Cellular and Subcellular Distribution of a cAMP-regulated Prestalk Protein and Prespore Protein in *Dictyostelium discoideum*: A Study on the Ontogeny of Prestalk and Prespore Cells

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Abstract. We have analyzed a developmentally and spatially regulated prestalk-specific gene and a prespore-specific gene from *Dictyostelium*. The prestalk gene, pst-cathepsin, encodes a protein highly homologous to the lysosomal cysteine proteinases cathepsin H and cathepsin B. The prespore gene encodes a protein with some homology to the anti-bacterial toxin crambin and has been designated beejin. Using the λgtll system, we have made polyclonal antibodies directed against a portion of the protein encoded by pst-cathepsin and other antibodies directed against the beejin protein. Both antibodies stain single bands on Western blots. By immunofluorescence and Western blots, pst-cathepsin is not present in vegetative cells or developing cells during the first ~10 h of development. It then appears with a punctate distribution in a subset of developing cells. Beejin is detected only after ~15 h of development, also in a subset of cells. Pst-cathepsin is distributed in the anterior ~1/10 of migrating slugs and on the peripheral posterior surfaces of slugs. Beejin is distributed in the posterior region of slugs. Expression of both pst-cathepsin and beejin can be induced in subsets of isolated cultured cells by a combination of conditioned medium and extracellular cAMP in agreement with the regulation of the mRNAs encoding these proteins. We have used the antibodies as markers for cell type to examine the ontogeny and the spatial distribution of prestalk and prespore cells throughout multicellular development. Our findings suggest that prestalk cell differentiation is independent of position within the aggregate and that the spatial localization of prestalk cells within the multicellular aggregate arises from sorting of the prestalk cells after their induction. We have also found a class of cell in developing aggregates that contains neither the prestalk nor the prespore markers.

VERY little is known about the molecular basis of differentiation and multicellular morphogenesis. We have chosen *Dictyostelium discoideum* to study these phenomena because of its simplicity. *Dictyostelium* normally exists as unicellular amebas that feed on bacteria; however, strains have been developed that can grow in axenic medium (Loomis, 1975). *Dictyostelium* initiates development upon the removal or disappearance of the food source in order to disperse spores to (hopefully) more favorable environments (Loomis, 1975; 1982). Amebas aggregate using relayed pulses of cAMP as the chemoattractant. Approximately 10 h after starvation, 10^2–10^5 cells have formed an aggregate which subsequently forms a tip. Typically, tipped aggregates elongate to form a slug which migrates to a spot favorable for spore dispersion, whereupon the slug develops into a fruiting body consisting of a mass of spores supported by a 1–2-mm-high stalk.

Within the aggregates, cells are thought to differentiate into either prespore or prestalk cells. These cell types can be purified from migrating slugs. cDNA and genomic clones of genes which are expressed in only one of these two classes of cells have been isolated (Mehdy et al., 1983; Barklis and Lodish, 1983; Reymond et al., 1984; Mehdy and Firtel, 1985). Many prespore and prestalk genes are coordinately regulated in wild-type and temporally deranged mutants (Saxe and Firtel, 1986). In cells plated at low density in medium conditioned by developing cells (referred to herein as conditioned medium), both prestalk- and prespore-specific genes are induced by exogenous cAMP (Mehdy et al., 1983; Mehdy and Firtel, 1985).

So as to be able to assay individual cells for cell type–specific gene expression and to examine the ontogeny of prestalk and prespore cells, we have used the λgtll expression vector system to make polyclonal antibodies directed against a portion of a cloned prestalk and a prespore gene. Since the expression of these genes are believed to represent good markers for these two cell types (Mehdy et al., 1983; Gomer et al., 1985 *b*; Saxe and Firtel, 1986), we have used the antibodies to examine the spatial distribution and ontogeny of prestalk and prespore cells.

Materials and Methods

Growth and Development of Dictyostelium discoideum

Strain KAx-3 was grown axenically in suspension (Firtel and Bonner, 1972) and used as a DNA source for cloning. KAx-3 was used for all experiments described, except for the separation of prestalk and prespore cells (Ratner and Borth, 1983). This procedure used NC-4 cells grown on *Klebsiella aerogenes* (Sussman, 1966).

General Procedures and Molecular Biologicals

Cloning procedures followed the methods described in Maniatis et al. (1982). Double-stranded M13 DNA was purified according to Birnboim and Doly (1979) and single-stranded M13 DNA was isolated as described by Messing (1981). The only major modification to generally used cloning methods was to eliminate the use of IPTG and X-gal when making the fusion protein gene to reduce the possibility of selecting for missense mutations.

λgtll DNA was a gift from Elliot Rosen (University of California at San Diego). *Escherichia coli* host strains were 71-18 for M13 and Y1088 and BNN103 for λgtll. Enzymes were from Promega Biotec (Madison, WI) and the sequencing primer was from Bethesda Research Laboratories (Gaithersburg, MD); the phosphorylated Eco RI linker was from New England Biolabs (Beverly, MA) and calf intestine alkaline phosphatase was from Worthington Biochemical Corp. (Freehold, NJ), and was further purified by chromatography over Sephadex G-75.

Cloning and Sequencing

The isolation and analysis of the prestalk-specific-cathepsin (pst-cath)[1] and prespore-specific beejin cDNA clones was previously described (Mehdy et al., 1983). In Mehdy et al. (1983) pst-cath is called 16-G1 and beejin is called 2-H3. A slug-stage cDNA library inserted in the Eco RI site of λgtll (Young and Davis, 1983; R. A. Firtel, personal communication) was screened with nick-translated pst-cath cDNA insert and a positively hybridizing cDNA designated 1-13 was isolated.

The cloning of the pst-cath gene has been previously described (Datta et al., 1986). The sequence and analysis of pst-cath is described in Datta and Firtel (manuscript submitted for publication). A 2.5-kb portion of the gene complementary to the beejin Bam HI-Hinc II cDNA fragment was isolated from both a Charon λ13 Eco RI library and a genomic library in pBR322 in a manner analogous to that used to isolate pst-cath. These genomic clones contained Bam HI-Hinc II fragments that were identical to the Bam HI-Hinc II cDNA fragment, as determined by DNA sequencing.

Genomic clones were mapped using single and double restriction digests. Some enzymes recognizing four base pair restriction sites were mapped on purified genomic insert or insert fragments. Localization of coding region was determined by restriction site clustering since *Dictyostelium* protein coding regions have a substantially higher GC content (~38%) than noncoding regions (15%) (Kimmel and Firtel, 1983), by hybridization of purified labeled cDNA insert to DNA blots of the appropriate restricted genomic clone, and by hybridization of labeled genomic clone fragments to RNA blots.

Transcription polarity of cDNA was established by cloning cDNA inserts into M13 in both orientations and probing RNA blots with single-stranded DNA. Transcription polarity of the genomic clones were obtained by comparison of restriction maps between the genomic clones and the cDNAs, and by direct sequence comparison.

Sequencing was accomplished either by the Maxam and Gilbert method (1980) using restriction fragments of the genomic clone or subclones labeled with polynucleotide kinase and $\gamma[^{32}P]$ ATP, or by a modification (Gomer et al., 1985*a*) of the dideoxy-chain termination method (Sanger et al., 1977). Manipulation of DNA sequences and determination of the putative amino acid sequence were done with some of the Staden computer programs (Staden, 1984). The derived amino acid sequence was compared to known protein sequences in the updated NEWAT data base (Doolittle, 1981).

Antibody Production

The 273-bp Dde I-Bgl II fragment from the 5′ end of the pst-cath coding region (between the curved arrows in Fig. 1) was excised and gel purified from the pst-cath genomic clone. The overhanging ends were filled in using the Klenow fragment of DNA polymerase I and deoxynucleotides. Double-stranded M13mp11 DNA was digested with Hinc II and the resulting ends dephosphorylated with calf intestine alkaline phosphatase. The blunt-ended Dde I-Bgl II fragment was ligated into the Hinc II site of M13mp11. The 420-bp Bam HI/Hinc II fragment of the Charon λ13 genomic beejin clone was similarly excised, blunt-ended, and ligated in the Hinc II site of M13mp11. Restriction analysis of DNA from *E. coli* transformants was used to identify M13 replicating-form DNA containing the insert in the proper orientation. The unique Hind III site in the M13 polylinker was then digested and the resulting ends were filled in and dephosphorylated as above. An Eco RI linker was then ligated into the site. The resulting DNA was digested with Hind III to linearize any DNA molecules which were not previously linearized with Hind III and then used to transform *E. coli*. Restriction analysis was performed to identify M13 clones containing an Eco RI fragment of the appropriate size. Single-stranded DNA was purified from M13 phage and partially sequenced using a modification (Gomer et al., 1985*a*) of the Sanger et al. (1977) dideoxy method to verify insert polarity and reading frame of the insert. An Eco RI fragment from a sequence-verified M13 clone was then excised, gel purified, and ligated into the Eco RI site of λgtll.

Restriction analysis was used to identify a recombinant phage containing a properly sized Eco RI insert in the λ DNA with the insert in the proper polarity. This was accomplished by using the Bam HI site in the polylinker near one end of the M13/pst-cath or M13/beejin Eco RI fragment and the Bam HI-Kpn I restriction maps of λgtll (Young and Davis, 1983).

Fusion protein was prepared according to Young and Davis (1983). Approximately 2×10^8 BNN103 bacteria were mixed with $\sim 2 \times 10^{10}$ pfu of λgtll/*Dictyostelium*-insert virus in 1.1 ml of 10 mM $MgSO_4$, 10 ml Tris-HCl, pH 7.5, and incubated at 30°C for 30 min. This was then added to 100 ml L broth. Infected bacteria were then grown and induced to synthesize fusion protein as described by Young and Davis (1983). Bacteria were then concentrated by centrifugation and solubilized by boiling in SDS sample buffer (Gomer and Lazarides, 1981). Fusion protein was isolated by preparative gel electrophoresis on 10% polyacrylamide gels as previously described (Gomer and Lazarides, 1981; Reymond et al., 1984). Fusion protein bands were excised from Coomassie-stained gels and washed in several changes of water. The fusion protein was electroeluted out of the gel matrix according to Hunkapiller et al. (1983) and then concentrated by lyophilization.

Female New Zealand white rabbits were immunized by injection of 20 μg fusion protein into popliteal lymph nodes and intradermal sites on the back. The 20 μg of protein was suspended in 250 μl of 100 mM NaCl and emulsified with a slightly greater volume of Freund's complete adjuvant. Rabbits were then boosted with 100 μg fusion protein 37 d after the first injection, and then with 200 μg fusion protein at 56 d. Both boosts were a combination of intradermal injections in the back and intramuscular injections in the hind legs. The protein for the boosts was suspended in 400 μl of 100 mM NaCl and emulsified as above with Freund's incomplete adjuvant. Rabbits were bled from an ear vein 7 d after the last boost.

Purification of Antiserum

Serum and DEAE-purified immunoglobins were prepared according to Garvey et al. (1977). For affinity purification, bacteria containing fusion protein were lysed by repeated freezing and thawing in 0.1 M $NaHCO_3$/0.1 mM phenylmethylsulfonyl fluoride (PMSF) and then dialyzed for 24 h against 0.1 M $NaHCO_3$. Bacterial protein was then coupled to Sepharose 4B according to Gomer and Lazarides (1981). Antiserum was affinity purified according to the procedures of Gomer and Lazarides (1981) with the material adsorbed to a fusion protein/bacterial lysate column and then passing it over a column containing a lysate of bacteria infected with insert-free λgtll.

