Specific Residues of the GDP/GTP Exchange Factor Bud5p Are Involved in Establishment of the Cell Type-specific Budding Pattern in Yeast*

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Cells of the budding yeast undergo oriented cell division by choosing a specific site for growth depending on their cell type. Haploid a and a cells bud in an axial pattern whereas diploid a/α cells bud in a bipolar pattern. The Ras-like GTPase Rsr1p/Bud1p, its GDP-GTP exchange factor Bud5p, and its GTPase-activating protein Bud2p are essential for selecting the proper site for polarized growth in all cell types. Here we showed that specific residues at the N terminus and the C terminus of Bud5p were important for bipolar budding, while some residues were involved in both axial and bipolar budding. These bipolar-specific mutations of BUD5 disrupted proper localization of Bud5p in diploid a/α cells without affecting Bud5p localization in haploid a cells. In contrast, Bud5p expressed in the bud5 mutants defective in both budding patterns failed to localize in all cell types. Thus, these results identify specific residues of Bud5p that are likely to be involved in direct interaction with spatial landmarks, which recruit Bud5p to the proper bud site. Finally, we found a new start codon of BUD5, which extends the open reading frame to 210 bp upstream of the previously estimated start site, thus encoding a polypeptide of 608 amino acid residues. Bud5p with these additional N-terminal residues interacted with Bud8p, a potential bipolar landmark, suggesting that the N-terminal region is necessary for recognition of the spatial cues.

Development of cell polarity is critical for the function of many cell types. In both animal and yeast cells, signaling pathways involving small GTPases regulate polarized organization of the actin cytoskeleton in response to intracellular or extracellular cues (1). During vegetative growth, cells of the budding yeast organize their actin cytoskeleton in a highly polarized manner by choosing a specific bud site on the cell cortex. Haploid a and a cells bud in an axial pattern in which both mother and daughter cells select a bud site immediately adjacent to their previous division site. Diploid a/α cells bud in a bipolar pattern; mother cells select a bud site adjacent to their daughter or on the opposite end of the cell, whereas daughter cells always choose a bud site directed away from their mother (2, 3). It is believed that cells respond to cortical cues that mark positions on the cell surface to establish these cell type-specific budding patterns (4). A GTPase module, composed of Rsr1p/Bud1p, Bud5p, and Bud2p, is essential for selecting the proper site for polarized growth in all cell types (3, 5–9). Our previous study indicated that Bud5p plays a key role in linking a spatial signal to polarity establishment (10). To gain insight into cell type-specific budding pattern, we explored whether distinct domains/residues of Bud5p are involved in a specific budding pattern by a series of mutagenesis and localization studies. In this study, we report that some residues of Bud5p are important only for bipolar budding, whereas other residues are involved in both axial and bipolar budding. We also report a new start codon of BUD5, which extends the ORF1 to 210 bp upstream of the previously estimated start site (6, 11).

EXPERIMENTAL PROCEDURES

Plasmids and Yeast Strains—The plasmids and strains used in this study are listed in Tables I and II. The bud5 point mutants were generated as described below using oligonucleotides as listed in supplemental Table I. Additional information about cloning is available in supplemental Methods. Standard methods of yeast genetics and growth conditions were used (21). The plasmid carrying each bud5 mutation was integrated into the BUD5 locus of IH2421 (a bud5Δ) to generate haploid a strains carrying each mutation. The resulting haploid strain was mated to IH2423 (a bud5Δ) to generate a diploid strain hemizygous for each mutation.

Rescue of the bud5-4 Allele—To identify the mutation site(s) of bud5-4, first, the DNA fragment carrying bud5-4 was amplified by PCR using primers 05-7R and 05-8R and genomic DNAs prepared from the strain YJJ219 (a kind gift from J. Pringle) as a template (12). The PCR product was cloned into YCp50, resulting in pPKY508. Haploid (HPY49) and diploid (HPY264) bud5 strains were transformed with pPKY508, and the transformants were examined for budding pattern. The haploid bud5Δ cells carrying pPKY508 exhibited an axial budding pattern, whereas the diploid bud5Δ cells carrying the same plasmid exhibited a random budding pattern, indicating that pPKY508 carries the bud5-4 allele.

