

Spatial and Temporal Regulation of a Foreign Gene by a Prestalk-Specific Promoter in Transformed *Dictyostelium discoideum*

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We analyzed a developmentally regulated prestalk-specific gene from *Dictyostelium discoideum* encoding a cathepsin-like protease. A hybrid gene was constructed by fusing 2.5 kilobases of 5' flanking sequences and part of the coding region of the gene in-frame to the *Escherichia coli* β -glucuronidase gene and was transformed into *D. discoideum* cells. In cells transformed with this vector, the gene fusion showed the same temporal regulation as the endogenous gene during multicellular development and, like endogenous prestalk genes, was highly inducible by cyclic AMP in *in vitro* cell cultures. Moreover, immunofluorescence studies showed that the fusion protein had the same spatial distribution within the migrating pseudoplasmodium as the endogenous gene. The results indicate that the regions of the *D. discoideum* prestalk-specific cathepsin gene contain all the necessary information for proper temporal, spatial, and cyclic AMP regulation of a prestalk cell-type gene in *D. discoideum* transformants and leads the way for experiments to identify the cell-type-specific regulatory elements.

Dictyostelium discoideum is an excellent system to study the molecular mechanisms regulating cell-type differentiation. In the presence of a food source, *D. discoideum* exists as unicellular, haploid vegetative amoeba. Upon removal of the food source, the cells undergo a relatively simple, multicellular developmental cycle. At ~6 h after starvation, cells start to stream together, using relayed pulses of cyclic AMP (cAMP) as the chemoattractant. At ~10 h, the cells have formed a tight aggregate and differentiate into either prestalk or prespore cells which then become spatially organized in the migrating slug or pseudoplasmodium. Markers for prestalk cells begin to appear at ~10 h after starvation, while markers for prespore cells begin to appear at ~15 h after starvation. A fruiting body containing mature spores and stalk cells is formed by ~25 h.

We have been interested in the mechanisms controlling cell-type-specific gene expression in *D. discoideum*. Sets of coordinately regulated genes have been isolated which are expressed preferentially in either prestalk or prespore cells (1, 9, 16), and culture conditions have been defined which allow specific induction of these genes in cultures of single cells. In fast-shaking cultures, prestalk-specific messages are induced in response to added cAMP but are not expressed in the absence of cAMP. In contrast, prespore-cell-specific messages are not expressed owing to lack of sustained cell-cell or cell-surface interactions. In cells plated at low density in the presence of a secreted factor, CMF, both prestalk- and prespore-specific genes are induced by exogenous cAMP (8, 9).

One of the cell-type-specific genes identified in our laboratory encodes a cathepsin-like cysteine protease called prestalk-specific cathepsin (pst-cath) which is preferentially expressed in prestalk cells and shows the same temporal, spatial, and cAMP regulation as other prestalk-specific genes (S. Datta and R. A. Firtel, manuscript in preparation). To examine sequences conferring cell-type specificity of this gene, we made use of the *D. discoideum* DNA-mediated transformation system (12, 13). We constructed a gene fusion containing the promoter and upstream sequences of

the *D. discoideum* cath gene (Datta and Firtel, in preparation) linked to the coding region of the *Escherichia coli* β -glucuronidase (β -gluc) gene (R. A. Jefferson, S. M. Burgess, and D. Hirsh, Biochemistry, in press). The gene fusion was transformed into *D. discoideum*. We show that the fusion gene is regulated coordinately with the endogenous gene during development and in fast-shaking cultures with exogenous cAMP in concert with other prestalk genes. Using antibodies against β -gluc, we show that the gene fusion demonstrates spatial and cell-type regulation similar to that of the endogenous gene. These results indicate that the *D. discoideum* genomic sequences used in the gene fusion carry all the necessary *cis*-acting sequences for proper regulation.

MATERIALS AND METHODS

Growth and development of *D. discoideum*. *D. discoideum* KAx-3 (14) was grown axenically in suspension (3) and used as a DNA source for transformation. This strain is derived from a single-colony isolate selected in our laboratory from an Ax-3 (5) cell line obtained from R. Kessin. Since the strain has different properties, such as a higher transformation frequency, than the original culture obtained from R. Kessin, it is designated KAx-3.

Cells were plated for development on buffer-saturated filter pads (3, 20). Fast- and slow-shaking cultures were prepared as previously described (9) with modifications (16).

Sequencing. Sequencing was accomplished by the Maxam and Gilbert method (7), using restriction fragments of the genomic clone labeled with polynucleotide kinase and [γ - 32 P]ATP (6).

Analysis of RNA levels in vegetative and developing cells. RNA was isolated from developing cells, sized on denaturing agarose gels, blotted onto GeneScreen, and analyzed by hybridization of nick-translated DNA probes as previously described (9).

***D. discoideum* transformation vector construction.** The pst-cath genomic clone was isolated as a 6.0-kilobase (kb) *Hind*III fragment cloned into pV3, a pBR322 derivative containing a DNA fragment from *D. discoideum* containing a *Bgl*III restriction site (Fig. 1). The pV3 vector was obtained

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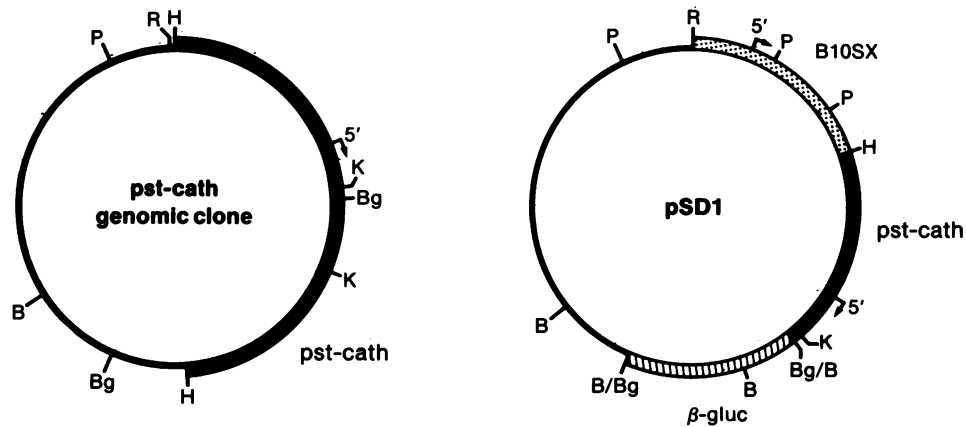


FIG. 1. Restriction maps of *pst-cath* genomic clone and *pSD1*. See Materials and Methods for discussion of the construction of *pSD1*. P, *Pst*I; H, *Hind*III; R, *Eco*RI; Bg, *Bgl*II; B, *Bam*HI; Bg/B or B/Bg, *Bgl*II-*Bam*HI fusion; K, *Kpn*I.

