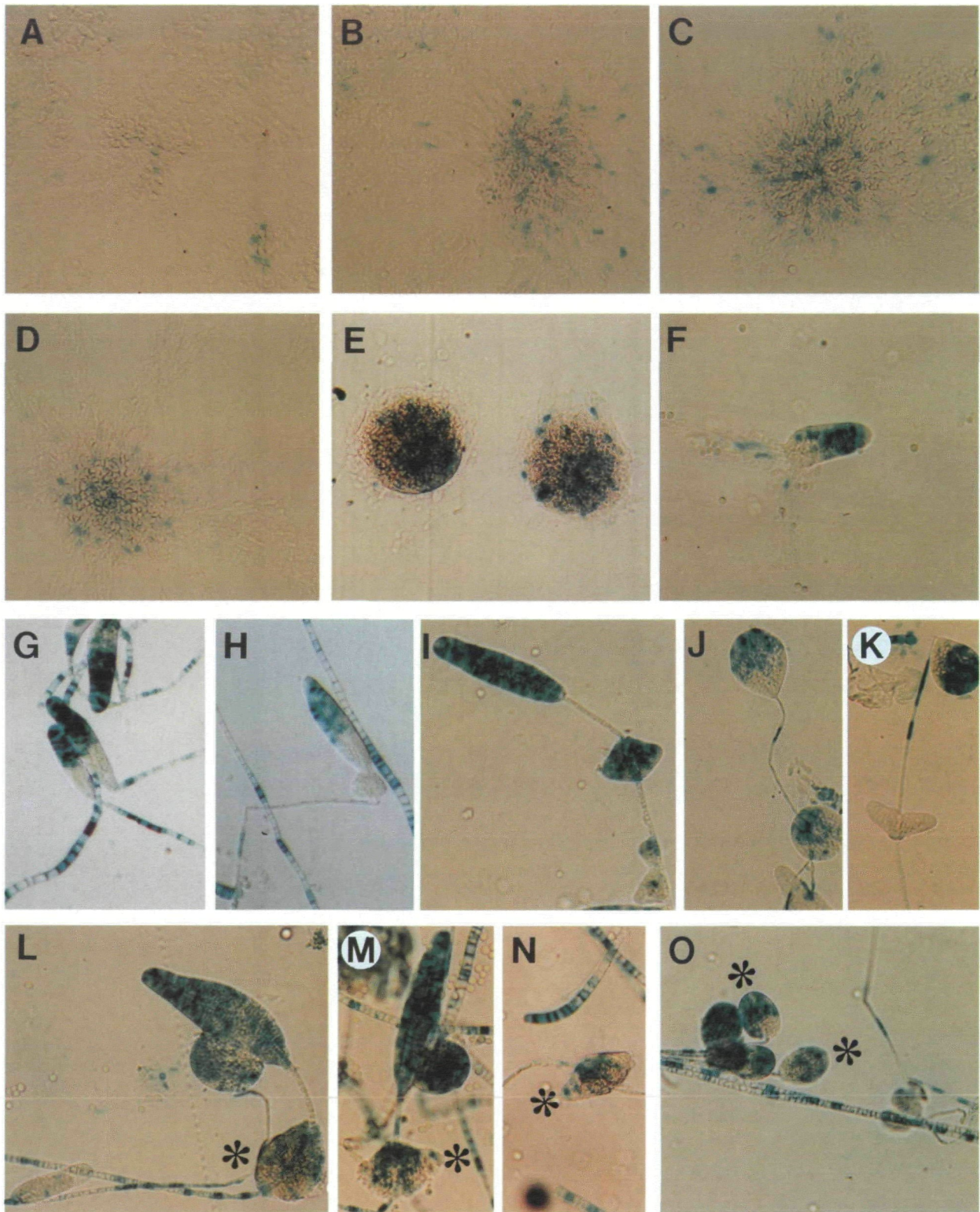


Fig. 1. Histochemical staining of developing p63/2.1 transformants. Cells were developed for (A) 1, (B) 3.5, (C) 4.5, (D) 5.5, (E) 6.5, (F) 16, and (G-O) 20-24 hours, and were then fixed and stained with 1 mM X-Gal. (G) 1° sorogens with no whorls. (H) 1° sorogen with one whorl. (I) 1° sorogen with 3 whorls. (J, K) 1° sorocarps with an unstained whorl of 2° sorogens. (L) 1° sorogen with a half-stained whorl mass (asterisk). (M, N) half-stained 2° whorl masses (asterisks). (O) half-stained 2° sorocarps (asterisks). Sample (A) was stained for 5 hours; (B-O) were stained for 1 hour. All magnifications $\times 167$.

