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Positional Information and Whorl Morphogenesis

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Cellular slime molds of the genus *Polysphondylium* periodically release cell masses from the base of culminating fruiting bodies. These masses quickly undergo a change in symmetry from spherical to radial as they differentiate into distinctive arrays of secondary fruiting bodies arranged about a primary axis of stalk cells. Here we show that a major event in whorl morphogenesis is the activation of a prestalk-specific promoter early and globally in newly forming whorls. With time, transcript synthesis and amplification become restricted to the equator of the whorl and then to patches which define where secondary tip morphogenesis will occur. The localization of early prestalk message synthesis depends on positional information, in contrast to the establishment of early prestalk/prespore patterns in both *Polysphondylium* and *Dictyostelium*.

INTRODUCTION

Polysphondylium pallidum cells begin their life cycle as free-living amoebae that feed on bacteria, grow, and divide. When the food is exhausted, cells signal to each other and form aggregation territories which further differentiate into fruiting bodies containing stalk and spore cells. During the formation of fruiting bodies, *Polysphondylium* releases cell masses from the base of the culminating sorogen, and these masses soon form whorls of secondary (2°) fruiting bodies arrayed symmetrically about the central or primary (1°) stalk (Figs. 1 and 2).

Antigens characteristic of sorogen tips (Byrne and Cox, 1986) first appear over the nascent whorl surface, just before it is released. With time, their distribution becomes restricted to a band around the circumference of the whorl, and this band breaks up into patches. Symmetrical patches become organizing centers for 2° tips, while asymmetrically arranged patches die away. At this point, there is no visible change in whorl shape. Visible sorogen tips then begin to form, positioned by the radial prepattern. The entire process, from the first appearance of a bulge at the base of the sorogen to the appearance of radially symmetric small visible tips on the whorl surface takes about 20 min. Secondary branch formation then proceeds, and the final result is a symmetrical whorl of fruiting bodies. Quantification of the spatial distribution of tip-specific antigens and numerical results suggest that whorl morphogenesis is regulated by a reaction–diffusion mechanism (Byrne and Cox, 1987).

Studies with monoclonal antibodies leave unanswered major questions, preeminetely the extent to which whorl prepatterns are explained either by the differentiation of prestalk cells at random followed by cell sorting or by specification of cell identity by gradients of positional information. To answer this question genetic markers for cell type are needed.

In this study we have transformed *Polysphondylium* with a *Dictyostelium discoideum* prestalk promoter P_{ecmB} fused to *lac*Z (P_{ecmB}:*lac*Z). *ecmB* is a pre-stalk- and stalk-specific gene that codes for an extracellular matrix protein EcmB, which is secreted into slime sheath and stalk tube (McRobbie and Ceccarelli, 1988; McRobbie *et al.*, 1988a,b). Using this promoter, we show that single and multiply integrated copies of this construct respond to cues in *Polysphondylium* that correspond in a homologous fashion to temporal and spatial cues in *Dictyostelium*. Analysis of the regulation of P_{ecmB}:*lac*Z reveals that a major early event in whorl morphogenesis is derepression of the prestalk pathway and that the early spatial restriction of prestalk cells to the equator of the whorl occurs by restriction of prestalk transcription in response to positional cues.

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MATERIALS AND METHODS

Growth and Development of Polysphondylium

Amoebae were propagated on *Escherichia coli* K12 WBS99 (Snyder and Silhavy, 1992). WBS99 carries a *lac* deletion and a Tn5 insertion. The insertion confers resistance to G418 and disrupts the synthesis of capsular polysaccharide, which normally prevents slime molds from growing on *E. coli* K12 (Farnsworth and James, 1972). Cells were grown on lawns of WBS99 until the lawn cleared. Starved cells were harvested and freed from bacteria by repeated low-speed centrifugation in 1× PB buffer (Vocke and Cox, 1992). Cells were then resuspended in 1× PB at a concentration of 5×10^8 cells ml^{-1} and plated for development on dialysis membranes placed on 2% agar plates (Vocke and Cox, 1992).