Solubilization of *Dictyostelium* cells for gel electrophoresis was as described by Reymond et al. (1984) except that the SDS sample buffer contained 0.3 mM PMSF, 5 μM leupeptin, and 5 μM chymostatin. Gel electrophoresis and transfer of proteins to nitrocellulose was performed according to Reymond et al. (1984), except that the transfer buffer did not contain SDS and the transfer was performed at 20 V/cm for 10 h. Blots were washed in 150 mM NaCl, 20 mM Tris/HCl (pH 7.5), 0.005% polyoxyethylene sorbitan monolaurate (Tween-20) [TBST] for 10 min. All operations were at room temperature. Blots were stained with affinity-purified anti-pst-cath at 1 μg/ml, affinity-purified anti-pst-cath preimmune serum, DEAE-purified anti-beejin immune or preimmune serum (10 μg/ml), or DEAE-purified anti-β-galactosidase (10 μg/ml) for 12 h, washed for 45 min with several changes, incubated with 10^6 cpm of ^{125}I-protein A in 30 ml for 2 h, washed as above, and autoradiographed as described in Reymond et al. (1984) for 1 wk. To check for total protein, the blots were then stained for total protein by rocking in 0.1% india ink in TBST overnight.

1. *Abbreviation used in this paper*: pst-cath, prestalk-specific cathepsin.

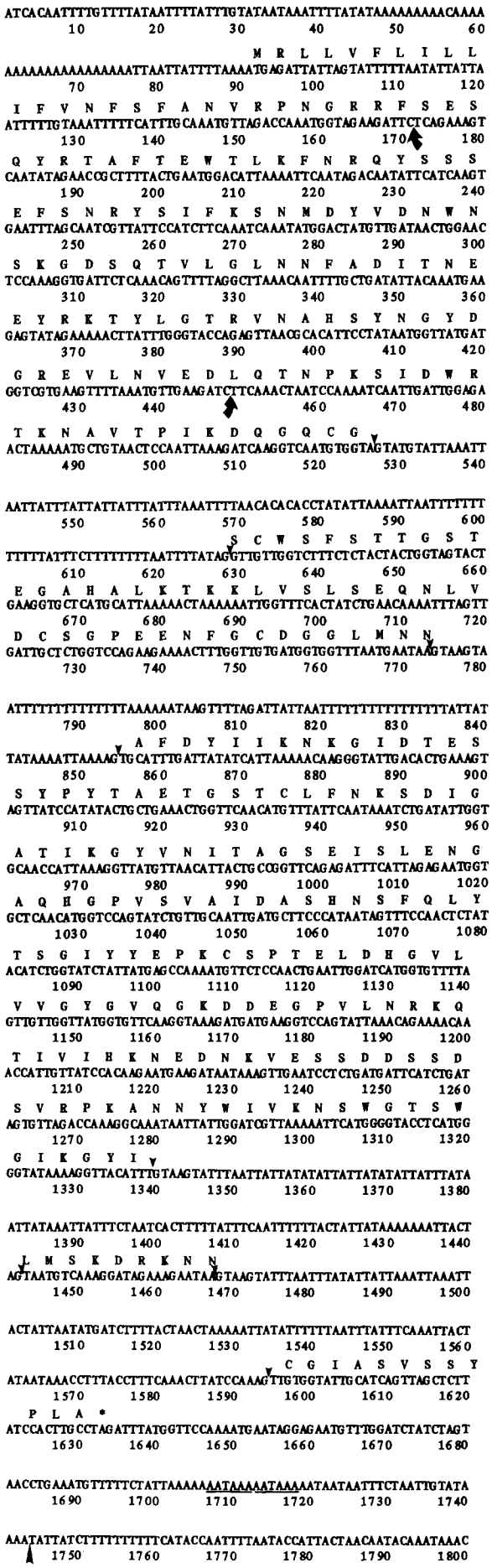


Figure 1. DNA and derived amino acid sequence of pst-cath. The sequence used for antibody production is between the curved arrows. The 5′ end of the sequence (nucleotide 1) is the mRNA Cap site (Datta S., and R. A. Firtel, manuscript submitted for publication). Downward pointing arrows are the mRNA splice sites as deduced from a comparison of the genomic and cDNA sequences. Underlining indicates a metazoan poly(A) addition recognition site (Fitzgerald and Shenk, 1981). The arrow at nucleotide 1741 is the 3′ end of the mRNA.

Cryosectioning, Whole Mounts, and In Vitro Cell Cultures

For cryosectioning, KAx-3 slugs migrating on 1.5% agar surfaces were fixed in 3.7% formaldehyde for 24–28 h at room temperature. Slugs were then embedded in Tissue-Tek II O.C.T. (Miles Laboratories, Inc., Naperville, IL), frozen and sectioned in a Slee cryostat (Gomer, 1986). Sections were picked up on subbed slides, permeabilized in 100% methanol at room temperature and air dried. Whole mount aggregates of KAx-3 cells were developed on plastic coverslips (Gomer, 1986). Aggregates were fixed in 100% methanol at room temperature for 20 min and then air dried.

For the immunofluorescence assays of pst-cath or beejin expression in individual cells, fresh medium and conditioned medium were prepared as described in Mehdy and Firtel (1985) except that no antibiotics were added to the medium (see below). Mid log-phase KAx-3 cells growing in axenic suspension culture were collected by centrifugation and washed according to Mehdy and Firtel (1985). Cells were resuspended and diluted in fresh or conditioned medium to a final density of $\sim 7.3 \times 10^4$ cells/ml; 200 μl of such a cell suspension was then added to a well of a Lab-Tek type 4818 8-chamber plastic slide (Miles Laboratories, Inc.). When the cells settled, this gave a surface density of 1.8×10^4 cells/cm^2 on the bottom of the chamber. To prevent formation of a meniscus and thus allow even spreading of the cells, no antibiotics were added to the plating medium. 6 h after starving the cells and plating them in wells, 50 mM cAMP was added to some of the wells to a final concentration of 300 μM; an equal volume of water or buffered salt solution was added to the remaining wells. 20 h after starvation and plating, the wells were drained, the gasket and well dividers removed from the slide, and the slide was immersed in 100% methanol at room temperature for 10 min and then air dried for 1 h to fix the cells.

Immunofluorescence

Cells or cryosections fixed on slides or coverslips were immersed in PBST (phosphate-buffered saline/0.01% Tween-20) for ~15 min. All reactions were at room temperature. DEAE-purified anti-pst-cath or anti-beejin, diluted in PBST to ~0.02 mg/ml was then added onto the slides or coverslips for 1–2 h. These were then rinsed for 1 h in PBST, and stained with 0.02 mg/ml fluorescein isothiocyanate-conjugated goat anti-rabbit for 1 h and finally rinsed as above. For double-label immunofluorescence, preparations at this stage would then be incubated with non-immune rabbit serum (~1:10 dilution in PBST) for 1 h to block remaining goat anti-rabbit binding sites. After draining off the blocking antibody, preparations were incubated with biotin-*N*-hydroxy succinimide ester-conjugated second antibody (Bethesda Research Laboratories product data sheet) and then washed; concentrations and times were similar to those used for the first antibody. Preparations were then incubated with Texas Red-conjugated streptavidin (Bethesda Research Laboratories; 5 μg/ml in PBST) and then finally washed in PBST. Slides or coverslips were mounted in PBS/glycerol containing *p*-phenylenediamine to prevent fading of images (Johnson and Araujo, 1981). Epifluorescence and phase-contrast images were photographed on Kodak Tri-X film which was then developed in Diafine.

Results

Structure of the Dictyostelium Prestalk Cathepsin Gene

We have previously reported the identification of cDNA clones complementary to developmentally regulated cell type-specific RNAs and have analyzed some of the physiological factors necessary for their regulation (Mehdy et al., 1983; Reymond et al., 1984; Mehdy and Firtel, 1985). The pst-cath cDNA clone is complementary to a developmentally

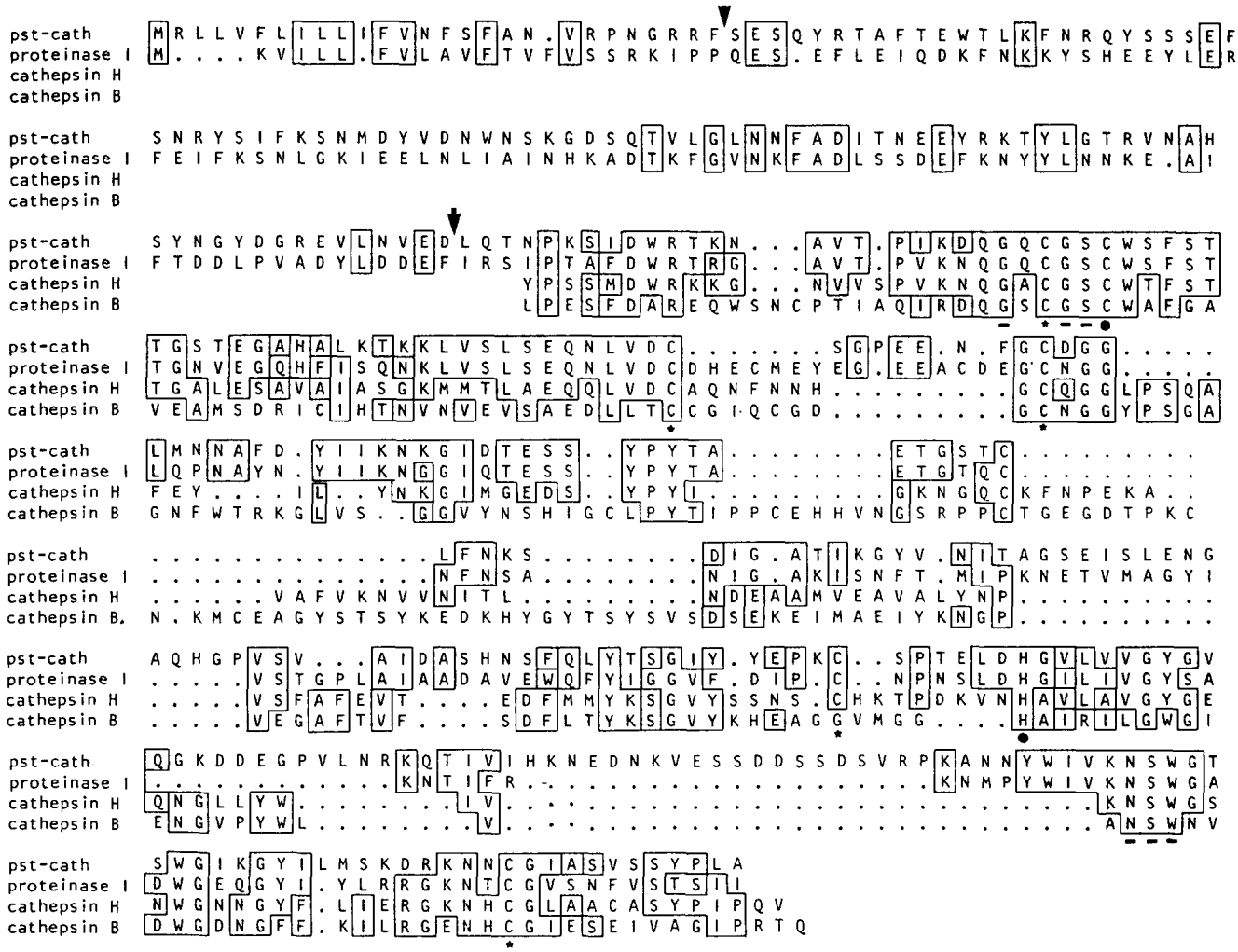


Figure 2. Amino acid comparison between pst-cath, cysteine protease I, cathepsin H, and cathepsin B. Amino acid sequences were derived from DNA sequence for pst-cath. Amino acid homologies are boxed in. Dots indicate gaps in sequence for the purpose of alignment. Large black circles indicate conservation of the active site amino acids. Bars indicate conservation of amino acids known to be important in catalysis. Asterisks indicate conservation of bridge-forming cysteine residues. Comparison is from Datta and Firtel (manuscript submitted for publication).

regulated prestalk-specific message and is regulated by extracellular cAMP (Mehdy et al., 1983, 1984; Mehdy and Firtel, 1985). The genomic clone encoding pst-cath mRNA has been isolated, mapped, and sequenced (Datta et al., 1986; Datta and Firtel, manuscript submitted for publication; Fig. 1). The derived amino acid sequence of the genomic clone was compared to known protein sequences compiled in the updated NEWAT data bank (Doolittle, 1981; and personal communication). Analysis revealed strong homology to the rat cysteine proteinase cathepsin H and lesser homology to rat cathepsin B (Datta et al., 1986; Datta and Firtel, manuscript submitted for publication). The nucleotide sequence of the coding region and derived amino acid sequence is shown in Fig. 1. A comparison of the *Dictyostelium* amino acid sequence with both rat cathepsins and another *Dictyostelium* cathepsin-like cysteine proteinase (called cysteine proteinase I) (Williams et al., 1985) is shown in Fig. 2 (Datta and Firtel, manuscript submitted for publication). Putative signal peptides of 28 and 24 amino acids are encoded by pst-cath and cysteine proteinase I, respectively (see Fig. 2). The NH_2-terminus of both mature proteases would then begin with Glu-Ser. In the case of pst-cath, immunofluorescence staining of cells suggests that the antigen is found in vesicles, consistent with the presence of a leader peptide necessary for insertion of the protein through a membrane (see below). The boundaries of the region of the pst-cath coding region used for fusion protein antigen is shown by the arrows in Figs. 1 and 2.