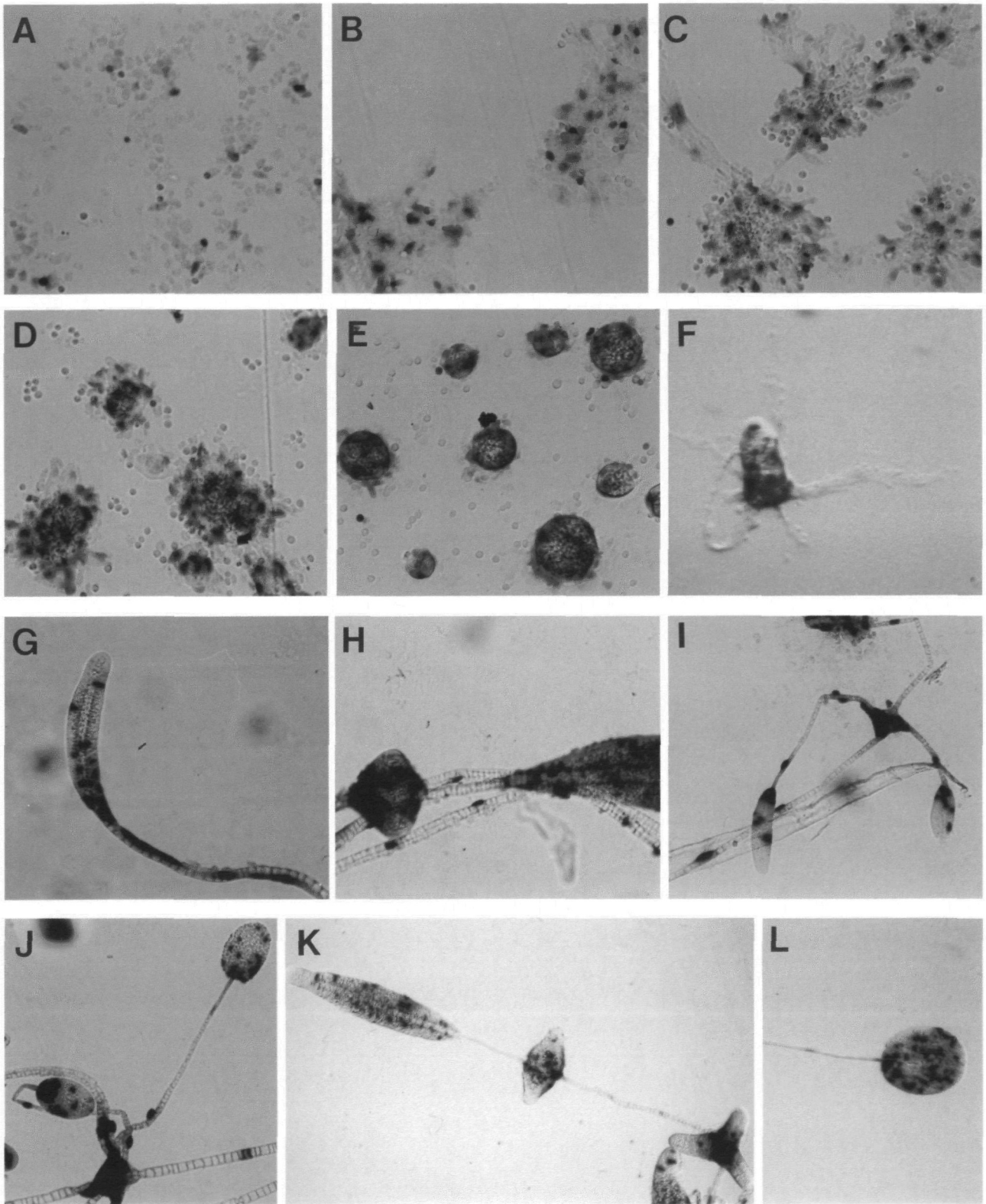


Fig. 2. Histochemical staining of developing p63/D transformants. Cells were developed for (A) 0, (B) 3.5, (C) 4.5, (D) 5.5, (E) 6.5, and (F-L) 20-24 hours, and were then fixed and stained with 100 mM X-Gal (G) 1° sorogen with no 2° whorls. (H) base of a 1° sorogen and first 2° whorl. (I) basal whorl of 2° sorogens (J) 2° sorocarps. (K) 1° sorogen with 2 whorls. (L) 1° sorocarp. All samples were stained for 1 hour. Magnifications: (A-H, J, L) $\times 167$, (I) $\times 133$; (K) $\times 108$.

apical region of a young 1° sorogen (Fig. 1I). If the fully differentiated fruiting body is relatively small and contains few sets of whorls, the staining boundary is commonly found within the mature 1° sorocarp (Fig. 1J, K). The apical/basal staining pattern is therefore stable from late aggregation through to the completion of development.

Occasionally, whorls are observed that contain stained cells only in the apical half (asterisk, Fig. 1L), even if 2° tips have begun to form (asterisks, Fig. 1M, N). Thus, the process of whorl mass formation does not disrupt the characteristic apical/basal staining pattern of the 1° sorogen. Whorls of fully differentiated 2° sorocarps have a staining boundary orthogonal to the 2° stalk (asterisks, Fig. 1O). This suggests that after 2° tip formation, marked cells alter their position relative to unmarked cells by moving toward the apex of the 2° sorocarp.

Early cell patterns in a basally staining transformant, p63/D

Transformant p63/D has a very high level of expression relative to p63/2.1; it was necessary to dilute the substrate tenfold to prevent overstaining of the specimens during the 1 hour staining assay. β -gal expression is clearly present in a subset of starved cells, even before the onset of aggregation (Fig. 2A). Many of the darkest staining cells are perfectly round rather than amoeboid.

During early to mid aggregation, the staining pattern of p63/D is similar to p63/2.1; marked cells are scattered at random within the aggregate (Fig. 2B, C; compare with Fig. 1B, C). However, in late streaming (Fig. 2D) and tight aggregate (Fig. 2E) stages, most of the marked cells are found outside the center of the mound. In fingers (Fig. 2F), they have formed a band at the base. Positioning of β -gal-expressing cells in transformant p63/D therefore occurs at about the same time as in p63/2.1, although the staining pattern is roughly reciprocal.