from A. Weiner, Yale University. The isolation and characterization of the *pst-cath* gene are described elsewhere (Datta and Firtel, in preparation). A more detailed map of the *Hind*III insert is shown in Fig. 2. The *pst-cath* genomic clone was digested with *Bgl*II, and the large fragment containing pV3 sequences and the 5' end of the *pst-cath* gene was purified on a 1% low-melting-temperature agarose gel. A clone of the *E. coli* β -gluc gene, as a *Bam*HI insert, was obtained from R. Jefferson and D. Hirsh (Jefferson et al., in press). The *Bam*HI fragment corresponding to the complete coding region was purified on a 1% low-melting-temperature agarose gel from a partial *Bam*HI digest. This insert contained no *Eco*RI or *Hind*III sites. The fragment carrying the *pst-cath* 5' end was ligated to the β -gluc-coding region and transformed into *E. coli*. This created an in-frame *pst*-

cath- β -gluc gene fusion which was confirmed by DNA sequencing across the fusion junction (data not shown). DNA was made from transformants and analyzed by restriction enzyme digests, and clones carrying the correct vector construct were identified. The *pst-cath*- β -gluc fusion gene was digested with *Hind*III and *Eco*RI and purified away from the small *Hind*III-*Eco*RI fragment seen in Fig. 1 on a 1% low-melting-temperature agarose gel. The Act6-Neo^R fusion gene carrying an actin 8 3' termination-polyadenylation region from the B10SX *D. discoideum* transformation vector (11-13) was isolated as a *Hind*III-*Eco*RI fragment and ligated into the gene fusion. DNA was made from transformants and checked for the presence of the Act6-Neo^R gene by restriction enzyme analysis of the DNA. This vector is designated *pSD1* and is shown in Fig. 1.

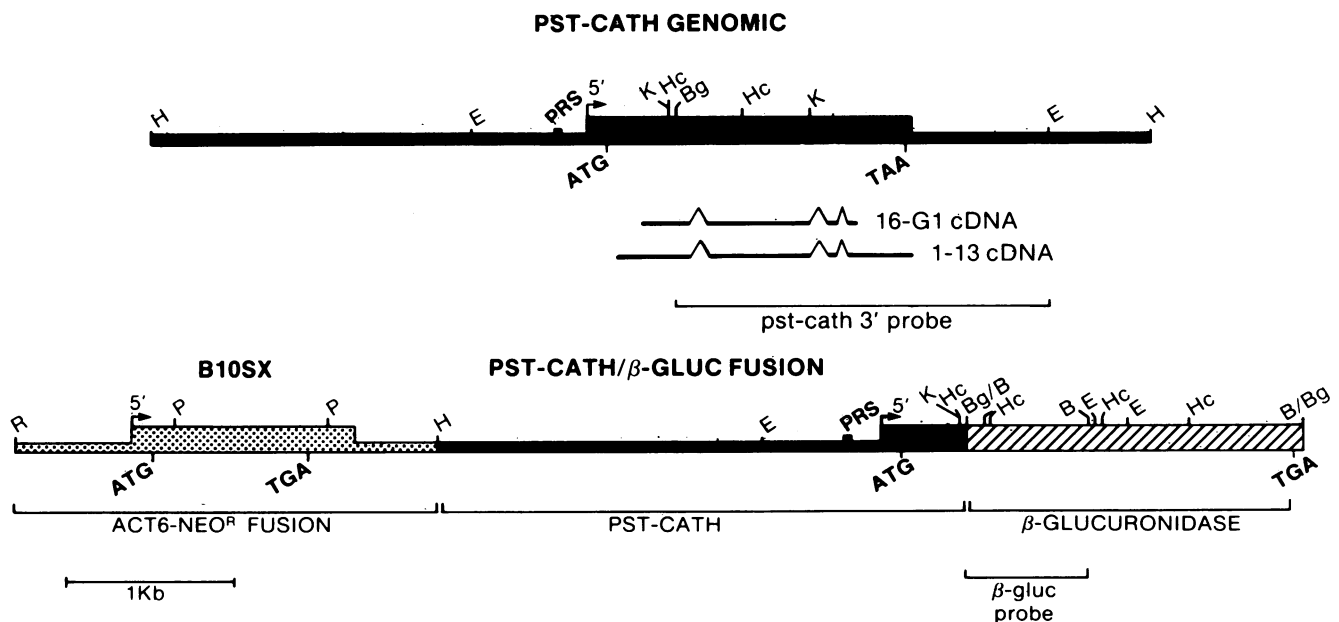


FIG. 2. Detailed restriction enzyme and transcription map of the *pst-cath*- β -gluc gene fusion transformation vector *pSD1*. Construction of *pSD1*, the *pst-cath*- β -gluc transformation vector, is described in Materials and Methods. E, *Eco*RV; R, *Eco*RI; H, *Hind*III; Hc, *Hinc*II; K, *Kpn*I (*Asp* 718); B, *Bam*HI; Bg, *Bgl*II; P, *Pst*I; Bg/B or B/Bg, *Bam*HI-*Bgl*II fusion; TGA, amino acid stop codon from *E. coli* genes. PRS indicates a GC-rich putative regulatory sequence ~200 bp upstream of the transcription start site. Origin of probes used to examine developmental and cAMP regulation are indicated.

Transformation of *D. discoideum* and single-colony isolation. The pst-cath- β -gluc transformation vector pSD1 was transformed into KAx-3 cells by the transformation procedure described previously (12, 13). Populations of transformed cells capable of growth in 20 μ g of G418 per ml were established. Samples were then plated for single colonies on SM plates in the presence of *Klebsiella aerogenes* (20). Single colonies were picked and tested for growth in axenic medium containing G418. Cultures derived from single colonies capable of growth in 20 μ g of G418 per ml were selected (11).

Immunostaining. Affinity-purified rabbit anti- β -gluc antibody and purified *E. coli* β -gluc were a generous gift of Richard Jefferson and David Hirsh. Preparation and characterization of the rabbit anti-cath antibody will be described elsewhere (Datta and Firtel, in preparation). This antibody recognizes a single ~45-kilodalton protein in Western blots of protein isolated from developing cells known to be expressing the pst-cath gene but does not react with proteins from vegetative or early developing cells (Gomer et al., in preparation). The pst-cath gene encodes a similarly sized protein as determined from the derived amino acid sequence of the gene (Gomer et al., in preparation). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting were performed as previously described (4, 16). All manipulations for cell fixation were at room temperature unless noted. For immunofluorescence on developing cells, aggregates were removed from filters and disaggregated by gently squirting in distilled water with a Pasteur pipette. Drops of cells were allowed to adhere to glass cover slips for 5 min. Excess liquid was drained off, and the cover slips were placed in room-temperature 100% methanol for 10 min and then air dried for 2 h.