Structure of the Dictyostelium Prespore Beejin Gene

The beejin cDNA clone is complementary to a prespore-specific mRNA which is regulated by cAMP. Labeled beejin insert was used to probe a genomic *Dictyostelium* DNA blot. Single bands in a variety of restriction digests suggest that the beejin cDNA is complementary to a single gene (data not shown). Labeled beejin insert was also used to screen an Eco RI *Dictyostelium* genomic library in λ Charon 13. Positively hybridizing plaques were re-screened at low density and phage DNA was isolated. A comparison between the cloned

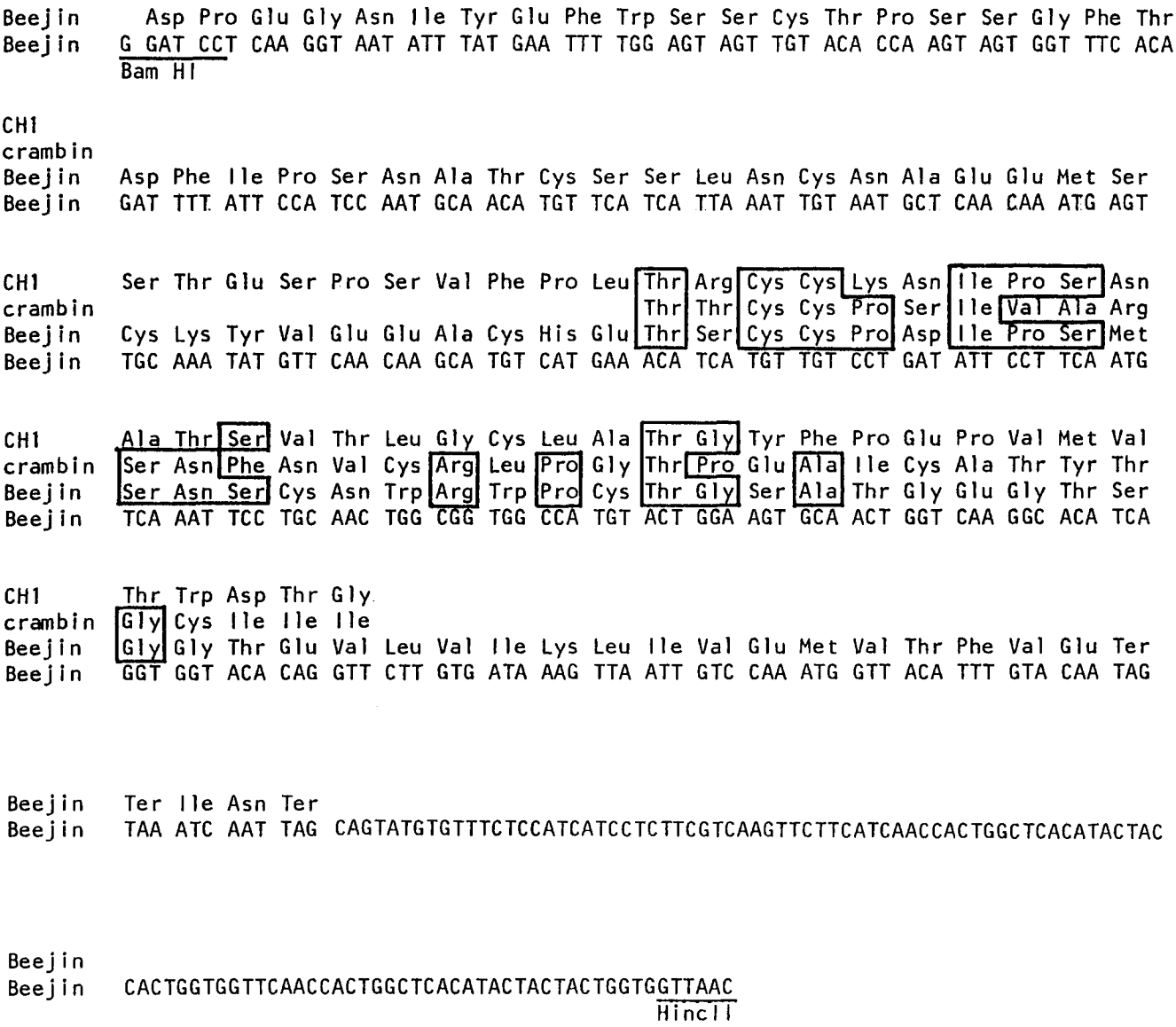


Figure 3. DNA and derived amino acid sequence of beejin genomic clone fragment. DNA sequence was established from both the genomic fragment and cDNA clone (see Materials and Methods). Boxes indicate conserved amino acids between beejin and crambin, or beejin and immunoglobin E (CH1).

insert size and the expected Eco RI genomic fragment size indicated that a deletion of ~5 kb had taken place in all five independent isolates. Further mapping showed that a Bam HI-Eco RI fragment containing the 3′ half of the gene, however, was the expected size.

In addition, all independent isolates of the Eco RI genomic fragment from plasmid banks showed the same 5-kb deletion. Attempts were made to clone a smaller genomic fragment using various restriction enzymes in combination. Three different banks showed no positive hybridization upon screening the same number of recombinant colonies which yielded multiple positive colonies when an Eco RI bank was used. Banks made in recA$^-$ and recBC$^-$ strains of *E. coli* were also screened without success. The 3′ Bam HI-Eco RI fragment was mapped using restriction enzymes, and areas which hybridized to the beejin cDNA were sequenced (Fig. 3). There appear to be no introns in the Bam HI/Hinc II genomic fragment as determined by comparison to cDNA sequence. Transcription polarity was postulated by determination of a single open reading frame in the cDNA sequence and confirmed by hybridizing strand-specific probes.

The derived amino acid sequence of a portion of the Bam HI-Eco RI fragment was compared to known protein sequences compiled in the updated NEWAT data bank (Doolittle, 1981; and personal communication). Analysis revealed weak homology to the anti-bacterial toxin crambin which is found in seed coats of *Crambe abyssinica* (Teeter et al., 1981), and to a portion of the human immunoglobin epsilon chain (Max et al., 1982). Because of its specific expression in prespore cells we have chosen to designate the gene and protein "beejin" after the Sanskrit "beejum," meaning seed or spore. The 420-bp Bam HI/Hinc II restriction fragment of beejin DNA (Fig. 3) was used to make fusion protein antigen (see below).

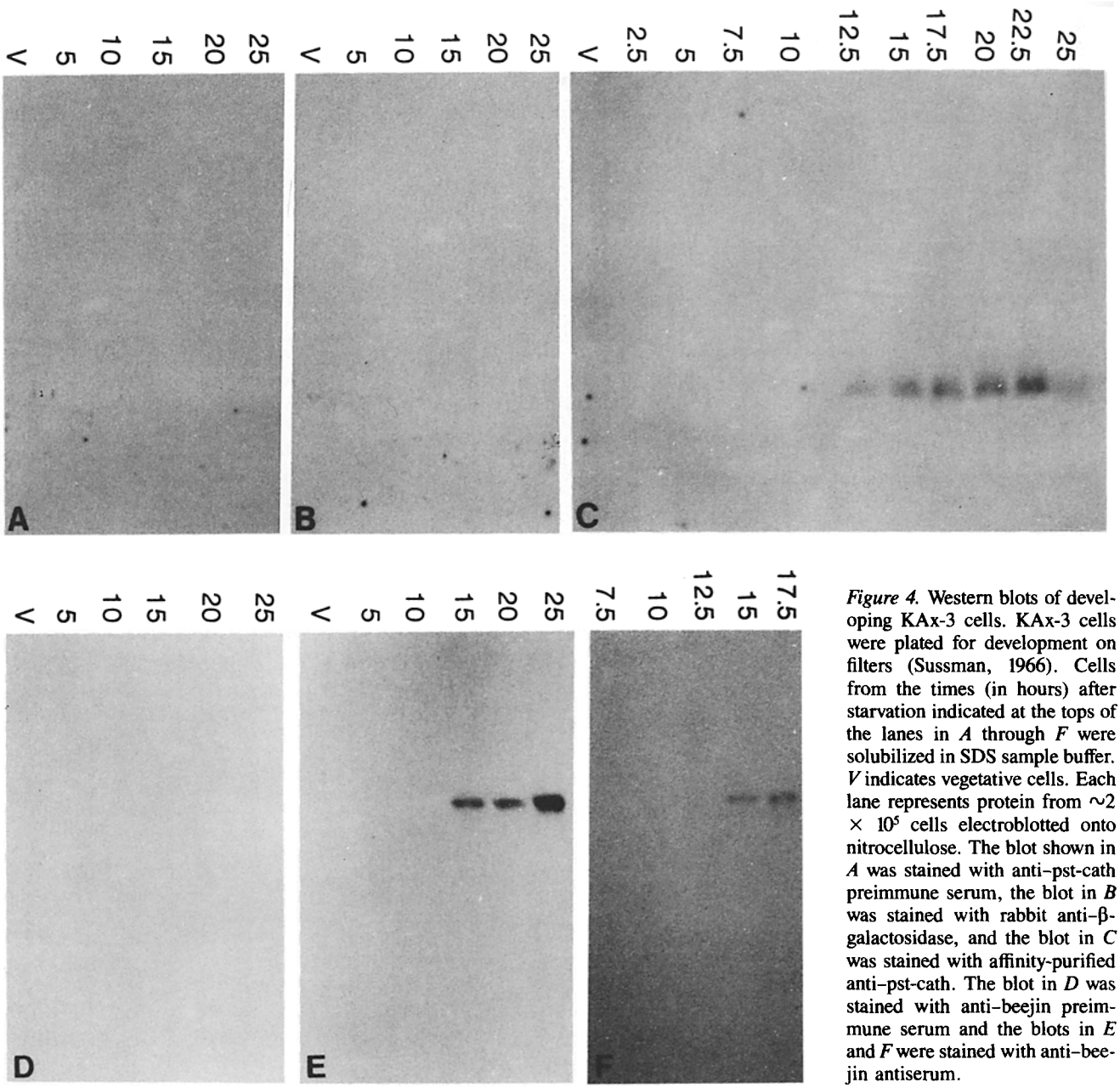


Figure 4. Western blots of developing KAx-3 cells. KAx-3 cells were plated for development on filters (Sussman, 1966). Cells from the times (in hours) after starvation indicated at the tops of the lanes in *A* through *F* were solubilized in SDS sample buffer. *V* indicates vegetative cells. Each lane represents protein from ~2 × 10^5 cells electroblotted onto nitrocellulose. The blot shown in *A* was stained with anti-pst-cath preimmune serum, the blot in *B* was stained with rabbit anti-β-galactosidase, and the blot in *C* was stained with affinity-purified anti-pst-cath. The blot in *D* was stained with anti-beejin preimmune serum and the blots in *E* and *F* were stained with anti-beejin antiserum.