Maintenance of the p63/D pattern mirrors that of the p63/2.1 pattern

Primary sorogens that have not formed whorls contain β -gal-expressing cells at the base; other marked cells are scattered throughout the sorogen (Fig. 2G). Additional characteristics of this transformant include staining of amorphous blobs along the sides of the stalk and some diffuse staining of the slime sheath (Fig. 2G-L). The blobs may be bits of extracellular matrix deposited at random along the stalk, since they do not appear to be defined by cell membranes.

The first whorl mass to be pinched off from the 1° sorogen invariably receives the greatest portion of marked cells (Fig. 2H). The most basal hemisphere of the whorl mass is abundantly marked; however, the apical hemisphere also contains marked cells. Secondary sorogens in the oldest whorls (Fig. 2I) re-establish the pattern characteristic of the 1° sorogen; a cobweb-like deposit of cells and slime is also commonly seen at

the junction of 1° and 2° stalks (Fig. 2I). Differentiated 2° sorocarps are basally stained (Fig. 2J).

As the culminant pinches off successive whorl masses, fewer and fewer marked cells remain with the 1° sorogen. In Fig. 2K, marked cells in the older whorl have remained at the base of the 2° arms. The youngest whorl has the fewest marked cells, and they are located basally. As in Fig. 2H, the basal surface is more darkly stained. Marked cells remaining in the 1° sorogen appear to be randomly scattered; they do not appear to sort to the base. Differentiated 1° sorocarps sometimes show slightly more basal staining (i.e., where they join the stalk), but the staining pattern is much more random for 1° sorocarps than for 2° (Fig. 2L, compare with Fig. 2J). The proportion of cells marked in 1° sorocarps is similar to the proportion of randomly distributed marked cells in older 1° sorogens; some diffuse staining is also commonly seen (Fig. 2L, compare with Fig. 2K).

