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Contrasting activities of the aggregative and late *PDSA* promoters in *Dictyostelium development*

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Abstract

Expression of the *Dictyostelium PdsA* gene from the aggregative (*PdA*) and late (*PdL*) promoter is essential for aggregation and slug morphogenesis, respectively. We studied the regulation of the *PdA* and *PdL* promoters in slugs using labile β-galactosidase (gal) reporter enzymes. *PdL* was active in prestalk cells as was also found with stable gal. *PdA* activity decreased strongly in slugs from all cells, except those at the rear. This is almost opposite to PdA activity traced with stable gal, where slugs showed sustained activity with highest levels at the front. *PdA* was down-regulated after aggregation irrespective of stimulation with any of the factors known to control gene expression. *PdL* activity was induced in cell suspension by cAMP and DIF acting in synergy. However, a DIF-less mutant showed normal *PdL* activity during development, suggesting that DIF does not control *PdL* in vivo. Dissection of the *PdL* promoter showed that all sequences essential for correct spatiotemporal control of promoter activity are downstream of the transcription start site in a region between −383 and −19 nucleotides relative to the start codon. Removal of nucleotides to position −364 eliminated responsiveness to DIF and cAMP, but normal *PdL* activity in prestalk cells in slugs was retained. Further 5′ deletions abolished all promoter activity. This result also indicates that the induction by DIF and cAMP as seen in cell suspensions is not essential for *PdL* activity in normal development.

Keywords: Dictyostelium discoideum; Cyclic nucleotide phosphodiesterase; Promoter analysis; Morphogenetic signaling; Differentiation inducing factor

Introduction

Extracellular cAMP signaling controls morphogenetic movement and prespore gene expression during multicellular development of *Dictyostelium discoideum* (Anjard et al., 2001; Bretschneider et al., 1995; Wang et al., 1988b). Three adenylyl cyclases, ACA, ACB, and ACG, have been identified that synthesize cAMP (Kim et al., 1998; Pitt et al., 1992; Soderbom et al., 1999) and one extracellular phosphodiesterase (PDE) that degrades it (Lacombe et al., 1986). The single *PdsA* gene, that encodes PDE, is regulated at the transcriptional level by three promoters that control expression during growth, aggregation, and multicellular development, respectively (Faure et al., 1990). The multicellular (late) promoter element is only active in the prestalk cell population (Hall et al., 1993). PDE enzyme activity is regulated by PDI, a secreted PDE inhibitor (Wu and Franke, 1990).

It has not been possible to associate any of the adenylyl cyclases with a specific role in either slug morphogenesis or prespore gene expression, because null mutants in any of them can still form migrating slugs and express prespore genes, although in the case of ACA null mutants, this requires overexpression of PKA or prestimulation with cAMP (Pitt et al., 1992, 1993; Soderbom et al., 1999; Wang

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and Kuspa, 1997). This is probably due to partial overlap in the function of these enzymes (Anjard et al., 2001). In contrast, more severe effects are observed when PDE expression is altered. Loss-of-function mutants in PDE cannot aggregate at all (Darmon et al., 1978). Aggregation can be restored by expression of PDE from the aggregative promoter (*PdA*), while restoration of slug and fruiting body formation requires expression from the late promoter (*PdL*) (Sucgang et al., 1997). The essential role of PDE in slug morphogenesis was also demonstrated by experiments where PDI was expressed from a prestalk promoter. This inhibited late PDE activity and blocked slug morphogenesis (Wu et al., 1995). Gain-of-function of PDE is equally disruptive in the slug stage. Overexpression of PDE accelerates the aggregation process, but arrests tip formation and cell differentiation (Faure et al., 1988).

Despite the crucial role of correct PDE expression for multicellular development, little is known about the regulation of the gene by the *PdL* promoter. It is also not clear whether expression from the *PdA* promoter plays a role in the slug stage. Similar to control of the ACA gene, transcription controlled by the *PdA* promoter is downregulated after aggregation (Faure et al., 1990; Pitt et al., 1992). In the case of ACA, this was due to selective cessation of transcription in posterior cells resulting in exclusive expression in the tip region (Verkerke-van Wijk et al., 2001). Other components of the oscillatory signaling system, such as cAR1, Gα2, Gβ, Aimless, and CRAC, become similarly enriched in the tip/prestalk region (Tsujioka et al., 2000) and this may also be the case for *PdA*.

To gain insight into the regulation of *PDE* expression during multicellular development, we studied the patterns of *PdA* and *PdL* promoter activity by using labile β-galactosidase reporter proteins (Detterbeck et al., 1994). *PdA* is downregulated after aggregation in most cells, except those at the rear-guard and basal disc. *PdL* is active in prestalk cells and is synergistically activated by cAMP and DIF in disaggregated cells. However, deletion studies of the *PdL* promoter and analysis of DIF-less mutants show that at least DIF is not the activating signal in normal development.

Materials and methods

Materials

Differentiation Inducing Factor (DIF) was obtained from Affinity Research Products (UK), and chlorophenylred β-D-galactopyranoside (CPRG) was from Roche (UK). Exonuclease III, mung bean nuclease, and pLITMUS29 were from New England Biolabs (USA). The DNA sequencing kit Sequenase 2.0 was obtained from US Biochemical (USA).

