The 'prespore-like cells' of *Dictyostelium* have ceased to express a prespore gene: analysis using short-lived β-galactosidases as reporters

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SUMMARY

In transgenic strains of *Dictyostelium discoideum* that express β-galactosidase under the control of a prespore-specific promoter, only early slugs show reporter confined to the prespore zone. As slugs migrate β-galactosidase-positive cells accumulate in the prestalk zone; ultimately, there may be so many that the prestalk-prespore boundary is no longer distinguishable (Harwood, A., Early, A., Jermyn, K. and Williams, J. (1991) *Differentiation* 46, 7-13). It is not clear whether these 'anomalous' reporter-positive cells currently express prespore genes; another possibility is that they are ex-prespore cells that have transformed to prestalk and sorted to the prestalk zone (Sternfeld, J. (1993) *Roux Archiv. Dev. Biol.* 201, 354-363),

while retaining their previously produced reporter. To test the activity of the prespore genes in these cells, we have made prespore reporter constructs whose products decay quickly; these are based on constructs used to investigate protein turnover in yeast (Bachmair, A., Finley, D. and Varshavsky, A. (1986) *Science* 234, 179-186). In strains bearing such constructs, β-galactosidase-positive cells do not appear in the prestalk zone. The apparent deterioration of the prestalk/prespore pattern in older slugs is thus an artefact of reporter stability.

Key words: protein breakdown, cell-type conversion, anterior-like cells, *Dictyostelium*, prespore-like cells, β-galactosidase

INTRODUCTION

A well-defined cell differentiation pattern is present in the slug stage of *Dictyostelium discoideum*. Prestalk cells occupy the anterior 20% of the slug, while the rear 80% contains prespore cells (Bonner, 1952) as well as anterior-like cells, which resemble prestalk cells but are scattered in the prespore zone (Sternfeld and David, 1981; Devine and Loomis, 1985). Further substructure is apparent in both the prestalk and prespore zones when one examines the expression of particular cell-type-specific genes (Jermyn et al., 1989; Haberstroh and Firtel, 1990). A major question in *Dictyostelium* research is how positional signals contribute to this spatial pattern (Schaap, 1991; MacWilliams, 1991; Nanjundiah and Saran, 1992).

Patterned extracellular signals are not necessary for the initial differentiation of prestalk and prespore cells. Thus both cell types can form in vitro at low cell density in the apparent absence of cell interactions (Gomer and Firtel, 1987). In vivo, prestalk and prespore cells are randomly intermixed at early developmental stages (Schaap et al., 1982; Williams et al., 1989) and the formation of the prestalk and prespore zones appears to proceed via sorting-out (Sternfeld and David, 1981; Williams et al., 1989; Bühl and MacWilliams, 1991; Bühl et al., 1993). It is nonetheless clear that the choice of differen-

tiation pathway in *Dictyostelium* is governed in part by extracellular factors (Blaschke et al., 1986; Inouye, 1989) and that different factors or factor combinations are required to maintain the expression of different cell-type-specific genes (Berks and Kay, 1990). A plausible hypothesis would appear to be that extracellular signals in the slug stage stabilize the cell-type differentiation pattern after sorting-out.

This hypothesis presumes that the prestalk-prespore pattern is in fact stable, but a recent experiment suggests that this may not be the case. When β-galactosidase is expressed under the control of the promoter of the prespore gene *PsA*, the reporter enzyme is only confined to prespore cells in young slugs. In older slugs reporter-positive cells appear in the prestalk zone; these cells can be so numerous that the prestalk-prespore demarcation "becomes somewhat arbitrary" (Harwood et al., 1991). Although the construct originally used to demonstrate these *prespore-like cells* (PLCs) is complex, containing (non-cell-type-specific) *actin-15* as well as *PsA* promoter sequences (Early and Williams, 1989; Dingermann et al., 1989), we show here that PLCs can be seen with other prespore reporter constructs, none of which contains foreign promoter fragments. Thus PLCs are not an artefact of the *PsA/actin-15* hybrid promoter.

Classical work confirms the existence of prespore-like cells in the prestalk zone. Such cells have been seen by immunos-

taining (Takeuchi et al., 1977) and electron microscopy (Schaap, 1983; see Discussion). Vital staining with neutral red (Bonner, 1952) also suggests that the prestalk-prespore pattern deteriorates in older slugs.

None of these results, however, establish that PLCs currently express prespore genes. Another possibility is that they are ex-prespore cells that have redifferentiated as prestalk (Sternfeld, 1993; Harwood et al., 1991). Since prestalk and prespore cells sort out from one another, such cells would be expected to move to the prestalk zone. Prespore gene products that turned over slowly could survive the sorting process and appear in the prestalk zone. In at least one case, bacterial β-galactosidase expressed in eukaryotic cells is stable (Cohen et al., 1993).

To investigate the role of marker stability in the PLC phenomenon, we have used β-galactosidase reporters with several different halflives (Bachmair et al., 1986; Gonda et al., 1989). Comparison of the staining patterns obtained with these reporters suggest that the the PLCs are indeed cells that once expressed prespore genes, but have ceased to do so.

