Abstract. Genomic studies in yeast have revealed that one eighth of genes are cell cycle regulated in their expression. Almost without exception, the significance of cell cycle periodic gene expression has not been tested. Given that many such genes are critical to cellular morphogenesis, we wanted to examine the importance of periodic gene expression to this process. The expression profiles of two genes required for the axial pattern of cell division, BUD3 and BUD10/AXL2/SRO4, are strongly cell cycle regulated. BUD3 is expressed close to the onset of mitosis. BUD10 is expressed in late G1. Through promotor-swap experiments, the expression profile of each gene was altered and the consequences examined. We found that an S/G2 pulse of BUD3 expression controls the timing of Bud3p localization, but that this timing is not critical to Bud3p function. In contrast, a G1 pulse of BUD10 expression plays a direct role in Bud10p localization and function. Bud10p, a membrane protein, relies on the polarized secretory machinery specific to G1 to be delivered to its proper location. Such a secretion-based targeting mechanism for membrane proteins provides cells with flexibility in remodeling their architecture or evolving new forms.

Key words: cell cycle • localization • secretory pathway • morphogenesis • budding yeast

Introduction

Genomic expression studies in budding yeast have suggested that coordinated cell cycle gene expression is critical to a wide variety of cellular processes (Cho et al., 1998; Chu et al., 1998; Spellman et al., 1998). However, experimental data directly testing this view is for the most part lacking. Many genes, whose expression is regulated by the cell cycle, are required for cell morphogenesis and polarization during cell division by budding. The potential exists that ordered periodic gene expression in the cell cycle is critical to morphogenesis. Here, we test this view by manipulating the expression profiles of two morphogenetic genes, BUD3 and BUD10, both of which are involved in controlling spatial patterns of cell division.

Yeast cells become highly polarized and maintain their polarity as the bud emerges and grows. During this time, DNA is replicated and segregated. Once the bud has grown to roughly the size of its mother, cytokinesis occurs. Budding can occur in two spatial patterns. Haploid cells exhibit the axial budding pattern in which mother and daughter cells bud adjacent to their previous site of cell division (Freifelder, 1960; Hicks et al., 1977; Chant and Pringle, 1995). Diploid cells exhibit the bipolar budding pattern in which both mother and daughter bud at their poles with overlaying biases for one pole or the other (Freifelder, 1960; Hicks et al., 1977; Chant and Pringle, 1995).

The axial budding pattern has been shown to specifically require four genes: BUD3, BUD4, AXL1, and BUD10/AXL2 (Chant and Herskowitz, 1991; Fujita et al., 1994; Halme et al., 1996; Roemer et al., 1996). Deletion of any of these genes in haploids results in a loss of axial budding in favor of bipolar budding (Fujita et al., 1994; Chant et al., 1995; Halme et al., 1996; Roemer et al., 1996; Sanders and Herskowitz, 1996). Apart from Axl1p, whose localization has not been reported, all of these factors localize to the mother–bud neck and form a double ring structure encircling the neck just before cytokinesis (Chant et al., 1995; Halme et al., 1996; Roemer et al., 1996; Sanders and Herskowitz, 1996). At cytokinesis, these double rings are split to endow each progeny cell with a single ring marking the previous site of attachment. It is thought that these rings act as a spatial memory designating the site of mother–bud attachment as the location for axial budding in the next cell cycle (Chant et al., 1995; Halme et al., 1996; Roemer et al., 1996; Sanders and Herskowitz, 1996). A complementary set of genes (BUD7, BUD8, BUD9, RAX1, and RAX2) is required for maintenance of the bipolar budding pattern in diploids (Fujita et al., 1994; Zahnner et al., 1996; Chen et al., 2000). The products of these genes likely comprise, at least in part, the cell surface landmarks used by the cell for the bipolar pattern of division.
A third class of genes (BUD1/RSR1, BUD2, and BUD5) is required for both the axial and bipolar budding patterns (Bender and Pringle, 1989; Chant and Herskowitz, 1991; Chant et al., 1991). The products of these genes, a Ras-like GTPase and its regulators, are thought to act on both axial and bipolar markers, coupling them with cellular components involved in generating an axis of polarity towards the incipient bud site (Chant and Herskowitz, 1991).

The timings of Bud3p and Bud10p localization to the mother–bud neck are not the same. Bud3p, a non-membrane protein, becomes detectable at the onset of mitosis, whereupon it localizes to the mother–bud neck (Chant et al., 1995). Bud10p, a membrane protein, becomes detectable at the onset of mitosis, whereupon it localizes to the mother–bud neck (Chant and Herskowitz, 1991). Bud10p localizes to the incipient bud site in G1 phase and then remains in the mother–bud neck as the bud grows outward (Halme et al., 1996; Roemer et al., 1996). Just before cytokinesis, both proteins localize as a double ring structure encircling the mother–bud neck (Chant et al., 1995; Halme et al., 1996; Roemer et al., 1996). Neither Bud3p nor Bud10p requires the other for localization to the mother–bud neck, though conversion of Bud10p localization from a loose concentration to tight double rings requires Bud3p (Halme et al., 1996).