To make slugs, mid-log-phase cells grown in axenic shaking culture were pelleted by centrifugation in a Sorvall SS-34 rotor at 2,000 \times g for 5 min, suspended in distilled water, and repelleted. Cells were then resuspended in distilled water to a density of approximately 5×10^7 /ml, and 0.1 ml was added along a chord of a 10-cm-diameter petri dish containing 1.5% agar in distilled water. The dish was sealed and placed in a blackened enclosed chamber with a small slit in it to admit light. The cells aggregated and formed slugs which were phototoxic toward the light source (15). Forty-eight hours after development was initiated, the slugs were fixed in 3.7% formaldehyde in distilled water. After 12 to 24 h of fixation, slugs were embedded in Tissue-Tek OCT compound, frozen, and cryosectioned at -18°C on a Slee cryostat. Sections were allowed to thaw onto subbed cover slips and were then placed in methanol for 10 min and finally allowed to air dry for 2 h.

For cells from shaking cultures, samples of fast and slowly shaken cells were removed 12 h after the addition of cAMP (see Results). Cells were allowed to adhere to glass cover slips for 5 min, excess liquid was drained, and the cover slips were immersed in methanol for 10 min and then air dried.

For low-density-plated cells, cells were pelleted, resuspended in fresh medium (buffer), repelleted, and resuspended to a density of approximately 10^7 cells per ml. The cell density was determined by counting in a hemacytometer. Samples of these cells were added to fresh or conditioned medium (8) to a final density of 1.8×10^4 cells per ml. The diluted cells (200 μ l) were added to the wells of a Miles eight-well multiwell chamber on a plastic slide. After 4 h, cAMP from a 50 mM stock (pH 7.2) was added to some of the wells to a final concentration of 300 μ M. Twenty-four hours after the initial addition of cells, the chambers were

drained and removed from the slides. The slides were methanol fixed and air dried as above.

Immunofluorescence. After air drying, cover slips were immersed in 20 mM Tris hydrochloride [pH 7.5]–100 mM NaCl–0.005% Nonidet P-40 at room temperature for 10 min. Primary antibody, either the affinity-purified rabbit anti- β -gluc at 7 μ g/ml or the rabbit anti-*D. discoideum* cath (1:30 dilution of ammonium sulfate-fractionated serum), was then added to the slides or cover slips for 2 h. These were then rinsed for at least 30 min, incubated with 0.2 mg of fluorescein isothiocyanate-conjugated goat anti-rabbit antibody (Sigma Chemical Co., St. Louis, Mo.) per ml for 2 h, and then washed as above. All washes, incubations, and dilutions were at room temperature. Cells or sections were photographed on Kodak Tri-X film through Zeiss epifluorescence optics (4).

RESULTS

Construction of gene fusion and transformation vector. A gene fusion was constructed to differentiate transformed pst-cath mRNA from the endogenous pst-cath message (see Materials and Methods and Fig. 1 and 2). The vector, pSD1, carried 2.5 kb of the 5' upstream sequences, the 5' untranslated region, and the first 350 base pairs (bp) of the coding region of the pst-cath gene fused in-frame to the *E. coli* β -gluc-coding region (see Materials and Methods) to allow production of a fusion protein detectable by anti- β -gluc antibodies. This gene fusion is denoted pst-cath- β -gluc. pSD1 also contained the *EcoRI*-*HindIII* fragment from the *D. discoideum* transformation vector B10SX, which carries the neomycin resistance gene under the control of the actin 6 promoter and the actin 8 3' flanking region containing a transcription termination or polyadenylation signal or both (11–13). Restriction enzyme and transcription maps of pSD1 are shown in Fig. 1 and 2.

The gene fusion-transformation vector pSD1 was introduced into *D. discoideum*, and transformants were selected for G418 resistance (11–13) (Materials and Methods). Transformed populations were grown in medium containing 20 μ g of G418 per ml to select transformants carrying a moderately high level of vector sequences. After the transformed-cell population was stably growing in G418, samples were plated at low density on *K. aerogenes* to isolate single colonies. DNA was isolated from both the population and a single colony capable of growth on G418 (11, 13), digested with *Asp* 718 and *HindIII*, size fractionated and transferred to nitrocellulose (18). The filter was probed with labeled pSD1 (Fig. 3). Comparison of hybridization intensity between pSD1 in transformed cells and plasmid controls led to an estimated gene fusion copy number of ~100 to 200 copies per cell in the population (data not shown) and ~100 copies in the single-colony isolate (Fig. 3).

Asp 718 linearized pSD1, while the *Asp* 718-*HindIII* double digest released the pst-cath 5' end (Fig. 1 and 2) from the transformation vector. Figure 3 shows bands present in lanes containing transformed DNA which correspond to linearized pSD1, the released pst-cath 5' end, and the remaining vector- β -gluc fragment, indicating that the majority of the vector sequences probably integrated in a head-to-tail tandem array. Additional bands are seen which may be a result of multiple integration sites, rearrangements, or some head-to-head tandem arrays. The lane labeled *EcoRI* contains DNA isolated from untransformed KAx-3 cells. Since the autoradiogram was exposed for a short period of time for proper exposure of the pSD1 DNA in transformants at 100 copies per cell, the restriction fragments containing the

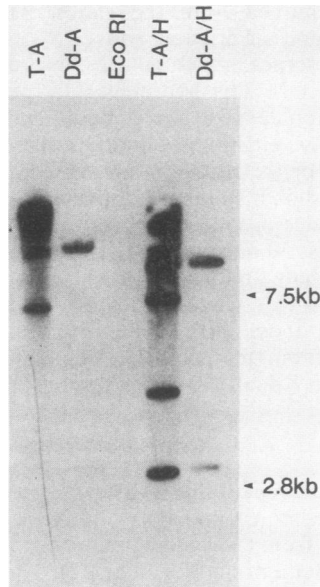


FIG. 3. Genomic southern blot of transformed single-colony DNA. Approximately 0.3 μ g of DNA from a pSD1-transformed single-colony isolate was digested with restriction enzyme *Asp* 718 (T-A) or *Asp* 718 and *Hind*III (T-A/H). As a control, 3.6 ng of pSD1 plasmid DNA were combined with 1.5 μ g of untransformed genomic *D. discoideum* DNA and digested with either *Asp* 718 (Dd-A) or *Asp* 718 and *Hind*III (Dd-A/H). Untransformed *D. discoideum* DNA (1.5 μ g) was also restricted with *Eco*RI (Eco RI) to provide a size marker. The restriction digests were size fractionated on a 0.7% agarose gel and blotted onto nitrocellulose. The filter was prehybridized for 6 h and hybridized with 10^6 Cerenkov cpm of nick-translated pSD1 plasmid. The filter was washed free of nonhybridized counts and exposed to XAR-5 film for 12 h.

single-copy endogenous *pst-cath* gene and the actin 6 5' flanking region are not visible.