MATERIALS AND METHODS

Vectors

In *CotB-gal* (Fosnaugh and Loomis, 1993) the promoter, 5′-untranslated region and nine amino-terminal codons of the *CotB* gene are fused via nine linker codons to *lacZ*. In *SP60-gal* (Haberstroh and Firtel, 1990) the *SP60* promoter, 5′-untranslated region and 7 amino-terminal codons are fused via 6 linker codons to *lacZ*. *PsA-gal*, obtained from D. Traynor, contains the promoter, 5′-untranslated region and 5 amino-terminal codons of the *PsA* gene fused via 11 linker-derived codons to *lacZ*. The vectors *PsA-X-gal* were constructed by amplifying a 1.1 kb fragment from the corresponding derivatives of pUB23 (Bachmair et al., 1986) using the primers 5′-GCAGATCTAAAATGCAGATTTTCGTCAA-3′ and 5′-GCGATCGGCATAACCA-3′; the first primer is complementary to the N terminus of ubiquitin and contains an additional 5′ *Bgl*II site, while the second primer is complemetary to a region just 3′ of the *Cla*I site of *lacZ*. The PCR products were treated with the Klenow-kinase-ligase procedure (Lorens, 1991) and ligated into *Bgl*II-*Cla*I restricted *PsA-gal*.

Strains and culture conditions

Dictyostelium discoideum strain AX2 (obtained from C. J. Weijer) was used in all experiments. Transformation was performed as described (Early and Williams, 1987). X-gal staining of colony blots (Bühl et al., 1993) showed all transgenic lines obtained to be unstable; this was also true of lines received from other laboratories. To obtain cells for experimental use, several SM/2 plates (Sussman, 1966) were inoculated with *Klebsiella aerogenes* and 10^5 amoebae from a freshly isolated, reporter-positive clone. The plates were incubated to a point just short of clearing and, when necessary, stored at 4° for up to 1 day.

β-galactosidase accumulation measurements

Cells and remaining bacteria were scraped from the plates, suspended in 50 mM potassium phosphate buffer, pH 6.2 (15 ml/plate) and shaken approx. 8 hours, by which time the bacteria had been exhausted. The cells were washed and suspended at $2×10^7$/ml in 20 mM potassium phosphate buffer, pH 6.2. 5 ml of this suspension was spread onto each of several levelled 1% agar plates containing 1 mM potassium phosphate, pH 6.2. After 5 minutes the fluid was decanted; the plates were then stood on edge for 10 minutes and excess fluid was removed. The plates were allowed to dry for a further 5 minutes before closing.

The dried plates were incubated at 7°C for 15 hours, then at 22°C for 4 hours. Aggregates (just before tip formation, finger stage of Soll (1979)) were harvested into 10 mM sodium/potassium phosphate buffer containing 1 mM cAMP (Schaap et al., 1986; Schaap and Van Driel, 1985), vortexed and the cells resuspended at $5×10^6$/ml. 6 ml of this suspension was pipetted into each of eight 15 ml disposable plastic centrifuge tubes, which were then agitated on a roll-tip shaker (Mixer 10, Denley Company, Sussex, England) for 0-8 hours, after which the cells were pelleted and frozen; there was a full exchange of medium at 4 hours. Pellets were thawed in 0.5 ml 100 mM phosphate buffer, pH 7.5 containing 10 mM benzamidine, and β-galactosidase activity was measured using the ONPG, CPRG (Dinger-mann et al., 1989) or Galacto-Light (Tropix, Bedford, Massachusetts, USA) methods. Protein was measured according to Bradford with BSA as standard and the measured enzyme activities were normalized to protein.

Accumulation experiments were performed 3-5 times with each transformed cell line. Replicate experiments showed significant variations in overall enzyme activity, presumably due to vector instability. Accordingly, each accumulation curve was normalized by dividing by its mean value before the data from replicate experiments were combined.

β-galactosidase breakdown measurements

Developing cells were prepared as for the accumulation measurements but allowed to develop at 22°C for several hours longer (early culminate I to maxifinger stage; Soll, 1979). Aggregates were harvested into 10 mM sodium/potassium phosphate buffer containing 10 mM caffeine, vortexed briefly, pelleted and resuspended at $5×10^6$ cells/ml in the same buffer. Incubation, harvesting and enzyme activity measurements were performed as described above except that the medium change was omitted.

Northern analysis

RNA was prepared (Maniak et al., 1989) from cells from one β-galactosidase accumulation experiment and one breakdown experiment, both performed with cells bearing *PsA-ile-gal*. Samples containing 10 μg of RNA were separated on 1% agarose gels (0.6% formaldehyde, 10 mM MOPS, pH 7.5) and blotted to a nylon membrane (Amersham). Probes specific for the endogenous *PsA* message and the β-galactosidase message were labelled with DIG (Boehringer, Mannheim) according to the manufacturer's protocol and hybridizations were performed as described (Engler-Blum et al., 1993). Relative quantification of the β-galactosidase mRNA was performed by comparison to a dilution series (factor-of-two steps) of the RNA sample two-hour time point of the accumulation experiment. Comparison of the films by eye gave essentially the same result as comparison of scanned images using IMAGE 1.47 (NIH Freeware).