We and others (Cho et al., 1998; Spellman et al., 1998) found that the expression profiles of BUD3 and BUD10 are cell cycle specific. To investigate the importance of periodic gene expression, the expression profiles of BUD3 and BUD10 were altered by promoter-swap experiments.

**Materials and Methods**

**Strains, Plasmids, Growth Conditions, and Genetic Methods**

Yeast strains and plasmids are described in Tables I and II. Standard yeast genetic procedures and media (Rose et al., 1990) were used, unless specified. For producing a bud3 deletion, plasmid pJC15 (Chant et al., 1995) carrying the bud3 deletion was linearized with BamHI and EcoRI and transformed into strain JC1030. Ura+ transformants that exhibited the bipolar pattern were isolated.

**Plasmid Construction**

pJC16 (prom4CSM-BUD10). An EcoRI/BamHI GALI promoter fragment was liberated from pRS316-GAL (E. Bi, University of Pennsylvania Medical School, Philadelphia, PA). An isolate of BUD3 in YCp50, p35-1 (Chant et al., 1995), was digested with BamHI and Sall to liberate the BUD3 region. The linearized YCp50 was then digested with EcoRI. The GALI promoter fragment and the BUD3 fragment were then double ligated into the EcoRI/SalI YCp50 to yield BUD3 under the control of the GALI promoter.

**Overexpression of BUD3**

The BUD3-overexpression construct (pJC16) and YCp50 (control vector) were transformed into EJY301. Ura+ colonies carrying the plasmids were selected. Each transformant was grown overnight in Ura- glucose complete synthetic medium (CSM). The cultures were divided into two samples, harvested, washed twice, and resuspended in either Ura- glucose or Ura- galactose CSM for 24 h. Morphological analysis of cells was performed by counting normally dividing cells versus cells producing elongated buds. For each sample, 600 cells were scored. Visualization of Cdc3-HA in all samples was performed by immunofluorescence, as described below.
Preparation of RNA Samples from Synchronized Cell Cultures

JC1362 (containing wild-type copies of BUD3 and BUD10) in rich medium, JC2123 carrying pJC117 (promoter-containing, p35-1) in LEU2- containing vector with the ADH promoter upstream of a single HA sequence, JC2133 carrying pJC256 (promoter-controlled BUD3) in Trp− CSM, and JC2133 carrying pJC1869 (promoter-controlled BUD3) in Trp− Met− CSM were grown up overnight in 500-ml cultures at 25°C. When cultures reached an optical density at 600 nm (OD600) of ~0.15, they were harvested and resuspended in 500 ml of their respective growth media that had been prewarmed to 37°C. cdc15-2based arrest was attained by incubation of cultures at 37°C for 4.5 h. Arrest was confirmed microscopically and cells were chilled on ice for 5–10 min before resumption of growth at 25°C (0 min after release from arrest). Samples (25 ml) were harvested every 15 min for JC1362 or 30 min (for all others), frozen in liquid nitrogen, and stored at −80°C at time points from 0–240 min after arrest. These 25-ml samples were used for subsequent RNA sample preparation. The Hot Phenol Method of total RNA preparation (Koher and Domdey, 1991) was used. In addition, at every time point, 1 ml of culture was fixed in 4% formaldehyde for 30 min at 30°C. These samples were used to determine the budding index (the number of budded cells versus unbudded cells). For the initial portions of the synchronizations (60 min for Figs. 1, 3, and 4, and 90 min for Figs. 2 and 6) a modification of this basic method was used. Cells were diluted to an OD600 of 0.3–0.5, cells were fixed in 4% formaldehyde at 30°C for 30–60 min and washed three times in PBS. Indirect immunofluorescence was performed as described by Pringle et al. (1991). A mouse anti-HA epitope monoclonal antibody (Jackson ImmunoResearch Laboratories) or rabbit anti-Bud3p antibody (Chant et al., 1995) was used to visualize the two proteins. The secondary antibodies used were CY3-conjugated goat anti-mouse IgG and FITC-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories). Microtubule staining was performed as described in Chant et al. (1995). Budding patterns were scored by staining bud scars with Calcofluor and observing with fluorescence microscopy (Pringle, 1991); 200–300 cells were scored for each set of counts. Fluorescence microscopy was performed using a Nikon Microphot SA microscope with a 63× Plan-apo objective.