Preparation of Antibodies

To make antibodies against the proteins encoded by pst-cath and beejin, we made pst-cathepsin or beejin fragment/β-galactosidase fusion protein in *E. coli* using the λgtll system (Young and Davis, 1983). Fusion protein was synthesized in *E. coli* infected with λ virus prepared in the above manner, as described by Young and Davis (1983), except that a ~100:1 ratio of virus to bacteria was used (see Materials and Methods). Fusion proteins were excised from preparative gels, and the fusion proteins electroeluted out of the gels and then used to immunize rabbits (see Materials and Methods). The initial screening of the sera from the immunized rabbits was by Ouchterlony immunodiffusion (Garvey et al., 1977). Lysates of *E. coli* synthesizing pst-cathepsin/β-galactosidase fusion protein were used as the antigen in these assays. Specificity of antisera found to give precipitation lines in the Ouchterlony assay was then tested by immunostaining Western blots of *Dictyostelium* cells.

Levels of Pst-Cathepsin and Beejin during Development

Western blots of *Dictyostelium* cells harvested at various stages of development were stained with the preimmune sera, the anti-pst-cath serum, the anti-beejin serum, or antiserum against *E. coli* β-galactosidase (see Materials and Methods). As shown in Fig. 4, *A* and *D*, no staining is observed with pst-cath or beejin preimmune sera. Since the antigen used to make the antibodies was a β-galactosidase fusion protein, it was necessary to check that antibodies against β-galactosidase did not stain *Dictyostelium* cells. A blot similar to that shown in Fig. 4 *A* was stained with anti-β-galactosidase antibody (Fig. 4 *B*); no staining can be seen at any developmental stage. A third Western blot was stained

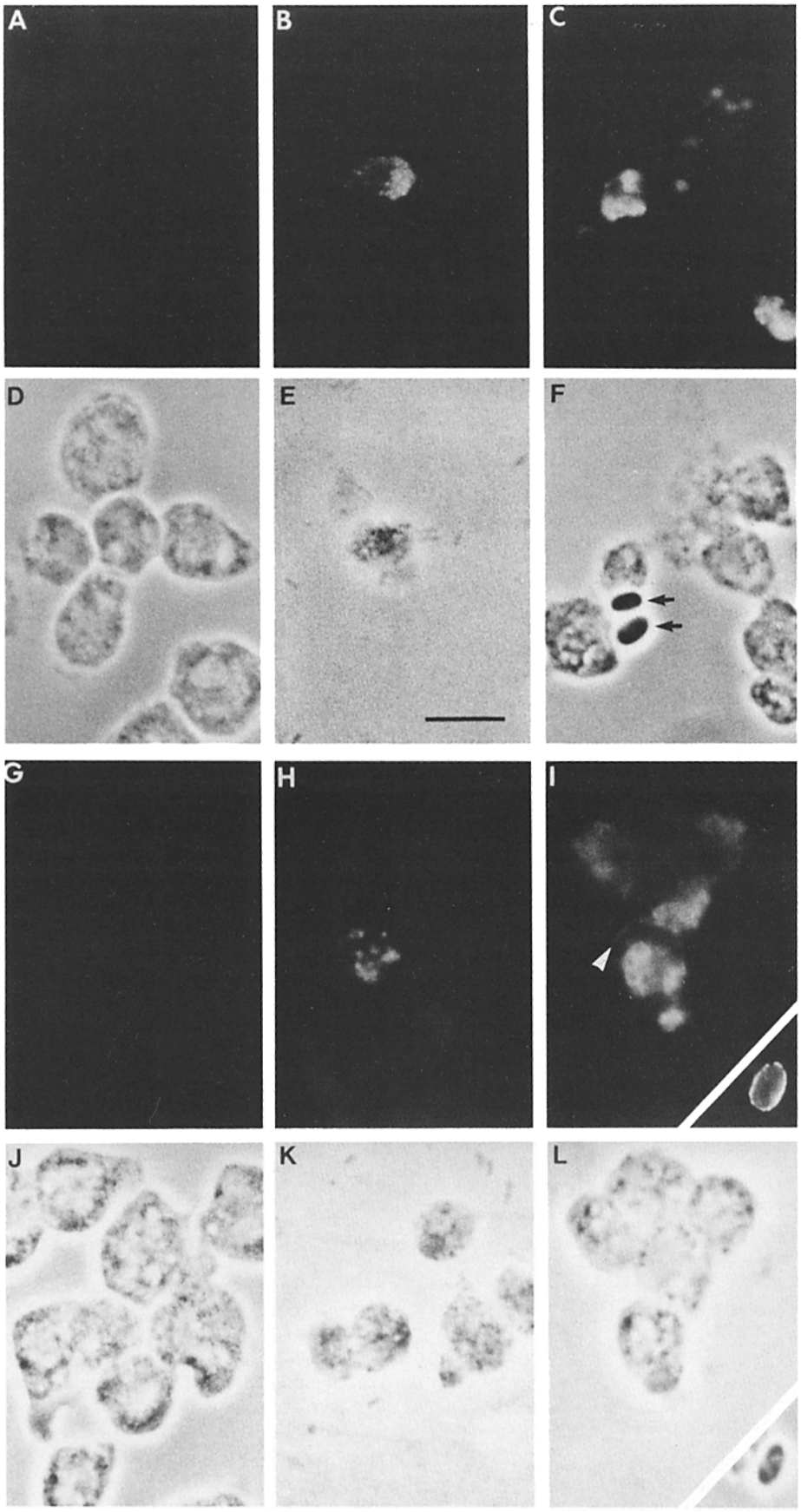
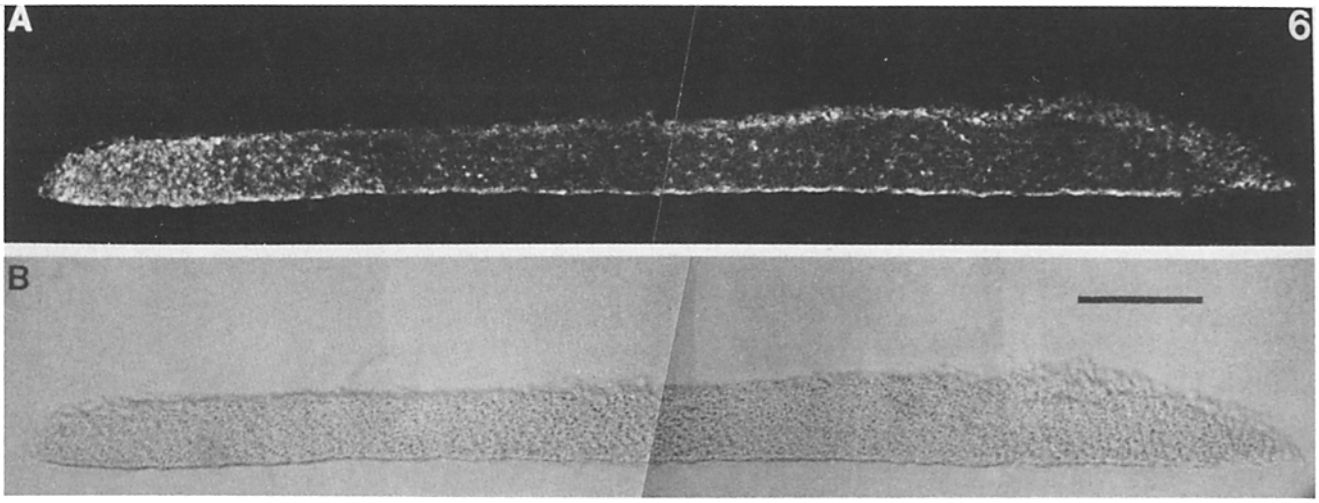
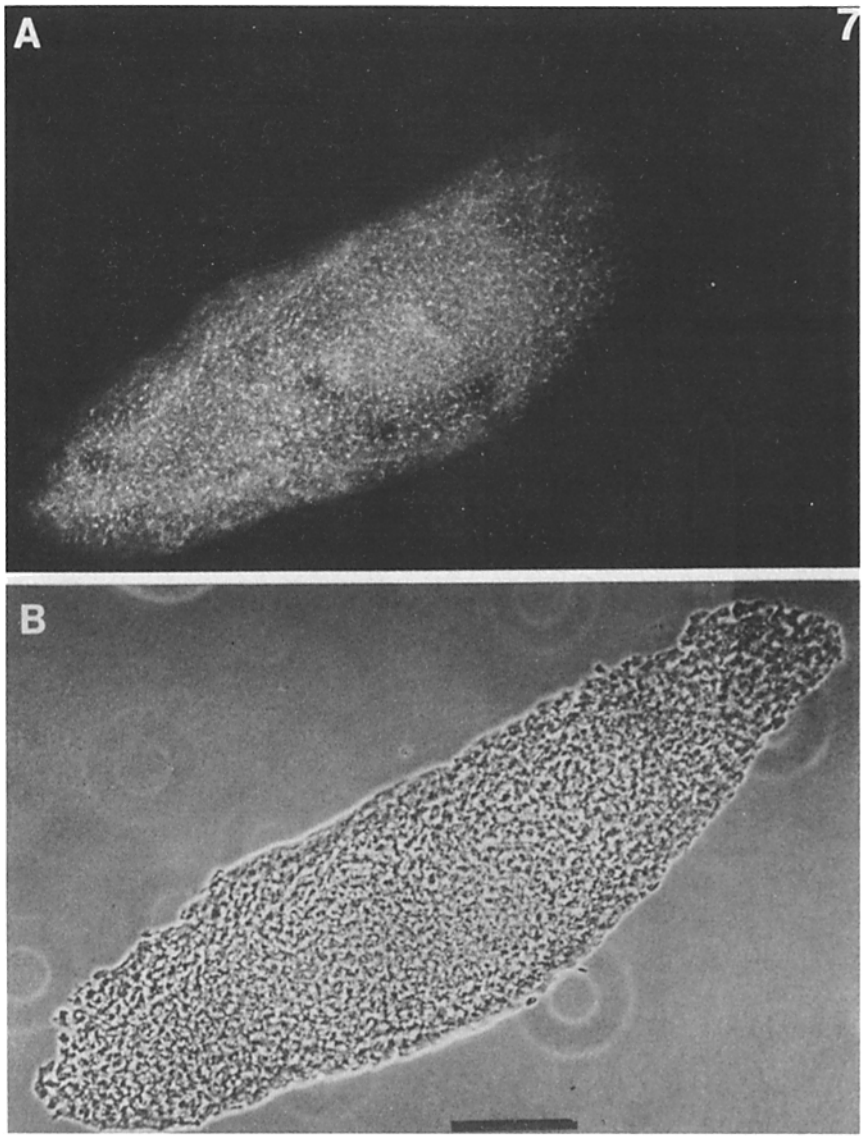


Figure 5. Subcellular localization of pst-cath and beejin in developing KAx-3 cells. Vegetative (*A*, *D*, *G*, and *J*), 15-h developing (*B*, *E*, *H*, and *K*), and 22-h developing (*C*, *F*, *I*, and *L*) KAx-3 cells were disaggregated, allowed to adhere to coverslips, and fixed for indirect immunofluorescence. The cells shown in the phase-contrast images *D–F* were stained with anti-pst-cath (*A–C*). The cells shown in the phase-contrast images *J–L* were stained with anti-beejin (*G–I*). Arrows in *F* indicate two spores, which are not stained with the antibody. Bar, 10 μm.