Expression of gene fusion during development. To examine the expression of the gene fusion, transformed cells were plated on filter pads to initiate the developmental cycle, and development was followed by observing morphogenesis under a dissecting microscope. Control cells transformed with B10SX formed tight aggregates at \sim 12 h, similar to untransformed KAx-3 cells, while a cell population or single-colony isolate carrying pSD1 formed tight aggregates at 21 h. After tight aggregates formed, morphological development continued at approximately the same rate as in control cells. Cells were harvested at various morphological stages, and their RNA was extracted and size fractionated on denaturing gels. The gels were blotted and probed with either a labeled β -gluc gene probe (Fig. 2), to follow expression of the *pst-cath*- β -gluc gene fusion, or with labeled *pst-cath* 3'-end probe (Fig. 2), to follow expression of the endogenous gene. A prespore-specific cDNA (3-E2) (9) was also used to assay for prespore gene expression in transformants. The results from a single-copy isolate are shown in Fig. 4 and are indistinguishable from the results obtained with RNA isolated from the whole population (data not shown). As can be seen, the β -gluc probe hybridized to a smear of RNA with some major visible bands. The largest band observed was at \sim 7 kb. The *pst-cath*- β -gluc gene fusion did not carry a *D. discoideum* 3' polyadenylation or transcription terminator site. The size of the 7-kb transcript suggests that it continues from the *pst-cath*- β -gluc cap site through most of the vector and may terminate in the 5' untranslated region of the

Act6-Neo^R 5' flanking region. We have previously shown that the transcript of the Act6-Neo^R gene fusion vector B10 which lacks the actin 8 3' end initiates at the actin 6 cap site and continues through the vector, terminating in the 5' flanking region of the downstream B10 gene in the tandem array (11). In addition to the \sim 7-kb RNA, a smear of

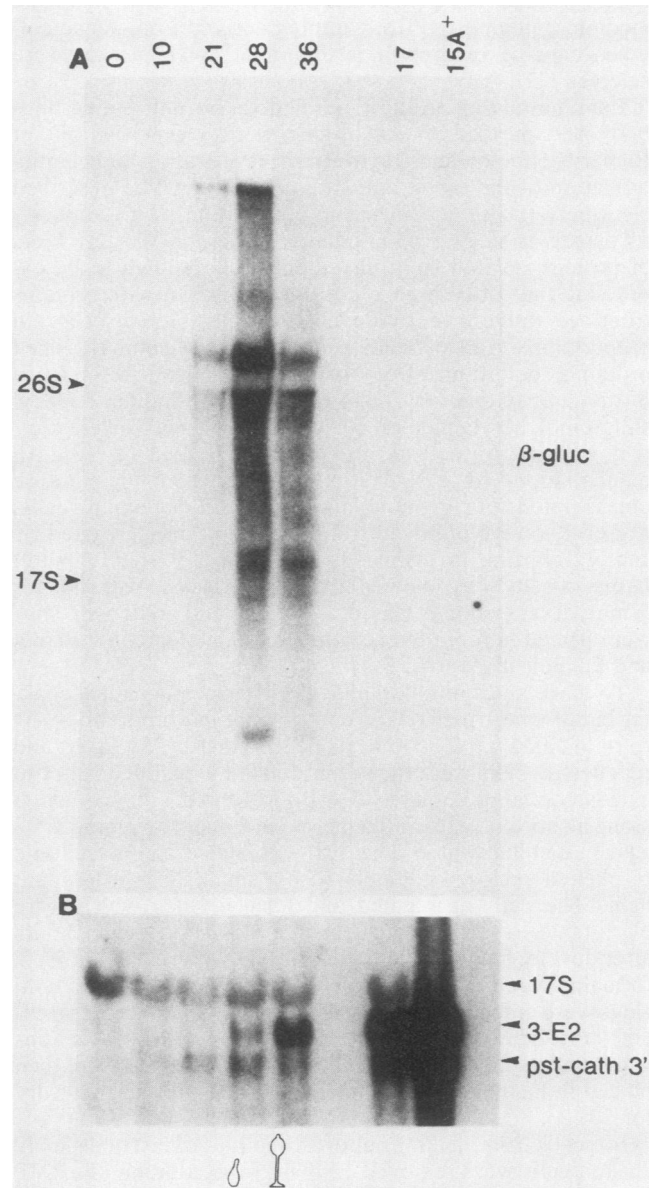


FIG. 4. Developmental regulation of *pst-cath*- β -gluc gene fusion in transformed cells. Transformed cells from a single-colony isolate were harvested and plated on filter pads for development. Samples were taken at defined morphological stages at the times indicated (hours), RNA was extracted, and 3 μ g per lane was size fractionated on a 1.5% agarose denaturing gel. RNA was blotted onto GeneScreen, baked, prehybridized for 6 h, and hybridized with either 1.0×10^7 Cerenkov cpm of nick-translated β -gluc probe (A) (Fig. 2) or 5×10^6 Cerenkov cpm each of nick-translated *pst-cath* 3' probe (Fig. 2) and 3-E2 insert (B). Control lanes consisting of 3 μ g of RNA from 17-h-developing untransformed KAx-3 cells or 2 μ g of poly(A)⁺ RNA from developing untransformed KAx-3 cells were included. Filters were exposed to XAR-5 film for 12 h for hybridization with the β -gluc probe or 48 h with an intensifying screen at -70°C when hybridized with *pst-cath* 3' or 3EC probe.

lower-molecular-weight RNAs was observed, including a major band at ~4.2 kb, slightly larger than the 26S rRNA. The RNAs smaller than the 7-kb transcript could result from either instability of the fusion RNA or termination at various points within the vector. The smear was not due to a general degradation of the RNA since other probes (see below) showed hybridization to discrete bands and since no visible degradation of the rRNA was seen after staining of the gel (data not shown).

The RNA levels of the *pst-cath-β-gluc* transcripts (Fig. 4A) changed coordinately with those from the endogenous *pst-cath* gene (Fig. 4B). RNA was not detectable in vegetative or preaggregative cells. The levels started to rise as the cells formed a multicellular aggregate, reached a maximal level before the onset of culmination, and then declined. As expected, the *β-gluc* probe did not hybridize to the control lanes carrying total and poly(A)⁺ RNA from untransformed aggregates. We also hybridized a prespore-specific gene probe to the blots. 3-E2 mRNA started to accumulate as *pst-cath* mRNA levels peaked (Fig. 4B), as has been observed in wild-type and a series of temporally deranged strains (8, 9; C. L. Saxe and R. A. Firtel, submitted for publication). These results indicate that although the initial stages of development are prolonged in the transformed cells, the relative timing of the cell-type-specific gene expression in relation to cellular morphogenesis is unchanged.