Mature spores and stalk cells contain β -galactosidase

The *ecmA* gene of *D. discoideum* encodes an extracellular matrix protein found in prestalk and anterior-like cells of migrating slugs, and in stalk, upper and lower sorocarp cups, and basal disc cells in culminants; it is not found in prespore or spore cells (Williams et al., 1987; Jermyn et al., 1989; Jermyn and Williams, 1991). In contrast, in both p63/D and p63/2.1, β -gal staining is observed in mature spore cells (Fig. 3, see also Figs 1, 2), as well as in mature stalk (Figs 1, 2). Thus, the prestalk specificity of the *ecmA* promoter has not been maintained in these transformants (and see Discussion).

A summary of the progression of staining patterns in p63/2.1 and p63/D through the various developmental stages is shown in Fig. 4.

Discussion

This is the first comprehensive *P. pallidum* study in which stable cell markers have been used to examine the establishment of spatial patterns in aggregation, and the subsequent fate of these patterns during the formation of the mature fruiting body. Each transformant expresses β -galactosidase early in development, establishes a distinctive pattern midway through aggregation and maintains this pattern throughout culmination. The boundaries between apical and basal cells, once established, are stably maintained in both the 1° and 2° sorogens (Fig. 5). This stability argues against random cell mixing, global cell circulation, or other large scale cell movements.

Cell movement during aggregation

In both p63/2.1 and p63/D, the number of marked cells and the intensity with which they stain remains approximately constant from mid aggregation onwards (see Figs 1, 2, other results not shown). Since cells are initially stained in an apparently random manner, we assume that induction of β -gal expression corresponds

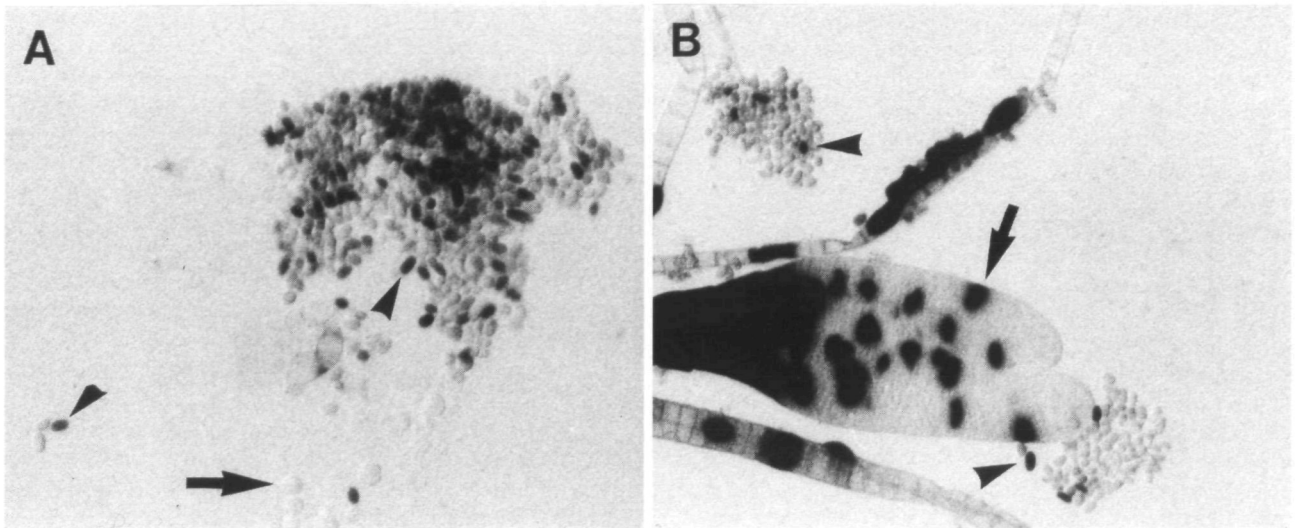


Fig. 3. Stained spores in p63/2.1 and p63/D transformants. Mature spores of p63/2.1 (A) and p63/D (B) were stained for 1 hour. Arrowheads point to mature spore cells stained with β -gal. Spores can be distinguished from amoeboid cells (arrows) by their smaller size and ovoid shape. Magnification $\times 330$.