Figures 6 and 7. Distribution of cells containing pst-cath or beejin in migrating slugs. KAx-3 cells were starved under conditions favoring slug formation. Slugs were fixed, embedded, and cryosectioned. (Fig. 6) A section of a slug migrating toward the left was stained with anti-pst-cath by indirect immunofluorescence (*A*); the corresponding phase-contrast image is shown in *B*. (Fig. 7) A section of a different slug migrating toward the upper right was stained with anti-beejin (*A*); the corresponding phase-contrast image is shown in *B*. Bars, 100 μm.

with affinity-purified anti-pst-cath antibody (see Fig. 4 *C*). Vegetative, 2.5-, 5-, 7.5-, and 10-h developing cells show no staining. A band at ~45 kD was stained in lanes carrying protein from 12.5-, 15-, 17.5-, 20-, 22.5-, and 25-h developing cells. The intensity of staining increases with time from 12.5 to 22.5 cells and then is decreased in the 25-h cells. Fig. 4 *E* shows staining of a blot with anti-beejin antiserum. Fig. 4 *F* is a similarly stained blot with greater temporal resolution. No staining can be seen in vegetative, 5-, 7.5-, 10-, and 12.5-h developing cells. 15-, 17.5-, 20-, and 25-h developing

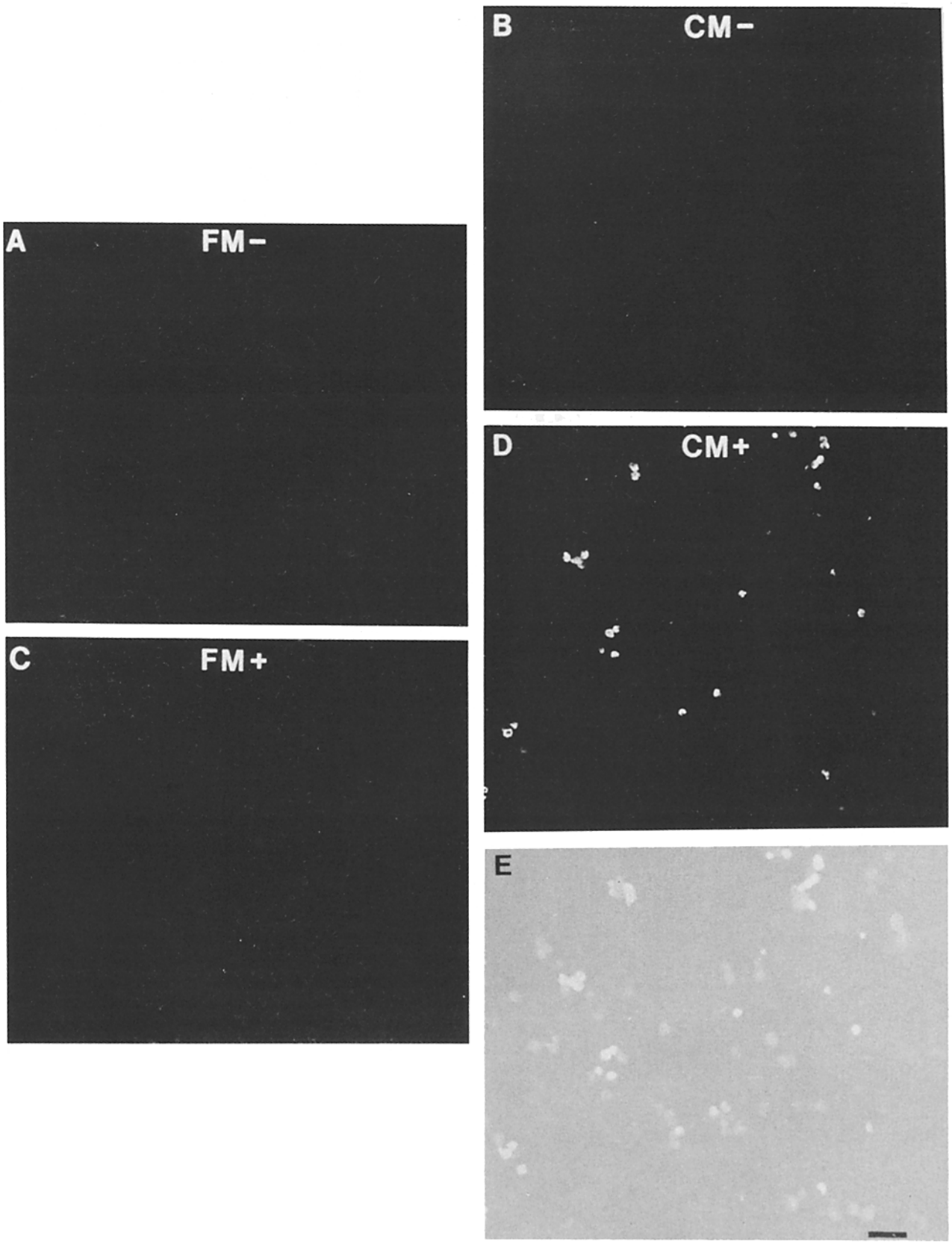


Figure 8. Induction of beejin expression in a subset of isolated cells. KAx-3 cells were starved and plated at low density in either fresh medium (*FM*; *A* and *C*) or in conditioned medium (*CM*; panels *B*, *D*, and *E*) according to Mehdy and Firtel (1985). 6 h after starvation, cAMP was added to the cells shown in *C–E*; an equal volume of water was added to the cells shown in *A* and *B*. 20 h after starvation the cells were fixed and stained for pst-cath by indirect immunofluorescence. *E* shows the field shown in *D*, photographed and printed to show the faint nonspecific staining of the remainder of the cells in the field. Bar, 40 μm.

cells show staining of a single band at ~78 kD. Two rabbits were immunized with the β-galactosidase/beejin fusion protein and showed identical preimmune and immune staining patterns.

Subcellular Distribution of Pst-Cath and Beejin

We also tested the antibodies by immunofluorescence staining of dissociated developing cells. Vegetative cells were not stained by the anti-pst-cathepsin antibody under a variety of buffer conditions (Fig. 5 *A*). Cells from 15-h developing aggregates showed a bimodal staining distribution: most cells were not stained, while ~10% of the cells showed a punctate staining pattern (Fig. 5 *B* and data not shown). The positive staining pattern appears as ~10–40 round dots, each 0.07–0.3 μm in diameter (the cells are 8–10 μm in diameter). Cells from 22-h aggregates also showed a bimodal staining distribution, with ~10% positive cells. The staining distribution in these positive cells appears as a diffuse network in the cells instead of distinct dots as seen previously (Fig. 5 *C*). Both the preimmune and the anti-β-galactosidase sera showed no staining of vegetative, 15-, or 22-h developing cells (data not shown). Beejin cannot be detected in vegetative or 0–12-h developing Ax-3 cells (Fig. 5 *G* and data not shown). 15 h after starvation, a few cells can be seen that contain ~0.8 μm diameter vesicles that are stained by the anti-beejin antibody (Fig. 5 *H*). The vesicles stain approximately as a solid sphere as determined by focusing the microscope up and down. As development proceeds, relatively more cells contain beejin-positive vesicles. Approximately 20 h after starvation the vesicles no longer appear solid. The apparent size of the vesicles increases to ~1.5 μm and in a given focal plane many vesicles appear as open circles (Fig. 5 *I*). By focusing the microscope up and down we see that the anti-beejin antibodies are staining a hollow sphere. Occasionally, some additional staining can be seen in patches on the surface of cells containing beejin-positive vesicles (arrow in Fig. 5 *I*). Finally, *Dictyostelium* spores from 26-h developing cultures show staining on their surface (Fig. 5 *I* [*inset*]). Western blots show that beejin is present in purified spore coats (a gift of C. Hong and W. F. Loomis [University of California at San Diego]; data not shown). No staining of cells could be seen at any developmental time with preimmune or anti-β-galactosidase antibody.

Cell Type Localization of Pst-Cath and Beejin

Pst-cath mRNA is mainly found in prestalk cells purified on Percoll gradients; very little can be detected in prespore cells (Mehdy et al., 1983). The opposite is true for beejin (2H-3) mRNA. We made use of the facts that prestalk cells are mostly localized to the anterior ~1/10 of migrating slugs and prespore cells are in the posterior of slugs to examine cell-type localization of pst-cath and beejin. Migrating KAx-3 slugs were fixed with formaldehyde, embedded, and cryosectioned (see Materials and Methods). Sections were allowed to adhere to subbed coverslips and stained for pst-cath or beejin by indirect immunofluorescence. Anti-pst-cath stains cells in the anterior tip of the slug and also cells on the periphery of the rest of the slug (Fig. 6). Little staining can be seen in interior regions in the posterior portion of the slug (see Discussion). This indicates that like its mRNA, pst-cath is present in prestalk cells and can be used as a specific marker for prestalk gene expression. Anti-beejin stains cells in the posterior region of slugs (see Fig. 7), indicating that beejin can be used as a marker for prespore gene expression. We also separated prestalk and prespore cells from migrating NC-4 slugs on percoll gradients as described by Ratner and Borth (1983). Immunofluorescence showed that anti-pst-cath stained cells in the prestalk fraction but not the prespore fraction; the opposite was true for anti-beejin (data not shown).

Assaying Pst-Cath and Beejin Expression in Individual Cells

Mehdy and Firtel (1985) showed that pst-cath or beejin mRNA expression can be induced in starved cells plated at very low densities such that >95% of the cells are not in contact with another cell. The induction requires two substances: conditioned medium factor, a factor secreted by starved cells, and cAMP. Cultures induced for pst-cath and beejin expression in the above manner (see Materials and Methods) show staining of beejin by immunofluorescence in a subset of the cells (Fig. 8, *D* and *E*). Cells plated in fresh medium in the absence (Fig. 8 *A*) or presence (Fig. 8 *C*) of cAMP are not stained by the anti-beejin antibody. Cells plated in conditioned medium in the absence of added cAMP (Fig. 8 *B*) also do not stain with the anti-beejin antibody. Expression of beejin thus required both conditioned medium factor and extracellular cAMP. These results were obtained with the plated cells grown in the dark or under standard fluorescent room lights, and with a variety of cAMP concentrations and addition times. Staining of similar preparations with anti-cathepsin gave essentially identical results (Datta et al., 1986). Like beejin, pst-cath is detected in only a subset of the induced cells.

Ontogeny of Prestalk and Prespore Cells

To determine the spatial distribution of prespore and prestalk cells during *Dictyostelium* development, we stained aggregates from various stages of development. Fig. 9 shows early aggregates at ~10 h of development. These are whole mounts instead of cryosections; the plane of the photograph is parallel to the plane of the surface on which the aggregates formed. Pst-cath is visible at the periphery of an aggregate but is not present in streaming cells (Fig. 9 *A*). No beejin staining is visible at this developmental stage (Fig. 9 *B*). A whole mount preparation of a tipped aggregate (~12–13 h of development) is shown in Fig. 10. The whole mount has been simultaneously stained for pst-cath and beejin by double-label immunofluorescence. Pst-cath is located in the body of the aggregate and at the periphery of the aggregate (*arrow*, Fig. 10 *A*). Less staining is observed in the tip than in the body of the aggregate. Beejin is distributed in the body of the aggregate (Fig. 10 *B*). Note that in the tip region that there are cells visible in the phase-contrast image (Fig. 10 *C*) that are not stained by either antibody. A cryosection of a tipped aggregate (in a plane perpendicular to that of the agar surface) from ~13 h of development is shown in Fig. 11. Staining with anti-pst-cath shows the distribution of prestalk cells seen in Fig. 10: they are found at the periphery of the aggregate and to a lesser extent scattered throughout the aggregate. As shown also in Fig. 10, there is less pst-cath staining in the tip than in the body of the aggregate.

