

A Ubiquitin-Conjugating Enzyme Is Essential for Developmental Transitions in *Dictyostelium*

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Submitted February 6, 1997; Accepted July 16, 1997
Monitoring Editor: James A. Spudich

We have identified a developmentally essential gene, *UbcB*, by insertional mutagenesis. The encoded protein (UBC1) shows very high amino acid sequence identity to ubiquitin-conjugating enzymes from other organisms, suggesting that UBC1 is involved in protein ubiquitination and possibly degradation during *Dictyostelium* development. Consistent with the homology of the UBC1 protein to UBCs, the developmental pattern of protein ubiquitination is altered in *ubcB*-null cells. *ubcB*-null cells are blocked in the ability to properly execute the developmental transition that occurs between the induction of postaggregative gene expression during mound formation and the induction of cell-type differentiation and subsequent morphogenesis. *ubcB*-null cells plated on agar form mounds with normal kinetics; however, they remain at this stage for ~10 h before forming multiple tips and fingers that then arrest. Under other conditions, some of the fingers form migrating slugs, but no culmination is observed. In *ubcB*-null cells, postaggregative gene transcripts accumulate to very high levels and do not decrease significantly with time as they do in wild-type cells. Expression of cell-type-specific genes is very delayed, with the level of prespore-specific gene expression being significantly reduced compared with that in wild-type cells. *lacZ* reporter studies using developmentally regulated and cell-type-specific promoters suggest that *ubcB*-null cells show an unusually elevated level of staining of *lacZ* reporters expressed in anterior-like cells, a regulatory cell population found scattered throughout the aggregate, and reduced staining of a prespore reporter. *ubcB*-null cells in a chimeric organism containing predominantly wild-type cells are able to undergo terminal differentiation but show altered spatial localization. In contrast, in chimeras containing only a small fraction of wild-type cells, the mature fruiting body is very small and composed almost exclusively of wild-type cells, with the *ubcB*-null cells being present as a mass of cells located in extreme posterior of the developing organism. The amino acid sequence analysis of the *UbcB* open reading frame (ORF) and the analysis of the developmental phenotypes suggest that tip formation and subsequent development requires specific protein ubiquitination, and possibly degradation.

INTRODUCTION

A significant amount of information has been obtained about the signal transduction pathways that control many aspects of *Dictyostelium* differentiation (Devreotes, 1994; Firtel, 1995). Cyclic AMP, functioning as

an extracellular ligand to a seven-span/serpentine class of receptors, mediates aggregation, the developmental switch to multicellular development, and cell-type differentiation through distinct signal transduction pathways. Subsequent morphogenesis and the initiation of culmination are also believed to be regulated by cAMP functioning both as an extracellular signaling molecule and intracellularly through the activation of PKA (Williams and Morrison, 1994; Firtel, 1995). While both molecular and biochemical ap-

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proaches have resulted in significant insights into these and other pathways controlling *Dictyostelium* differentiation, it is clear that many other regulatory networks participate in controlling differentiation of this organism.

Genetic analysis in other systems has identified ubiquitination and deubiquitination as important mechanisms controlling a diversity of cellular regulatory processes, including ubiquitin-mediated protein turnover, protein targeting, and cell fate decisions. Ubiquitin conjugating enzymes UBC/E2 are thought to be involved in substrate recognition: the multiplicity of UBC identified in a number of organisms suggests that each UBC may be important in controlling the ubiquitination of a specific subset of proteins (Hershko and Ciechanover, 1992; Varshavsky, 1992; Ciechanover, 1994). For example, a mammalian UBC/E2 protein is involved in p53 degradation; UBC6 and UBC7 are involved in degrading MAT α 2 in yeast; UBC2 has been implicated in degradation of the α protein subunit GPA1 in yeast; UBC9 is implicated in cyclin degradation essential for the progression of the cell cycle in yeast; the Huntingtin protein interacts with hE2; PAS2 is required for peroxisome biogenesis; and the *Drosophila bendless* gene is involved in synaptic transmission (Dohmen *et al.*, 1991; Glotzer *et al.*, 1991; Wiebel and Kunau, 1992; Chen *et al.*, 1993; Muralidhar and Thomas, 1993; Chowdary *et al.*, 1994; Ciechanover *et al.*, 1994; Madura and Varshavsky, 1994; Oh *et al.*, 1994; Palombella *et al.*, 1994; Seufert *et al.*, 1995; Kalchman *et al.*, 1996). In addition, ubiquitination has been shown to be a component of the pathway by which the yeast MDR transporter Pdr5, the yeast α -factor receptor, and the mammalian growth hormone receptor are targeted for endocytosis (Egner and Kuchler, 1996; Hicke and Riezman, 1996; Strous *et al.*, 1996), while ubiquitination/deubiquitination controls some cell fate decisions during development of the *Drosophila* eye (Jermyn and Williams, 1995) and larval development in *Caenorhabditis elegans* (Zhen *et al.*, 1996). In *Dictyostelium*, the structure and regulation of expression of genes encoding ubiquitin have been examined (Muller-Taubenberger *et al.*, 1988a and 1988b; Ohmachi *et al.*, 1989), and the *in vitro* ubiquitination and degradation of *Dictyostelium* calmodulin have been reported in a reticulocyte extract (Gregori *et al.*, 1985). However, little is known about the role of protein ubiquitination during *Dictyostelium* development.

In this article, we report the characterization of the gene *UbcB* that, when disrupted, results in developmental arrest during the multicellular stages of development. *UbcB* encodes a putative ubiquitin-conjugating enzyme designated UBC1. Disruption of *UbcB* affects tip formation, cell-type differentiation, the ability of *ubcB*-null cells to participate in normal development in chimeras containing wild-type cells, and the pattern of protein ubiquitination during development.

Our results suggest that protein ubiquitination at the mound and finger stage mediated by UBC1 is required for subsequent development and may be an essential component of the developmental transition between the induction of postaggregative gene expression and subsequent cell-type differentiation and morphogenesis.

MATERIALS AND METHODS

REMI mutagenesis using uracil selection, DH1 uracil auxotroph, and the pRHI30 vector, was done as previously described (Kuspa and Loomis, 1992; Dynes *et al.*, 1994; Insall *et al.*, 1994). Cell culture, transformation, and selection of clones expressing *lacZ* reporters were performed as previously described (Dynes *et al.*, 1994; Gaskins *et al.*, 1996). All other techniques, including the isolation and cloning of the insertion vector from the REMI mutant, construction of vectors, sequencing, analysis, and RNA, Southern, and Western blot techniques are standard and were performed as described previously (Dynes *et al.*, 1994; Gaskins *et al.*, 1994, 1996). Standard Southern hybridization conditions for *Dictyostelium* genomic DNA, which is AT-rich, are 3 \times SSC, 60 mM sodium phosphate (pH 6.8), 10 mM EDTA (pH 7.2), 0.2% SDS, 4 \times Denhardt's solution, and 50% deionized formamide at 37°C as described previously (Nellen *et al.*, 1987). For lower stringency hybridizations (see text), the salt concentrations were kept constant during the hybridization; however, the salt concentrations in the wash solutions were doubled and the last wash of 5 mM EDTA, pH 7.2, and 1% SDS was replaced with 1 \times SSC, 1% SDS.

Western blot analysis was performed as described previously (Gaskins *et al.*, 1994).