Northern Blotting

Standard methods were employed (Sambrook et al., 1989) with the following modifications. 5 µl of RNA samples were mixed with 10 µl of RNA loading buffer (Ambion) and run on 1% agarose-MOPS gels containing 6% formaldehyde. Running buffer was 1× MOPS. Gels were washed five times in 0.1% diethyl pyrocarbonate-treated water then by a 2× equilibration in 20× SSC. RNA was subjected to capillary transfer overnight onto Zeta Probe (Bio-Rad Laboratories) nylon membranes. Membranes were washed for 5 min in 6× SSC and dried at room temperature for 30 min on paper towels before baking them in a vacuum oven at 80°C for 1.5 h. Membranes were prehybridized for 1 h in UltraHyb (Ambion) at 55°C then hybridized overnight at 37°C with the appropriate radioactive labeled probes. 0.5–1.5 kb of BUD3, BUD10, LEU2, or ACT1 were PCR amplified, gel purified, and used as templates for the manufacture of probes through the use of the “Prime-a Gene” Labeling System (Promega). After hybridization, membranes were washed twice at 55°C for 15 min in 2× SSC,0.1% SDS, and then by two washes for 30 min in 0.1× SSC,0.1% SDS. Blots were exposed to a BAS-III Imaging Plate (Fuji) for appropriate times. The Imaging Plate was processed by a Fujix BAS 2000 Imager (Fuji). Images were analyzed by MacBAS V2.5 (Fuji). Blots were stripped for reprobing by washing three times for 20 min in 0.1× SSC,0.5% SDS at 95°C.

Immunofluorescence and Calcofluor Staining

JC1997 carrying pJC117 (promoter-controlled BUD3) was grown overnight in Leu− Met− CSM. JC1296 carrying pJC246 (promoter-controlled BUD10) or pJC256 (promoter-controlled BUD10) was grown in Trp− CSM overnight. JC1296 carrying pJC1869 (promoter-controlled BUD10) was grown in Trp− Met− CSM overnight. At an OD600 of 0.3–0.5, cells were fixed in 4% formaldehyde at 30°C for 30–60 min and washed three times in PBS. Indirect immunofluorescence was performed as described by Pringle et al. (1991). A mouse anti-HA epitope monoclonal antibody (Jackson ImmunoResearch Laboratories) or rabbit anti-Bud3p antibody (Chant et al., 1995) was used to visualize the two proteins. The secondary antibodies used were CY3-conjugated goat anti-mouse IgG and FITC-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories). Microtubule staining was performed as described in Chant et al. (1995). Budding patterns were scored by staining bud scars with Calcofluor and observing with fluorescence microscopy (Pringle, 1991); 200–300 cells were scored for each set of counts. Fluorescence microscopy was performed using a Nikon Microphot SA microscope with a 63× Plan-apo objective.

![Figure 1. Cell cycle expression profiles of BUD3 and BUD10.](image)

A. Northern blot of BUD3 and BUD10 expression during the cell cycle of wild-type cells (JC1362). Synchronization of the cell cycle was achieved through use of a cdc15-2ts background (Benton et al., 1997). LEU2 is a cell cycle–constitutive control. (B) Cell cycle stages of the synchronized culture defined by the budding index (scored by counting unbudded versus budded cells; see Materials and Methods for details). SE denotes the initiation of spindle elongation. (C) Cell cycle stages of the synchronized culture were scored by microtubule morphologies.
Western Blot Analysis

15 OD600 units of cells were harvested and washed with distilled water. Cells were resuspended in 150 µl lysis buffer (1% SDS, 2 mM PMSF plus protease inhibitors) and lysed with glass beads by vortexing on ice for a total of 6 min. 100 µl of lysis buffer was added to the extracts that were then centrifuged at 500 g for 2 min. Lysates were decanted and stored at -80°C if they were not required immediately. The protein concentrations of the lysates were determined using Pierce Coomassie Plus protein reagent (Pierce Chemical Co.). Equal protein levels from each lysate were loaded onto an SDS-PAGE gel and immunoblotted by standard methods (Sambrook et al., 1989). Mouse anti-HA epitope monoclonal primary antibodies (Jackson ImmunoResearch Laboratories) were used at a dilution of 1:500. Secondary antibodies were goat anti–mouse antibodies conjugated to horseradish peroxidase (Sigma–Aldrich) used at a dilution of 1:2,500. Blots were developed using the ECL Western blotting detection system (Amersham Pharmacia Biotech). Autoradiographs were scanned and the protein bands were quantitated using the MacBAS V2.5 program.

Pulsed Expression Experiments

Unsynchronized Cells. JC1296 carrying pJC1869 (promMET3-BUD10) was grown in repressing conditions (4 mM methionine, Trp+ CSM) overnight. At an OD600 of ~0.25, cells were spun down and washed three times in inducing medium (Trp+ Met+ CSM), and followed by a 30 min incubation in the same medium. Induction of BUD10 expression was terminated by the addition of 4 mM methionine. Samples were harvested and fixed in formaldehyde (as described above) at 0, 60, and 120 min after cells were removed from inducing conditions. All samples were washed three times in PBS. Samples were subjected to analysis by immunofluorescence, as described above. Typically, 100 cells were scored per sample.