A whole mount of a late slug stage aggregate (~17 h of de-

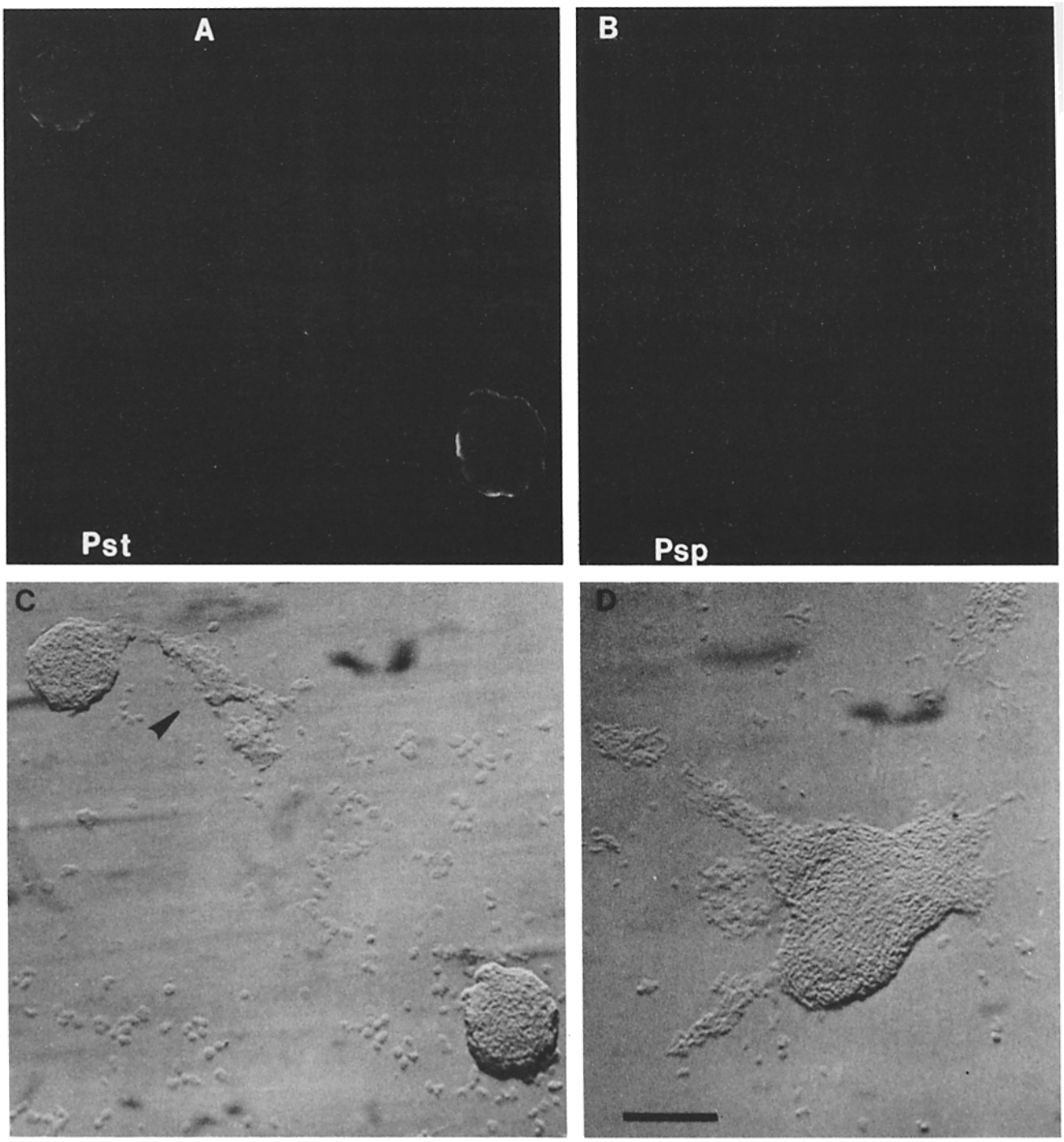


Figure 9. Distribution of pst-cath and beejin in early aggregates. Whole mounts of 10-h developing KAx-3 cells were stained for pst-cath (*A*) or beejin (*B*) by immunofluorescence. Corresponding phase-contrast images are *C* and *D*. Arrow in *C* shows cells streaming in toward an aggregate. *Pst*, prestalk. *Psp*, prespore. Bar, 100 μm.

velopment) is shown in Fig. 12. As in Fig. 10, the aggregate was stained for pst-cath (*A*) and beejin (*B*) by double-label immunofluorescence. Pst-cath and beejin show the same distribution in the whole mount as they do in cryosections (Figs. 6 and 7). Note that, as in the tipped aggregates, there are cells visible in the phase-contrast image (arrow in *C*) that are stained by neither antibody. It is possible that the unstained cells seen in Fig. 10 may be the progenitors of the unstained slug cells. If this is the case, then these cells have apparently moved from the tip of tipped aggregates to a region in the interior of the slug behind the tip.

Finally, whole mounts of fruiting bodies are shown in Figs. 13–15. While most of the prestalk cells have differentiated into stalk, some prestalk cells or cells containing pst-cath are present as a smooth covering around the spore mass (Fig. 13). In some preparations, this covering is lost giving the spore mass a rough appearance (Fig. 14 *C*); concentrations of the pst-cath staining cells on the radial axis above and be-

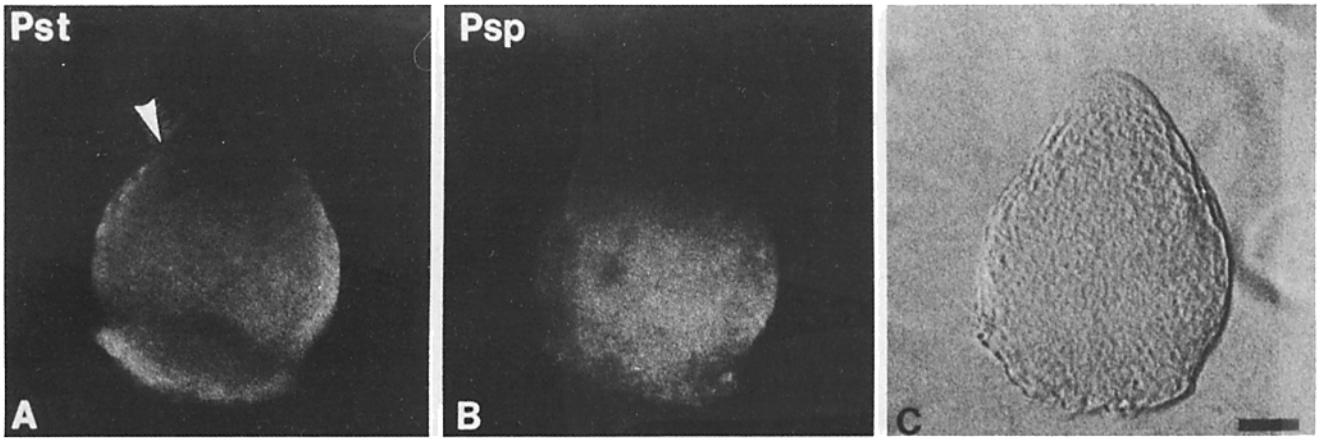


Figure 10. Distribution of pst-cath and beejin in tipped aggregates. Whole mounts of tipped aggregates (~12–13 h of development) were stained by double-label immunofluorescence. Staining for pst-cath is shown in *A*; staining for beejin is shown in *B*, and *C* is the phase-contrast image. Arrow in *A* shows pst-cath staining at the periphery of the aggregate. *Pst*, prestalk; *Psp*, prespore. Bar, 100 μm.

low the spore mass can then be seen (Fig. 14 *A*). Pst-cath is not present in the stalk proper (*arrow*), beejin is present in the spore mass (Fig. 13 *B*, 14 *B*, and 15 *B*). A whole mount of a fruiting body, viewed partially along the axis of the stalk, is shown in Fig. 15. A ring of cells not stained by either antibody can be seen in the spore mass next to the pst-cath–containing tip (arrows in 15, *A–C*).

Discussion

Use of Antibodies As an Assay for Prestalk and Prespore Cells

We have made antibodies against β-galactosidase fusion proteins containing a portion of pst-cath or beejin. The anti–pst-cath and anti–beejin antisera have several advantages over previous markers for prestalk or prespore cells. Each antibody is directed against a single protein rather than a mixture of proteins. Second, the antisera are directed against a polypeptide backbone: the bacterially synthesized antigen will not contain the *Dictyostelium* posttranslational modifications and thus the resulting antisera do not contain antibodies against a posttranslational modification, such as a glycosyl group, that might be found on products of more than one gene or that might be regulated in a manner other than that of pst-cath or beejin transcription and translation (Grant and Williams, 1983; Loomis et al., 1983; Knecht et al., 1984). Third, the antisera are against the product of cloned cell type–specific genes (Mehdy et al., 1983). Finally, the antisera are polyclonal and are (theoretically) directed against a number of epitopes spread over many amino acids of their protein antigens. This is an advantage over monoclonal antibodies which can give misleading results due to modification or inaccessibility of the binding site (Blose et al., 1982; Franke et al., 1983; Danto and Fischman, 1984).

Pst-Cath

The pst-cath open reading frame that was inserted into λgtll codes for 92 amino acids. An examination of Fig. 2 shows that there are only 16 amino acids, all fairly widely separated, which match between this region of pst-cath and cysteine proteinase I. We therefore feel that there is little chance that anti–pst-cath will cross-react with cysteine proteinase I. In addition, our anti–pst-cath antibody stains only a single band on Western blots (Fig. 4). By both Western blots and immunofluorescence, pst-cath is not present in vegetative cells or aggregating developing cells (Figs. 4, 5, and 9 and data not shown); it appears only after the aggregate (~10 h of development) stage. This is in agreement with the time of appearance of pst-cath mRNA (Mehdy et al., 1983). The antibody stains cells in the anterior tip and the periphery of migrating slugs (Figs. 6 and 7). These results show that the anti–pst-cath antibody is a useful marker for prestalk cells. The antibody allows detection of prestalk cells by Western blots or immunofluorescence. Previous markers for prestalk differentiation in *Dictyostelium* have included neutral red staining, acid phosphatase, and monoclonal antibodies (Bonner, 1952; Oohata, 1983; Tasaka et al., 1983; Noce and Takeuchi, 1985). Both neutral red and the monoclonal antibody stain cells in the anterior tip of the slug, and the monoclonal antibody additionally stain cells at the periphery of the slug (Tasaka et al., 1983). This antibody, however, appears to stain an epitope present in vegetative cells that disappears from prespore cells rather than an epitope that specifically appears in prestalk cells.

The punctate appearance of pst-cath in ~15-h developing cells, combined with the sequence homology of pst-cath to known cysteine proteases, suggests that the protein may be sequestered in vesicles, possibly lysosomes, at this developmental stage. This is in agreement with a putative leader sequence found at the NH_2-terminal end of the derived amino acid sequence of pst-cath (Datta and Firtel, manuscript submitted for publication). Later in development, pst-cath appears to be distributed over a wider area of the cell. One hypothesis might be that this distribution is caused by the pst-cath–containing lysosomes digesting large areas of the prestalk cells in order to allow them to vacuolate.