Promoter deletions and plasmid construction

The 1.5-kb *PdA* promoter was amplified by PCR from vector pGalA (Hall et al., 1993) using oligonucleotides that generate *Xba*I and *Bg*/II sites at the 5′ and 3′ ends of the promoter, respectively. The *Xba*I/*Bgl*II-digested product was ligated into the *Xba*I/*Bgl*II-digested vector 63-iDQgal to generate *PdA*-ile-gal.

The GalL cell line used previously (Hall et al., 1993) was cotransformed with plasmid pB10T, which carries the neomycin selection cassette, and plasmid pGalL, which contains a gene fusion of *PdL* and *lacZ*. In our hands, β-galactosidase expression in this cell line was weak and declined progressively during cell culture. We made two new vectors, *PdL*-gal and *PdL*-ile-gal, in which *PdL* was fused to stable and labile variants of *LacZ*, respectively. *PdL*-gal was made by replacing the *Kp*nl/*Xh*ol *lacZ* fragment of pDdgal17 (Harwood and Drury, 1990) with the *Kpn*l/*Xho*l *PdL-lacZ* fragment from pGalL. *PdL*-ile-gal was made subsequently by fusing the *Xba*l-*Bgl*II fragment from *PdL*-gal that contained the *PdL* promoter to ubi-ile-gal in the *Xb*al/*Bgl*II-digested vector 63-iDQgal.

To construct promoter deletions, the 0.72-kb *PdL* promoter was subcloned into the pLITMUS29 vector by using the *Eco*RI/*Hin*dIII restriction sites. This yielded the plasmid *PdL*-pLIT29, which contained an exonuclease III protected 3′ overhang (*Nsi*I) and an exonuclease III digestible 5′ overhang (*Eco*RI). Five micrograms of *PdL*-pLIT29 plasmid, digested with *Nsi*I/*Eco*RI, were treated under standard conditions for the preparation of exonuclease III-generated 5′ deletions (Ausubel et al., 1990). Samples from selected time points from the deletion reactions were used to transform *E. coli* DH5 cells. Estimates of the deletion end point were made by agarose gel electorophoresis and precisely determined by DNA sequencing. The *Bgl*II/*Hin*dIII promoter fragments of selected deletion constructs were fused to the *lacZ* gene in vector pDdgal17. Promoter fragments were also constructed by using PCR. PCRs were performed by using the oligonucleotide primers indicated in Fig. 7. All 5′ primers contained a *Xba*I site and all 3′ primers contained a *Bg*III site to allow fusion of the amplified DNA to the *lacZ* gene in pDdgal17 or 63iDQgal. Correct amplification of all PCR-generated constructs was determined by DNA sequencing. *Dictyostelium* AX3 cells were transformed with all constructs, and AX2 and HM1030 cells (Thompson and Kay, 2000) were transformed with *PdL*-ile-gal.

Growth and incubation conditions

All cell lines were grown in standard axenic medium, which was supplemented with 20 μg/ml G418 for cells transformed with Pde promoter fusions with stable or labile gal and with 5 μg/ml blasticidin for the DIFless mutant HM1030. To modulate *PdA* or *PdL* promoter activity in cell

suspension, exponentially growing cells were harvested, washed in 10 mM Na/K-phosphate buffer, pH 6.5 (PB), and distributed on 1.5% agar in PB at 3×10^6 cells/cm^2. Cells were incubated at 22°C until aggregation territories or loose aggregates had formed. Aggregates were dissociated by vigorous pipetting, resuspended in PB at 3×10^6 cells/ml, and challenged with variables at 22°C and 150 rpm for 6–10 h.

For detection of *PdA* or *PdL* promoter activity in developing structures, cells were harvested from growth medium, distributed at 10^6 cells/cm^2 over nitrocellulose filters, supported by PB agar, and incubated at 22°C until the stage of interest had been reached.

Spectrophotometric and histochemical β-galactosidase assays

For spectrophotometric quantitation of β-galactosidase activity, cells were lysed by freeze-thawing, and 100-μl aliquots of lysate were incubated at 22°C in microtiter plate wells with 30 μl of 2.5× Z-buffer and 20 μl of 40 mM CPRG (Dingermann et al., 1989). The OD_{574} was measured at regular time intervals by using a microtiter plate reader. Activity was expressed as the increase of OD_{574} per hour per mg protein.

For histochemical staining, nitrocellulose filters supporting developing cells were fixed for 20 mins with 0.25% glutaraldehyde and 2% Tween-20 in Z-buffer, washed thoroughly, and subsequently incubated with 1 mM X-gal in Z-buffer containing 5 mM $K_3[Fe(CN)]_6$ and 5 mM $K_4[Fe(CN)]_6$ for 1–4 h (Dingermann et al., 1989).