Transformation

P. pallidum PN500 (Francis, 1975) was grown axenically in standard HL5 culture medium (Knecht *et al.*, 1986) and transformed with a *D. discoideum* P_{ecmB}:*lac*Z fusion construct (Jermyn and Williams, 1991) by a standard calcium phosphate procedure (Nellen *et al.*, 1984). Transformants were selected for G418 resistance on lawns of *E. coli* B/r-1 (Hughes *et al.*, 1992) spread on LP plates (Cox *et al.*, 1988) containing 200 μg ml^{-1} G418. They were cloned and maintained on these same plates (Vocke and Cox, 1992).

Histochemical Staining for β-Galactosidase

The expression of β-gal at different developmental stages was visualized by staining fixed whole-mounts with 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside (X-gal) (Dingermann *et al.*, 1989). Whole-mounts on the dialysis membrane were fixed in 1% gluteraldehyde, 0.1% Triton X-100, and 10 m*M* EGTA in Z buffer (Vocke and Cox, 1992). By fixing specimens on membranes, the entire life cycle can easily be followed. In some experiments, fixed samples were treated with 100% ethanol for 1 min at room temperature. This treatment removes β-gal from some cells, but allows the substrate to penetrate the sorogen, so that centrally located stalk cells can be stained and visualized. Amoebae were stained for 12 hr; aggregates and fruiting bodies for 30 to 60 min.

Whole-Mount in Situ Hybridization

*lac*Z expression in whole-mounts was also revealed by *in situ* hybridization (Escalante and Loomis, 1995), with some modifications. Cells were allowed to culminate on dialysis membranes on 2% agar. The dialysis membranes were moved into 24-well ''Cell Wells'' (Corning Glass Works) and fixed, first in cold methanol for 15 min and then in freshly made 4% paraformaldehyde in PBS for 5 hr at room temperature. Fixed samples were washed 3×5 min in PBS and then treated with 500 μg ml^{-1} proteinase K (BRL) in PBS for 10–20 min at room temperature. They were washed briefly in PBS and then fixed again in 4% paraformaldehyde at room temperature for 2 hr. Hybridization and antibody staining followed the protocol in Escalante and Loomis (1995), using 150 ng ml^{-1} of the labeled probe.

Isolation of Genomic DNA

Cells for DNA isolation were grown on lawns of WBS99 and harvested as described above. Genomic DNA was isolated by lysing cells directly in 4 *M* guanidine thiocynate buffer containing 0.5% Sarkosyl and 100 m*M* Tris–HCl, pH 7.5 (Nelson and Krawetz, 1992). This mixture was extracted repeatedly with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) until a nominal interface layer was present. The aqueous phase was collected, and 2 vol of ethanol were added to precipitate the DNA in the presence of 0.25 *M* sodium acetate.

Southern Blots

Restriction enzyme digests were separated on 1% agarose gels in TAE buffer (TAE, 40 m*M* Tris, 1 m*M* Na_2EDTA, 5 m*M* sodium acetate, pH 8.2). Nucleic acids were transferred and fixed to membranes by UV cross-linking, hybridized, and washed as described in Cox *et al.* (1990).

Pulsed-Field Gel Electrophoresis

The chromosomes of *Polysphondylium* were analyzed by pulsed-field gel electrophoresis as previously described (Cox *et al.*, 1990) modified to account for the smaller average chromosome size of *Polysphondylium*. Briefly, cells at a concentration of 5×10^8 ml^{-1} in 1× PB were mixed with an equal volume of 2% ''InCert'' agarose (FMC). Aliquots of 25 μl were then pipetted into rectangular molds to generate plugs, which were treated with 1 mg ml^{-1} proteinase K (BRL)/50 m*M* EDTA/1% Sarkosyl, pH 9.5, at 50°C for 48 hr. They were then stored in 0.5 *M* EDTA, pH 9.5, at 4°C. Electrophoresis was in 0.9% agarose (BRL) using 0.5× TBE as running buffer. The pulse times were 1250 sec for 96 hr at a voltage of 2.8 V cm^{-1}, followed by 2000 sec for 72 hr at 2.7 V cm^{-1}. Separated chromosomes were transferred to a nylon membrane, cross-linked, and probed (Cox *et al.*, 1990).