Next, domain swapping experiments were carried out using YCp-BUD5 (pHP556) and pPKY508 to locate the mutation site(s). The hybrid clone containing the C-terminal HindIII fragment of pPKY508 exhibited the same mutant budding pattern as the original bud5-4 mutant, indicating that the mutation was located in this C-terminal HindIII fragment of pPKY508. In contrast, the two other hybrid clones carrying different portions of the DNA fragments of pPKY508 exhibited the same budding pattern as that of the wild type BUD5 plasmid (data not shown). Subsequent DNA sequencing identified a single nucleotide change from G to A, resulting in A596T in Bud5p. Finally, to confirm that the A596T mutation is responsible for the phenotype of bud5-4, the G to A substitution was introduced by PCR-based site-directed mutagenesis using the QuikChange site-directed
Residues of Bud5p Involved in Specific Budding Patterns

Fig. 1. Identification of a new start codon of BUD5. A, the numbers indicate the position of the nucleotide relative to A (+1) of the newly defined start codon of BUD5. The first ATG in the frame of the BUD5 ORF overlaps with the stop codon (TGA) of the MATa2 gene. B, immunoblot analysis of an epitope-tagged Bud5p. Bud5p with six copies of an HA epitope at the C terminus (lanes 1 and 6), the same Bud5p-HA with a frameshift mutation at the BamII site (lane 2), untagged Bud5p (lanes 3 and 5), and Bud5p with six copies of an HA epitope at the N terminus (right after the second ATG) (lane 4) were detected with anti-HA antibody. Bud5p with a protein C epitope at the C terminus (Bud5-(C) lane 7) and the same Bud5p-PC with a frameshift mutation at the AatII site (lane 8) were detected with anti-protein C antibody. The asterisk indicates a protein non-specifically cross-reacting with the anti-HA antibody. C, the additional N-terminal region of Bud5p is necessary for interaction with Bud8p. The membrane fractions prepared from cells carrying the HA-BUD8 plasmid and the GST-BUD5 (full-length), GST-Rho5, or GST-BUD5-N3 (deletion of the N-terminal 70 amino acids) plasmid were solubilized with 2% Triton and 200 mM NaCl (lanes 4–6). GST-Bud5p (lane 1), GST-Rho5p (lane 2), and GST-Bud5-N3 (lane 3) were pulled down with glutathione-Sepharose, and eluates were analyzed by immunoblotting with the anti-HA antibody (top panel) or with antibodies against GST (bottom panel).

Fig. 2. A, schematic representation of the predicted domains of Bud5p. A domain with a sequence similarity to the Ras GEF family of proteins is indicated as RasGEF; a region with a sequence similarity to a domain commonly found in the 5’ region of Ras GExs is indicated as RasGEX-N. The asterisk indicates the position (at 596) of a mutation found in bud5-4. B, the budding pattern of deletion mutants of BUD5. C3 to C3 and N2 to N3 denote deletions at the C terminus and the N terminus of Bud5p, respectively. The budding pattern was determined in haploid a or diploid a/a bud5-3 cells carrying either the wild type BUD5 or each mutant on YCp50. At least 200 cells with more than three bud scars were counted for each sample. Ax, more than 85% budding in the axial pattern; Bi, more than 70% budding in the bipolar pattern; R, more than 85% budding in a random position.

Fig. 3. A, the positions of the alanine scanning mutations of BUD5. The amino acid sequence of Bud5p is shown from 71 to 120. Mutations b1 to b7 carry amino acid substitutions in more than one residue; mutations m8 to m12 carry single amino acid substitutions. B, budding pattern of bud5 point mutants. The budding pattern was determined in a haploid a strain carrying each mutation (left panel) and also in a diploid a/a strain hemizygous for each mutation (right panel). At least 300 cells were counted for each strain, and the percentage of each budding pattern is given. The hatched bar, black bar, and gray bar denote axial, bipolar, and random bud site selection from left to right, respectively. The asterisk indicates mixed budding patterns including random budding (see “Results”). C, Bud scars in the bud5-m9 and bud5-m8 mutants. Representative images of bud scars of haploid a and diploid a/a cells carrying each mutation and wild type cells are shown. The arrow indicates disconnected chains of bud scars in an a bud5-m9 cell (see “Results”).