RESULTS

Isolation of UbcB and Identification of Transcripts

We identified *UbcB* (see below) as a developmentally essential gene in a REMI mutagenesis (Kuspa and Loomis, 1992) screen for genes required for multicellular development. REMI mutagenesis was performed using an insertion vector (pRHI30) carrying the *Dictyostelium Pyr5-6* gene (uracil prototroph) transformed into the uracil auxotroph DH1, selecting for uracil prototrophs (Kuspa and Loomis, 1992; Insall *et al.*, 1994). The insertion vector was mapped to a single site in the *Dictyostelium* genome by Southern blot analysis (Figure 1A; our unpublished observations). There was an apparent shift in the mobility of the endogenous *UbcB* band in the mutant strain, consistent with a *UbcB* gene disruption. The vector and \sim 1.7 kb of flanking DNA were excised by digestion with the restriction enzyme *EcoRI*, circularized by ligation, and cloned into *Escherichia coli* (see MATERIALS AND METHODS). Restriction maps of the genomic and cDNA clones (see below) are shown in Figure 2, A and B. To confirm that the developmental phenotype resulted from insertion at this locus, we retransformed the isolated DNA into *Dictyostelium* after linearizing it by digestion with *EcoRI*. Approximately 60% of the clones had the same developmental phenotype as the original REMI mutation, while the remainder were

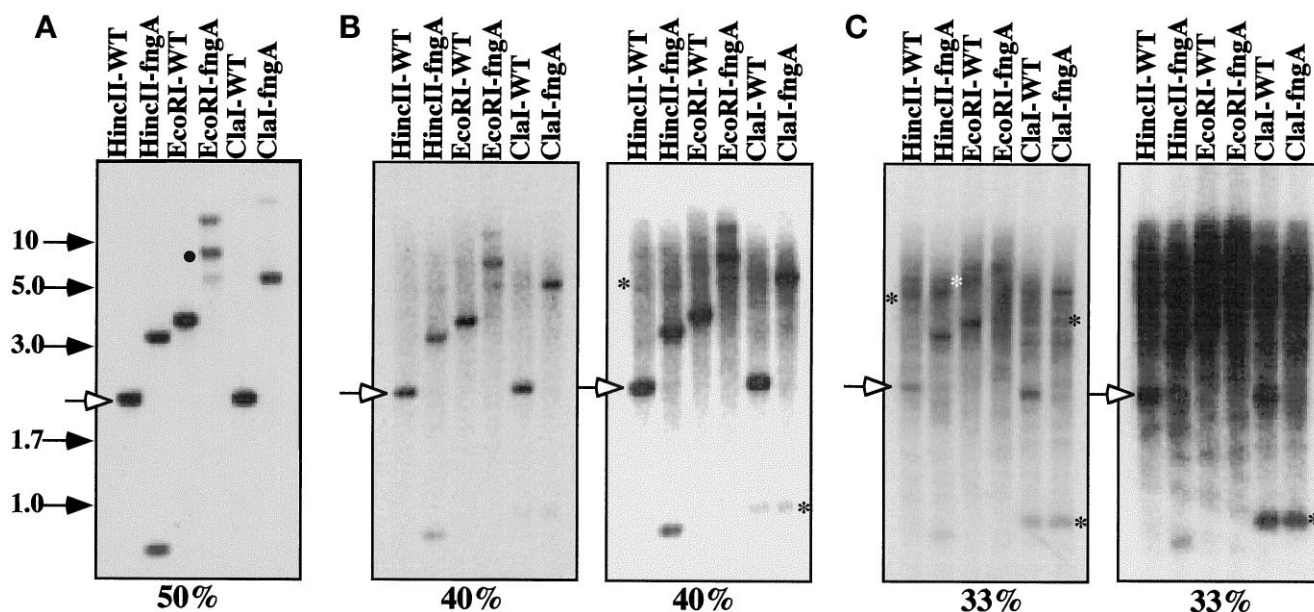


Figure 1. Southern blot of wild-type and *ubcB*-null cells. Genomic DNA isolated from wild-type cells and *ubcB*-null cells was digested with the restriction enzymes shown and analyzed by Southern blot hybridization as described previously at different hybridization stringencies (Nellen *et al.*, 1987). (A, 50% formamide) The solid circle marks the proper *UbcB* gene band for this digest. (B, 40% formamide) The two panels are different exposures of the same filter. (C, 33% formamide) The same batch of restriction enzyme-digested DNA was used for each hybridization. The two 40% and 33% formamide panels are different exposures of the same filter. The positions of size markers (in kb) are shown. See MATERIALS AND METHODS for details. The open arrowhead marks the position of a common band (wild-type *HincII* band). The asterisks mark the positions of some bands not seen in the high stringency (50% formamide) hybridization blot but which appear in the lower stringency hybridizations.

wild type. Southern blot analysis showed a 1:1 correlation with the phenotype of the original REMI strain (see below) and insertion of the DNA at the same site (our unpublished observations).

DNA from both sides of the insertion site of the genomic clone hybridized to two transcripts of ~0.65 and 0.75 kb (Figure 2C; our unpublished observations). The larger transcript was expressed during growth and development, with levels decreasing in the multicellular stages. The smaller transcript was expressed at very low levels during vegetative growth, and the levels increased during the time of aggregation. This transcript was present throughout the remainder of development. When the *ubcB* mutant strain is allowed to develop for 8 h (early mound formation) and RNA is isolated and hybridized to either probe, no hybridization is observed, implying that both transcripts are encoded by the same gene (our unpublished observations). From the lack of detectable transcripts in the mutant strain, we conclude this is an *ubcB*-null strain. Expression of the *UbcB* cDNA downstream from the *Act15* promoter complemented the null phenotype.

Hybridization probes were then used to screen a λ Zap library (Schnitzler *et al.*, 1994). Several sets of cDNA clones ranging from 0.6 to 0.8 kb were identified. The sequence of a larger and smaller clone

showed that both encoded the same open reading frame (ORF) but had different lengths of 5' and 3' untranslated sequences. This suggests that differences in the two transcripts probably result from different start and/or termination sites and are not due to splicing variants within the ORF (see below). Moreover, the different temporal patterns of expression of the two RNAs suggest that *UbcB* transcription is regulated in a complex manner.

UbcB encodes a putative ubiquitin-conjugating enzyme

The two cDNAs of different lengths were completely sequenced in both directions and a single full-length ORF was identified (accession number U67838). Comparison of the sequence to the GenBank databases using the BLAST program showed that the *UbcB* ORF has very high amino acid sequence identity to E2/ubiquitin-conjugating enzymes from yeast, various metazoans, and plants (Figure 3). The extremely high amino acid sequence conservation throughout the entire protein strongly suggests that *UbcB* encodes a ubiquitin-conjugating enzyme. Because multiple ubiquitin-conjugating enzymes have been identified in a variety of other species, we expect that *Dictyostelium* also contains a complex multigene family. We have

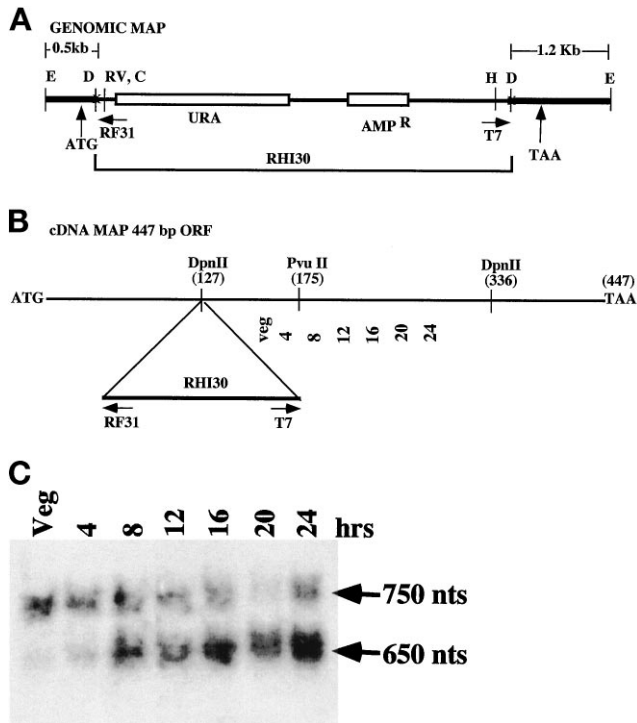


Figure 2. Restriction maps of the *UbcB* genomic clone and the cDNA ORF and RNA blot hybridization. (A) The map of genomic clone shows the positions of the *UbcB* ORF translation initiation site (ATG) and termination site (TAA). Restriction sites for the genomic clone: E, *EcoRI*; D, *DpnII*; RV, *EcoRV*; C, *Clal*; HP, *HpaI*. The transformation vector is RHI30 carrying the *Pyr5-6* gene required for uracil prototrophy. The parental strain is the uracil prototroph DH1. The vector and strain were gifts from R. Insall and P. Devreotes. RF31 and T7 are sequencing primers. RF31 was obtained from W. Loomis. (B) The map of cDNA clones shows the position of the *UbcB* ORF translation initiation site (ATG) and termination site (TAA). The *DpnII* insertion site for RHI30 in the *UbcB* REMI mutant strain is shown. (C) Developmental RNA blot of *UbcB* expression. Time in development is given. Veg, vegetative cells.

thus designated the protein encoded by *UbcB* as UBC1.