Results

Expression patterns of PdA and PdL

The patterns of activity of the aggregative and late promoters of the PDE gene have been studied previously by using a reporter gene that encodes a stable form of β-galactosidase (Hall et al., 1993). In cases where promoter activity is transient, stable reporters can provide misleading information because the activity of the reporter protein persists, when the mRNA has already dissappeared (Detterbeck et al., 1994). To avoid this problem, the *PdA* and *PdL* promoters were fused to a chimeric DNA encoding ubiquitin-isoleucine-β-galactosidase to give vectors PdA-ile-gal and PdL-ile-gal. After translation, the ubiquitin is cleaved off and exposes the isoleucine at the N terminus of β-galactosidase, which reduces its half-life to about 60 min (Bachmair et al., 1986; Detterbeck et al., 1994; Gonda et al., 1989). The ile-gal constructs cannot be used when promoter activity is low, because they yield considerably lower absolute activities of β-galactosidase than the stable constructs. For deletion studies of the PdL promoter, we therefore also used a stable PdL-gal construct.

When measured with the ile-gal reporter, *PdA* activity becomes detectable after a few hours of starvation and is visible in most of the cells when they are collecting into aggregation streams (Fig. 1A). Once cells have formed tight mounds, expression begins to decline. This decline is first visible in the tip region (Fig. 1B) and later spreads over the anterior 75% of the slug. During culmination, *PdA* activity is concentrated at the basal disc/lower cup area (Fig. 1D, arrow) and becomes restricted to the basal disc when fruiting bodies have formed (Fig. 1E, arrow). The prestalk and stalk cells show almost no *PdA* activity (Fig. 1C and E). This pattern is quite different from that observed with the stable gal reporter. In this case, all cells continue to show high activity after aggregation, but particularly those at the tip (Hall et al., 1993).

The pattern of *PdL* promoter activity measured with the ile-gal reporter is not very different from that observed with the stable gal reporter (Hall et al., 1993). *PdL* is first active at the tip and in a few scattered cells in mounds (Fig. 2B). During slug formation, activity becomes most pronounced in the anterior prestalk region and in cells scattered throughout the prespore region (Fig 2C). In fruiting bodies, only the apical prestalk cells show activity, and there is no significant activity in the stalk and basal disc (Fig. 2D).

A quantitative assay for β-galactosidase in cell lysates shows that *PdA* activity increases after 4 h of starvation to reach a peak at 10 h of development. *PdL* activity increases after cells have formed mounds, to reach a maximum during fruiting body formation (Fig. 3). These temporal expression patterns closely follow those of the *PdA* and *PdL* mRNAs, respectively (Faure et al., 1990), which indicates that the labile reporter gene traces promoter activity reliably.

Signals that up- and down-regulate PdA and PdL promoter activity

Upon starvation, *PdA* activity is induced by the prestarvation factor PSF (Clarke and Gomer, 1995) and further upregulated by cAMP (Franke et al., 1987). After aggregation, *PdA* activity is down-regulated dramatically in the prestalk cell population, while *PdL* activity is induced in these cells. We examined the signals most likely to trigger both events. The predominant signals for postaggregative gene regulation are micromolar cAMP, which induces prespore gene expression (Kay, 1982; Wang et al., 1988b), and DIF, which induces prestalk gene expression (Morris et al., 1987; Williams et al., 1987). We also included nanomolar cAMP pulses, isopropylidene adenosine (IPA), and a combination of micromolar cAMP and DIF. cAMP acts in synergy with DIF to induce the prestalk gene *ecmA;* cAMP pulses are likely to be responsible for tip-specific expression of *ACA* in slugs (Verkerke-van Wijk et al., 2001), while adenosine acts as an inhibitor of prespore differentiation (Schaap and Wang, 1986; Weijer and Durston, 1985). IPA is a metabolically stable analog of adenosine (Soede et al., 1996).