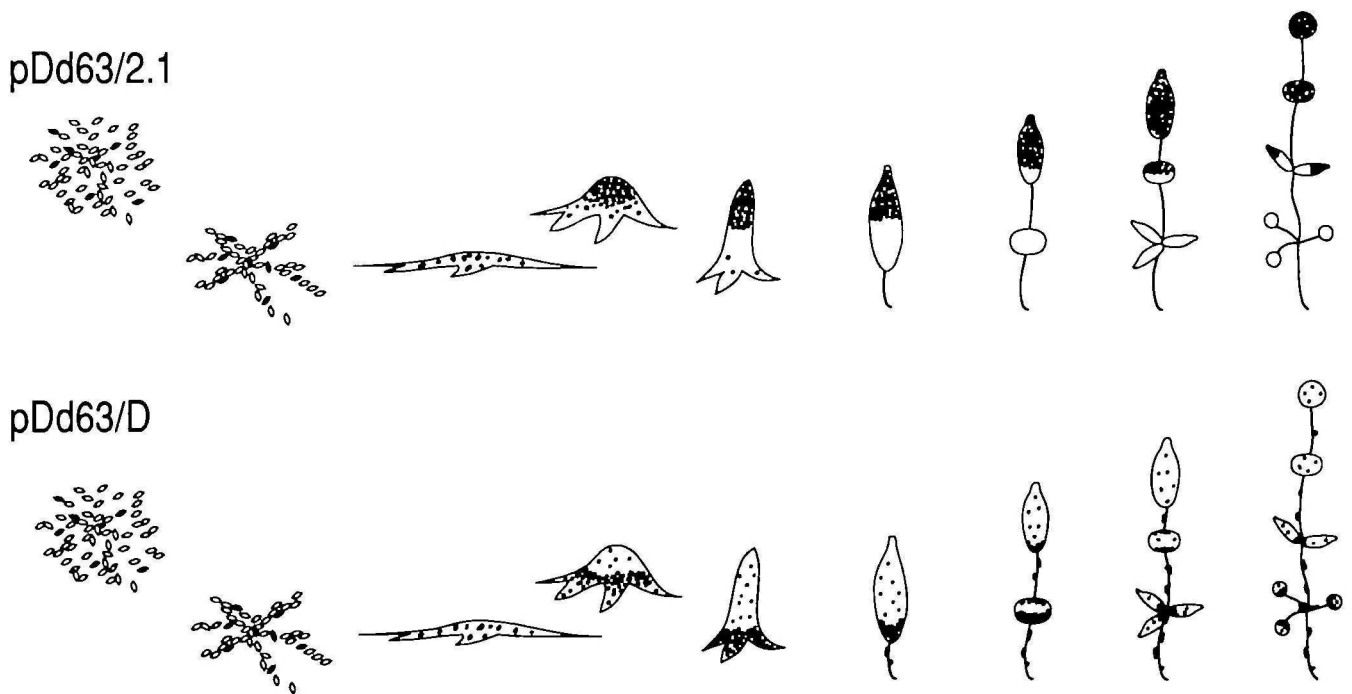


Fig. 4. Schematic drawing of the staining patterns in transformants p63/2.1 and p63/D during aggregation and culmination.

to a stage in differentiation which biases these cells to migrate to a particular location, and that these transformants reveal regions in the genome related to this decision. One possibility is that β -gal expression in p63/2.1 and p63/D is driven by cell-cycle-specific elements that determine commitment to apical or basal regions of the sorogen. This would be analogous to *D. discoideum*, in which prestalk and prespore fates are thought to be influenced by the point in the cell cycle at which cells become starved (Weijer et al., 1984;

McDonald and Durston, 1984; McDonald, 1986; Gomer and Firtel, 1987).