Synchronized Cells. JC2133 carrying pJC1869 (promMET3-BUD10) was grown up at 25°C in repressing conditions (4 mM methionine, Trp+ CSM) overnight. Cell synchronization was achieved as described earlier using the cdc15-2ts mutation. The synchronized culture was divided in two: one half for late G1 induction and the other half for S/G2 induction. All subsequent incubations were performed at 25°C. 35 min after release from cell cycle arrest, late G1 induction was performed by the following method: cells were washed three times in inducing medium (Trp+ Met+ CSM) and incubated 45 min in the same medium. Induction was terminated by addition of 4 mM methionine and an additional brief incubation. At 90 min after release from arrest, cell samples were taken and fixed (as performed above). Cells were washed three times in PBS and subjected to analysis by immunofluorescence. S/G2 induction was performed in identical fashion, but 110 min after release from cell cycle arrest.

Results

BUD3 and BUD10 Expression Are Cell Cycle Regulated

Microarray experiments indicated that the expression profiles of the axial budding–specific genes, BUD3 and BUD10, are tightly cell cycle regulated, being expressed in S/G2 and late G1, respectively (Cho et al., 1998; Spellman et al., 1998). We confirmed this observation by Northern
blot analysis. BUD3 mRNA levels peaked after bud emergence at the S/G2 phase of the cell cycle (Fig. 1, A and B). BUD10 mRNA levels peaked at the start of the cell cycle just before bud emergence in late G1 phase (Fig. 1, A and B). Both budding index (Fig. 1 B) and an assessment of microtubule morphology (Fig. 1 C) confirmed that this method of synchronization was effective.

Constitutive Expression of BUD3 Does Not Affect Its Localization or Function

The importance of a pulse of BUD3 expression at the S/G2 phase of the cell cycle was tested by expressing BUD3 from the MET3 promoter under steady state inducing conditions (methionine-deficient medium). Northern blot analysis of BUD3 confirmed that BUD3 expression was now uniform throughout the cell cycle (Fig. 2 A). Immunofluorescence was employed to analyze Bud3p localization (Fig. 2 B). Bud3p was able to localize to the mother–bud neck as observed in the wild-type situation. However, the normal temporal regulation of Bud3p localization was lost. In wild-type cells, Bud3p localizes to the mother–bud neck coincident with the onset of mitosis (Fig. 2 B, cells 1–4). In the constitutive strain, Bud3p localized to the mother–bud neck at all phases of the cell cycle (Fig. 2 B, cells 5–8). Premature Bud3p localization had little consequence on Bud3p function: constitutive expression of BUD3 under the MET3 promoter complemented a bud3 null mutation for the axial pattern (Fig. 2 C). Thus, the periodic expression of BUD3 during the cell cycle is the basis for the tight temporal control of Bud3p localization, but this control is not critical for Bud3p function.

Possible Morphogenetic Consequences of BUD3 Misexpression

To further test the consequence of altered BUD3 expression, we overexpressed BUD3 throughout the cell cycle by using a GAL1 promoter–BUD3 construct. Galactose-induced overexpression of BUD3 resulted in a cell division defect in 42% of cells (Fig. 2 D): cells failed to divide correctly and displayed large, elongated buds, a defect similar to that of mutants defective in the septins (Longtine et al., 1996). Immunofluorescence was employed to analyze the consequences of BUD3 overexpression on the localization of the septin Cdc3p. In glucose medium, cells exhibited wild-type Cdc3p localization as rings present at division sites (Fig. 2 D). In galactose medium, many cells displayed aberrant Cdc3p localization (Fig. 2 D), which was visible as multiple rings found throughout elongated buds or as a patch at the tips of elongated buds. Upon closer examination of the strain carrying the prom<sup>MET3</sup>-BUD3 construct,
we were able to observe a similar morphological defect in a minority of cells (3 versus 0% in wild-type cells). Thus, inappropriate expression of BUD3 can have deleterious consequences (see Discussion).

Constitutive or Delayed Periodic Expression Affects Bud10p Localization and Function