Cells expressing the *PdA* or *PdL* ile-gal constructs were

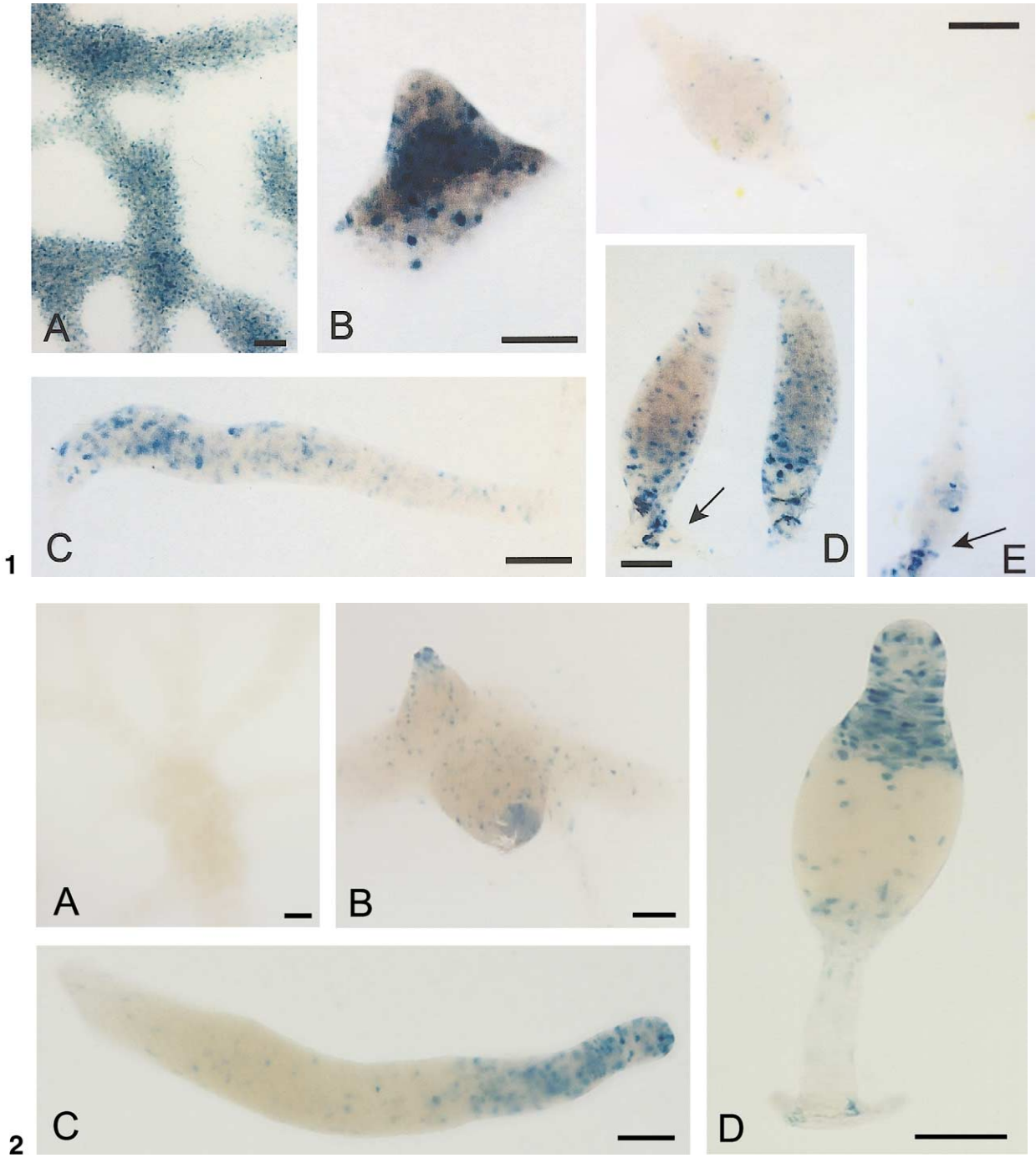

Fig. 1. Patterns of *PdA* promoter activity during normal development. *PdA*-ile-gal cells were distributed on nitrocellulose filters supported by PB agar and incubated at 22°C. Cells were fixed and stained with X-gal after they had formed streaming aggregates (A), tipped aggregates (B), migrating slugs (C), early culminants (D), and mature fruiting bodies (E). Scale bar, 100 μM.

Fig. 2. Patterns of *PdL* promoter activity during normal development. *PdL*-ile-gal cells were incubated at 22°C on nitrocellulose filters and stained with X-gal after they had formed streaming aggregates (A), tipped aggregates (B), migrating slugs (C), and mature fruiting bodies (D). Scale bar, 100 μM.

developed to the loose aggregate stage. The aggregates were dissociated into single cells and incubated for 10 h with the different stimuli. Fig. 4A shows that *PdA* activity decreased strongly, regardless of any added stimulus. However, after an initial decline, cAMP pulses induced a modest transient increase in activity. A stronger but also transient increase was induced by the combination of cAMP and DIF. *PdL* was induced to moderate levels with micromolar cAMP. Neither DIF alone nor any of the other stimuli induced any significant *PdL* activity. However, high levels of activity were induced by the combination of cAMP and DIF (Fig. 4B). This combinatorial effect of cAMP and DIF can either

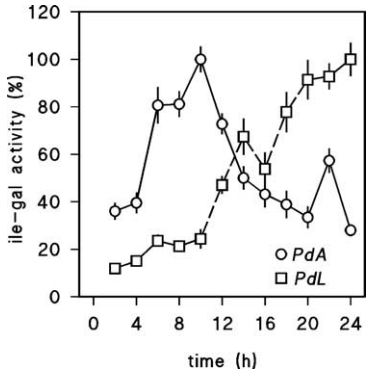

Fig. 3. Developmental regulation of *PdA* and *PdL* promoter activity. *PdA*-ile-gal and *PdL*-ile-gal cells were distributed on PB agar and incubated for 24 h at 22°C. Cells were harvested at 2-h intervals, and β-galactosidase activity was measured in cell lysates. Data were standardized on the protein content of the cell lysate and are expressed as percentage of enzyme activity at $t = 10$ h for *PdA* and at $t = 24$ h for *PdL*. Means and s.e.m. of three time courses, assayed in quadruplicate, are presented.