Estimation of protein halflife in accumulation experiments

A computer simulation was used to derive best-fit estimates of the β-galactosidase protein halflife for each accumulation curve. For the *PsA-ile-gal* curve, this routine employed the mRNA levels measured as described above (25%, 50%, 100%, 25%, 25%, 37%, 12% at time points t0 through t6); for the other curves, we allowed a displacement of this time course along the time axis, corresponding to the idea that the different cell lines initiate PsA promoter activity at slightly different times. Predicted accumulation curves were generated for all reasonable combinations of protein halflife and time displacement. Each predicted data set was normalized by dividing by its mean, and the goodness of fit to the actual data then judged by the sum of squared deviations between prediction and data, weighted by the uncertainties of the data points (MacWilliams et al., 1970; Cohen and MacWilliams, 1975). Plotted against protein halflife and time displacement, the weighted sum of squared deviations was a smooth well-shaped surface; the best-fit halflife was taken as the value at the
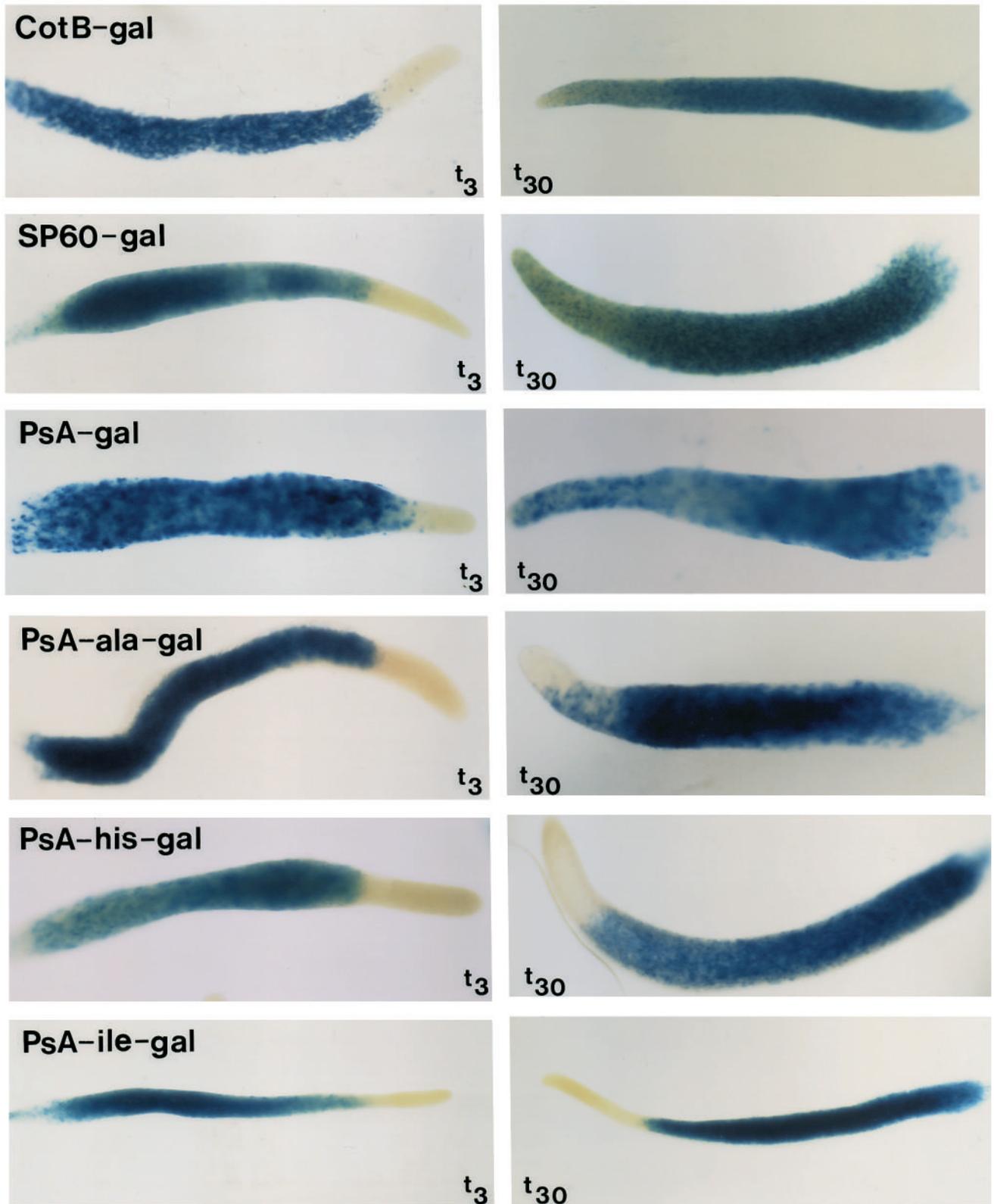
Fig. 1. Prespore-like-cells (PLCs) as visualized with various reporter constructs. Shown are slugs carrying 6 different constructs, in each of which a prespore promoter drives the expression of β-galactosidase (x-gal staining). In all cases, 3 hour old slugs show well-defined prestalk zones (unstained areas on the right of the slugs). In 30-hour-old slugs, β-galactosidase-positive cells are visible in the prestalk zones (here on the left) using the top 4 constructs, all of which appear to produce stable proteins. Using the bottom two constructs, whose products appear to be unstable, the PLCs are not detectable.

minimum. Statistical evaluation of the fit was carried out by reference to a chi-squared distribution (MacWilliams et al., 1970; Cohen and MacWilliams, 1975), in this case with 4 degrees of freedom (8 measurements minus three fitted parameters (normalization, halftime, time displacement) minus 1).