RESULTS

Distribution of β-Gal in the Transformant 56.6

In order to obtain a stalk-specific marker, we transformed *P. pallidum* with a *Dictyostelium* P_{ecmB}:*lac*Z construct. A collection of transformants with distinctive staining patterns was isolated. One of them, 56.6, expresses β-gal with stalk cell specificity. β-gal expression is first detected in the center of multicellular aggregates (Fig. 1B). Later, stained cells are localized primarily at the tips of fingers (Fig. 1C). In the fruiting body, the strongly staining cells are at the tip of the 1° sorogen, at the tips of a nascent whorl, and in stalk cells (Figs. 1D, 1E, and 1F). When a cell mass starts to release from the 1° sorogen, clear β-gal staining is not observed early during whorl release (Fig. 1E). Later, strong β-gal activity is detected as evenly spaced patches around the equator of the releasing whorl (Fig. 1F). The staining time required for β-gal detection in the aggregates is about 10 times longer than that in sorogens, suggesting that the level of β-gal production at the culmination stage is much higher. β-Gal production at both 1° and 2° tips marks a

region similar to the prestalk zone of *Dictyostelium*, revealing the existence of a prestalk region in *Polysphondylium*.

The sorogen in Fig. 1D was treated with ethanol, allowing X-gal to penetrate into its center. Although this treatment causes loss of β-gal staining in the sorogen body, it reveals β-gal activity in stalk cells at the center of the sorogen. The sorogens in both Figs. 1E and 1F were not treated with ethanol, and they show a few stained cells in the body of the sorogen as well as in the tips. β-Gal activity is also detected in about 30% of the spores in both 1° and 2° spore heads (Figs. 1G and 1H).

lacZ mRNA Distribution

P_{ecmB}:*lac*Z expression was further analyzed by *in situ* hybridization. *lac*Z mRNA is first detected in the tips of 1° sorogens (Fig. 2A) and then uniformly in a releasing whorl (Fig. 2B). Later, synthesis is restricted to a band around the equator of the nascent whorl (Fig. 2C). This equatorial band breaks up into patches where 2° tips will form (Fig. 2D). Transcripts are also seen in newly formed 2° tips (Figs. 2E and 2F) and in both 1° and 2° stalks (Figs. 2A, 2C, and 2D). They are not detectable in controls probed with plasmid sequences alone (Fig. 2G). As the 2° tip prepattern evolves, the intensity of the stain increases with time (Figs. 2B–2E). The unambiguous stain in centrally located 1° stalk cells also demonstrates that our fixation procedure adequately permeablizes the entire fruiting body.

Because the *in situ* hybridization protocol clears the specimen, we have been able for the first time to visualize how 2° stalks form. Nascent 2° tips originate in patches of prestalk cells (Figs. 2F and 2H). Immediately after visible tips form, a few cells differentiate into stalk cells and form a tongue-shaped structure, the ''stalk bud'' (Fig. 2I, arrow). As cells are partitioned within the whorl, the stalk bud elongates and moves toward the center of the whorl and finally forms mature 2° stalks (Figs. 2J, 2K, and 2L). The fact that the growth of the stalk bud is highly directed toward the center suggests the existence of polar signals directed from the center of the whorl to the surface and therefore a third morphogenetic axis.

Insertion of P_{ecmB}:lacZ in the Genome

*lac*Z expression and β-gal production in 56.6 show stalk cell specificity. Is *lac*Z expression in this cell line regulated by the *Dictyostelium ecmB* promoter or by an endogenous promoter trapped by the insertion? To answer this question, we first localized the transforming DNA to a single chromosome by probing Southern blots of pulsed-field gels with different fragments of the construct. All probes hybridized to chromosome 2 (Fig. 3), showing that the construct, either intact or in fragments, inserted into a single chromosome.