Beejin

The anti–beejin antibody stains a single band of 78 kD on Western blots of *Dictyostelium* cells. Since we have been unable to clone the 5′ region of the beejin gene, we cannot compare predicted and observed molecular weights of the protein. Because of the homology of beejin to an anti–bacterial

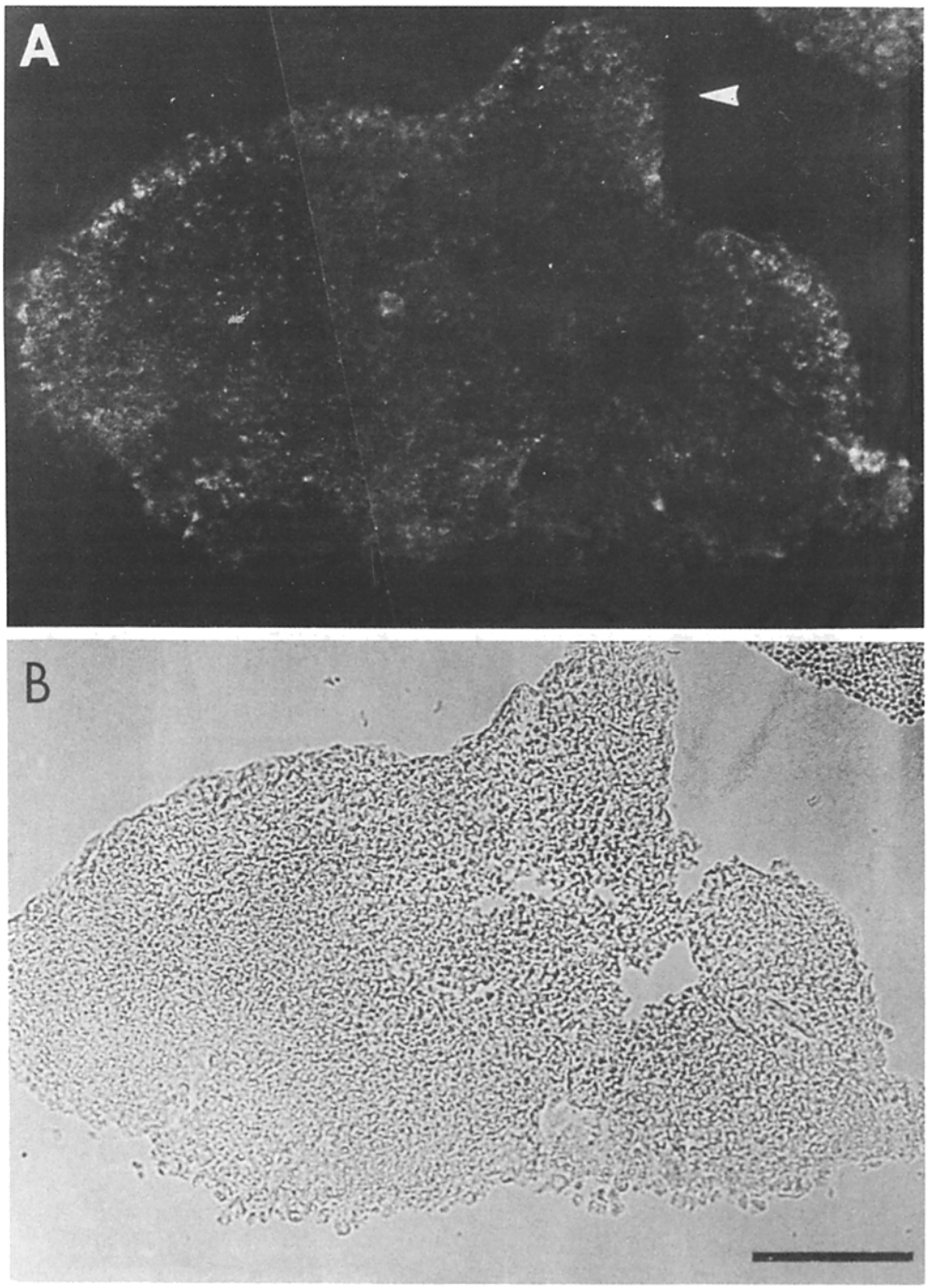


Figure 11. Distribution of pst-cath within tipped aggregates. A tipped aggregate of the same approximate stage as shown in Fig. 10 was cryosectioned in a plane perpendicular to the supporting surface. A section was stained for pst-cath (*A*); the phase-contrast image is shown in *B*. Arrow in *A* indicates the tip. Bar, 50 μm.

toxin, instability of the promoter region of the beejin gene may be the result of the gene being expressed in *E. coli* and the product being deleterious to the *E. coli* host. By both immunofluorescence and Western blots, beejin can be detected only after 15 h of development, in agreement with the time of appearance of beejin (2-H3) mRNA (Mehdy et al., 1983; Reymond et al., 1984). Like other antibodies against prespore-specific antigens (Devine et al., 1983; Tasaka and Takeuchi, 1983), anti-beejin stains cells in the posterior ~80% of migrating slugs. These results show that the anti-beejin antibody is a useful marker for prespore cells. At the time of its appearance, beejin is localized to solid, approximately spherical bodies inside cells. By ~22 h of development beejin is distributed at the surface of larger vesicles and also at peripheral regions of the prespore cells; it later can be seen at the surface of spores. A possible interpretation of these observations is that beejin is synthesized in the smaller solid vesicles. The larger vesicles might then mediate the transfer of beejin to the surface of spores, where it may act as an antibacterial toxin to protect the spores. It is possible that beejin is an integral structural element of the spore coat.

Localization of Anterior-like Cells

From assays of prestalk-specific enzymatic activities in microdissected slugs, it has been known that there is a population of anterior-like cells in the posterior section of migrating slugs (see Devine and Loomis, 1985). Our immunofluores-

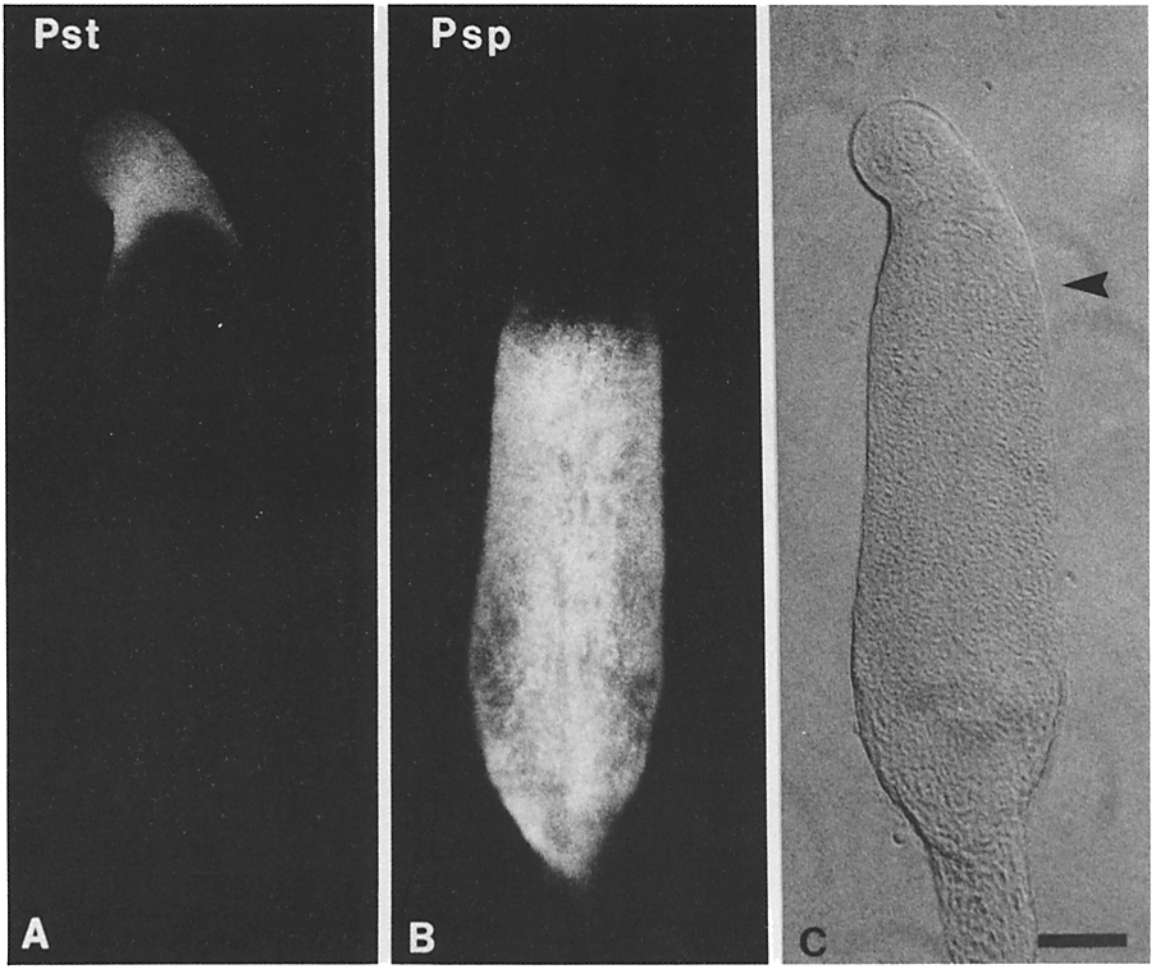


Figure 12. Distribution of pst-cath and beejin in the same slug. A slug from ~17 h of development migrating toward the top was stained for pst-cath (*A*) and beejin (*B*) by double-label immunofluorescence. *C* is the phase-contrast image. Arrow in *C* indicates cells that are not stained by either antibody. Bar, 50 μm.

cence data suggest that these anterior-like cells or more precisely cells expressing this prestalk marker are located primarily at the periphery of the slug. We also observe a few anti-pst-cath reactive cells scattered throughout the central regions of the posterior of the slug; these may have been dislodged from their anterior or peripheral locations in the slug by the flow of cells within the slug, or else may be a subpopulation of cells in the posterior central region of the slug that normally express prestalk genes. They are probably not an artifact of cryosectioning since the slugs were fixed before sectioning.

The Origin of Prestalk and Prespore Cells

Using immunofluorescence, we have been able to examine the spatial distribution of prestalk and prespore cells during *Dictyostelium* development. Both markers are not present in vegetative or aggregating cells. Prestalk cells appear at the periphery of the aggregates. The existence of some prestalk cells in the interior of these aggregates (Fig. 11) suggests that differentiation into prestalk cells may arise from factors other than a cell's position within the aggregate, and that the prestalk cells may then migrate to the anterior region in the slug. The maintenance of the prestalk/prespore tissue patterns could then conceivably arise by differential cell cohesiveness (Bonner, 1959; Feinberg et al., 1979; Lam et al., 1981). Whether differentiation into prestalk or prespore cells depends on a cell's position within the cell cycle at the time of starvation (Weijer et al., 1984) or some other mechanism cannot be examined in these experiments. We have previously examined the localization of a pst-cath/*E. coli* β-galactosidase gene fusion and showed that a pst-cath/β-glucuronidase fusion protein has the same spatial distribution as pst-cath and that the fusion protein is very stable during development (Datta et al., 1986). This suggests that during normal development, there is not a substantial amount of interconversion between prestalk and prespore cells in slugs. We observe the first appearance of prespore cells in the posterior regions of tipped aggregates. Later, during culmination, prestalk cells are in front of and surround the mass of prespore cells (Figs. 11–15). Whether this has any morphogenetic significance (the surrounding layer of prestalk cells might control the shape of the prespore mass) is as yet unknown.