The best-fit protein halflives fell into three groups (approx. 1 hour, approx. 3 hours and infinite); all fits were statistically acceptable (see text). The curves that suggested a stable protein (*CotB-gal, SP60-gal, PsA-gal, PsA-ala-gal*) could not be fitted acceptably assuming a 1-hour halflife ($P<0.02$). The data from *PsA-ile-gal* (best-fit halflife = 1 hour) could not be fitted assuming a stable protein ($P<0.01$). The *PsA-his-gal* data (best-fit halflife = 3 hours) could not be fitted acceptably assuming either a 1-hour halflife or a stable protein ($P<0.01$ in both cases).

Prestalk regeneration

Cells were harvested as for the accumulation measurements, washed once in water and the cell pellet suspended in 1 pellet volume of water. 20 μl droplets were deposited onto well-dried, 60 mm plates containing 1% agar and 1 mM potassium phosphate, pH 6.2. The plates were incubated at 22°C in the dark until slugs had formed. 1×1 cm squares of washed nitrocellulose membrane were placed on the agar; prestalk zones were removed by cutting slugs at the midpoint, and the rear halves were transferred to the nitrocellulose membranes. After 2 or 4 hours, the membranes bearing slug fragments were immersed in 0.5% glutaraldehyde, 2% Tween-20 in Z-buffer (Dingermann et al., 1989) and fixed for 20 minutes at room temperature. The filters were then washed several times in Z-buffer and held in this buffer on ice until the end of the experiment.

All filters from one experiment were stained simultaneously; staining was as described by Dingermann et al. (1989), except that the concentrations of $K_3[Fe(CN)_6]$ and $K_4[Fe(CN)_6]$, which appear to inhibit the enzyme to some degree, were reduced fivefold with all strains except *PsA-gal* and *CotB-gal*. In the latter strains, which showed very high enzyme activity, staining was conducted on ice to avoid possible artefacts associated with incomplete substrate penetration. Staining times were 1-3 hours for *CotB-gal* and *PsA-gal*, 6-12 hours for *SP60-gal* and *PsA-ala-gal*, and one to several days for *PsA-his-gal* and *PsA-ile-gal*.

Prolonged slug migration

Slugs were prepared as above except that the diluted pellet was deposited directly on 49 mm round nitrocellulose membranes which had been placed onto the agar. After slug formation (approx. 18 hours) and migration for 3 or 30 hours, the slugs were fixed by transferring the membranes to filter supports (Schleicher and Schuell #2294) saturated with glutaraldehyde/Tween 20 (above) for 5 minutes, then immersing them in the same solution for 15 minutes. Staining was conducted as above.

RESULTS

Prespore-like cells can be seen with several reporter constructs

To determine how generally PLCs can be detected, we performed X-gal staining on slugs bearing three prespore reporter constructs: *SP60-gal* (Haberstroh and Firtel, 1990), *CotB-gal* (Fosnaugh and Loomis, 1993) and *PsA-gal* (D. Traynor, personal communication) (which is similar to the construct originally used by Harwood et al., 1991, but lacks actin-15 promoter sequences). The slugs were fixed after 3 or 30 hours of migration and staining times were varied to obtain a comparable staining intensity in the prespore zone (deep blue but retaining some transparency; not black).

The prestalk zones of the newly formed slugs were generally devoid of β-galactosidase-positive cells (Fig. 1). After 30 hours of migration, large numbers of PLCs were visible in all cases, so that the prestalk-prespore boundary was not readily distinguishable. PLCs are thus seen in a variety of prespore promoter/β-galactosidase fusions; they do not result from a peculiarity of the original *PsA/Actin 15* hybrid promoter.

'N-terminal rule' β-galactosidases have differing halflives in *Dictyostelium*

Little can be said a priori about the halflives of the proteins coded for by the above fusions; each has a different N terminus and the effects of these sequences on protein breakdown are unknown. To obtain reporters with well-defined halflives, we have therefore turned to a family of β-galactosidases whose halflives have been measured in yeast (Bachmair et al., 1986) and in mammalian cells (Gonda et al., 1989). These proteins undergo posttranslational processing, with the result that the N terminus is removed and an originally internal amino acid residue appears at the N terminus. The protein halflife depends on this amino acid and varies from 2 minutes to 24 hours or more.

We placed the genes for three such proteins (N-terminal amino acid = *ala*, *his* or *ile*) under the control of the PsA promoter (see Materials and Methods). For simplicity, the *Dictyostelium* constructs will be referred to as *PsA-ala-gal*, *PsA-his-gal* and *PsA-ile-gal*. In yeast *ala-gal* is stable, whereas *his-gal* and *ile-gal* are labile. We carried out three experiments to estimate the halflives of these molecules in *Dictyostelium*: (1) We followed the accumulation of enzyme activity after the PsA promoter is activated with cAMP; (2) we followed the disappearance of enzyme activity after the PsA promoter is shut off with caffeine; (3) we induced slugs bearing the various constructs to regenerate their prestalk zones, a process in which prespore cells are thought to be converted to prestalk cells.