To investigate the possibility of additional genes encoding UBC in *Dictyostelium*, we performed Southern blot analysis of DNA from wild-type and *ubcB*-null cells under different stringencies. As shown in Figure 1A, hybridization under standard conditions (see MATERIALS AND METHODS) identifies a single *UbcB* gene in wild-type cells. When the stringency is reduced by lowering the formamide concentration from 50% to 40% while keeping the salt concentration and hybridization and wash temperatures constant (Figure 1B), additional hybridizing bands are observed, suggesting the presence of genes that are related at the level of DNA sequence. When the stringency was reduced further (33% formamide, constant salt and temperature), some additional strong bands and a signifi-

cant amount of apparently nonspecific background hybridization are observed (Figure 1C). The presence of additional bands that are observed in the 40% formamide hybridization and are stronger at 33% formamide is consistent with the possibility that there are multiple *UbcB*-related genes in *Dictyostelium*.

Developmental Phenotype of *ubcB*-Null Cells

To examine the developmental morphologic phenotype of the *ubcB*-null mutant, cells were grown axenically, washed of nutrients, and then plated for multicellular development on sodium/postassium phosphate-containing agar plates. *ubcB*-null cells aggregate and form a mound at 7–8 h of development, ~1 h earlier than the wild-type parental axenic strain. At the same plating densities, the aggregate sizes of the mutant and wild-type strains were approximately the same [our unpublished observations; Figure 4, compare panels C (wild type) and D (*ubcB* null)]. Wild-type mounds start to form tips at 10–11 h (Figure 4C) and begin to culminate by 18 h (Figure 4, A and B), forming a fruiting body at ~24 h (Figure 4J). In contrast, *ubcB* cells arrest at the mound stage for ~10–12 h (Figure 4D), whereupon development reinitiates with the formation of multiple tips on the same aggregate (Figure 4, E and F). These tips elongate to form fingers with a majority of the cells remaining in a mound and not participating in finger formation. Development then arrests under these conditions at ~36 h with no migrating slugs being formed (Figure 4, H and I). At a higher cell density, not all of the cells aggregate. The mounds that form also have multiple tips and protrusions that often produce aerial tangles (Figure 4G). When cells are plated on Millipore filters, multiple fingers arise from each mound after a delay of ~10–12 h. These fingers comprise only approximately one-half of the cells in the original mound, with the remainder found as an undifferentiated mass (see below; Figure 7B, panel B). At ~30–36 h, some of the fingers form migrating slugs; however, none of these initiate culmination. When cells from terminal structures plated on Na/K agar or Millipore filters are disaggregated after 36 and 48 h, respectively, and assayed for heat and detergent-resistant spores, no viable cells are found. Expression of the *UbcB* cDNA from the *Act15* promoter complements the developmental phenotypes of the *ubcB*-null cells, consistent with the developmental abnormalities being the result of a disruption of the *UbcB* gene (our unpublished observations). Overexpression of the cDNA in wild-type cells does not result in any observable morphologic defect (our unpublished observations).

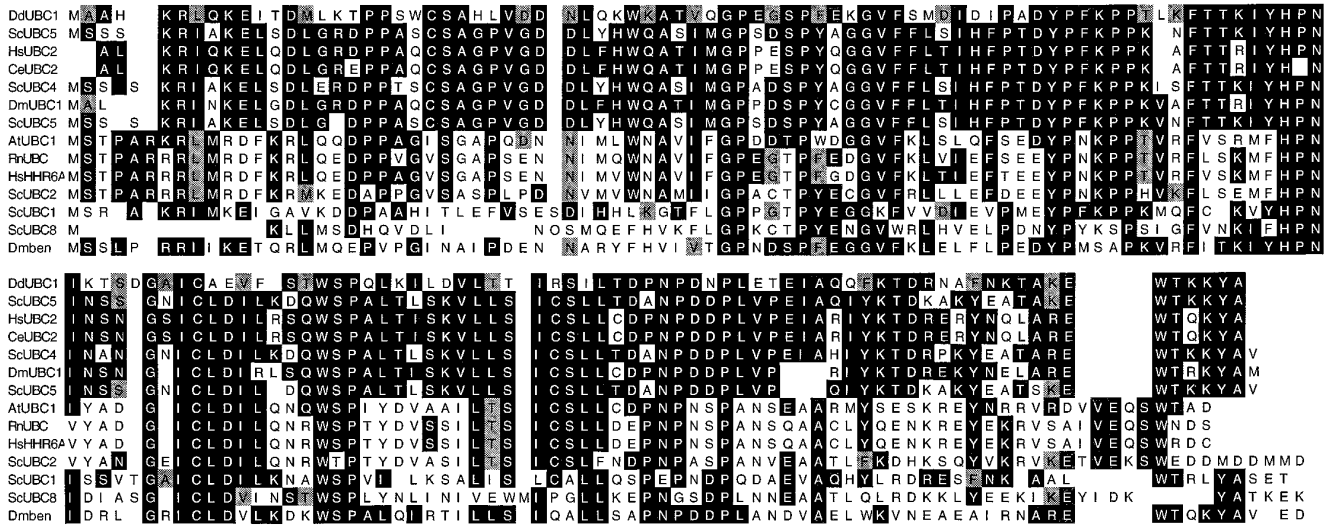


Figure 3. Amino acid sequence of the *UbcB* ORF and comparison to UBCs from a variety of organisms. Names and accession numbers are given below. UBC1, this article; ScUBC5, *Saccharomyces cerevisiae* UBC5, accession no. P15732; HsUBC2, human UBC2, accession no. P35129; CeUBC2, *C. elegans* UBC2, accession no. P35129; ScUBC4, *S. cerevisiae* UBC4, accession no. P15731; DmUBC1, *Drosophila melanogaster* UBC1, accession no. P25867; ScUBC5, *S. cerevisiae* UBC5, accession no. P15732; AtUBC1, *Arabidopsis thaliana* UBC1, ac. # P25865; RnUBC, rat, accession no. P23567; HsHHR6A, human UBC, accession no. P06104; ScUBC21, *S. cerevisiae* UBC21, accession no. P06104; ScUBC1, *S. cerevisiae* UBC1, accession no. P21734; ScUBC8, *S. cerevisiae* UBC8, accession no. P28263; Dmben, *D. melanogaster* Bendless (UBC), accession no. P35128.

Changes in the Pattern of Protein Ubiquitination in *ubcB*-Null Cells

Because *UbcB* encodes a putative UBC, we have investigated potential changes in the pattern of ubiquitinated proteins during development on Western blots using an anti-ubiquitin antibody. While most of the pattern of ubiquitinated proteins is similar between wild-type and *ubcB*-null cells (Figure 5), three proteins that react strongly with the antibody show significant differences between the two strains. In wild-type cells, a protein of ca. 73 kDa (Figure 5, band a) from its mobility reacts weakly with the antibody through 12 h of development, the time at which the *ubcB* phenotype is first observed. Afterward, the intensity of a band of the same mobility (and thus possibly the same protein) increases, suggesting that this protein undergoes either a reduction in the rate of degradation and/or an increase in the level of ubiquitination and/or expression. Our observations cannot exclude the possibility that band a represents two proteins of the same electrophoretic mobility but with different developmental kinetics. In contrast with observations in wild-type cells, in the mutant strain, this band does not decrease in intensity at 12 h but shows strong staining throughout development. Another protein (band b, ca. 59 kDa) shows a reciprocal staining pattern in wild-type cells: it is detected through 12 h of development cells, after which the level of the protein or the level of ubiquitination of the protein rapidly declines. In *ubcB*-null cells, the band continues to stain strongly for a longer time in development. A third protein (band c, ca. 51

kDa) increases in staining intensity at 4 h and remains high throughout the remainder of development in wild-type cells. The staining is generally weaker in *ubcB*-null cells and the level of staining decreases after the 12-h time point. The level of ubiquitination of other proteins may also be affected but is not detectable on our blots. While these data do not directly address whether any of these proteins are direct substrates of UBC1, they indicate that *ubcB*-null cells show observable changes in the quantitative and/or qualitative pattern of protein ubiquitination as compared with wild-type cells (see DISCUSSION).