Expression of fusion protein. To test for proper translation of the *pst-cath-β-gluc* mRNA, Western blots of transformed cells were probed with anti-*β-gluc* antiserum (Fig. 5). The anti-*β-gluc* antibody stained purified *β-gluc* (lane 2) but did not appreciably stain any bands in 15-h-developed KAx-3 cells (lane 1). The antibody stained a major band in 15-h-developed cells from three different single colonies of the *pst-cath-β-gluc* transformant (lanes 3, 4, and 5). This band had a molecular size of 90 kilodaltons compared with 75

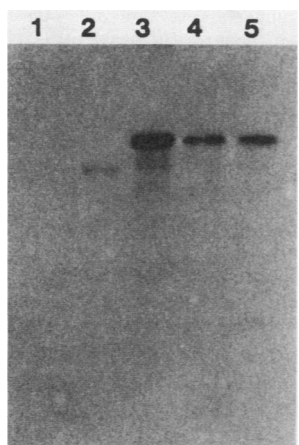


FIG. 5. Autoradiograph of Western blot stained with anti-*β-gluc*. Cells or protein were solubilized in sodium dodecyl sulfate sample buffer and electrophoresed on a 15% polyacrylamide-sodium dodecyl sulfate gel. Proteins from the gels were electroblotted to nitrocellulose which was then incubated with antibody, washed, incubated with ¹²⁵I-labeled protein A, washed, and autoradiographed. Lane 1, 15-h-developing KAx-3 cells. Lane 2, 0.01 μg of purified *β-gluc*. Lanes 3 to 5, 15-h-developing *pst-cath-β-gluc*-transformed cells: lane 3, Cells derived from single colony no. 5; lane 4, cells from single colony no. 1; lane 5, cells from single colony no. 3. Some faint lower-molecular-weight bands can be seen in lane 3 which has the strongest signal. These could be due to proteolysis or processing of the fusion protein within the cell.

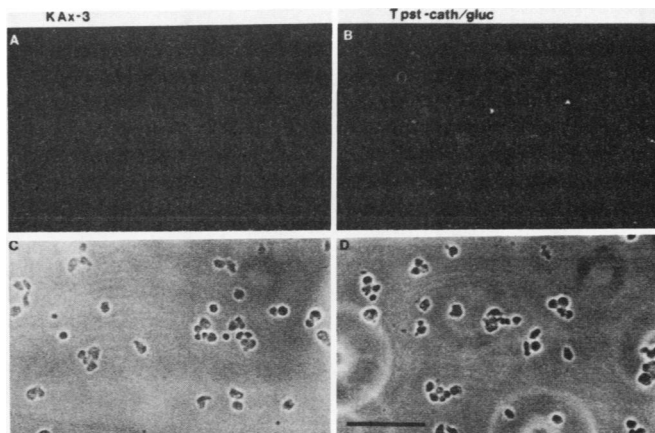


FIG. 6. Indirect immunofluorescence staining of KAx-3 (wild-type) and T *pst-cath-β-gluc* (transformed) cells with anti-*β-gluc* antibody. (A) 15-h-developing KAx-3 cells stained for *β-gluc*; a phase-contrast image of this field is shown in panel C. (B) 15-h-developing transformed cells stained for *β-gluc*; a phase-contrast image of this field is shown in panel D. Bar in panel D is 100 μm.

kilodaltons for *β-gluc*. The difference can be accounted for by the 350 bp of *pst-cath*-coding sequence in the *pst-cath-β-gluc* gene fusion, assuming approximately 37 kilodaltons of protein per 1,000 bp of coding DNA. The fusion protein could not be seen by Coomassie staining of 15-h transformed cells electrophoresed on a one-dimensional sodium dodecyl sulfate-polyacrylamide gel (data not shown).

The *pst-cath-β-gluc* fusion protein could also be detected by immunofluorescence. Figure 6 shows staining of dissociated 15-h-developed KAx-3 cells (Fig. 6A) and transformed *pst-cath-β-gluc* cells (Fig. 6B). None of the KAx-3 cells showed any staining with anti-*β-gluc* antibody, while transformed cells had a bimodal staining distribution: some cells showed no staining while other cells were stained with the antibody. Since the gene fusion would only be expected to be expressed in prestalk cells (see below), staining of only a subpopulation of cells from aggregates is expected.

Using a variety of assays, no *β-gluc* enzymatic activity could be detected in aggregates containing the fusion protein (see Discussion).

Localization of fusion protein within slugs. To examine the spatial distribution of fusion gene expression, we used immunofluorescence to stain migrating slugs. In migrating slugs, the prestalk cells are spatially localized in the anterior 10 to 20%, while the posterior 80 to 90% contains the prespore cells. KAx-3 and transformed cells were starved under conditions favoring migratory slug formation, and the slugs were fixed, embedded, and cryosectioned (see Materials and Methods). Sections were stained for immunofluorescence with either the anti-*β-gluc* or anti-*pst-cath* antibody (see Materials and Methods) and also photographed with phase-contrast optics (Fig. 7E, F, G, and H). Figure 7D shows a slug composed of transformed cells stained for *β-gluc*. The antibody stained cells predominantly in the anterior region of the slug. The cells in this region showed a fairly even staining distribution. The region ends abruptly at a plane normal to the axis of the slug. Additional cells in the outer radial regions of the slug were also stained with the anti-*β-gluc* antibody (see below). Similar slugs composed of transformed cells were stained with anti-*cath* antibody which should stain both the endogenous *pst-cath* and the fusion protein, since the antibody against *pst-cath* was made with a