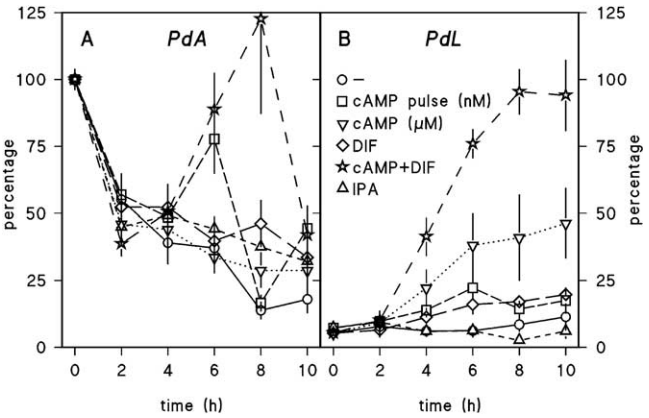

Fig. 4. Effects of cAMP, DIF, and IPA on *PdA* and *PdL* promoter activity. *PdA*-ile-gal and *PdL*-ile-gal cells were incubated on PB agar until loose aggregates had formed ($t = 10$ h). Cells from dissociated aggregates were resuspended in PB and incubated for 10 h with 30 nM cAMP pulses, 300 μM cAMP/h, 100 nM DIF added once, 300 μM cAMP/h plus 100 nM DIF, 1 mM IPA, or without additive. Cells were lysed and assayed for β-galactosidase activity, which was standardized to the protein content of the lysates. Data are expressed as percentage of activity at $t = 0$ h for *PdA* and activity at $t = 10$ h after the cAMP plus DIF treatment for *PdL*. Means and s.e.m. of three experiments performed in quadruplicate are presented.

be due to the signals acting in synergy, or to one signal inducing responsiveness to the other. After prestimulation of cells with cAMP or DIF, maximal induction still required both signals acting together. Furthermore, responsiveness of the cells to either signal was decreased rather than increased (data not shown). This suggests that cAMP and DIF act in synergy on PdL activation.

Is PdL regulated by DIF and cAMP during normal development?

To answer this question, we introduced the *PdL*-ile-gal construct into a mutant that cannot synthesize DIF (Thompson and Kay, 2000). Fig. 5 shows that, in the DIF-less mutant HM1030, *PdL* activity is high in anterior prestalk cells and scattered cells in the prespore region of slugs and in the apical region of fruiting bodies. This is identical to the *PdL* activity pattern in wild-type cells and suggests that DIF is not required to induce *PdL* expression in the developing organism.

As a second approach, we identified regions in the *PdL* promoter that mediate its expression during normal development and its induction by cAMP and DIF in cell suspension. A set of *PdL* promoter modifications was prepared as nested 5′ deletions (Δ constructs) or as PCR-amplified promoter fragments (P constructs) (Fig. 6) and fused to stable or labile reporter genes. We measured the activity of the promoter fragments during normal development and during induction in cell suspensions with DIF and cAMP. All 5′ deletion constructs up to 4Δ, in which the entire untranscribed region is deleted, showed the same pattern of expression in slugs as the intact *PdL* promoter (Fig. 7A and B), with high levels of expression in the prestalk region and in the anterior-like cells. However, the intensity of staining decreased with the shorter constructs and was too low for the ile-gal gene fusions to remain detectable. During incubation in suspension, all constructs showed high levels of induction by the combination of DIF and cAMP and some induction by cAMP alone. The absolute levels of β-galactosidase decreased 3- to 4-fold when the region between −554 and −462 bp was deleted (Fig. 6C). In constructs 5P and 6P, that lacked most of the transcribed region, β-galactosidase activity was undetectable in slugs and could no longer be induced by cAMP and DIF. This indicates that regions downstream of the start site of transcription are essential for promoter function.

Constructs 7P and 8P, which contain the predicted

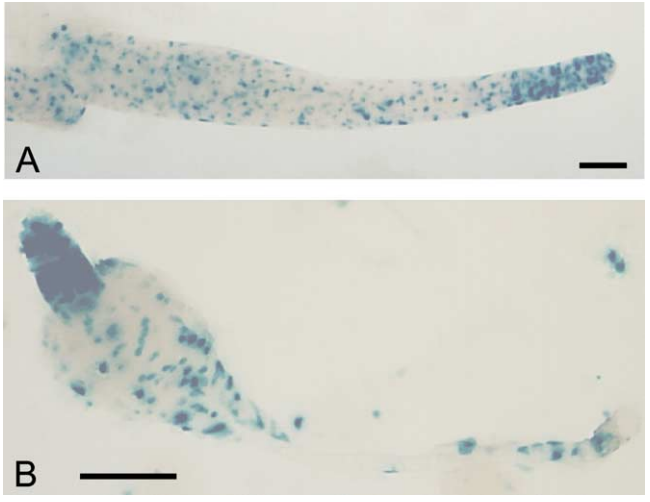

Fig. 5. *PdL* promoter activity in the DIFless mutant. The DIFless mutant HM1030 was transformed with the *PdL*-ile-gal vector. Transformed cells were incubated at 22°C on nitrocellulose filters and stained with X-gal after they had formed migrating slugs (A) and mature fruiting bodies (B). Scale bar, 100 μM.