Control of Gene Expression in *ubcB*-Null Organisms

To better understand regulatory changes that might be occurring in the *ubcB*-null cells, we examined the developmental regulation of genes preferentially expressed during multicellular development. The expression of *LagC* and *CP2/cprB* was used as a molecular marker for postaggregate gene regulation during mound formation (Pears *et al.*, 1985; Datta *et al.*, 1986; Dynes *et al.*, 1994). In wild-type cells, both genes are induced by a high, constant level of extracellular cAMP that is thought to occur as the mound forms (Abe and Yanagisawa, 1983; Firtel, 1995). *CP2/cprB* encodes a cysteine protease and has been used as a marker for postaggregate gene expression (Pears *et al.*, 1985; Datta *et al.*, 1986). *LagC*, which is similarly regulated, encodes a putative transmembrane protein that is essential for development past the mound stage

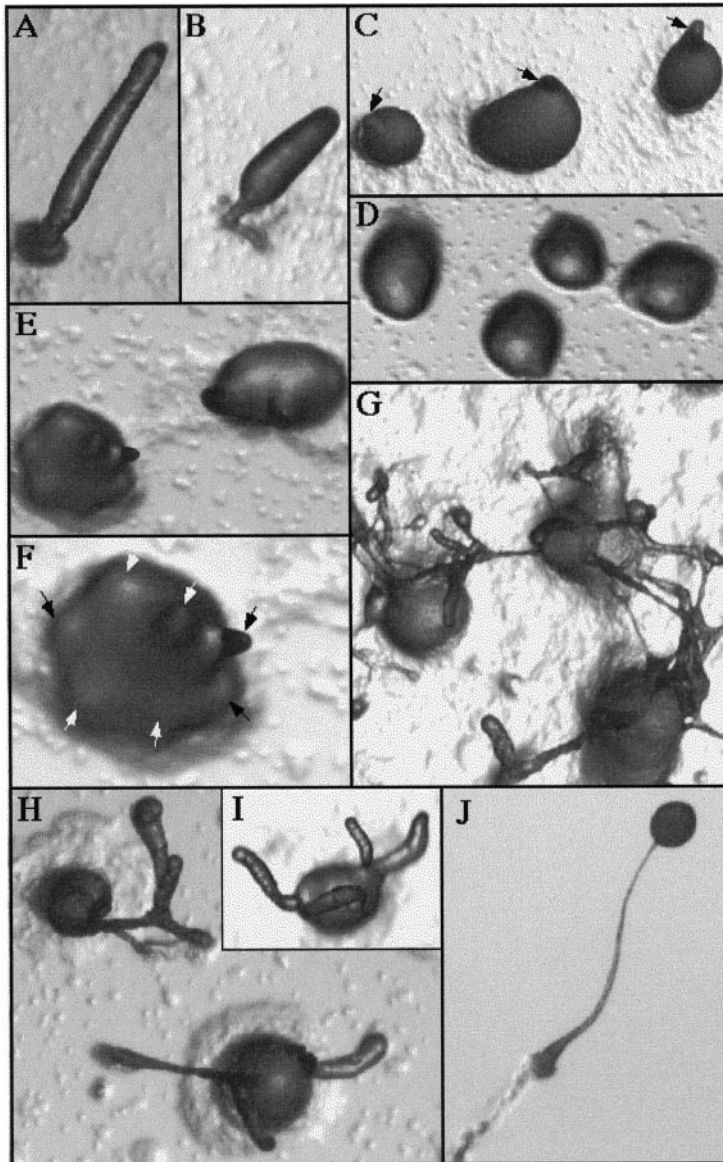


Figure 4. Developmental morphology of *ubcB*-null cells plated on Na/K P₀₄ agar plates. (A–B) Wild-type cells at the second finger/early culmination stage (18 h). (C) Wild-type tight aggregates forming tips (12 h), which are indicated by arrowheads. Note that each aggregate has only one tip. (D) *ubcB*-null cells at 18 h. Mounds first form at ~8 h. Notice that no tips are present. (E) *ubcB*-null cells at 19 h as tips are first forming. (F) Enlargement of the lower left aggregate in E. The multiple tips are indicated by arrowheads. (G) *ubcB*-null cells plated at a higher density at 36 h. (H–I) *ubcB*-null cells at 36 h. (J) Mature wild-type fruiting body (26 h).

and is required for prestalk and prespore cell differentiation (Dynes *et al.*, 1994). In wild-type cells, *CP2/cprB* and *LagC* transcripts start to accumulate late in aggregation as the mound forms. Transcript levels remain high for 4–6 h through the tipped aggregate and first finger stages (~11–13 h), after which levels of the transcripts decrease significantly (Figure 6). In *ubcB*-null cells, both genes are induced during mound formation, but in contrast to observations in wild-type cells, the level of transcript accumulation is >10-fold higher and remains high for ~10 h, until the time *ubcB*-null cells start to form tips, before decreasing only moderately. This extended expression of *CP2/cprB* and *LagC* is consistent with the developmental delay of the mutant at the mound stage and its sub-

sequent arrest at the finger stage. The high, extended level of expression suggests that the regulatory mechanism involved in down-regulating these genes during finger formation is blocked in *ubcB*-null cells.

Analysis of the expression of the endogenous prestalk-specific gene *ecmA* demonstrated that the gene was induced ~12 h later in *ubcB*-null cells than wild-type cells and showed a similar level of expression in both wild-type and *ubcB*-null strains (Figure 6). The time of *ecmA* induction corresponds approximately to the time of tip formation in the mutant strain. Expression of the prespore-specific gene *SP60/cotC* shows a similar delay in induction, indicating that the onset of expression of both prestalk and prespore genes is equally affected. However, in contrast

to *ecmA* expression, *SP60/cotC* mRNA accumulation in *ubcB*-null cells is significantly lower than in wild-type cells.

Spatial Patterning of Prestalk and Prespore Cells in *ubcB*-Null Cells and *ubcB*-Null/Wild-Type Cell Chimeras

The effect of the *ubcB*-null mutation on spatial patterning of prestalk and prespore cells in the multicellular organisms was examined using cell-type-specific *lacZ* reporters and histochemical staining. Several cell-type-specific and cell-type-enriched reporters were examined. The cloned promoter of the prestalk-specific gene *ecmA* has been dissected into two domains by deletion analysis using whole-mount histochemical staining of *lacZ* reporters to define spatial patterning into regulatory elements that are preferentially expressed in prestalk A and AB cells and in prestalk O and anterior-like cells (ALC), respectively (Early *et al.*, 1993, 1995). The vector containing the entire promoter driving *lacZ* (*ecmA*O/*lacZ*) exhibits maximal expression in the anterior prestalk A and AB cells and is expressed at a lower level in prestalk O cells in the slug. It is also expressed in ALC (Early *et al.*, 1993), a regulatory cell population comprising ~5–10% of the cells that are scattered throughout the slug. The prestalk gene *ecmB* is expressed primarily in a central core of prestalk AB cells localized in the very anterior of the slug but is also expressed in the ALC population (Jermyn *et al.*, 1989; Jermyn and Williams, 1991). *rasD* is a prestalk-enriched gene that is expressed predominantly in prestalk A, prestalk O, and ALC in the slug (Esch, 1991), while *SP60/cotC* is a prespore-specific gene expressed in the posterior 80% of the slug (Haberstroh and Firtel, 1990). Figure 7A shows the staining pattern of *ecmA*O/*lacZ*, *SP60/cotC*, *ecmB*/*lacZ*, and *rasD*/*lacZ* in wild-type slugs.