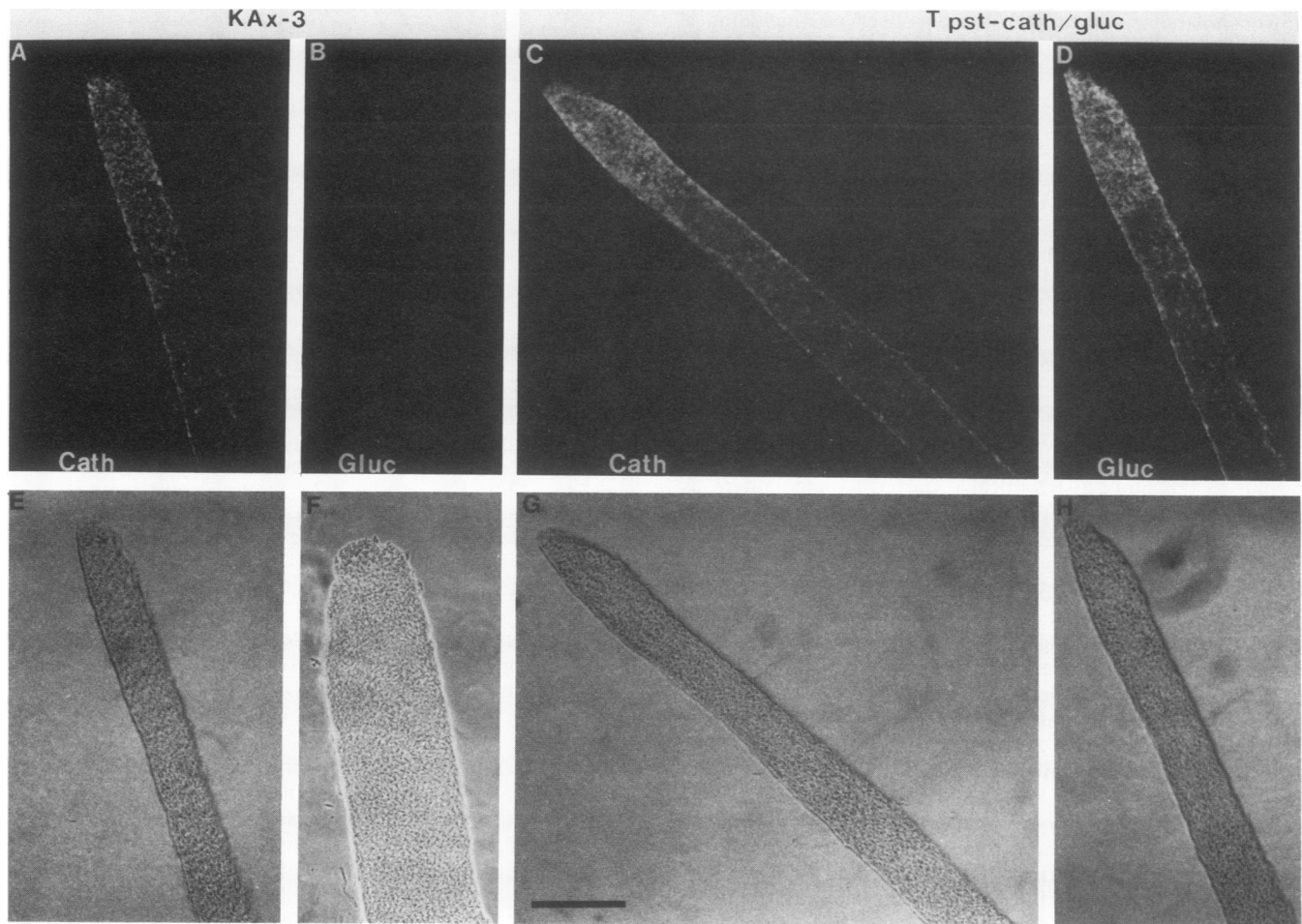


FIG. 7. Indirect immunofluorescent staining of cryosections of migrating slugs. (A) A cryosection of a KAx-3 slug was stained for cath. A phase-contrast image of this section is shown in panel E. (B) A KAx-3 slug stained for β -gluc; the corresponding phase image is shown in panel F. (C) A pst-cath- β -gluc-transformed slug stained for cath; the phase-contrast image is shown in panel G. (D) A pst-cath- β -gluc-transformed slug stained with anti- β -gluc antibody; panel H shows the phase-contrast image. Bar in panel G is 100 μ m.

β -galactosidase fusion protein carrying a portion of the same coding sequence present in the pst-cath- β -gluc fusion protein. The staining with this antibody was very similar to the pattern seen with anti- β -gluc (Fig. 7C).

Untransformed KAx-3 slugs were also sectioned and stained. Staining with anti-cath antibody (Fig. 7A) showed a pattern very similar to that seen in the transformed cells. As expected, no staining was seen with the anti- β -gluc antibody (Fig. 7B).

A small number of stained cells were seen in the posterior region of slugs from both transformed and untransformed cell populations. These could be anteriorlike cells found in this region of the slug (19) which are known to express some prestalk markers (2). In addition, stained cells were observed on the surfaces of the posterior in slugs from transformants and control cells stained with either antibody. This was observed in most sections. These cells could be prestalk cells which are naturally localized in this portion of the slug. A more complete analysis of the distribution and ontogeny of prestalk cells will be published elsewhere (Gomer et al., in preparation). The similarity of staining between transformed slugs stained for β -gluc and untransformed slugs stained for cath indicates that the spatial expression pattern of the transformant fusion gene is the same as the endogenous cath gene.

cAMP regulation of fusion gene. We have previously shown that prestalk-specific mRNAs, including that encoding pst-cath, are expressed in fast-shaking, high-density cell cultures in response to exogenous cAMP but are not induced in the absence of added cAMP (9). Under these conditions, the cells do not have the sustained cell-cell or cell-surface interactions which are necessary for prespore gene expression (8, 9). To examine the expression of the pst-cath- β -gluc gene fusion under these conditions, vegetative transformed cells were placed in a fast-shaking culture for 6 h. The culture was then divided into four aliquots which were shaken either slowly (70 rpm), which allows agglomerate formation, or fast (230 rpm), in which the cells remain as single cells, with or without the addition of exogenous cAMP to 200 μ M every 2 h. Northern blots were performed with RNA extracted from cells taken at various times after initiation of starvation. The β -gluc probe was used to assay for pst-cath- β -gluc expression, and the pst-cath 3'-end probe was used to verify expression of the endogenous gene. A labeled prespore-specific cDNA was used to monitor prespore gene expression. The results are shown in Fig. 8A and B.

As can be seen, there was negligible expression of the pst-cath- β -gluc fusion or the endogenous pst-cath gene in fast-shaking culture lacking cAMP but a high level of expres-