Accumulation time course

In an ideal protein accumulation experiment, in which protein synthesis proceeds at a constant rate, a stable protein will accumulate linearly for an indefinite period, while a labile protein will approach an early plateau. Our experiments (Fig. 2) were complicated by the fact that the message levels, and thus presumably the rate of protein synthesis, were not constant; instead, the message increased to an early peak and then declined (see Fig. 3 and Materials and Methods). Under these circumstances, a stable protein is expected to accumulate at a rate that initially increases and then slows; a labile protein should increase to a peak and decline. With four reporter constructs (all previously existing constructs and *PsA-ala-gal*), the accumulation pattern suggested a stable protein, while in *PsA-his-gal* and *PsA-ile-gal* the accumulation pattern suggested that the protein was labile.

We used computer simulations to extract protein halflives from these data (see Materials and Methods). The accumulation curve for *PsA-ile-gal* was best fitted using a protein halflife of 1.0 hours; the fit was statistically acceptable ($P≈0.60$). For *PsA-his-gal*, the optimal protein halflife was 3.0 hours ($P≈0.90$), while the assumption of stable proteins gave the best fit to the data for *PsA-ala-gal*, *CotB-gal*, *SP60-gal* and *PsA-gal*) ($0.30<P<0.70$). The best-fit curves are given in Fig. 2.
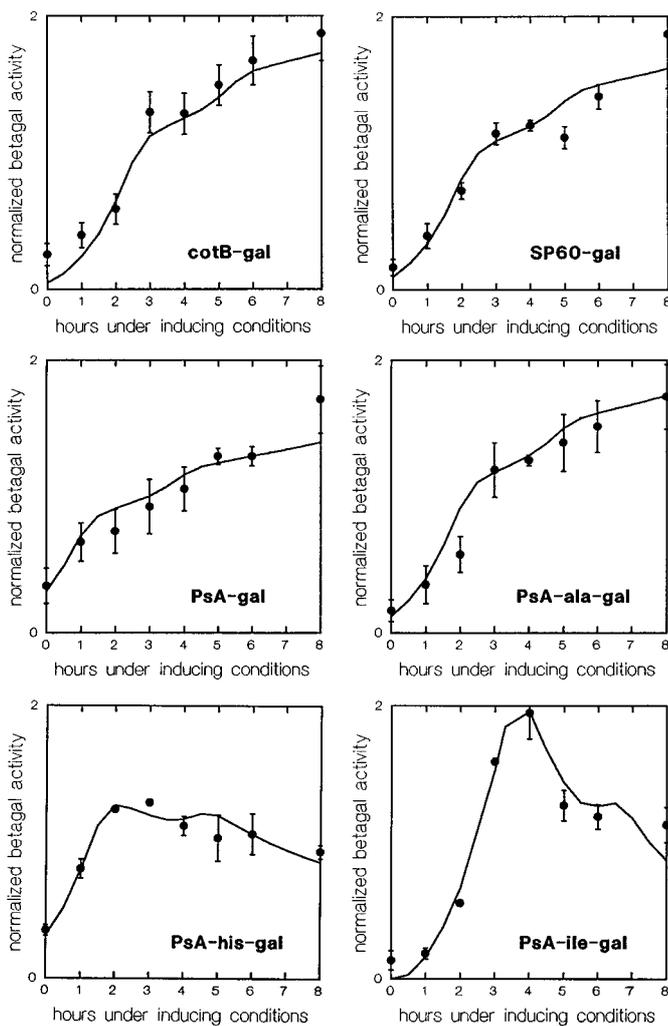
Fig. 2. Accumulation of β-galactosidase in cells bearing various reporter constructs. Prespore genes were activated by shaking cells in cAMP and the response followed over 8 hours. The data points give the mean enzyme activity ± s.e.m. in 3-5 replicate experiments. The fitted curves were calculated from measured messenger levels assuming an infinite protein halflife (top four panels), a 3-hour halflife (PsA-his-gal) and a halflife of 1 hour (PsA-ile-gal).

Decay measurements

To observe protein breakdown more directly, we allowed *PsA* expression to begin, then blocked further *PsA* promoter activity with caffeine (Weijer and Durston, 1985; Schaap et al., 1986). Northern analysis (Fig. 3) confirmed the effectiveness of the caffeine treatment. Among the putatively stable reporters, the only hint of protein breakdown was seen with *CotB-gal*, whose level declined by half during an 8-hour period (Fig. 4). With the putatively labile reporters *PsA-his-gal* and *PsA-ile-gal*, there was an initial lag and then, after the message had dissappeared, a sharp decline. With *PsA-ile-gal*, the decline conformed almost exactly to an exponential decay with a 1-hour halftime, matching the value deduced from accumulation data. With *PsA-his-gal* the decline was compatible with the halflife (3 hours) suggested by the accumulation experiment, but the measurements scattered too much to allow a clear confirmation.
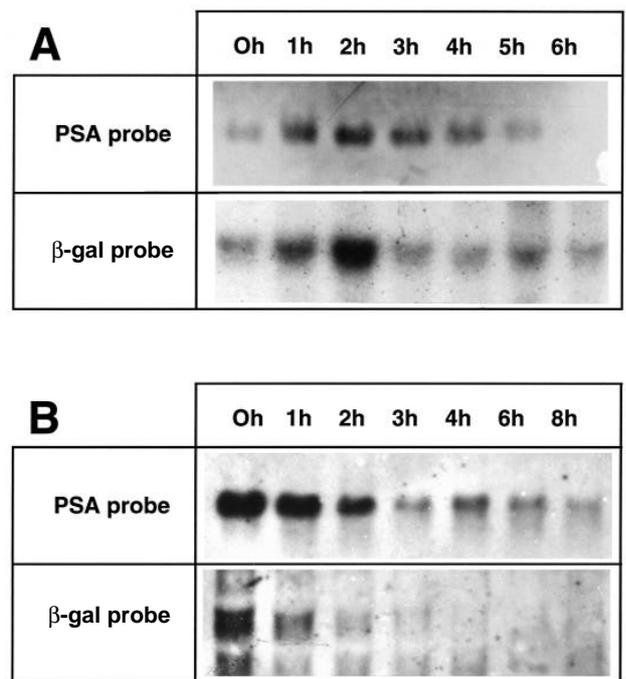