Marked cells in each transformant exhibit distinctive migration properties. Under our experimental conditions, cells are relatively densely packed when first starved. This means that cells destined to become positioned at the center of a mound must migrate farther, on average, than those destined for the periphery. This is much like the filtration of 'faster' *D. discoideum* prestalk cells through the 'slower' prespore

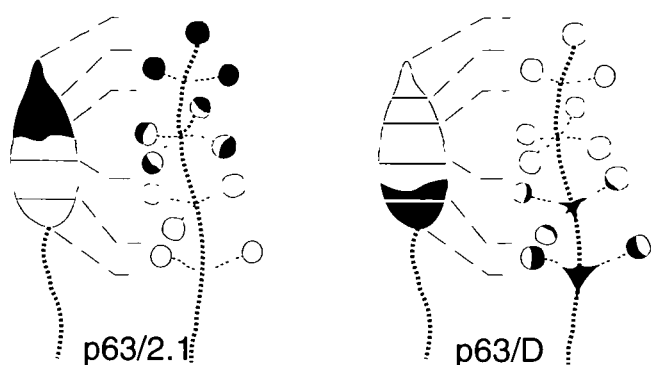


Fig. 5. Maintenance of cell position throughout late morphogenesis

region to the anterior when they have been grafted onto a slug posterior (Bonner, 1952, 1959). Factors correlated with relative rates of cell movement in *D. discoideum* prestalk and prespore cells are differences in densities of individual cells (Lam et al., 1981; Schaap et al., 1982), cell adhesiveness (Lam et al., 1981; Lam and Siu, 1982), and chemotaxis of prestalk cells to cAMP or other factors (Takeuchi, 1969; Matsukuma and Durston, 1979; Inouye and Takeuchi, 1982; Mee et al., 1986). Perhaps the relative positions of the marked cells observed here are determined by similar forces. Interestingly, the properties exhibited by the β -gal expressing cells bear no obvious relationship to ultimate cell fate (i.e., stalk or spore). Rather, they are involved only in cell localization.

There is no large-scale mixing during P. pallidum culmination

Once the marked cells of either transformant have found their "niche", they do not continue to move relative to unmarked cells found in the same region. Thus, randomly distributed marked cells of p63/D remaining within an older 1° sorogen that has lost basal staining to 2° whorls, do not subsequently sort to the base. One possible explanation for this is that β -gal is regulated in two populations: one that exhibits a base preference and one that has no apparent spatial preference. Alternatively, those marked cells scattered throughout the apical regions of the sorogen may have ceased expression and lost their base preference, but are nonetheless permanently marked. Additional experiments would have to be performed to distinguish between these possibilities.

In p63/2.1, only one staining boundary ever exists along the axis of a 1° sorogen. It moves basally with time. This confirms that marked and unmarked apical cells in the 1° sorogen do not sort out during culmination. If it were otherwise, recurring apical/basal boundaries would be generated as each whorl mass is deposited. In addition, the ratio of marked to unmarked cells near the apex, and therefore in the stalk itself, would increase as culmination progresses. In fact, neither of these phenomena is observed, suggesting that cells remain in a fixed position relative to the rest of the 1° sorogen, regardless of how many 2° branches are

produced. This results in the observed stable pattern, and is summarized in Fig. 5.

Small-scale cell movements may still occur

Our results do not rule out the possibility of directed migrations of small numbers of cells. During *D. discoideum* culmination, for example, anterior-like cells, which had been randomly distributed during slug migration, accumulate either at the prestalk-prespore boundary or at the base; they ultimately form an upper cup and a lower cup, respectively, on the mature spore mass (Sternfeld and David, 1982; Jermyn et al., 1989; Jermyn and Williams, 1991; Ceccarelli et al., 1991). Similar cell movement by a minority population could also occur during *P. pallidum* culmination; it would not have been detected in the experiments reported here.

Secondary tip compared to primary tip formation

β -gal is not re-expressed during 2° sorogen formation in these transformants. Instead, β -gal distribution in 2° cell masses appears to be dependent only on whorl position relative to the 1° sorogen axis: basal whorl masses of the apically staining p63/2.1 transformant are unmarked and do not express β -gal as 2° tips are formed; basal 2° sorocarps are also unmarked. Even after a 24 hour staining assay, β -gal expression was not observed in basal whorls, arguing against the possibility that there is a low level of de novo β -gal expression in 2° tips. In addition, nascent whorl masses that contain marked cells are stained apically relative to the 1° sorogen, not the 2° sorogen. This differs from the regulation of a number of tip-specific antigens identified by monoclonal antibodies previously studied in this laboratory. Antigens Tp200, Tp423 and Pg101 are expressed apically in 1° sorogens, and are then re-expressed in 2° tips of all whorls, regardless of whorl position (Byrne and Cox, 1986, 1987).