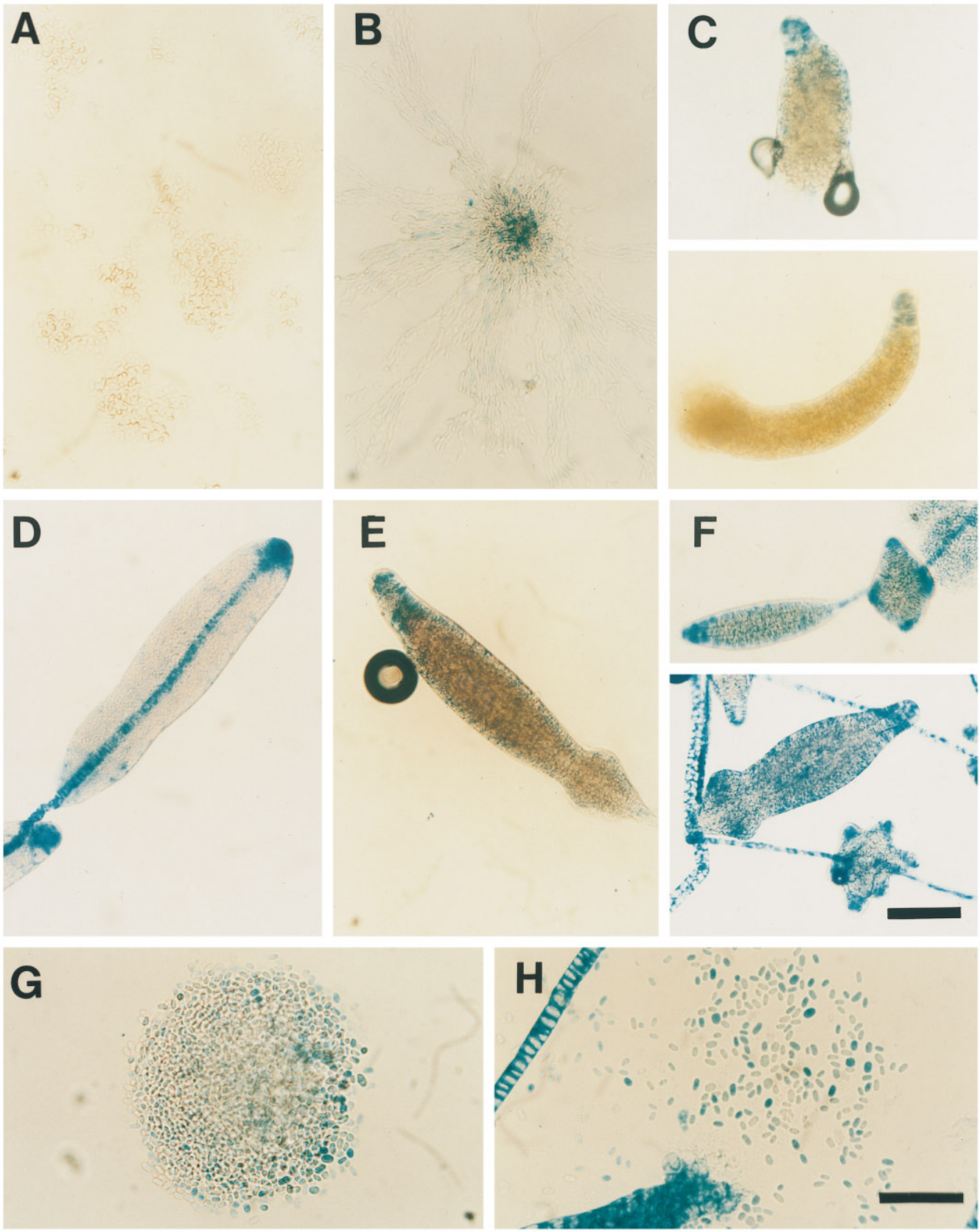
Southern blots were used to analyze plasmid integration further. *Cla*I, which cuts the transforming plasmid once, and *Bgl*II, which cuts it twice, were used in these experiments (Fig. 4A). Both *Cla*I- and *Bgl*II-cut genomic DNA was probed with different probes from the transforming plasmid. Plasmid cut with *Cla*I and *Bgl*II was used as a control. If the insertion were a multicopy tandem repeat, then a common band equal in size to the *Bgl*II or *Cla*I fragment derived from the transforming vector should be detected by all probes. For multicopy insertions at different sites on chromosome 2, multiple bands would be expected. In sum, the results show that 56.6 contains a single copy insertion since all probes hybridize mainly to a single band, whose size varies from probe to probe, none of which has the same size as the transforming plasmid (Fig. 4B). Close attention to Fig. 4 will also convince the reader that the recombination site is located between the *actin-6* promoter and the *amp* gene in a 0.46-kb fragment lying between the *Hae*II and *Bsp*HI restriction sites (and see the caption to Fig. 4).