When *ecmA*O/*lacZ*- and *ecmB*/*lacZ*-expressing *ubcB*-null cells were examined, little or no staining was observed until ~15 h after the time of mound formation (our unpublished observations). In *ubcB*-null migrating slugs, the spatial pattern of *ecmA*O/*lacZ* expression was similar to that seen in wild-type cells (Figure 7B, panels E and F). *ecmB* expression, however, was abnormal. Staining is significantly stronger than in wild-type cells at similar copy numbers of the reporter vector and is observed throughout the developing first finger slug (Figure 6B, panel A; our unpublished observations). This may be an indication of an increase in the level of staining in each expressing cell or an increase in the number of ALC. Similarly, when *rasD* was examined, *ubcB*-null mounds produced a finger in which almost the entire finger was stained intensely (Figure 7B, panel B), instead of the anterior, prestalk zone staining that is seen in wild-type cells at this same stage (Esch and Firtel, 1991; our unpub-

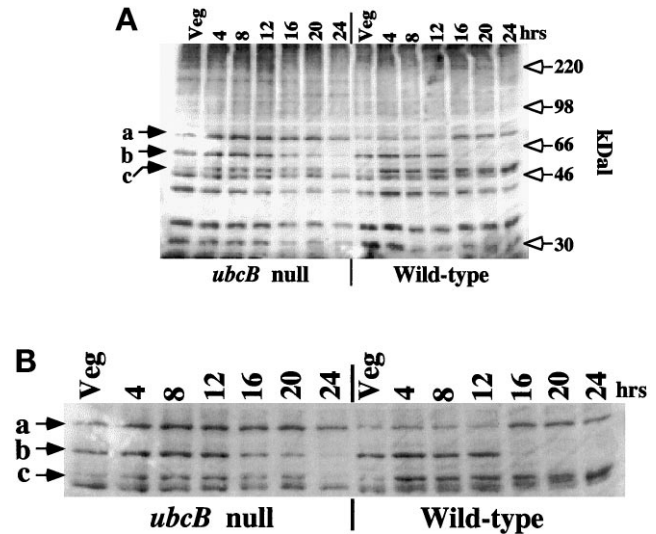


Figure 5. Anti-ubiquitin Western blot. (A) Cells were taken at the times in development indicated, lysed in PAGE-SDS gel buffer, sized on a 10% gel, blotted to Immobilon-P membrane (Millipore), and probed with a commercial antiubiquitin antibody (Sigma, St. Louis, MO). The position of the size markers are shown. Band a, ca. ~73 kDa; band b, ca. ~59 kDa; band c, ca. ~51 kDa. (B) Enlargement of the section of the blot containing bands a–c.

lished observations). Cells remaining in the mound that did not participate in finger formation showed only scattered staining. In the migrating slug, the very anterior of the slug stained the strongest (prestalk A domain) but *ubcB*-null slugs also showed strong staining through the posterior of the slug (Figure 7B, panel C). Wild-type slugs expressing *rasD*/*lacZ* showed strong staining in the prestalk A/O region but only

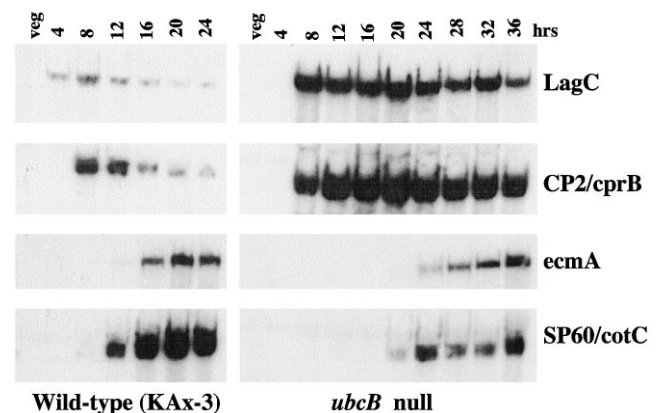


Figure 6. RNA blot hybridization of reporter genes during development. Expression of developmentally regulated genes in wild-type and *ubcB*-null strains: *LagC* and *CP2/cprB*, postaggregative genes; *ecmA*, prestalk-specific; *SP60/cotC*, prespore-specific. The filters assaying the expression level of the same gene were probed together.

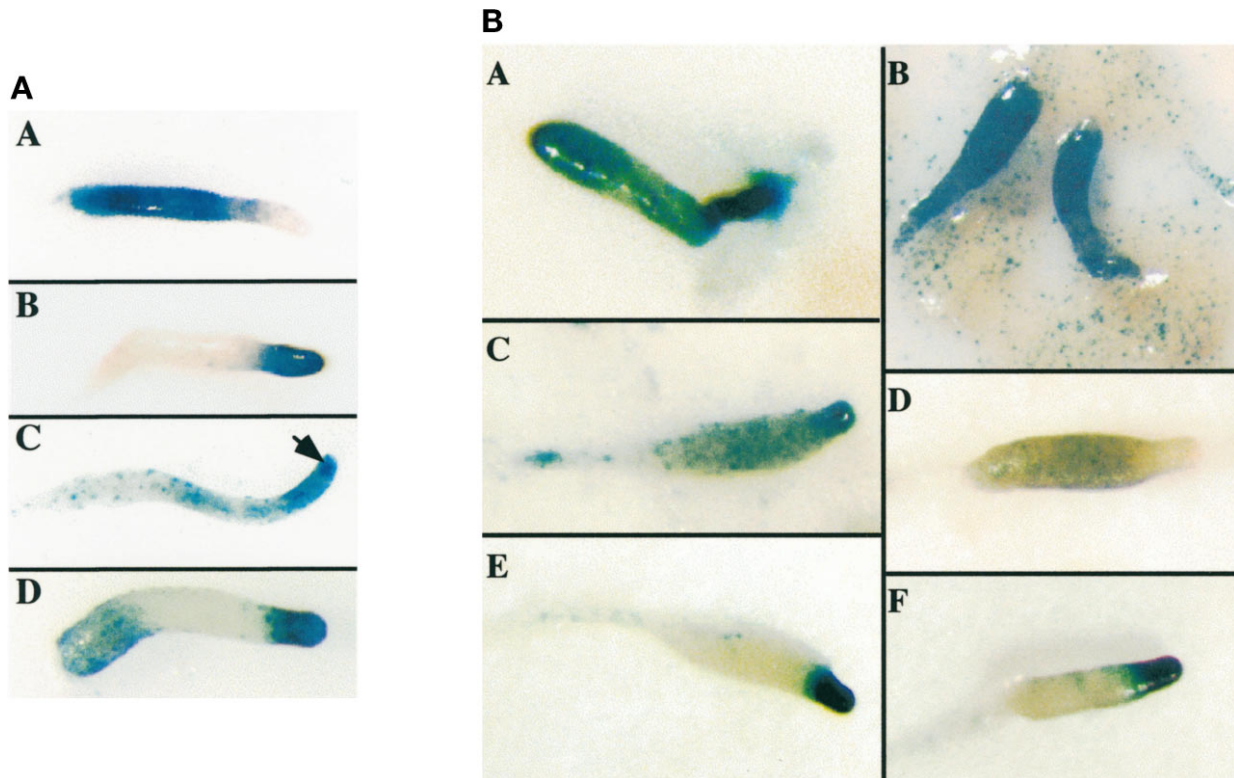


Figure 7. Spatial patterning of cell types in wild-type and *ubcB*-null cells. (A) Staining of wild-type cells at the slug stage with: panel A, *SP60/lacZ* (prespore); panel B, *ecmAO/lacZ* (prestalk A, AB, and O and ALC); panel C, *ecmB/lacZ* (prestalk AB and ALC). Arrowhead points to the core of prestalk AB cells. Panel D, *rasD/lacZ* (prestalk and ALC). (B) Pattern of *lacZ* reporter staining of *ubcB*-null slugs using various reporters: panel A, *ecmB*; panel B, *rasD*; panel C, *rasD*; panel D, *SP60*; panel E, *ecmAO*; F, *ecmAO*.

light scattered staining of ALC in the prespore region and moderate staining of the posterior-most rearward zone (Figure 7A, panel D; Esch and Firtel, 1991), which forms the basal disk in the mature fruiting body. A similar pattern was observed for all clonal isolates studied. As *rasD/lacZ* stains predominantly prestalk and ALC in wild-type slugs (Esch and Firtel, 1991), the more intense staining in the posterior may indicate a more intense staining of the ALC or an increase in the number of *ubcB*-derived ALC. It has been suggested that *rasD* is initially expressed in all cells and then the expression becomes significantly weaker in prespore cells (Jermyn and Williams, 1995). However, the observed *rasD/lacZ* staining pattern cannot simply be due to a continued expression of *rasD* in all *ubcB*-null cells, as there is negligible staining in the cells left in the mound.

When the prespore-specific marker *SP60/cotC* was examined, staining was seen in the posterior 80% of the slug, as in wild-type cells (Figure 7B, panel D). However, the staining was weaker in the mutant compared with that in wild-type cells. This pattern was also reproducible in multiple clones from independent transformations. When wild-type and *ubcB*-null slugs were dissociated into single cells and the number of

prespore cells counted (*SP60/lacZ* staining cells), a similar fraction of cells stained in both strains (our unpublished observations), indicating that the number of prespore cells in the slugs is not altered significantly in the *ubcB*-null mutant.