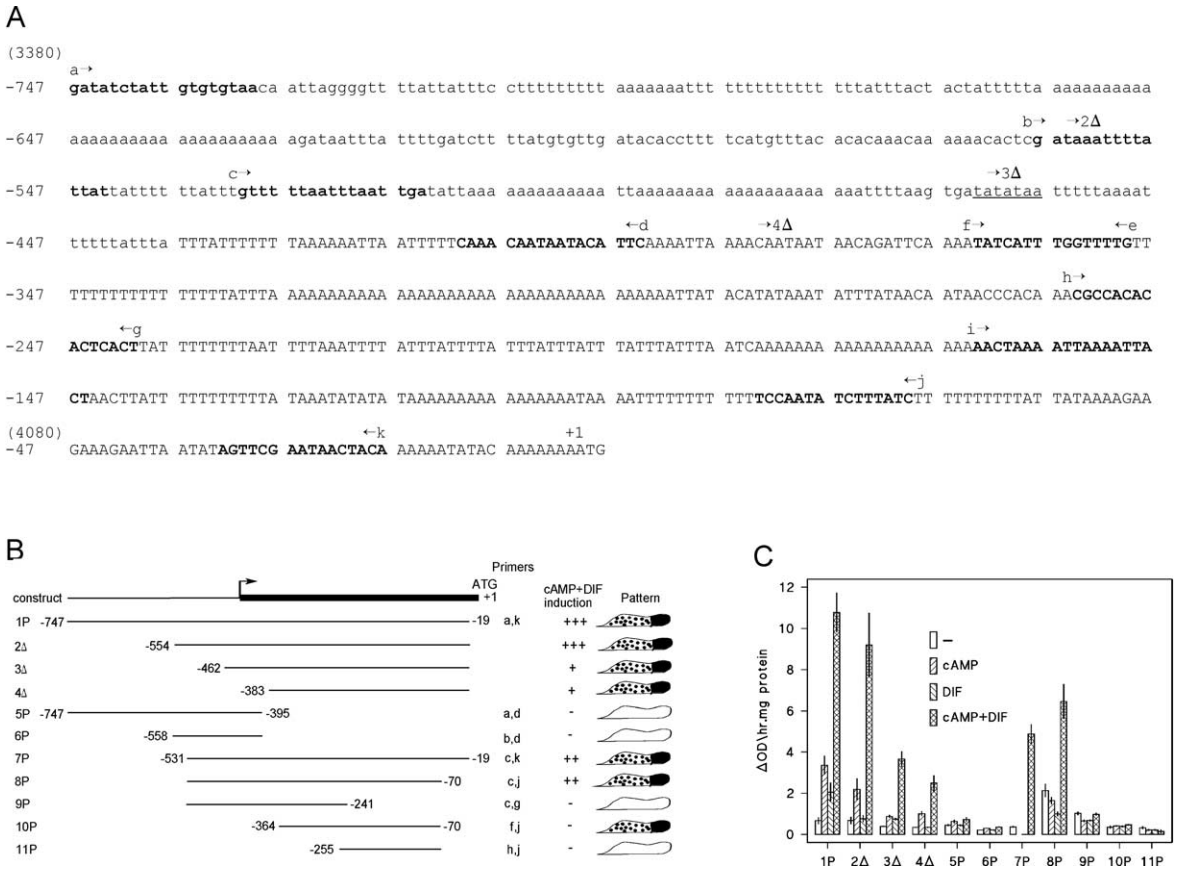

Fig. 6. Overview of promoter deletions and their effect on expression patterns and induction in vitro. (A) Nucleotide sequence of the PdL promoter. The promoter sequence was retrieved from Faure et al. (1990) and renumbered for convenience with the start codon at position +1. The original numbering is indicated between parentheses. Deletion end points of *Exo*III-generated constructs 2Δ, 3Δ, and 4Δ are indicated by arrows. The sequences of PCR primers, a–k, are indicated in bold and their direction by arrows. The TATA box predicted by Faure et al. (1990) is underlined. Transcribed regions are shown in upper case and nontranscribed regions in lower case. (B) Schematic of deletions. The first column provides construct names, the second column shows a schematic view of the constructs and, in the case of PCR-generated constructs, the position of primers used in their creation. The next column indicates relative induction by 100 μM cAMP plus 100 nM DIF (−, no significant induction; +, ++, +++, moderate, strong, and very strong induction, respectively). The last column summarizes the expression pattern of β-galactosidase in the transformants. (C) Response of deletion constructs to cAMP and DIF. Dissociated loose aggregates of cells transformed with deletion constructs were incubated without additives (−), 100 μM cAMP/h, 100 nM DIF, or a combination of 100 μM cAMP/h with 100 nM DIF (cAMP + DIF) and assayed for β-galactosidase activity and protein levels. Two or three independently transformed clones were tested twice for each construct in an assay performed in triplicate. The absolute β-galactosidase activities (Δ OD/h mg protein) obtained after incubation of individual clones for each construct with the different variables were averaged, and means and s.e.m. of the pooled data are indicated.

TATA box and most of the 5′ leader sequence, showed the same expression pattern in migrating slugs as the intact promoter as well as good inducibility by cAMP plus DIF (Figs. 6C and 7E). Expression in slugs and inducibility by cAMP plus DIF was completely lost from the shorter construct 9P (Figs. 6C and 7F), indicating that the essential promoter sequences reside between −70 and −383 bp from the start codon. A deletion from −383 to −364 (compare constructs 4Δ and 10P) retained the normal expression pattern in migrating slugs, but lost cAMP and DIF inducibility (Figs. 6C and 7G). In a further deletion, up to −255, all promoter activity was lost (Fig. 7H). This indicates that one region of the promoter controls normal expression in slugs and another is implicated in induction by cAMP plus DIF in cell suspension.

Discussion

Opposite expression patterns for the aggregative and late PdsA promoters

The *PdsA* gene, that encodes PDE, has an elaborate structure with different promoters controlling expression of the gene during growth, aggregation, and postaggregative development (Faure et al., 1990). The function of PDE during growth is not clear, but it is essential for aggregation and later morphogenesis (Darmon et al., 1978; Sucgang et al., 1997; Wu et al., 1995).