The importance of temporally regulated BUD10 expression in late G1 phase was tested by two perturbations: expression from the constitutive MET3 promoter and expression from the periodic BUD3 promoter. BUD10 expression under the MET3 promoter appeared to occur uniformly throughout the cell cycle, barring an initial lag at 0 and 30 min after release from arrest (Fig. 3 A). The initial low levels of BUD10 expression, which quickly recovered, were likely a consequence of cells exiting from the cdc15-2-ts-based arrest. Immunofluorescence was undertaken to investigate whether constitutive expression of BUD10 affected Bud10p localization. Constitutive BUD10 expression resulted in uniform distribution of Bud10p throughout the plasma membrane at all points in the cell cycle (Fig. 3 B). In rare instances, Bud10p was found somewhat concentrated in small buds (Fig. 3 B, 8 and 9). This effect can be accounted for if we consider the fact that during constitutive expression, some BUD10 is still expressed at the normal time at late G1. Expression at this point in the cell cycle apparently allows Bud10p to occasionally concentrate at small buds. The uniform localization of Bud10p resulting from constitutive expression affected the axial pattern of budding (Fig. 3 C). First bud-scar analysis revealed a small reduction in the number of cells budding at the proximal pole (80 versus 86% in wild-type cells) (Fig. 3 C). Four bud-scar analysis portrayed a larger effect: only 45% of cells budded in an axial manner as compared with 78% in the wild-type control (Fig. 3 C). The levels of Bud10p in wild-type cells and cells expressing BUD10 from the MET3 promoter were compared by Western blot analysis: approximately sevenfold more Bud10p was produced via the constitutive MET3 promoter than via the native promoter in wild-type cells (results not shown). However, it seemed unlikely that the defects in the axial budding pattern apparent in cells expressing BUD10 constitutively were due to excessive levels of Bud10p causing interference: expression of BUD10 from the MET3 promoter plasmid was recessive in a wild-type haploid (JC1293) background (results not shown).

In a second set of experiments, BUD10 expression was delayed until S/G2 by placing BUD10 under control of the BUD3 promoter. The pulsatile nature of BUD10 expression was preserved, but its timing was altered. Northern blot analysis confirmed that expression of BUD10 from the BUD3 promoter occurred in a pulse that was delayed to the S/G2 phase of the cell cycle (Fig. 4 A). Just as with constitutive expression, delayed expression resulted in a uniform distribution of Bud10p throughout the plasma membrane (Fig. 4 B). Expression of BUD10 under the BUD3 promoter in haploids resulted in a similar defect in the axial pattern as observed when BUD10 was placed under the MET3 promoter. Only 57% of cells exhibited proximal bud-site selection (versus 86% of wildtype haploids) (Fig. 3 C) on the basis of first bud-scar analysis (Fig.
On four bud-scar analysis, only 54% of cells budded in an axial manner as compared with 78% in the wild-type control (Figs. 4 C and 3 C). As observed above for the prom\text{MET3}\text{BUD10} construct the prom\text{BUD3}\text{BUD10} construct was recessive in its effects (results not shown). To eliminate the possibility that a significantly altered level of Bud10p expression from the \text{BUD3} promoter was the basis for the defects in its localization, Western blot analysis was performed. As shown in Fig. 4 D, expression of \text{BUD10} under the \text{BUD3} promoter resulted in levels of Bud10p very similar (72–81%) to the levels apparent in wild-type cells. The possibility remained that reduction in Bud10p levels generated the defects reported above. Therefore, we examined the budding pattern of a heterozygous \(a/\alpha\) \text{BUD10/bud10} strain (JC2312), which behaves as an \(\alpha\) cell. This strain, which in principle expresses 50% the normal levels of Bud10p, exhibited a wild-type axial budding pattern identical to that of the wild-type control, JC2313 (results not shown). Therefore, the quantity of Bud10p produced from the \text{BUD3} promoter appeared sufficient for carrying out its function. Based on these two promoter-swap experiments, we conclude that a \text{G1} pulse of \text{BUD10} expression is critical to Bud10p localization and function.

**Pulsed Expression of BUD10 in Late G1 Phase Restores Correct Localization of Bud10p**

The experiments above suggest that a cell cycle–specific temporal pulse of \text{BUD10} expression is critical to Bud10p localization. According to this view, it should be possible to restore Bud10p localization by artificially producing a pulse of \text{BUD10} expression in \text{G1}. Through use of the construct in which \text{BUD10} was under the control of the \text{MET3} promoter, pulsed \text{BUD10} expression could be produced by transferring cells grown in medium with methionine (repressing) to a brief incubation in methionine-deficient medium (inducing). Optimization indicated that a pulse of 30 min in methionine-deficient medium was sufficient to stimulate detectable Bud10p in 20–25% of cells (results not shown). 30 min is a relatively short pulse as the cell cycle time is \(\sim 150\) min in this medium at 30°C. Induction experiments were performed on unsynchronized or synchronized cell cultures.