Since the *ecmB* promoter is intact and flanked by *neo* and *lac*Z, it is unlikely, but not proven, that β-gal expression in this cell line is under the control of an endogenous promoter. A second transformant (56.17) containing a multicopy tandem insertion of the same construct yields an identical *lac*Z expression pattern (data not shown). Because a multiple insertion should buffer the effects of potential *cis*-acting flanking sequences, *lac*Z expression in 56.17 is most likely controlled by the *Dictyostelium ecmB* promoter itself. Thus we conclude that the *Dictyostelium ecmB* promoter can be expressed and regulated in *Polysphondylium* with similar tissue specificity in both organisms.

DISCUSSION

The results described here reveal that a prestalk promoter isolated from *Dictyostelium* responds to developmental cues in a prestalk-specific manner in *Polysphondylium*. This has allowed us to define a prestalk zone and to show that whorl patterning begins as the regulation of a stalk-specific promoter on the entire nascent whorl. The transition from a uniform distribution of transcripts proceeds by

FIG. 1. β-Galactosidase staining of 56.6 through its developmental cycle. Whole-mounts at different developing stages were stained with X-gal. (A) Starved amoebae. (B) Aggregate. (C) Fingers. (D) 1° sorogen treated with 100% ethanol to reveal central stalk staining. (E) 1° sorogen about to release a whorl showing some stained cells in the sorogen body and there is no strong staining in the whorl. (F) (Top) 1° sorogen and a recently released whorl. (Bottom) A whorl with tips and a sorogen about to release a whorl. β-Gal staining is in both 1° and 2° tip cells and in the newly formed 2° tip prepattern of a releasing whorl. (G–H) β-Gal-negative and -positive spores, 1° sorogens. Scale bar, A–F, 100 μm; G–H, 50 μm.