When measured with a stable gal reporter, *PdA*-driven β-galactosidase activity increases strongly during aggregation and remains very pronounced in slugs with highest apparent activity in the anterior prestalk cells (Hall et al.,

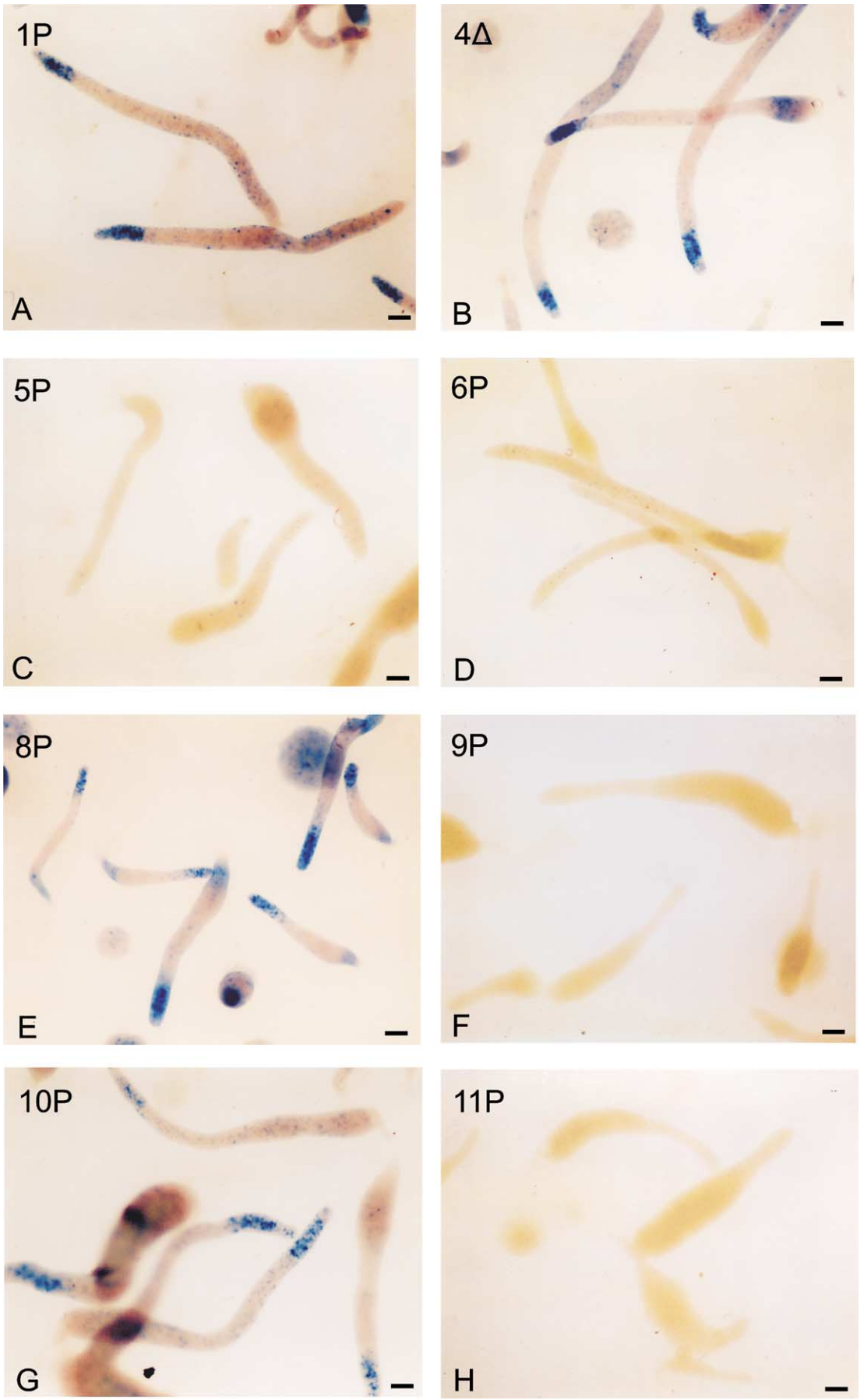

Fig. 7. Expression patterns of *PdL* promoter deletion constructs. Cells transformed with the different deletion constructs were developed on nitrocellulose filters until migrating slugs had formed and stained for β-galactosidase activity. The construct code (see Fig. 7B) is indicated on each photograph. The transformant carrying the full-length *PdL* promoter was photographed after 1 h of staining, and all others after 4 h. Scale bar, 100 μM.

1993). A labile reporter presents quite a different pattern. In this case, *PdA* also increases during aggregation, but decreases once mounds have formed. This decrease spreads as an anterior to posterior wave until only the rear-guard and basal disc cells show activity (Fig. 1). *PdA* transcripts also decrease markedly after aggregation (Faure et al., 1990), so the activity measured with the labile ile-gal construct is likely to follow *PdA* promoter activity closely. The prestalk-enriched expression that was detected with the stable gal is most likely a memory effect, reflecting a tendency for prestalk sorting in cells that strongly expressed *PdA* during aggregation and never lost the stable β-galactosidase enzyme later. This is supported by findings that prestalk sorting and high *PDE* expression during aggregation are both properties of cells that enter development in early cell cycle phase (Wang et al., 1988a; Weijer et al., 1984). No significant differences were found for the stable and labile lacZ versions of the *PdL* promoter (Fig. 2) (Hall et al., 1993). This is probably due to the fact that this promoter is expressed so late that memory effects do not have time to become established.