Fig. 3. Levels of the β-galactosidase message and, for comparison, the endogenous *PsA* message in accumulation and decay experiments using *PsA-ile-gal* cells. In the accumulation experiment (above) both messages increase to a maximum and subsequently decline. In the decay experiment (below) both messages dissappear after the cells are transferred to caffeine. In both cases the β-gal message appears to decrease faster than the *PsA* message, suggesting that it may be more labile.

Prestalk regeneration experiments

When the prestalk zone of a *Dictyostelium* slug is removed, the remaining prespore fragment regenerates a new prestalk zone. The new prestalk cells are thought to be formed in part by re-differentiation of prespore cells (Sternfeld and David, 1982). In all four strains with putatively stable reporters (*PsA-gal*, *SP60-gal*, *CotB-gal* and *PsA-ala-gal*), regenerated prestalk zones contained β-galactosidase-positive cells (Fig. 5). With *PsA-his-gal* and *PsA-ile-gal*, in contrast, no such cells were seen. With severe overstaining, a few positive cells were sometimes apparent in the prestalk zones of *PsA-his-gal* regenerates (not shown); none were ever seen with *PsA-ile-gal*.

PLCs are not apparent with labile reporter constructs

Cells bearing each of the three new constructs were allowed to develop to the slug stage; the slugs were fixed after approx. 3 or 30 hours of migration, and stained (Fig. 1, bottom half). In slugs fixed at 3 hours, no prespore-like cells were seen with any construct. After 30 hours, many positive cells were seen with the (stable) *ala* construct. Slugs bearing the (moderately labile) *PsA-his-gal* reporter showed at most a few PLCs after normal staining, but after severe overstaining a handful of weakly positive cells could often be seen near the prestalk-prespore boundary (not shown) No positive cells were seen in the prestalk zone with the (most labile) *PsA-ile-gal* construct.
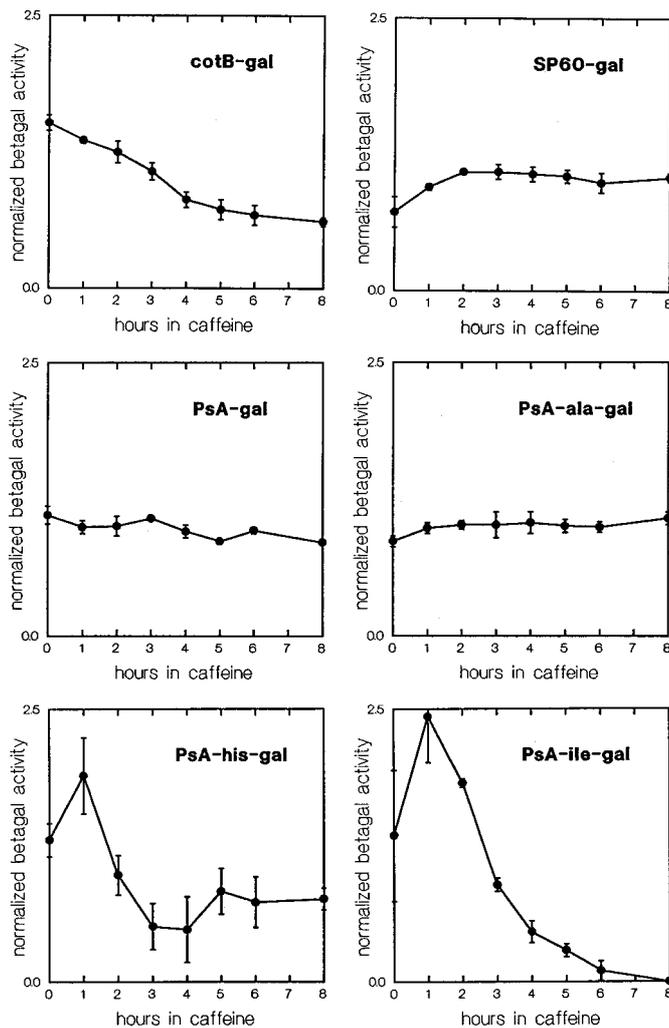
Fig. 4. Decay of the β-galactosidase reporter in cells bearing various reporter constructs. Cells expressing the reporter were transferred to caffeine and the enzyme activity followed over 8 hours. Only the bottom two panels show extensive breakdown. In *PsA-ile-gal*, the enzyme activity disappears with a halftime of 1 hour after an initial lag.