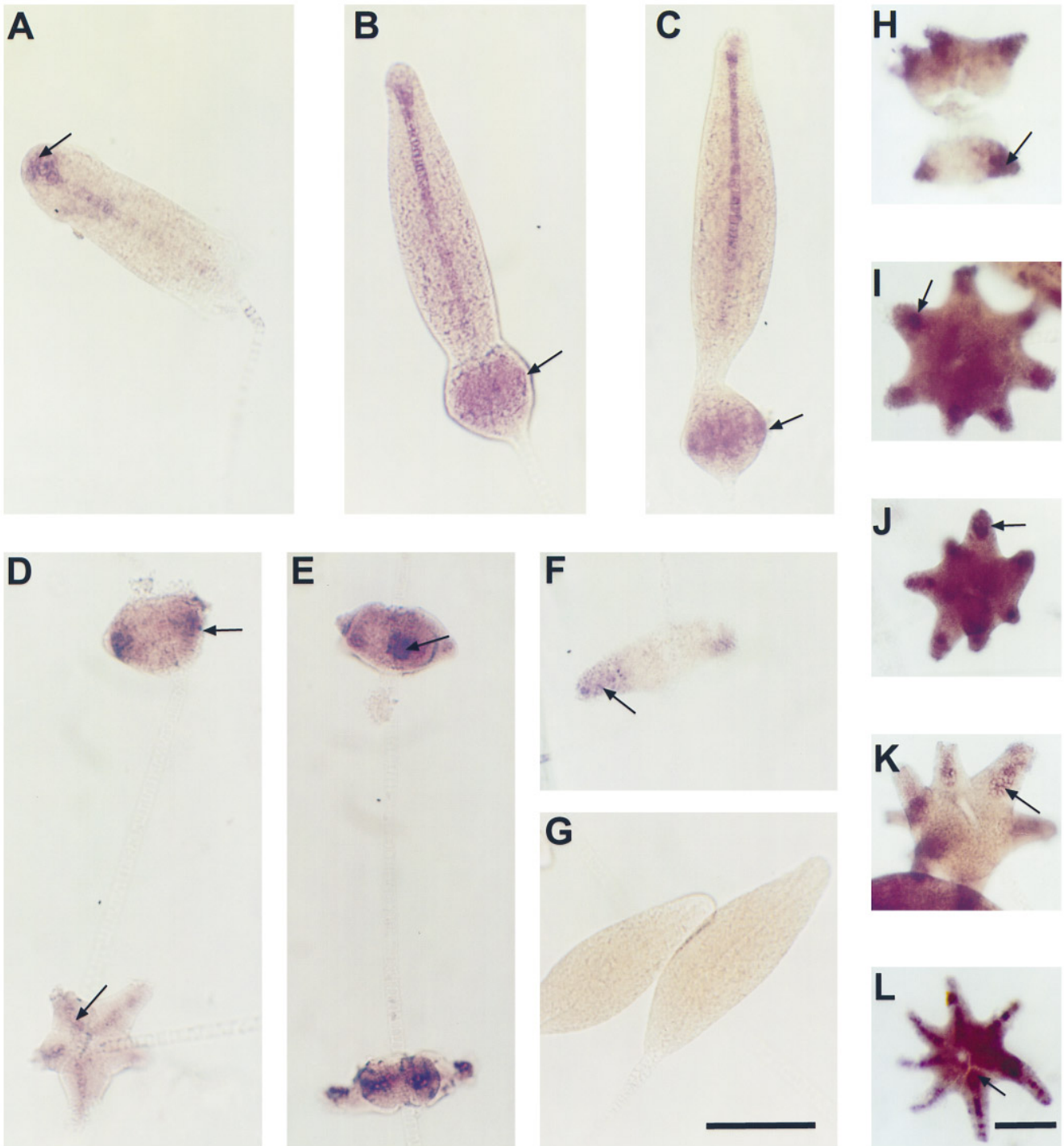


FIG. 2. Spatial distribution of *lac*Z mRNA detected by *in situ* hybridization. (A) A 1° sorogen. (B) A whorl forming at the base of a 1° sorogen. *lac*Z mRNA is uniformly distributed in the releasing whorl. (C) A whorl about to detach from a 1° sorogen. *lac*Z mRNA appears as a band about the equator. (D) A newly released whorl (top) shows patches of *lac*Z mRNA. A later stage (bottom) shows that 2° stalk cells contain *lac*Z mRNA. (E) Two developing whorls have strong *lac*Z expression in their newly formed tip cells. (F) *lac*Z expression in the tip cells of a whorl with 2° tips. (G) A control probed with the transforming vector carrying a *lac*Z deletion. (H) Two whorls with visible tips containing high concentrations of *lac*Z mRNA. (I) A whorl with 2° tips showing that a few cells differentiate into vacuolated stalk cells and form a tongue-like structure, the ''stalk bud.'' (J) A whorl with 2° tips showing that the stalk bud starts to elongate toward the 1° stalk. (K) A later stage showing that the stalk bud elongates and moves toward the center of the whorl. (L) A whorl with 2° fingers showing that the 2° stalk finally reaches the center. Scale bar, A–G, 100 μm; H–L, 50 μm.

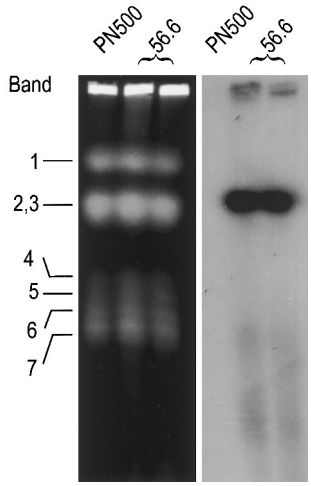


FIG. 3. The P_{ecmB}:*lac*Z construct is inserted into chromosome two. Chromosomes were separated by pulsed-field electrophoresis, blotted to a membrane, and probed by different fragments of the construct (Fig. 4A, fragments 3–7). All the probes hybridized to a single chromosome. Here we show the *lac*Z probe. Note that the probe hybridizes at the top of the chromosome 2, 3 doublet.

a series of restrictions, first to the equator of the whorl and then to radially positioned patches that define where shape changes will occur. The initial appearance of new transcripts clearly responds to positional information in the 1° sorogen. The expression of the P_{ecmB} promoter appears to be a relatively late event in whorl morphogenesis, since the translation of message under control of this promoter first occurs in radially symmetric patches where 2° tips will form, in contrast to other, presumably early, antigens such as Tp423.