To obtain a better understanding of the properties of the *ubcB* cells, experiments were performed in which *lacZ*-tagged *ubcB*-null and wild-type cells were mixed in a ratio of 1:3 and allowed to coaggregate, forming chimeric organisms. These chimeras were then histochemically stained for β -galactosidase activity for localization of *lacZ*-expressing *ubcB*-null cells. Such studies allow the examination of both the spatial localization of the *ubcB* cells and the ability of the cells to participate in different terminal structures of the fruiting body. When *ecmAO/lacZ* and *ecmB/lacZ* expression was examined in the chimeric slug, no staining was detectable (our unpublished observations). For *rasD/lacZ*, staining was significantly less intense compared with that seen in *ubcB*-null slugs. The stained cells were concentrated in the posterior of the slug, and no staining was detected in the anterior prestalk region (Figure 8B). When *SP60/lacZ* was examined, staining was seen in the prespore zone in migrating slugs (Figure 8C), but in second fingers at the onset of

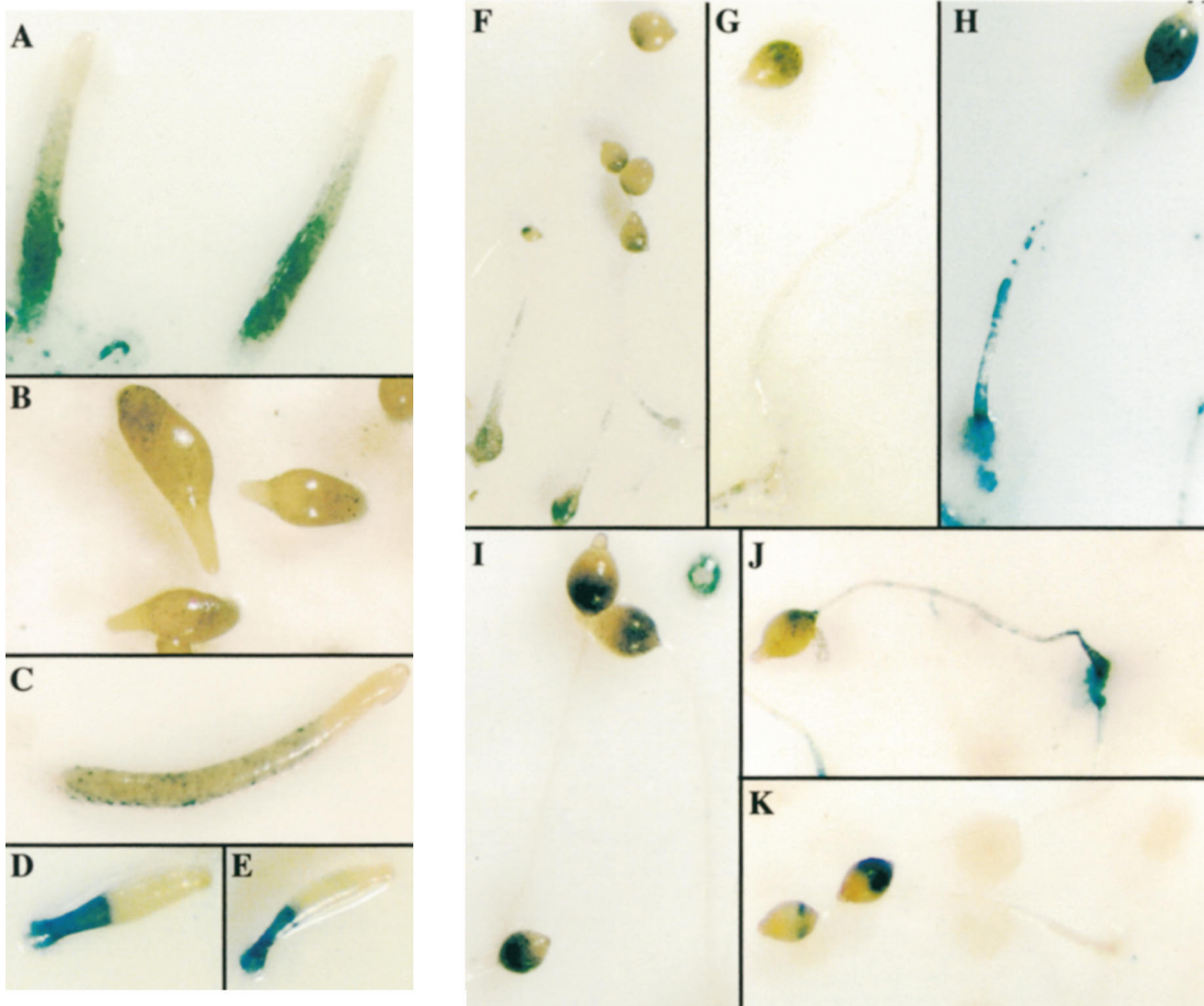


Figure 8. Localization of *ubcB*-null cells expressing *lacZ* reporters in chimeras of wild-type and *ubcB*-null cells (ratio of 3:1). Wild-type KAX-3 cells and *ubcB*-null cells expressing various reporters were mixed in a ratio of 3:1 and allowed to develop. Multicellular organisms were stained at the slug/finger stages (A–E) and culminants (F–K). *Act15*, panels A, D, E, and H; *rasD*, panels B and I; *SP60*, panels C and G. *ecmAO*, panel F (no staining is seen in the slug stage); *ecmB* Δ 89, panel J; *SpiA*, panel K.

culmination, staining was restricted to the posterior half of the prespore zone (our unpublished observations). The *Act15/lacZ* reporter driven by the Actin 15 (*Act15*) promoter is expressed in all cell types (Mann and Firtel, 1991). At the slug stage, *Act15/lacZ* staining was found predominantly in the posterior 50% of the slug with the level of staining, and thus the proportion of *ubcB*-null cells, decreasing toward the anterior of the slug (Figure 8A). During early culmination, staining was seen almost exclusively in the posterior 70% of the chimera, indicating *ubcB* cells were being excluded from the anterior part of the prespore and prestalk zones (Figure 8, D and E).

In the mature fruiting body the *ubcB* cells were, as determined from *Act15/lacZ* staining, found in the basal disk, lower portions of the stalk, and the lower cup and lower regions of the sorus, but were absent from the tip (Figure 8H). Much of the staining seen on the sorus represents surface staining of a layer that is probably an extension of the lower cup and derived from the ALC population. When the sorus was dissociated, only ~8% of the spores stained (our unpublished observations). In mature fruiting bodies, *ecmAO/lacZ* staining is observed in the basal disk, the lower portion of the stalk, and the lower cup, but no staining was observed in the upper cup and little staining was

seen in the upper portion of the stalk tube (Figure 8F). *rasD*, which displays a similar staining pattern in wild-type fruiting bodies to that of *ecmA*O, also showed a similar pattern in the *ubcB* chimeras as the *ecmA*O/*lacZ* reporter with no staining seen in the upper cup and tip, a strongly staining region in wild-type organisms (Figure 8I). *lacZ* expressed from the *ecmB*Δ89 stalk-cell-specific promoter (Ceccarelli *et al.*, 1991) had a similar pattern of expression: staining cells were more concentrated in the basal disk and lower stalk than throughout the stalk (Figure 8J). Some staining was also seen in the lower cup. *SP60/lacZ* and *lacZ* expressed from the spore cell-specific promoter *SpiA* (Richardson *et al.*, 1994) showed staining localized to the lower portion of the sorus in contrast to expression throughout the entire sorus, as seen in wild-type strains (Figure 8, G and K, respectively). The absence of *ubcB*-null cells in the upper cup or the upper part of the stalk tube may be due to the absence of *ubcB*-null cells in the anterior of the chimera.