The existence of cells that stain with neither anti-pst-cath nor anti-beejin throughout development (Figs. 9, 10, 12, and 15) suggests that there may be a third cell type in developing *Dictyostelium* aggregates. This putative third cell type is

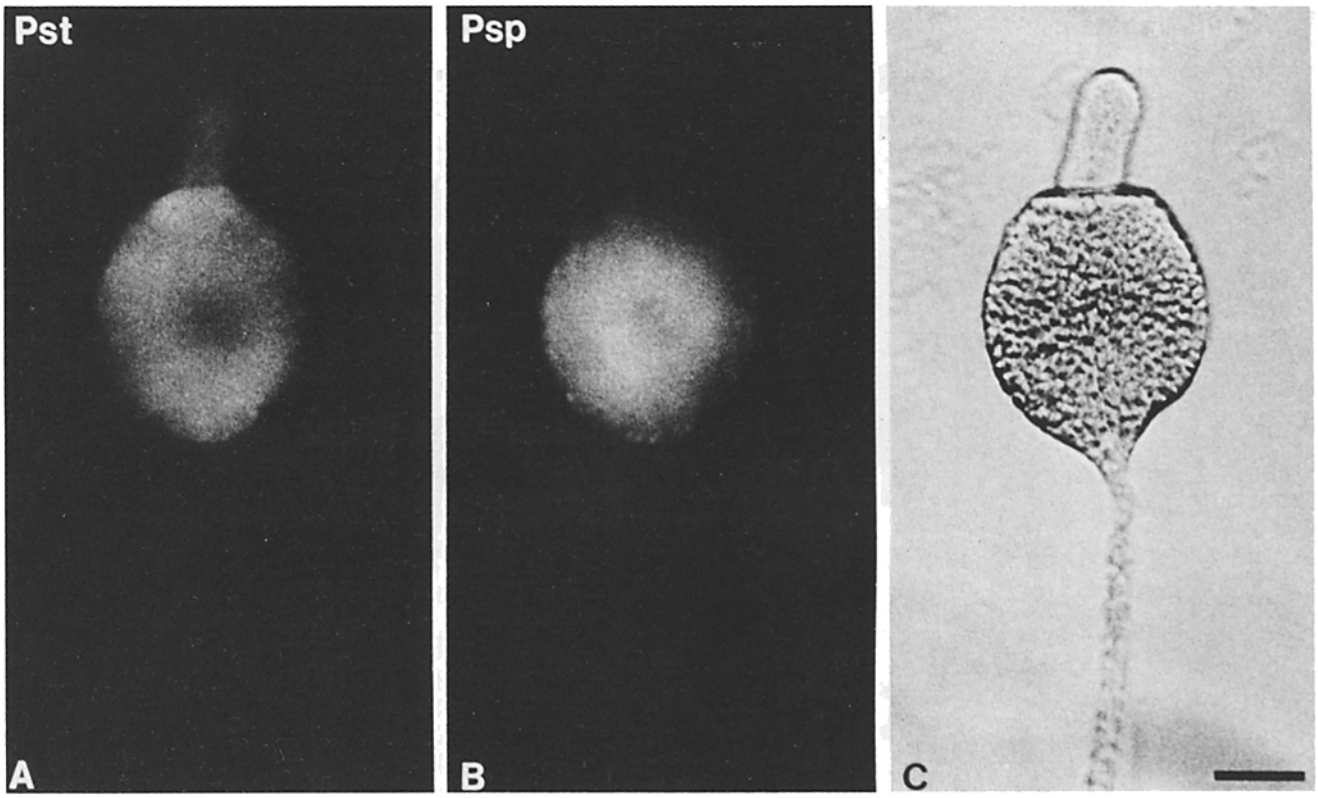


Figure 13. Distribution of pst-cath and beejin in a fruiting body. A fruiting body whose base is below the bottom of the figure was stained for pst-cath (*A*) and beejin (*B*) by double-label immunofluorescence. *C* is the phase-contrast image. Bar, 100 μm.

either undifferentiated vegetative-like cells or cells that differentiate and express genes for which probes do not yet exist. Interestingly, the region of culminating aggregates where no staining is observed is approximately that where 5′-AMP nucleotidase activity is localized (Armant and Rutherford, 1979). The observation that isolated prestalk cells show heterogeneity (Lam et al., 1981; Ratner and Borth, 1983; Weijer et al., 1984) may be due either to this cell type, or to the anterior-like cells that we see at the periphery of aggregates.

The ratio of prestalk to prespore cells in an aggregate is regulated over a wide range of aggregate sizes. Slug dissection and other types of experiments show that the developmental fate of cells in a slug is plastic (see MacWilliams and Bonner, 1979 for review; Weijer and Durston, 1985). This suggests that an individual cell may have a propensity to develop into a given cell type and that differentiation is maintained by a homeostatic mechanism. Two distinguishable hypotheses have been presented as to the mechanism whereby a starved vegetative *Dictyostelium* cell becomes a prestalk as opposed to a prespore cell. The first is that extracellular physiological factors, such as a cell's position within a morphogen gradient in the aggregate determines its fate (for review see MacWilliams and Bonner, 1979). The second is that the probability of whether a cell differentiates into a prestalk or prespore cell may depend on the cell's position within the mitotic cycle of the cell at the time of starvation (Weijer et al., 1984) or some other mechanism which is independent of the cell's position within the aggregate. When *Dictyostelium* cells were plated at very low densities and induced to express prespore and prestalk genes (following Mehdy and Firtel, 1985), we found that ~10% of the cells were positive for our prestalk marker. Under similar conditions, up to 50% of the cells express beejin. While this could be an artifact of the in vitro culture assay, it may mimic the normal developmental fate of cells. Since the assay allows us to examine the cell type of individual cells, we can devise experiments aimed at differentiating between the above two hypotheses.

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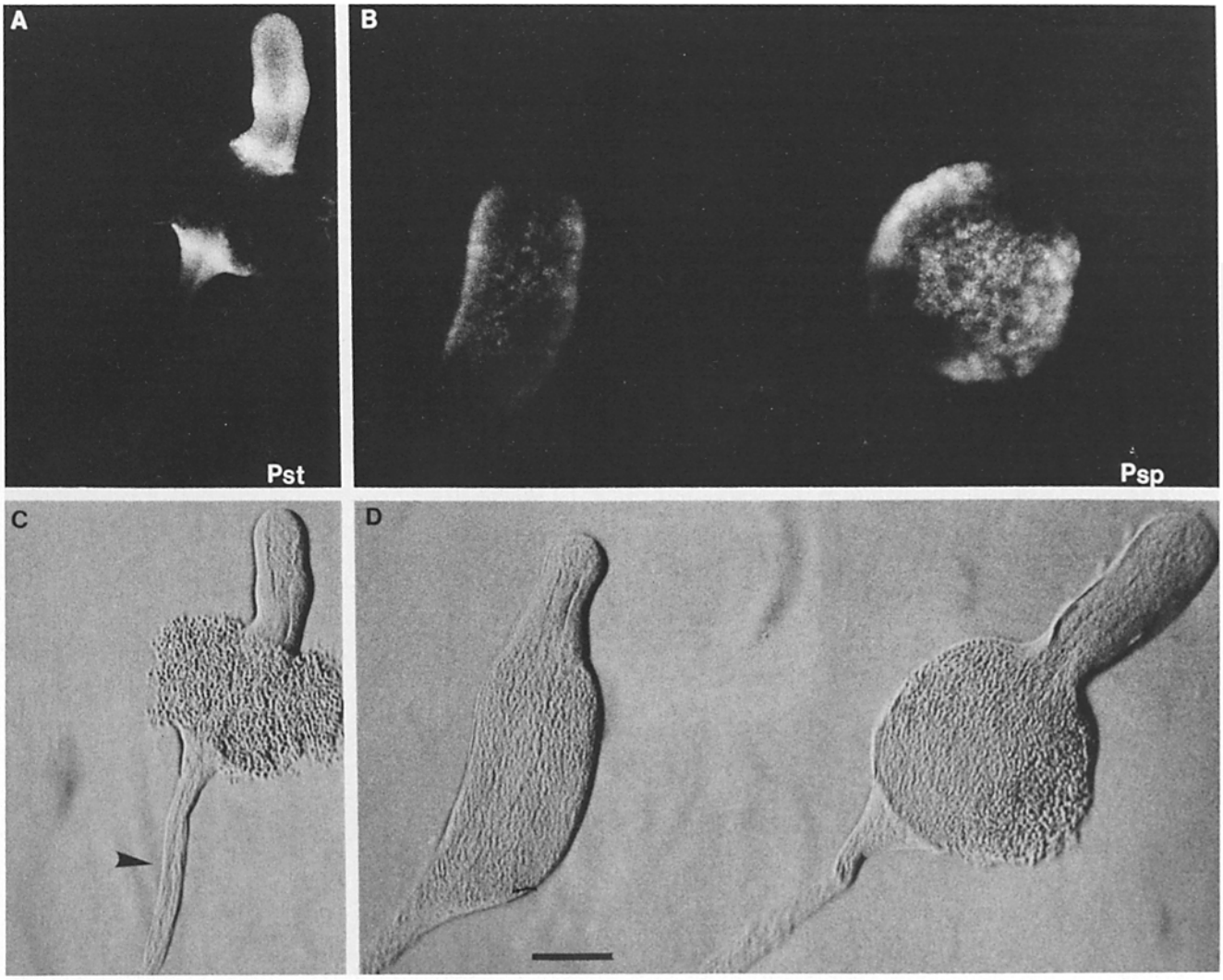

Figure 14. Distribution of pst-cath and beejin in fruiting bodies. Fruiting bodies were stained for pst-cath (*A*) or beejin (*B*) by immunofluorescence; phase-contrast images are shown in *C* and *D*. Arrow in *C* indicates the stalk, which is not stained by anti-pst-cath (see also Figures 13 and 15). Note the absence of a smooth covering over the spore mass in *C*. Bar, 100 μm.

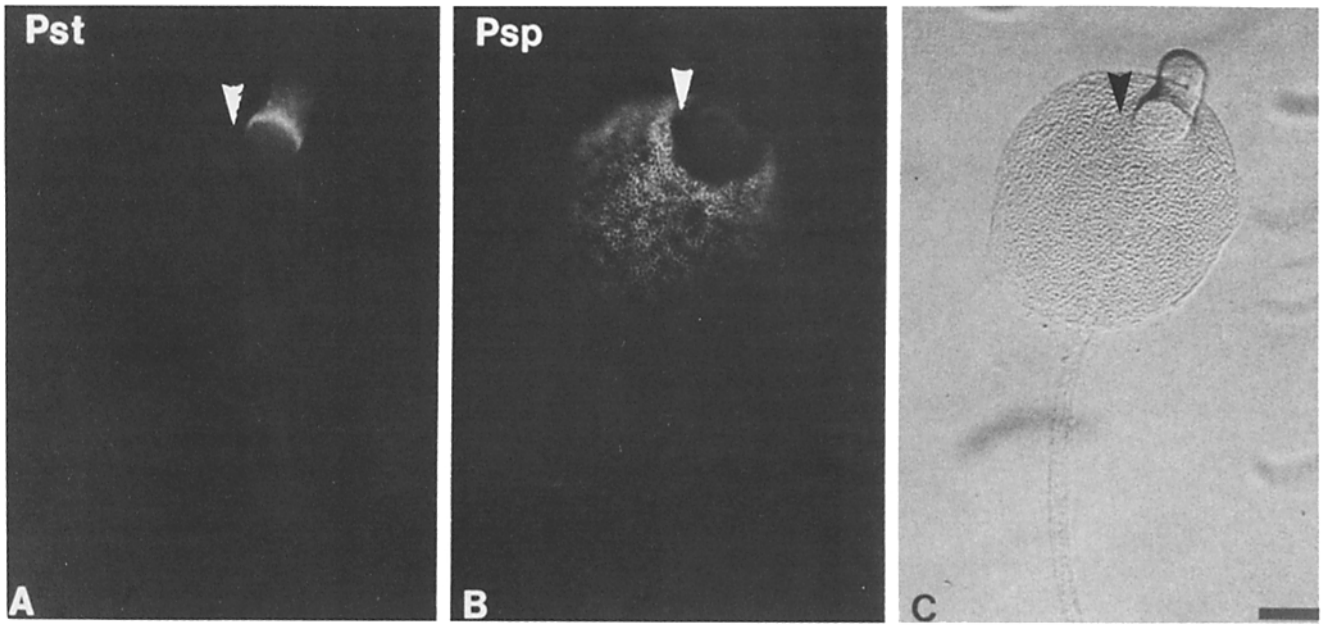

Figure 15. Oblique view of a fruiting body. A fruiting body was stained for pst-cath (*A*) and beejin (*B*) by double-label immunofluorescence. The phase-contrast image is shown in *C*. Arrows indicate cells that are stained by neither antibody. Bar, 75 μm.

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