When *Polysphondylium* cells first begin to aggregate, prestalk cells appear randomly distributed in aggregating streams and centers. They sort out later to the center at the mound stage, the precursor of the 1° sorogen (Vocke and Cox, 1992). This behavior is similar to early events in *Dictyostelium* stalk-cell differentiation and morphogenesis, where prestalk cells are also found scattered throughout early aggregates, sorting later to the mound center (reviewed in Williams and Morrison, 1994).

One might have expected a similar mechanism to function during the establishment of 2° tips on the whorl, as has been suggested by Fukushima and Maeda (1991). If cell sorting were the explanation, however, we would expect to find a few tens or perhaps hundreds of cells expressing *lac*Z transcripts at the base of the sorogen. These cells would then sort out to the equator and eventually to radially symmetric patches. Instead, we find very little *lac*Z message at the base of the culminating sorogen, followed by a sudden turn-on of message synthesis in the whorl as it begins to break free from the 1° sorogen. This is particularly clear in Figs. 2B and 2C. For this reason, positional signals appear to dominate this phase of morphogenesis. This interpreta-

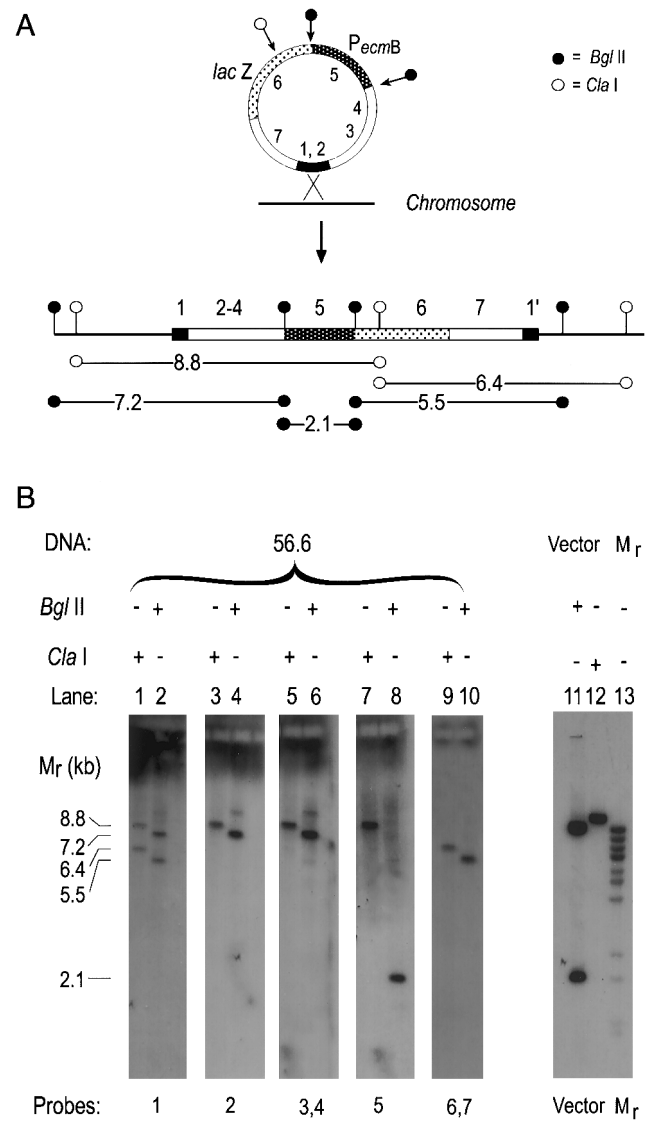


FIG. 4. Genomic structure of 56.6. (A) The structure of the transforming plasmid with the probes used for hybridization labeled on the inside: 1, a 0.96-kb *Bsp*HI–*Eco*RV fragment located between the *actin-6* promoter and *amp* gene; 2, a 0.5-kb *Hae*II–*Eco*RV fragment which is adjacent to the *actin-6* promoter and is part of probe 1; 3, a 0.7-kb *actin-6* promoter fragment generated by PCR; 4, a 0.9-kb *Pst*I fragment of the *neo* gene; 5, a 2.1-kb *Bgl*II fragment of the *ecm*B promoter; 6, a 2.2-kb *Cla*I–*Xho*I fragment of *lac*Z; and 7, a 1-kb *Bsp*HI fragment of *amp*. The transformation vector inserted into the genome as an intact single copy with its insertion site in a 0.46-kb *Bsp*HI–*Hae*II fragment located between the *actin-6* promoter and the *amp* gene. *Bgl*II digestion generates three fragments: a 7.2-kb fragment hybridizing to probes 1–4; a 2.1-kb fragment hybridizing to probe 5; and a 5.5-kb fragment hybridizing to probes 6, 7, and 1′. *Cla*I digestion generates two fragments: an 8.8-kb fragment hybridizing to probes 1–5 and a 6.4-kb fragment hybridizing to probes 6, 7, and 1. For a restriction map of the vector see Jermyn and Williams (1991). (B) Southern blots documenting the above. Genomic DNA of 56.6 was cut with *Cla*I or *Bgl*II and separated on 1% agarose (lanes 1–10). The blots were probed with the seven probes described above.