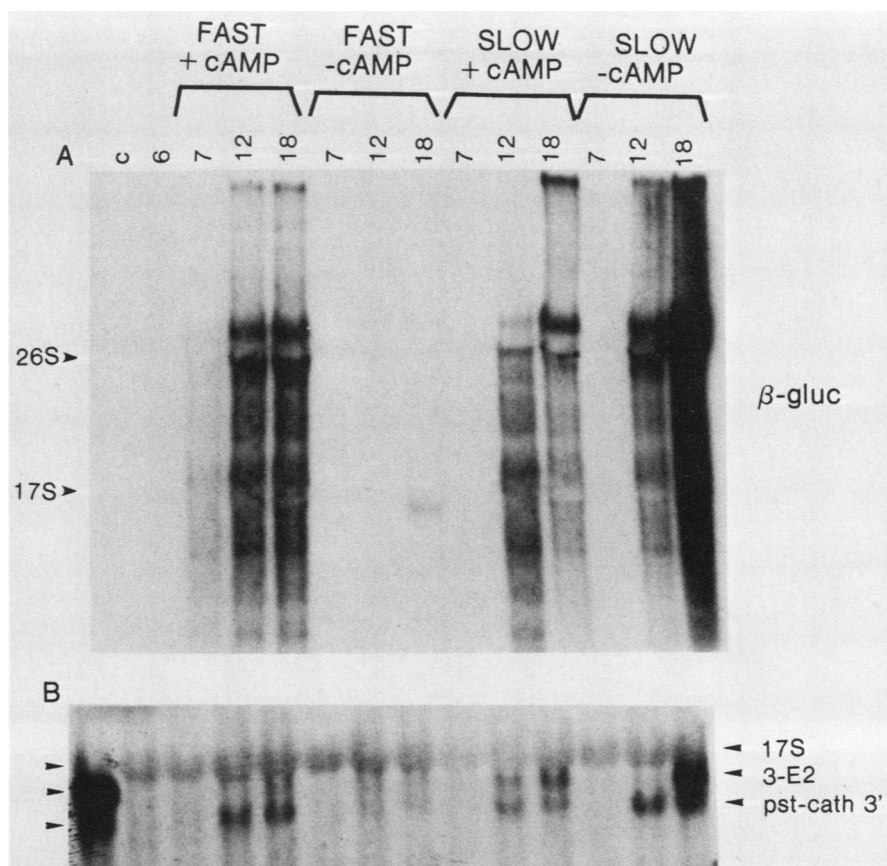


FIG. 8. cAMP regulation of *pst-cath- β -gluc* gene expression. Transformed cells from a single-colony isolate were harvested and starved in fast-shaking (230 rpm) culture for 6 h. After 6 h a sample was taken, and the culture was split into four parts: fast shaking with added cAMP; fast shaking without exogenous cAMP; slow shaking (70 rpm) with cAMP; and slow shaking without exogenous cAMP. Cultures with cAMP were given cAMP to 200 μ g every 2 h starting at 6 h. Fast-shaking cultures received 1 mM EDTA to help eliminate aggregate formation. See Mehdy et al. (9) and Reymond et al. (16) for details. Samples were taken at the times indicated (hours) after the initiation of starvation, RNA was extracted, and 3 μ g of RNA per lane was run on a 1.5% agarose denaturing gel. The RNA was blotted onto GeneScreen, baked, prehybridized for 6 h, and hybridized with 1×10^7 Cerenkov cpm of nick-translated β -gluc probe (A) or 5×10^6 Cerenkov cpm each of nick-translated *pst-cath* 3' probe and 3-E2 insert (B). Nonhybridized counts were washed off, and the filters were exposed to XAR-5 film as described in the legend to Fig. 4. A control lane (c) containing 2 μ g of poly(A)⁺ RNA from 15-h-developing untransformed KAx-3 cells was also included as a marker.

sion in the culture receiving cAMP. These results indicate that the *pst-cath- β -gluc* gene fusion is also regulated by exogenous cAMP in parallel with the endogenous *pst-cath* gene. As expected, there was no expression of the prespore mRNA (3-E2 probe) in either culture (8, 9). In slow-shaking cultures, all three genes were expressed, as expected from previous results (9).

Expression of the *pst-cath- β -gluc* fusion protein can also be used to assay for correct promoter activity of the gene construct under a variety of conditions. Figure 9 shows cells from fast-shaking cultures that were allowed to adhere briefly to cover slips. The cells were then fixed and stained for β -gluc by immunofluorescence. In the fast-shaken cultures lacking exogenous cAMP, a very small number of cells reacted with the anti- β -gluc antibody (Fig. 9A). In the cultures containing added cAMP, a large number of cells contained expressed β -gluc (Fig. 9B), while some cells did not express the antigen. A similar staining pattern is obtained when untransformed cells are stained with anti-*pst-cath* after similar experimental manipulations (Gomer et al., in preparation).

We have recently shown that prestalk and prespore cells plated at a very low density (<2,000 cells per cm²) can be

induced to express prestalk and prespore mRNAs with exogenous cAMP if they have previously been cultured in the presence of CMF, a soluble factor secreted by developing cells (8; unpublished observation). Transformed cells were washed and plated in small wells on a plastic slide in fresh medium or conditioned medium (buffer containing CMF) with or without cAMP (see Materials and Methods). After 20 h, the cells were fixed and stained for β -gluc by immunofluorescence. Very few if any of the cells grown in fresh medium with or without cAMP or in conditioned medium without cAMP showed appreciable amounts of staining for β -gluc (data not shown). However, many cells developed in conditioned medium with cAMP expressed β -gluc. As before, a bimodal distribution could be seen, with some cells showing no staining and other cells showing a positive staining. No cells could be found that showed, as judged by eye, intermediate levels of staining (data not shown).

DISCUSSION

We used the *pst-cath* promoter, ~2.5 kb of upstream sequences, and 350 bp of the N-terminal-coding region (see