The unsynchronized experiment was performed as follows. A log phase cell culture was induced for 30 min of \text{BUD10} expression. Samples were taken immediately, 60 and 120 min after induction was completed. Most of the cells analyzed at the three different time points exhibited uniform localization of Bud10p, rather than the tight bud site and mother–bud neck localizations seen in wild-type cells (Fig. 5, A and C, cells 5–8, all three time points). Considering the previous set of experiments, mislocalization in most cells was presumably due to expression having occurred at the wrong time in the cell cycle, i.e., at a time other than late \text{G1} phase. A fraction of cells (20–26%) did display correct localization of Bud10p. Localization was scored as correct if it appeared as expected for a wild-type cell at the corresponding phase of the cell cycle. Correct Bud10p localization was presumably a result of a pulse of expression having occurred in those cells traversing \text{G1} when induction occurred (Fig. 5, A and C, cells 1–4 at 0, 60, and 120 min). Correctly localized Bud10p signal was examined at 0, 60, and 120 min after induction and divided into five subcategories based on the cell cycle stage at

![Figure 5](image-url)
which the correctly localized signal was seen (Fig. 5 B). A correlation was apparent between the time after induction and the point in the cell cycle at which Bud10p was observed to be correctly localized. At 0 min after induction, correctly localized Bud10p signal was mostly concentrated at incipient bud sites or in small buds (Fig. 5, B and C, cells 1–4); 60 min after induction, a majority of the correctly localized signal was evident at the bases of medium-sized buds (Fig. 5, B and C, cells 1–4); 60 min after induction, most of the correctly localized Bud10p signal was seen as

double rings in large budded cells or as single rings in newly divided cells (Fig. 5, B and C, cells 1–4). This correlation is consistent with the interpretation that a pulse of BUD10 expression early in the cell cycle, just before bud emergence, established localized Bud10p, which was then maintained for the duration of the cell cycle.

Experiments were performed on synchronized cell cultures to test directly whether the appearance of a correctly localized signal was dependent on the cell cycle phase at which the pulse occurred, as suggested by the above experiments. Two inductions of BUD10 expression were carried out: one pulse centered on late G1 and a second centered on S/G2 phase (Fig. 6 A). Of cells exhibiting a detectable Bud10p signal in the G1 induction experiment, a majority (65%) displayed correctly localized Bud10p with concentrations at the incipient bud sites of unbudded cells or at the small buds of newly budded cells (Fig. 6 B, cells 2 and 3). Some mislocalized signal (13%) was apparent after G1-centered induction; however, this signal was mostly found in medium-sized budded cells, apparently a result of imperfect synchrony (Fig. 6 B, cell 4). The remainder of the cells (22%) exhibiting Bud10p signal in intracellular compartments likely reflected the transit of Bud10p through the secretory pathway (Fig. 6 B, cell 1).

On induction of BUD10 at the S/G2 phase, Bud10p was mislocalized throughout the plasma membrane of most cells (63%). In some cases Bud10p was seen concentrated at the tips of medium-sized buds (Fig. 6 C, cells 3 and 4). Bud tip localization was never seen in wild-type cells, suggesting that pulsed gene expression can create a novel localization pattern. A fraction of cells (25%) displayed a correctly localized Bud10p signal, but these cells were small budded, a result of imperfect synchrony. (Fig. 6 C, cell 2). This proportion (25%) corresponded very closely with the proportion of cells out of synchrony in this induction experiment (Fig. 6 A). 12% of cells exhibited Bud10p signal in intracellular compartments (Fig. 6 C, cell 1), just as observed above.

The results obtained from experiments performed on unsynchronized and synchronized cells demonstrate that correct localization of Bud10p can be restored in cells expressing BUD10 under the MET3 promoter. This correct localization is dependent on a pulse of BUD10 expression taking place in late G1 phase.

**Discussion**

Genome-wide expression studies in yeast have demonstrated that the expression of a large fraction of genes is cell cycle regulated (Cho et al., 1998; Spellman et al., 1998). We sought to investigate the role of cell cycle–regulated gene expression in morphogenesis. Our study focused on BUD3 and BUD10, two cell cycle–regulated genes involved in maintenance of the axial budding pattern in yeast. The phenotypes of these two genes and the subcellular localizations of their products have been well documented (Chant et al., 1995; Halme et al., 1996; Roemer et al., 1996). As such, BUD3 and BUD10 provide ideal candidates for cell cycle expression studies seeking to understand the importance of periodic expression to function.
Cell Cycle Regulation of BUD3 and BUD10 Transcription

Our observations argue strongly that regulated expression of BUD3 and BUD10 mRNAs is controlled principally at the level of transcription. This view was first suggested by analysis of the upstream sequences of BUD3 and BUD10, which revealed elements predicted to be common promoter recognition sites for cell cycle-specific transcription factors. BUD3 has been classed with a small group of genes expressed at G2, all of which possess a common upstream sequence speculated to mediate cell cycle regulated transcription (Spellman et al., 1998). The upstream sequence of BUD10 contains three variant “Swi4/6-cell cycle-box” promoter elements, as well as one consensus and two variant “MluI-cell cycle-box” elements. Both elements are associated with genes expressed in late G1 (Koch and Nasmyth, 1994; Cho et al., 1998; Spellman et al., 1998).