The pattern of expression of *PdL* is exactly opposite to that of *PdA* with predominant expression in the prestalk region and in the anterior-like cell population. These different distributions are in good agreement with the distribution of PDE enzyme activity in slugs as detected by microdissection. Also here, high activity was detected in both the anterior prestalk region and rear-guard/basal disc region with little activity in the intervening prespore region (Brown and Rutherford, 1980). In situ hybridization also shows the presence of *PdsA* mRNA in the prestalk and rear-guard/basal disc regions (Tsujioka et al., 2001).

Signals that regulate postaggregative PdA and PdL promoter activity

Consensus models for regulation of cell-type specification in *Dictyostelium* indicate micromolar cAMP and DIF as inducers of prespore and prestalk cell differentiation, respectively. Micromolar cAMP can potentially be generated by the adenylyl cyclases ACA, ACB, and ACG, which are all expressed in slugs (Alvarez-Curto and Scehaap, unpublished results; Soderbom et al., 1999; Verkerke-Van Wijk et al., 2001). DIF is generated by prespore cells to trigger conversion of a proportion of their number into anterior-like cells (Kay and Thompson, 2001). These cells then sort to the anterior to form the prestalk region (Sternfeld and David, 1982). Earlier work showed that *PdL* mRNA was elevated in cells that were treated with DIF after a 10-h pretreatment with 5 mM cAMP (Franke et al., 1991). We show here that DIF also induces *PdL* activity in cells that have developed into mounds under more physiological conditions. However, they only do so when cAMP is present at the same time.

We attempted to identify the signal that triggers the precipitous decline in PdA promoter activity once aggregates have formed. We found that dissociating aggregated cells did not block the decline in PdA promoter activity, making it unlikely that cell–cell contact or a diffusible signal secreted in the aggregate is the trigger.

DIF activates PdL in cell suspension, but not in the intact organism

PdL is strongly upregulated by DIF and cAMP in suspensions of dissociated cells, but two lines of evidence indicate that at least DIF does not regulate expression of the gene in slugs: (1) a mutant that is defective in DIF synthesis showed normal expression of *PdL* in slugs and fruiting bodies (Fig. 6); (2) Deletion analysis of the *PdL* promoter indicated that a region from −383 to −364, relative to the start codon, is required for DIF/cAMP induction in disaggregated cells. However, when this region was deleted, *PdL* expression in prestalk and anterior-like cells in slugs was not diminished. The *PdL* promoter showed another unusual feature; primary transcriptional regulatory elements lie in the 5′ leader sequence of the gene. Deletions of this region eliminated promoter activity, whereas deletions that removed regions upstream of the transcription start site only reduced the efficiency of transcription.

Prestalk genes share no common inductive signal

A number of genes, such as *ecmA, ecmB, rasD, carB,* and *PdL,* are first expressed in the prestalk cell population (Reymond et al., 1984; Saxe III et al., 1993; Williams et al., 1987). Other genes, such as *gpaB (Ga2), gpbA (Gβ), carA, acaA, dagA* (CRAC), and *aleA* (aimless), are expressed in most cells during aggregation and later become prestalk specific (Carrel et al., 1994; Tsujioka et al., 2001; Verkerke-van Wijk et al., 2001). This is most likely because their expression is down-regulated in prespore cells.

In contrast to prespore genes, which are dependent on cAMP for expression in cell suspension and in intact slugs (Anjard et al., 2001; Kay, 1982; Wang et al., 1988b), the prestalk genes share no common mode of induction. *EcmA, ecmB,* and *PdL* are all DIF-inducible in suspension (Williams et al., 1987), but only the pstO subdomain of the *ecmA* promoter requires DIF for expression in slugs (Thompson and Kay, 2000). *carB* and *rasD* are induced by micromolar cAMP in suspension (Reymond et al., 1984; Saxe III et al., 1993), while *AcaA* expression is maintained in dissociated slug cells by cAMP pulses (Verkerke-van Wijk et al., 2001).

This heterogeneity in regulation is most likely a consequence of the multifunctionality of cells in the anterior region of the slug. The posterior prespore cells loose the chemotactic functions of aggregative cells and show a concerted pattern of gene activities that prepare them for encapsulation. In contrast, the anterior cells continue to play a very active role in cAMP signaling and chemotaxis; in addition, they acquire novel roles in phototaxis, thermotaxis, and synthesis of the mucopolysaccharide sheath. The

gene products that are required for this plethora of activities are probably under control of several regulatory feedback loops, using any of a number of intercellular interactions.

The overriding characteristic of the prestalk region may be that these cells are inhibited to enter the spore pathway and can therefore remain fully competent for signaling and motility. The function of DIF in this scheme could be to act as a prespore inhibitor rather than a prestalk inducer. The expression of *PdL* and probably other prestalk genes are likely to require signaling interactions that remain to be identified.

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