DISCUSSION

Classical observations of PLCs in *Dictyostelium* slugs

Cells with characteristics reminiscent of PLCs were observed on at least two occasions prior to the introduction of β-galactosidase reporters. Takeuchi and coworkers (1977) found cells containing an antigen characteristic of prespore vesicles (see Müller and Hohl, 1973) in the prestalk zones of older slugs. Using electron microscopy, Schaap and coworkers (1982, 1983) confirmed that occasional cells in the prestalk zone contain prespore vesicles; these cells also possessed an electron density more typical of prespore than prestalk cells. Prespore vesicles appear to be a relatively labile marker of the prespore state; they are lost with a halftime of about 3 hours following the addition of caffeine (Weijer and Durston, 1985). One would thus expect them to behave roughly like the *PsA-his-gal*

reporter. Consistent with this idea, both Schaap and Takeuchi reported the number of cells bearing prespore vesicles to be small, and in Takeuchi's preparations these cells could be seen to be concentrated near the prestalk-prespore boundary, as is sometimes seen with *PsA-his-gal* slugs. Our observations are thus in good agreement with previous results.

Nature of PLCs

Using our most labile reporter, we find no sign that prespore genes are ever expressed in the prestalk zone of *Dictyostelium* slugs, even after many hours of migration. A labile reporter should mainly reflect current gene expression; *Dictyostelium* slugs can thus maintain a sharply defined pattern of prespore gene activity for long periods. This observation appears to rule out two otherwise plausible explanations for the PLCs: (1) that PLCs are prestalk cells that begin to express prespore genes and (2) that prespore cells invade the prestalk zone. The fact that PLCs are readily demonstrable with stable reporters, but not with labile ones, can only mean that PLCs are ex-prespore cells, cells that did once but no longer do express prespore genes.

It has been previously suggested (Sternfeld, 1993) that some prespore cells in *Dictyostelium* slugs deactivate their prespore genes and move to the prestalk zone. Our results suggest that this is a sufficient explanation of the PLC phenomenon.

PLCs and cell-type transformations in *Dictyostelium*

A most interesting question is when and where the PLCs deactivate their prespore genes. If this occurred as PLCs crossed the prestalk-prespore boundary, one could imagine the change in gene expression to be a response to morphogen-like positional signals. It appears more likely, however, that the change takes place while the cells are still in the prespore zone. This idea is supported by three arguments.

(i) Previous experiments with the vital dye neutral red (Sternfeld, 1993) suggest that some cells in the prespore zone acquire a prestalk or anterior-like identity after slug formation. Neutral red is taken up by autophagic vacuoles, the characteristic organelles of prestalk and anterior-like cells (Yamamoto and Takeuchi, 1983). Cells are normally stained early in development and then allowed to form slugs. After a period of slug migration, the prespore zones characteristically contain three cell populations: in addition to stained (anterior-like) and unstained (prespore) cells, a third class of cells is present which are not stained but are able to take up neutral red if it is provided anew. These 'restainable' cells are presumably former prespore cells which have acquired autophagic vacuoles during slug migration.