The promoter-swap experiments confirmed experimentally that transcription is largely, if not entirely, responsible for the cell cycle periodicity of BUD3 and BUD10 mRNA expression. When placed under the control of the MET3 promoter, the expression of both genes became cell cycle constitutive. Perhaps most convincing was the observation that placement of BUD10 under the control of the BUD3 promoter caused a pulse of BUD10 mRNA expression to occur in S/G2, the period during which BUD3 mRNA is normally expressed. These results confirmed that the regulation of BUD3 and BUD10 mRNA levels was dependent on cell cycle-specific transcription with the degradation of BUD3 and BUD10 mRNAs likely occurring constitutively throughout the cell cycle.

Regulated BUD3 Expression Determines the Timing of Bud3p Localization

Replacement of periodic BUD3 expression in favor of uniform expression did not affect the function of Bud3p. Bud3p supported the axial budding pattern and localized to its correct position at the mother–bud neck. The only observed change was that Bud3p localized prematurely. We conclude that the temporal regulation of Bud3p localization to the mother–bud neck is driven by a rise in BUD3 mRNA abundance and the corresponding rise in protein levels. It is known that Bud3p localization at the mother–bud neck depends upon the septin ring, which assembles at the incipient bud site and remains encircling the mother–bud neck for the remainder of the cell cycle (Byers and Goetsch, 1976; Haarer and Pringle, 1987; Ford and Pringle, 1991; Kim et al., 1991; Chant et al., 1995; Carroll et al., 1998; Mino et al., 1998). Therefore, it is not surprising that Bud3p is able to localize prematurely, since the septin ring is always present. These results are entirely consistent with a simple mechanism for Bud3p localization relying on direct, or indirect, affinity for the septin ring (Fig. 7). Accordingly, the temporal regulation of Bud3p localization observed in wild-type cells reflects the presence of Bud3p protein.

If Bud3p localizes normally to the mother–bud neck and supports axial budding when expressed constitutively, why is BUD3 expression so tightly temporally regulated? One possibility is that S/G2-specific expression of BUD3 prevents deleterious interactions with other factors. Overexpression of BUD3 under the strong GAL1 promoter led to cytokinesis defects in >40% of cells, defects reminiscent of a septin mutant phenotype. Interestingly, BUD3 overexpression resulted in septin mislocalization as measured by the aberrant distribution of Cdc3p. Perhaps expression of BUD3 during G1 permits Bud3p to interact with the septins during the time at which they are still assembling into the septin ring structure. Premature interaction could interfere with septin assembly. Similar but milder defects were observed when BUD3 was expressed from the MET3 promoter. Under normal expression conditions, such an effect, even minor, could confer a considerable selective disadvantage on cells.

Figure 7. Proposed localization mechanisms of Bud3p and Bud10p. Cell cycle expression of BUD3 leads to the production of cytoplasmic Bud3p (red), which localizes by diffusion and docking at the septin ring. BUD10 is expressed in G1 at which time secretion is tightly focused on the nascent bud site. Bud10p (green) is delivered to the bud site and remains for the duration of the cell cycle in this position, which becomes the mother–bud neck.
Cell Cycle Regulated BUD10 Expression Directs the Localization of Its Product

Constitutive BUD10 expression from the MET3 promoter and delayed periodic expression from the BUD3 promoter both affected Bud10p function. Cells displayed a reduction in the ability to direct the axial budding pattern, and Bud10p was not properly localized. In wild-type cells, Bud10p concentrates to the incipient bud site, in the mother–bud neck, and at the division sites of newly divided cells. Both alterations led to uniform distribution of Bud10p throughout the plasma membrane. Taken together, these results imply that a cell cycle–specific pulse of BUD10 expression in late G1 is critical for Bud10p localization and subsequent function. To examine this hypothesis further, we artificially induced pulses of BUD10 expression. An artificial pulse of BUD10 expression around late G1 restored correct localization of Bud10p, whereas pulses of expression outside this time led to mislocalization of Bud10p throughout the plasma membrane.

Why is a pulse of BUD10 expression in late G1 so critical to its localization? The most economical explanation for our observations is that establishment of the Bud10p localization pattern relies on the timing of its passage through the secretory pathway (Fig. 7). A pulse of Bud10p production in late G1 allows delivery of Bud10p via the secretory pathway during exactly the period when secretion is very tightly focused on the future bud site (Kilmartin and Adams, 1984; Lew and Reed, 1993). Once Bud10p is delivered to this location, the affinity of Bud10p for cell wall components or other bud-site factors retains Bud10p in this location for the duration of the cell cycle. As the bud grows outward, the position of the bud site becomes the mother–bud neck.