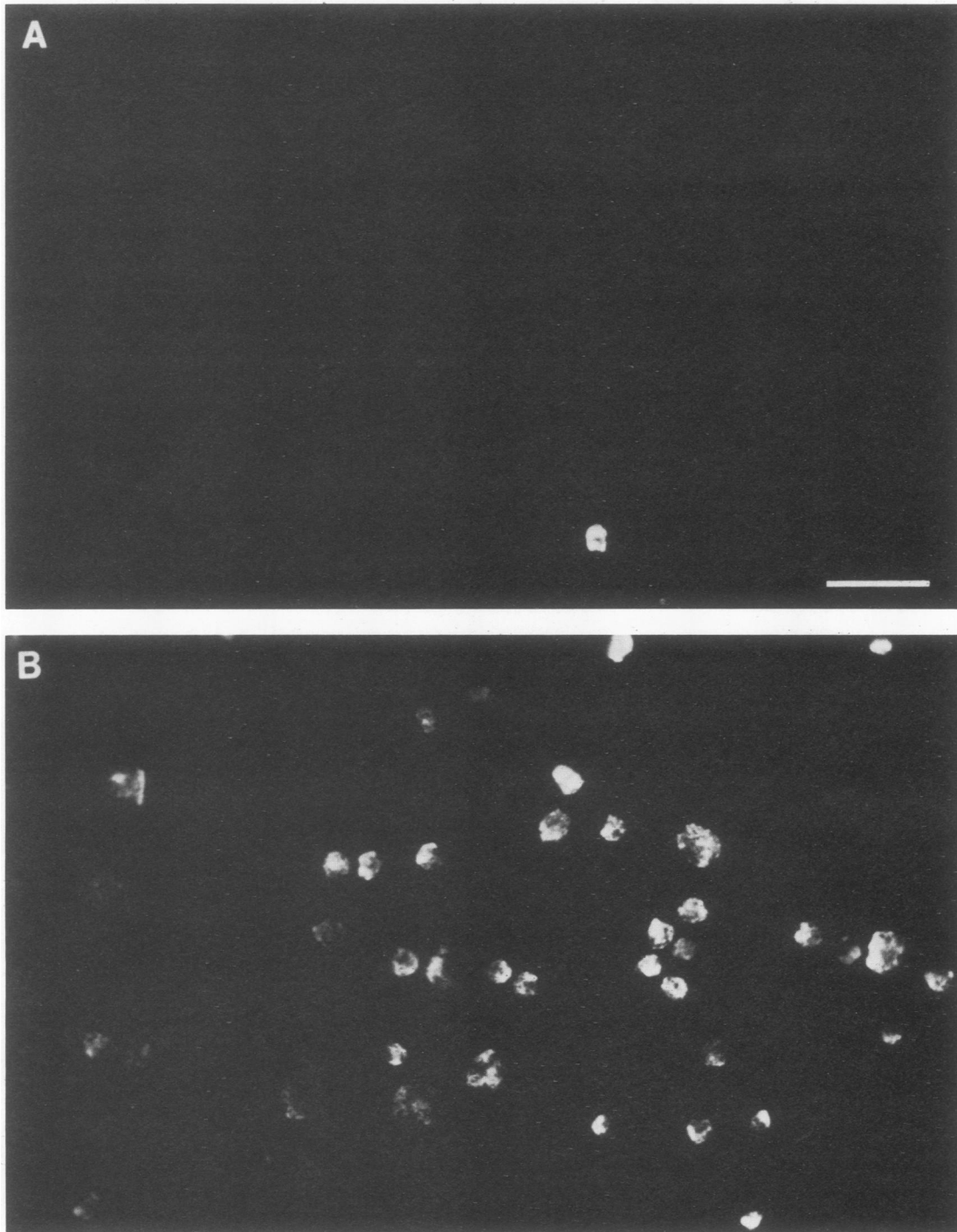


FIG. 9. Indirect immunofluorescence staining of transformed cells with anti- β -gluc antibody. KAx-3 cells transformed with *pst-cath- β -gluc* were starved and then placed in fast-shaking cultures to inhibit formation of cell-cell contacts. After 6 h the culture was divided in half; cAMP was added every 2 h to one of the cultures (see the legend to Fig. 8). After 12 h of cAMP addition, cells from each flask were briefly allowed to adhere to cover slips, fixed, and stained for β -gluc. Cells shown in panel A had no added cAMP; cells shown in panel B had added cAMP. Bar in panel A is 30 μ m. Phase-contrast images (not shown) showed that there are approximately 70 cells in each field.

Materials and Methods) to create an in-frame gene fusion with the *E. coli* β -gluc gene. Transformation of the gene fusion into *D. discoideum* resulted in a cell line carrying \sim 100 copies of pSD1 per cell. Restriction enzyme analysis of transformed DNA indicated that the bulk of pSD1 sequences was integrated into the genome in a head-to-tail tandem

array (Fig. 3) (unpublished data) similar to that seen in previously characterized transformants (11–13). The presence of extra bands was probably due to rearrangements, some head-to-head arrays of pSD1 during integration, or possibly multiple integration events. Rearrangement of transformation vectors in *D. discoideum* has not previously

been seen (W. Nellen, C. Silan, and R. A. Firtel, unpublished observation).

Developmental studies of the pSD1 transformants showed that pst-cath- β -gluc mRNA was regulated coordinately with the endogenous pst-cath mRNA and was induced at the same morphological stage as in control, untransformed cells. Development of transformed cells was delayed with respect to control cells until the time of tight aggregate formation, which is a couple of hours after the pst-cath gene is induced. It is possible that the presence of a large number of prestalk-specific promoters may be causing the delay owing to a limiting concentration of a *trans*-acting regulatory factor, although transformants containing ~100 copies per cell of other developmentally regulated promoters such as discoidin I α or γ and the *Dictyostelium ras* gene (*Dd-ras*), a gene expressed first in vegetative cells and later in prestalk cells with the same kinetics as pst-cath, have not shown delayed development (11, 17; T. Crowley, W. Nellen, R. H. Gomer, and R. A. Firtel, Cell, in press). Induction of prespore gene expression in developing transformants occurred at the same morphological stage as in control cells, indicating that the high copy number of the pst-cath promoter in the transformant and the developmental delay do not adversely affect prespore regulation.

Expression of the fusion protein was followed with anti- β -gluc antibodies. We showed that the fusion protein is spatially localized to the anterior region of migrating, transformed pseudoplasmodia, which contains prestalk cells. The staining pattern with anti- β -gluc antibody on transformed pseudoplasmodia was indistinguishable from that of the anti-cath antibody on untransformed pseudoplasmodia, indicating that the gene fusion shows the proper spatial and cell-type-specific expression.

Cell extracts containing the pst-cath- β -gluc fusion protein had no detectable β -gluc activity when assayed with a series of substrates. R. Jefferson and D. Hirsh (personal communication) have made a similar fusion gene containing an appreciably shorter region from the N-terminal portion of a *Caenorhabditis elegans* collagen-coding region fused to β -gluc. This gene encodes a fusion protein having β -gluc activity. It is unclear whether the lack of enzymatic activity is due to the length of the *D. discoideum* coding sequence attached to the N terminus of β -gluc or the specific amino acid sequence. In other systems, cath is a lysosomal enzyme (21). We have noted that pst-cath has a putative leader sequence and that the protein is localized in vesicles in prestalk cells (Gomer et al., in preparation). It is possible that the pst-cath- β -gluc fusion protein is targeted to lysosomes and that the low pH in the lysosomes irreversibly inactivates the enzyme.

Examination of the developmental regulation of the gene fusion showed that the mRNA is coordinately expressed with the endogenous gene and occurs at the same morphological stages as in control cells. In addition, the mRNA and fusion protein were induced in fast-shaking culture with added cAMP but were not expressed in fast-shaking culture without cAMP, similar to endogenous pst-cath regulation (9). Prespore gene expression was assayed in the transformant and found to be identical with prespore gene expression in untransformed *D. discoideum* in shaking culture.

Our immunofluorescence studies on pst-cath- β -gluc expression in response to cAMP showed that only a fraction of the transformed cells express the fusion protein. Similar results were obtained by using the anti-pst-cath antibody to stain untransformed cells under the same experimental conditions. We know that during development only ~20% of the

cells differentiate into prestalk cells which would express the pst-cath gene product. One model proposed to explain the fraction of cells which become prestalk cells suggests that cells within a certain part of the cell cycle when development is initiated have a greater tendency to become prestalk cells (22). It is also possible that only a fraction of *D. discoideum* cells are capable of responding to cAMP at a given time. The pst-cath- β -gluc gene fusion in conjunction with the anti- β -gluc antibody will be extremely useful in investigating these questions since they can be used as a molecular marker for prestalk-cell differentiation. Strains with different genetic backgrounds which form aggregates consisting of different ratios of prestalk to prespore cells (H. MacWilliams, personal communication) can be examined.

From our results, it is clear that the portions of the pst-cath gene used to construct the gene fusion carry all the necessary *cis*-acting information for the cell-type-specific, spatial, and cAMP regulation of the pst-cath gene expression during *D. discoideum* development. This and similar constructions can now be used to identify the *cis*-acting regulatory regions. Analysis of DNA sequences 5' to the cap region of pst-cath (S. Datta and R. A. Firtel, unpublished observation) and *Dd-ras* (C. D. Reymond and R. A. Firtel, unpublished observation) have shown a relatively GC-rich region in the midst of a sequence which is >85% A+T and which lies ~150 to 200 bp 5' to their respective cap sites. Within each is a region of sequence homology which could be involved in prestalk-specific gene expression. Subsequent deletion analysis should define the functions of these sequences. The successful introduction of a foreign gene into *D. discoideum* by using our DNA-mediated transformation system (12, 13) and its proper expression at both the mRNA and protein level under the control of a *D. discoideum* cell-type-specific promoter will be the basis of a powerful system for the elucidation of the molecular mechanism of cell-type differentiation.

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