Together, these results suggest to us that β -gal expression in p63/2.1 and p63/D define very early events in a morphogenetic pathway and that the expression of the tip-specific antigens occurs later. Alternatively, β -gal expression may define a morphogenetic pathway for 1° tip formation that is not recapitulated in 2° sorogens. Most tip cells in 2° but not 1° sorogens contain prespore vesicles (Hohl et al., 1977), are electron opaque (Schaap et al., 1985) and are stained by antispore sera (O'Day, 1979; Vocke and Cox, 1990); thus 1° and 2° tips are not identical.

Our results provide convincing evidence for directed cell migration in 2° sorogens. For example, marked cells in newly formed whorls of p63/2.1 are not found at 2° tips, but are initially oriented toward the apex of the 1° sorogen (Fig. 1L-N). The fact that one hemisphere of a young whorl mass may contain marked cells, while the other one does not, makes it unlikely that one of the driving forces for whorl detachment is random movement of basal cells as they lose their chemotactic or chemokinetic attraction to the 1° tip, as we have postulated (Cox et al., 1988). After 2° tips form, however, the marked cells move toward the apices of the 2° sorogens, maintaining cell identities that were

established early in aggregation. The reciprocal phenomenon is observed with p63/D.

P. pallidum compared to *D. discoideum*

The *D. discoideum* *ecmA* promoter regulates an extracellular matrix protein exclusive to prestalk cell and anterior-like cells of slugs, and to stalk, upper and lower sorocarp cups, and basal disc cells in culminants; it is not found in prespore or spore cells (Jermyn and Williams, 1991; Williams et al., 1987; Jermyn et al., 1989). Although this promoter is present in the vector used to create *P. pallidum* transformants p63/2.1 and p63/D, the transformants express β -gal in cells destined to become spores as well as in those destined to become stalk (Fig. 3). Thus the spatial specificity of the *ecmA* promoter has been lost in these transformants.

Early starvation and aggregation: Our results in *P. pallidum* resemble most closely those obtained by Tasaka and Takeuchi with *D. discoideum* (1981). They used ^3H thymidine-labeled cells to mark subpopulations first purified from glucose-deprived cells (largely prestalk) and unlabeled cells grown in glucose (enriched for prespore cells). They reported that cell sorting occurs at about the time tipped aggregates form, and this is similar to *P. pallidum*. Other experiments in the literature, while broadly similar to those reported here, used cell marker methods such as vital dyes or antibody staining of prespore vesicles (reviewed in Weijer et al., 1987), or electron opacity (Schaap et al., 1985), which are by their nature somewhat ambiguous due to cell type interconversion and/or turnover rates of gene products.

Sorogen movement and culmination: Early studies in *Dictyostelium discoideum* made use of *Serratia marcescens* (Raper, 1940) or the vital dye Neutral Red (Bonner, 1952, 1959) to mark cells situated in the anterior of the slug. Grafting and dissociation experiments (Raper, 1940; Bonner, 1952; Sternfeld and David, 1981) with stained and unstained cells provided evidence for cell sorting of prespore and prestalk populations to their respective zones in migrating slugs. Odell and Bonner (1986) believe that there may also be some global cell circulation along the length of a migrating slug, and that this movement may be responsible for locomotion. Some evidence is available for such circulation, again using grafts of Neutral Red-stained cells (Odell and Bonner, 1986). Cells stained with vital dyes, however, can interconvert; thus, these dyes are not stable cell-specific markers. Using β -gal-stained transformants in experiments similar to those reported here, Harwood et al. (1991) showed that there is some cell mixing during *D. discoideum* slug migration, and that the degree of mixing is directly proportional to the time of slug migration.

In our experiments *P. pallidum* slugs do not migrate. Culmination proceeds directly from the center of the aggregate. It seems possible, then, that if *D. discoideum* were studied under similar conditions with the appropriate cell-specific marker, the results would be similar to ours. Neither the presence of prespore vesicles nor electron opacity have served as a suitable marker for

cell sorting in *Polysphondylium*, because prespore cells are constantly being converted into stalk during culmination (Hohl et al., 1977), and because electron-opaque cells are distributed randomly in both aggregates and 1° sorogens (Schaap et al., 1985).

A mechanism for coordinated cell movement during sorogen morphogenesis in *P. pallidum* must now be sought. Those involving global cell mixing are ruled out by our results.

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