Three lines of evidence support this timed delivery hypothesis. First, all perturbations that caused BUD10 expression outside of the late G1 window resulted in Bud10p mislocalization. Such observations make sense since during other phases of the cell cycle secretion is not directed to the bud site or the mother–bud neck (Lew and Reed, 1993). Second, correct localization of Bud10p was restored by creating an artificial pulse of BUD10 expression in G1. Third, if the initial localization of Bud10p is dictated by the pattern of cell surface growth at the instant of BUD10 expression, novel patterns of Bud10p localization should be produced by expression of BUD10 in other windows of the cell cycle. This prediction was fulfilled: in the S/G2 centered pulse experiment, some cells exhibited a patch of Bud10p protein tightly localized to the bud tip (Fig. 6 C, cells 3 and 4). Such a localization pattern is presumably produced by expression of BUD10 in a short window of the cell cycle when cell surface growth is directed to the bud tip. In addition to this evidence, it is well established that Bud10p is delivered to the cell surface via the secretory pathway (Halme et al., 1996; Roemer et al., 1996; Powers and Barlowe, 1998; Sanders et al., 1999).

Our hypothesis does not explain one aspect of Bud10p localization, the conversion of a loose concentration of Bud10p in the mother–bud neck to a tight double ring structure toward the end of the cell cycle. How this remodeling occurs remains unknown, though it likely involves Bud3p (Halme et al., 1996).

Despite the fact that altered timing of BUD10 expression resulted in uniform membrane localization of Bud10p, half of the cells were able to maintain an axial pattern of budding. These results suggest the possibility that an interaction with a ligand found in the vicinity of the mother–bud junction allows Bud10p to be more active in generating a signal than the Bud10p in other locations. We support the notion that at least two levels of control produce the high degree of spatial specificity in Bud10p signaling: high local concentration, resulting from pulsed expression in combination with secretory targeting, and local activation of Bud10p, possibly through interaction with a ligand.

Do Pulses of Cell Cycle Expression Direct the Localization of Other Morphogenetic Factors?

The expression of several other genes involved in cellular morphogenesis are cell cycle regulated, including BUD4, BUD8, BUD9, and RAX2. Bud4p is a nonmembrane protein that acts in similar fashion to Bud3p. BUD4 expression occurs at M phase (Cho et al., 1998; Spellman et al., 1998), and Bud4p forms rings encircling the mother–bud neck in a septin-dependent manner (Sanders and Herskowitz, 1996). Interestingly, the BUD4 promoter shares sequence similarity to those of BUD3 and CLB2 (Sanders and Herskowitz, 1996), and all three promoters contain a putative Fkh1p-binding site (Zhu et al., 2000). We consider it likely that the pulse of BUD4 expression controls the timing of its localization to the mother–bud neck, as observed for Bud3p.

Three genes required for the bipolar budding pattern, BUD8, BUD9, and RAX2 (Zahner et al., 1996; Chen et al., 2000) are also expressed periodically in the cell cycle (Cho et al., 1998; Spellman et al., 1998). Like Bud10p, the products of BUD8, BUD9, and RAX2 are membrane proteins; therefore, timing of expression could play a large part in directing the localizations of these morphogenetic marker proteins as well. Finally, a very recent report suggests that the localization of Crh1p, a secreted cell wall protein, is determined by the cell cycle timing of CRH1 expression (Rodriguez-Peña et al., 2000).

The Importance of Timed Gene Expression and Cell Cycle Regulation

Given the prevalence of cell cycle–dependent transcription in yeast, it seems likely that periodic transcription will serve as a general mechanism of regulation within the cell cycle. In very few instances has the importance of the periodic expression been directly tested. DNA replication is dependent on the late G1 expression of CLB5 and CLB6 (Epstein and Cross, 1992; Schwob and Nasmyth, 1993), and it is thought that the specific timing of expression of these cyclins is critical. Indeed, the inappropriate expression of CLB2, encoding a mitotic cyclin, interferes with the initiation of DNA replication (Detwiler and Li, 1998). In addition, the initiation factor Cdc6p must be synthesized in G1 to control the initiation of DNA replication effectively (Pittati et al., 1996). Beyond these studies and those presented here, it shall be of interest to learn precisely the role of cell cycle–specific transcription in diverse aspects of cellular function now that we know the cell cycle expression profile of every yeast gene (Cho et al., 1998; Spellman et al., 1998).
Conclusion
We have examined the importance of timed gene expression in cellular morphogenesis in two test cases. For Bud10p, a secreted protein, the timing of BUD10 expression played a major role in directing its localization. In principle, pulsed gene expression may direct the localization of any secreted protein in a cell type that has spatially regulated patterns of cell surface growth.

A localization mechanism that relies on the delivery of a protein through the secretory pathway, rather than specific affinity for a docking site, affords a high degree of flexibility in changing patterns of protein localization within cells. Changes in the timing of expression would allow for the evolution of a novel protein localization pattern without structural changes in the protein. In the case of cells that must frequently remodel, a secretory targeting mechanism of protein localization could allow tremendous plasticity in altering cellular architecture such as occurs during changes in synaptic connectivity in neurons.

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