To determine whether a smaller proportion of wild-type cells is able to direct the differentiation of *ubcB* nulls in chimeras, we mixed *ubcB*-null cells with wild-type cells carrying either the *ecmA*O/*lacZ* or *SP60/lacZ* reporter in ratios of either 3:1 or 9:1 (*ubcB*:wild type). As shown in Figure 9 (A and B, panels A-C), even a small proportion (10%) of wild-type cells is able to direct tip formation in a chimeric organism. However, most, if not all, of the wild-type cells appear to be localized in the very anterior of the organism as observed in the panels in which the wild-type cells are tagged with *Act15/lacZ*, which marks all wild-type cells (Figure 9, A and B, panel A). This is also observed when the wild-type prespore cells are marked by *SP60/lacZ*. As is seen in the panel showing the 3:1 ratio mix, the *SP60/lacZ* expressing cells are found as a band immediately posterior to the position of the wild-type prestalk cells identified by the *ecmA*O/*lacZ* marker (Figure 9A, panel E). No *ecmA*O/*lacZ* expressing cells are observed in the posterior bulk of the chimera, which also does not show significant staining with the *Act15/lacZ* reporter except at the very posterior of the organism. These results indicate that the wild-type cells are localized predominantly in the anterior of the chimera (Figure 9, A and B, panel A). When the *ubcB*-null cells were tagged with *Act15/lacZ*, no *ubcB*-null cells were observed in the anterior of slugs that contained the wild-type cells in either the 3:1 or 9:1 *ubcB*-null:wild-type chimeric organisms (our unpublished observations).

In the final structure (Figure 9, A and B, panels D-F), the wild-type cells appear to form a fruiting body with little participation of the *ubcB*-null cells and <1% of the mature spores derived from the *ubcB*-null strain. The patterns are very similar for 3:1 and 9:1 cell ratios with the patterning and morphology being more severe in the chimera containing only 10% wild-type

cells. A very small anterior structure is seen, which does not appear to contain the spore cells, as it is not labeled with the *SP60/lacZ* reporter. These may be ALC or prestalk cells that have not entered the stalk tube, as they stain strongly with *ecmA*O/*lacZ* (Figure 9, A and B, panels E). In contrast, in many of the mature organisms, a central swelling in the stalk appears to contain *SP60/lacZ*-expressing cells (Figure 9, A and B, panels F). These data are consistent with a model proposing that as the fraction of wild-type cells in the chimera decrease, the wild-type cells are unable to orchestrate the differentiation of the mutant cells, suggesting that the *ubcB*-null phenotype is predominantly cell autonomous.

DISCUSSION

UbcB Protein Is Required for Developmental Transitions during Multicellular Development

We have identified a developmentally essential gene, *UbcB*, that is required at two stages during multicellular differentiation. *ubcB*-null cells form mounds and remain at this stage for ~10 h before the mounds develop multiple tips and then finally arrest at the finger stage when plated on agar. When the cells are plated on Millipore filters, development proceeds slightly further, with a fraction of the fingers forming migrating slugs. In both cases, not all of the cells participate in finger and slug formation and under the conditions in which cells are plated on agar, most of the cells remain in the mound. The nature of these cells is unclear. They do not stain efficiently with *rasD/lacZ* or cell-type-specific *lacZ* reporters, suggesting that these cells arrest before or very early during cell-type differentiation. The observation that *ubcB*-null cells are able to form tips and fingers after an extended delay at the mound stage indicates that they are eventually able to bypass the initial block at the mound stage. The pathway required for tip formation may proceed, albeit very slowly, without the processes mediated by the *UbcB* protein, which we have designated UBC1. Alternately, another ubiquitin-conjugating enzyme may substitute for UBC1 function at a low efficiency at the mound stage during tip formation, but cannot substitute later in development after finger/slug formation. Low stringency hybridization suggests the presence of additional genes encoding UBCs in *Dictyostelium*, which would be consistent with published data showing that in organisms ranging from yeast to humans in which UBCs have been identified, multiple UBCs are found (see INTRODUCTION).

In wild-type cells, postaggregative genes such as those encoding CP2 and LagC proteins are maximally expressed at the mound stage, after which their expression decreases. The higher level and extended time of expres-

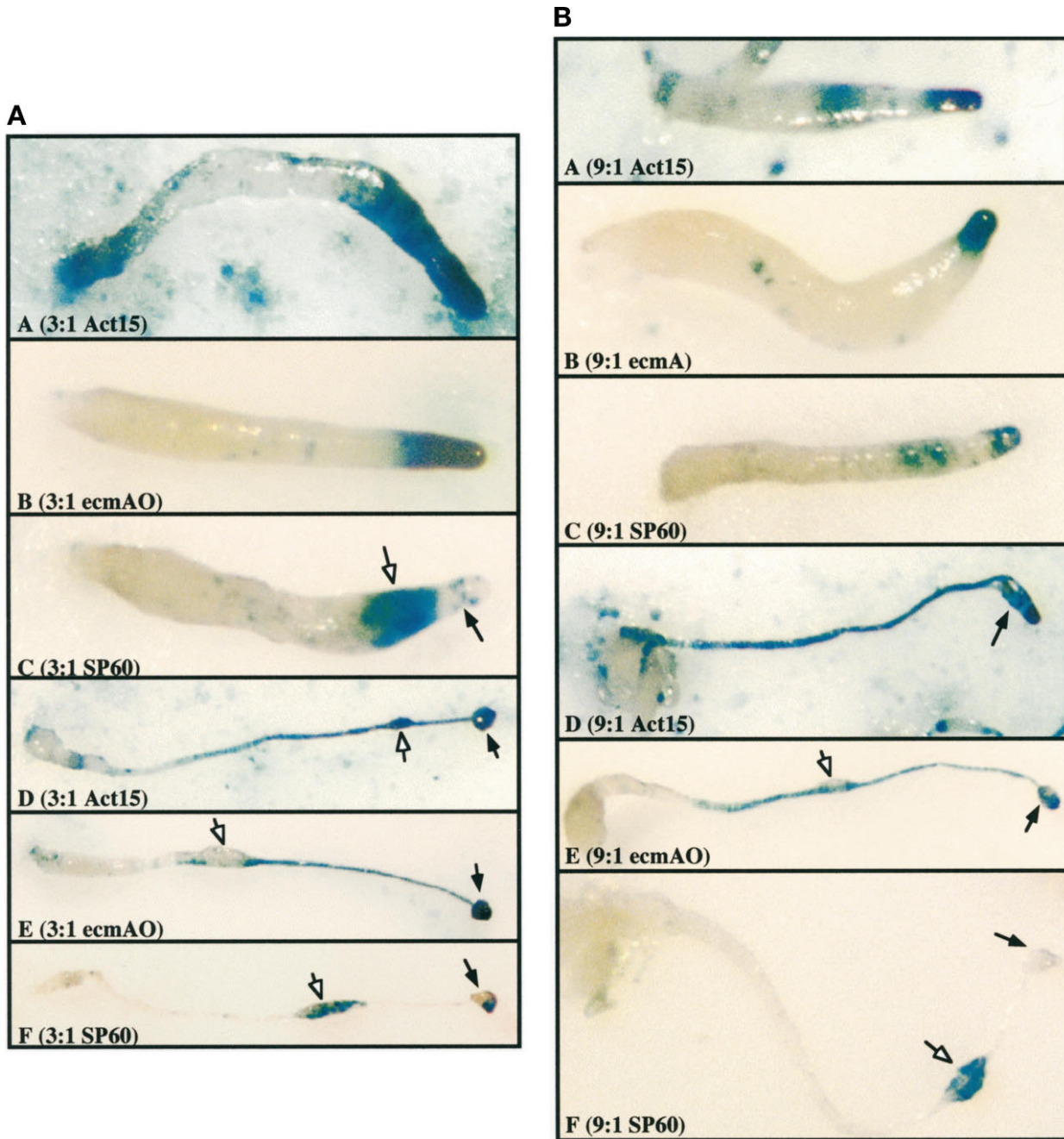


Figure 9. Localization of *ubcB*-null cells expressing *lacZ* reporters in chimeras of *ubcB*-null and wild-type cells at ratios of 3:1 and 9:1. (A) *ubcB*-null and wild-type cells a ratios of 3:1. Panel A, slug stage, *Act15/lacZ*; panel B, slug stage, *ecmAO/lacZ*; panel C, slug stage, *SP60/lacZ*; panel D, final structure, *Act15/lacZ*; panel E, final structure, *ecmAO/lacZ*; panel F, final structure, *SP60/lacZ*. (B) *ubcB*-null and wild-type cells at ratios of 9:1. Panel A, slug stage, *Act15/lacZ*; panel B, slug stage, *ecmAO/lacZ*; panel C, slug stage, *SP60/lacZ*; panel D, final structure, *Act15/lacZ*; panel E, final structure, *ecmAO/lacZ*; panel F, final structure, *SP60/lacZ*. In A and B, panels D-F, closed arrowheads point to enlarged region on stalk that does not stain with the prestalk/stalk marker (*ecmAO/lacZ*) but does stain with the prespore/spore marker (*SP60/lacZ*). The open arrowhead points to a small "sorus-like" structure. In A, panel C, the open arrowhead points to a wild-type prespore domain; the closed arrowhead points to a wild-type prestalk domain.