(ii) The prestalk zones of slugs bearing our labile constructs are 'cleaner' than one would expect if the PsA promotor were deactivated at the moment PLCs cross into the prestalk zone. Even with the most labile construct *Psa-ile-gal*, substantial amounts of β-galactosidase are present hours after the PsA promotor is turned off (Fig. 4). If the cells in caffeine experiments are stained with X-gal, about half of the cells which are positive initially can still be stained after 3 hours (Detterbeck, unpublished thesis). Thus if PLCs did not deactivate prespore genes until entering the prestalk zone, a substantial number of β-galactosidase-positive cells should be seen in the prestalk zone even with *Psa-ile-gal*. Since this is not the case, it appears that PLCs deactivate their PsA promotor earlier.
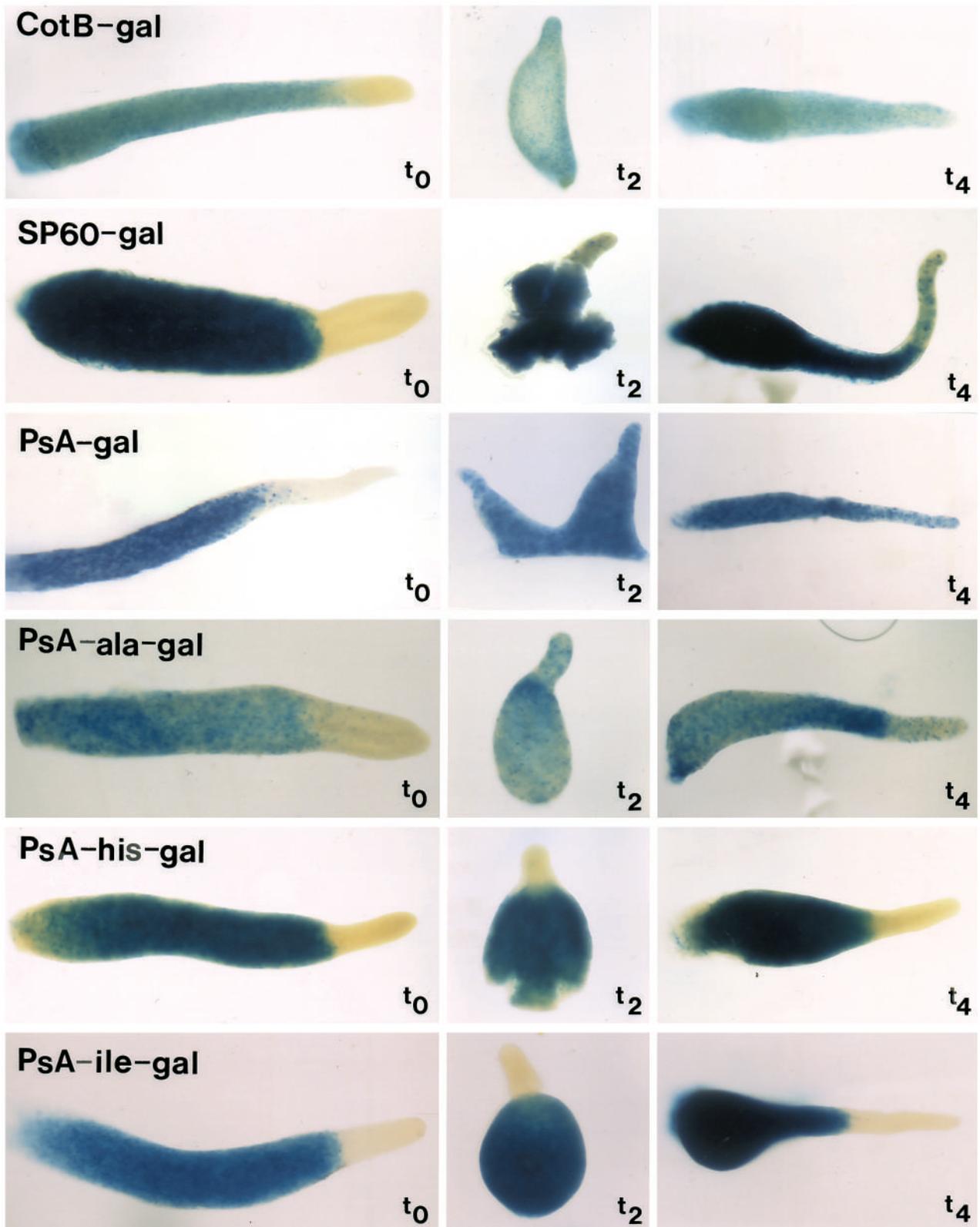
Fig. 5. Regeneration of the prestalk zone in strains bearing different reporter constructs. Several-hour-old slugs (t_0) were bisected and the front halves discarded. In the first four strains, the regenerated prestalk zones (t_2,t_4) contain cells positive for the prespore reporter. In the last two cases, these cells are presumably present but are reporter-negative. This supports the idea that the reporter is stable in the first four cases, but labile in the last two.

(iii) Similarly, prestalk zones regenerated by slugs bearing labile constructs are 'cleaner' than one would expect if the new prestalk cells had deactivated their prespore genes at the moment regeneration began. Prestalk zones are first distinguishable after two hours of regeneration; at this point β-galactosidase-positive cells are plentiful in slugs bearing stable reporters but completely absent when either of our labile reporters is used. Two hours is less than the halflife of *PsA-his-gal*, and clearly too short for this reporter to break down completely. The cells that are stainable with stable, but not labile reporters thus appear to have deactivated their prespore genes before regeneration was initiated, i.e., in the undisturbed prespore zone.

Our experiments thus confirm previous suggestions that a population of prespore cells deactivate their prespore genes while resident in the prespore zone. These cells do not appear to transform to prestalk cells, but rather to anterior-like cells. This is clearly seen in the slugs used in our regeneration experiments (Fig. 5, t_0); the prespore zones of these slugs must contain many ex-prespore cells, but none have moved to the prestalk zones. Prestalk cells in the prespore zone would move rapidly to front of the slug (Bühl and MacWilliams, 1991). As has been shown for anterior-like cells, the ex-prespore cells convert rapidly to prestalk cells if the prestalk zone is removed. These phenomena are consistent with previous suggestions that the transformation from prespore to anterior-like and that from anterior-like to prestalk are independently regulated (MacWilliams et al., 1985; Blaschke et al., 1986).

Conclusions

At the outset of the work reported here, it appeared that the prestalk-prespore pattern in *Dictyostelium* might be unstable. Thus Harwood et al. (1991) suggested, as one of several interpretations of the PLC phenomenon, that 'the demarcation' (between prestalk and prespore zones) "becomes somewhat arbitrary after prolonged periods of slug migration". Our experiments show that this is not true; *Dictyostelium* slugs maintain an immaculate prestalk-prespore pattern over at least 30 hours of migration. The PLC phenomenon nonetheless suggests an instability of a sort: some of the prespore cells transform to anterior-like cells, which later may become prestalk cells. Further information about these transformations – exactly when and where they occur, and whether they represent bidirectional exchange (Kakutani and Takeuchi 1986) or ordered cell flow through the various differentiation states (Sternfeld, 1993) – may be available through the use of two reporters simultaneously (Early et al., 1993), if one of these is stable and the other labile. This may allow us to relate these phenomena to the system of positional signals that various authors have proposed exists in the slug (Schaap, 1991; Kay et al., 1993; Steinbock et al., 1993).

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