tion is consistent with earlier results based on the distribution of stalk-specific antigens detected with monoclonal antibodies (Byrne and Cox, 1987). Although the results presented here do not allow us to distinguish between cell sorting and positional cues for later morphogenetic steps, it seems likely that the continued restriction of the prepattern from an equatorial band to radially positioned patches also occurs by restriction and amplification of message, since cells tagged with green fluorescent protein and followed by confocal microscopy are stationary while whorl prepatterns form (Fey and Cox, unpublished results).

Secondary sorogen morphogenesis differs from primary in other respects as well. Although 2° tips express prestalk message (Figs. 2D–2F) and protein, they do not have a prestalk zone by morphological criteria. Thus all of the nonstalk cells in 2° sorogens have prespore vesicles, and they stain with spore-specific polyclonal (O'Day, 1979) and monoclonal (Vocke and Cox, 1990) antibodies. This is not surprising, since cells at the base of the sorogen clearly express prespore antigens and vesicles throughout culmination. Consequently, they are marked by both prespore and prestalk gene products as the whorl is released. These cells must then transdifferentiate into stalk cells, unlike those in the 1° sorogen prestalk zone.

The distribution of *lac*Z mRNA marks the same pathway as the tip-specific antigen Tp423 (Byrne and Cox, 1986) (Fig. 2), whereas β-gal synthesis occurs predominately within patches already correctly positioned on the equator, but not before (Figs. 1E and 1F). Since *lac*Z is an imported gene, there is no reason to believe it carries signals for translational regulation, and thus the time lag between message appearance and β-gal synthesis is probably the time needed for translation and product accumulation. Thus, the regulation of P_{ecmB}, marked by *lac*Z expression, is a relatively late event, lying downstream of, for example, both the antigen Pg101, which first appears equatorially (Byrne and Cox, 1987), and Tp423.

Cells expressing β-gal move into the stalk tube and mature into vacuolated stalk cells (Figs. 1 and 2, especially 2H–2L). In *Dictyostelium*, stalk cells are derived from pstA cells, marked by the expression of *ecmB* at the entrance of the stalk tube (Jermyn and Williams, 1991). Apparently the tip of the sorogen in *Polysphondylium* is equivalent to the entrance of the stalk tube in *Dictyostelium*. The regulation of the *ecmB* promoter thus marks stalk cell differentiation in both genera. *D. minutum*, a species thought to be more closely related to *D. discoideum* than *P. pallidum*, contains a homologue of *Dictyostelium ecmB*. The *ecmB* promoter of *D. minutum* is also regulated in *Dictyostelium* with stalk cell specificity (Van Es *et al.*, 1994). The fact that P_{ecmB} seems interchangeable between the different species suggests that the *cis* regulatory elements governing *ecmB* expression are conserved. Whether or not they respond to similar intracellular signals in *Polysphondylium* is not known.

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