sion of these two genes in *ubcB*-null cells suggest that there is a regulatory mechanism in wild-type cells, leading to the down-regulation of both of these genes after

the mound stage and that this is absent in the *ubcB*-null cells. The delay of induction of prestalk and prespore cell-type-specific genes in *ubcB* cells suggests that the

developmental arrest at the mound stage occurs between the induction and down-regulation of the postaggregative genes and the induction of the cell-type-specific genes. *UbcB* function is essential for the developmental transition required for the down-regulation of postaggregative gene expression.

ubcB-null cells show normal spatial patterning of prespore cells in slugs, although the intensity of the staining is reduced, consistent with the lower level of expression of the endogenous *SP60/cotC* gene, indicating prespore gene expression is affected. The *ecmA*O/*lacZ* staining pattern also appears similar to that of wild-type cells; however, for *ecmB/lacZ*- and *rasD/lacZ*-expressing cells, the level of their expression is very high and/or an unusually high number of cells express these two reporters in comparison to what is observed in wild-type cells. The high level of staining with both *ecmB/lacZ* and *rasD/lacZ* suggests that the *ubcB*-null strain may have an unusually high proportion of ALCs. It is possible, as with *LagC* and *CP2/cprB*, that expression of *rasD* may continue to increase in the subset of *ubcB*-null cells that form the slug, resulting in a higher, more extended expression pattern. However, *rasD* expression is not induced in the loose mass of cells that remain at the base of the developing finger, and thus, the mechanisms by which *UbcB* protein controls spatial patterning are complex. The phenotypes observed in *ubcB*-null aggregates have not been previously described in other mutant strains.

Analysis of chimeric organisms containing varying ratios of wild-type and *ubcB*-null cells in which the two strains are marked with *lacZ* reporters indicates that when there is a high enough proportion of wild-type cells, a small fraction of *ubcB*-null cells are able to form spores and participate in stalk formation. However, it is clear that *ubcB*-null cells do not effectively participate in later morphogenesis, and in chimeras containing predominantly mutant cells, the null cells are found predominantly in the posterior/basal regions and do not participate in either stalk or spore formation. The results are consistent with the inability of these cells to efficiently undergo the developmental transition that occurs at the time of cell-type differentiation. While *ubcB*-null cells are unable to efficiently form a tip, this alone is not responsible for the *ubcB*-null phenotype; the addition of a small proportion of wild-type cells leads to tip formation in chimeras but this is unable to promote the proper development of the majority of the *ubcB*-null cells.

***ubcB*-Null Cells Show an Altered Pattern of Ubiquitin-containing Proteins**

The very high level of amino acid sequence identity between the *UbcB* ORF and UBCs from a variety of organisms strongly suggests that *UbcB* encodes a UBC, which we have named UBC1. Our finding that

UbcB is an essential gene for progression past the finger stage suggests that protein ubiquitination as an essential step in this process. This is consistent with our observations of an altered pattern of ubiquitinated proteins in *ubcB*-null cells compared with wild-type cells. At least three ubiquitinated proteins exhibit changes in their developmental pattern of staining with the antiubiquitin antibody on Western blots between wild-type and *ubcB*-null cells. One major ubiquitinated protein stains weakly in wild-type cells during early development, and then stains more intensely starting at the time of tip formation (12 h), the same time that the *ubcB*-null phenotype is first observed. In *ubcB*-null cells, this band shows a more constant staining starting at the preaggregation stage (4 h). Another protein stains strongly through 12 h and then disappears in wild-type cells. In *ubcB*-null cells, this band does not disappear, although the intensity of staining decreases. A third band exhibits a constant in wild-type cells from 4 h. In *ubcB*-null cells, this band is significantly weaker in *ubcB* cells and this intensity decreases even further starting at 12 h. It is of interest that the observed changes in the developmental pattern of these three proteins between wild-type and *ubcB*-null cells are different from each other. We do not know if the changes in staining represent changes in the level of ubiquitination, the rate of protein degradation, the kinetics of protein expression, or combinations of the three. Nor do we know whether either of these proteins are direct substrates of the *UbcB* protein UBC1, whether the putative protein ubiquitination directed by UBC1 targets that protein for degradation by the proteasome pathway, or whether the ubiquitination functions in some other way. The timing of the changes is consistent with UBP1 being required either directly or indirectly. Our data do not distinguish between changes in protein ubiquitination that are a direct function of UBP1 activity or pleiotrophic changes that are indirectly the result of the *ubcB*-null mutation. Nonetheless, these results indicate an effect of disruption of *UbcB* on protein ubiquitination that correlates temporally with the *ubcB*-null cell phenotype.

From the role of some UBCs in ubiquitin-mediated protein degradation, one possible explanation of the developmental phenotypes exhibited by the *ubcB*-null mutant is that the mutant cells are unable to degrade one or more proteins through the proteasome pathway whose turnover may be essential for development to proceed. This could either be the programmed degradation of an endogenous inhibitor of development that regulates tip formation or a protein whose degradation is required to activate a developmental pathway. Ubiquitination has also been linked to other cellular functions, including protein targeting and cell fate decisions. In the case of the yeast MDR transporter Pdr5, yeast α -factor receptor, and mammalian

growth hormone receptor, ubiquitination is involved in regulating turnover via targeting of the protein to the endosome (Egner and Kuchler, 1996; Hicke and Riezman, 1996; Strous *et al.*, 1996). In *Drosophila*, the *fat facets* gene, which encodes a deubiquitinating enzyme, is required for cell fate decisions in the eye (Jermyn and Williams, 1995), opening up the possibility that ubiquitination is involved in mediating a diverse set of cellular pathways.

At present, it is not known which ubiquitination-mediated pathway may be altered in *ubcB*-null cells, although the possibility that UBP1 may be involved in targeting specific proteins for degradation is consistent with previous results of mutations affecting a *Dictyostelium* homolog of the human Tat-binding protein, DdTBP α (Cao and Firtel, 1995). Members of the TBP family are also known to be components of the 26S proteasome involved in protein degradation. The *DdTBP α* gene appears to be essential for both vegetative growth and multicellular development. Disruption of one of the two copies of the gene results in developmental abnormalities that can first be observed at the finger stage. Expression of an antisense construct in the partial knockout results in additional morphologic abnormalities, while DdTBP α over-expression leads to a cytokinetic defect. These over-expressing cells also show abnormal morphogenesis during the multicellular stages, with development arresting at the finger stage. These results suggest that protein degradation through the 26S proteasome pathway(s) may be important for controlling specific stages during growth and development in *Dictyostelium*. Whether UBP1 and DdTBP α regulate the same developmental processes or whether either is involved in protein turnover is not known.

A variety of proteins in addition to UbcB have been shown to be required for cells to proceed normally past the mound stage, form a finger, and continue through morphogenesis. These include: the transcription factor GBF; LagC, a putative cell surface signaling molecule; TagB, a subunit of an MDR with a putative serine protease domain; and the cAMP receptor cAR2 (Saxe *et al.*, 1993; Dynes *et al.*, 1994; Schnitzler *et al.*, 1995; Shaulsky *et al.*, 1995). In addition, RasD has been implicated in tip formation (Reymond *et al.*, 1986). Cells lacking myosin II and cells in which cytosolic calcium has been depleted by expressing a constitutively active Ca²⁺ membrane pump also arrest or are delayed at this stage (Springer *et al.*, 1994; A.B. Cubitt, I. Reddy, J.G. McNally, and R.A. Firtel, unpublished data). The diversity of the pathways that might be affected by these gene products suggests that multiple regulatory pathways are required to control these transitions.

ACKNOWLEDGMENTS

We thank W. Loomis, A. Kuspa, and R. Hampton for helpful discussions during the process of this work. The *UbcB* cDNAs were sequenced by the *Dictyostelium* sequencing core supported by United States Public Health Service Grant PO1HD30892. This work was supported in part by United States Public Health Service grants to R.A.F.

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