Mutagenesis of the BUD5 gene was carried out by PCR-based site-directed mutagenesis using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) and primers 05-15 and 05-16. The resulting plasmid carrying the A596T mutation (pPKY517) was confirmed by DNA sequencing. This plasmid was introduced into haploid and diploid bud5a strains, and the budding pattern was determined. The budding pattern confirmed that the bud5a596T mutant exhibited the same phenotype as bud5-4.

Alanine Scanning Mutagenesis—The bud5 alanine scanning mutations were generated by PCR-based site-directed mutagenesis using the QuikChange site-directed mutagenesis kit (Stratagene) and pHP763 (pRS304-BUD5-GFP) (10) as a template using primers M1-T to M12-B listed in supplemental Table I. Each mutation was confirmed by DNA sequencing.

Microscopy and Staining of Bud Scars—To view Bud5p fused to green fluorescent protein (Bud5p-GFP), cells were grown to early log phase and visualized using a Nikon E800 microscope fitted with a 100× oil immersion objective (N.A. = 1.30) as described previously (13). Images were collected using IPLab software (Signal Analytics Corp., Vienna, VA) with a Hamamatsu ORCA-2 CCD camera, and the exposure time was 2 s. To determine the budding pattern, bud scars and birth scars were visualized by staining with Calcofluor as described previously (13). For Fig. 3C, cells with bud scars were visualized by
fluorescence microscopy with a 4,6-diamidino-2-phenylindole dihydrochloride filter and at the same time by phase contrast microscopy with dim light to show the outline of the cells, and the exposure time was 0.05 s.

Preparation of Yeast Lysate and Immunoblotting—Yeast lysate preparation and immunoblotting were carried out as described previously (10, 14). The hemagglutinin epitope-tagged Bud5p (Bud5p-HA) and the pro-}

TABLE I

Plasmids used in this study

| Plasmid | Description | Source |
|---------|-------------|--------|
| pHP556  | YCp50-BUD5 (pMIN1)                        | (6)    |
| pHP780  | YCp50-BUD5 with NotI site at the C terminus| (10)   |
| pHP783  | pRS304-BUD5-GFP (C-terminal fusion)       | (10)   |
| pHP1315 | YCp50-budd5-N41                           | This study |
| pPKY502 | YCp50-budd5-N42                           | This study |
| pPKY503 | YCp50-budd5-N33                           | This study |
| pPKY505 | YCp50-budd5-C1                           | This study |
| pPKY506 | YCp50-budd5-C2                           | This study |
| pPKY507 | YCp50-budd5-C3                           | This study |
| pPKY508 | YCp50-budd5-4                            | This study |
| pPKY511 | YCp50-budd5-4*                           | This study |
| pPKY517 | pRS304-bud5-4-A596T-GFP                   | This study |
| pPKY518 | pRS304-bud5-b11BDA,BDA,GFP                | This study |
| pPKY519 | pRS304-bud5-b3R102A,K103A,R104A-GFP       | This study |
| pPKY520 | pRS304-bud5-b4D110A,R111A,GFP             | This study |
| pPKY521 | pRS304-bud5-b4H110A,D111A-GFP             | This study |
| pPKY522 | pRS304-bud5-b4R94A-GFP                    | This study |
| pPKY523 | pRS304-bud5-b5R77A,R90A-GFP               | This study |
| pPKY524 | pRS304-bud5-b5H110A,R111A,R104A-GFP       | This study |
| pPKY525 | pRS304-bud5-m10R104A-GFP                  | This study |
| pPKY526 | pRS304-bud5-m9R104A-GFP                   | This study |
| pPKY528 | pRS304-bud5-m8R110A-GFP                   | This study |
| pPKY529 | pRS304-bud5-m11R90A-GFP                   | This study |
| pPKY530 | pRS304-bud5-m12R94A-GFP                   | This study |
| pPKY531 | YCp50-BUD5-HA (C-terminal tag)            | (10)   |
| pPKY532 | YCp50-BUD5 (BanII mutant)                 | This study |
| pPKY533 | YCp50-HA-BUD5 (N-terminal tag)            | This study |
| pPKY534 | pGST-BUD5-ΔN (in pRD56)                   | This study |
| pPKY535 | pGST-BUD5 (in pRD56)                      | This study |
| pPKY536 | pGST-BUD5-ΔN in BanII                     | This study |
| pPKY537 | pRS305-bud5 protein C epitope at the C terminus | This study |
| pPKY538 | pRS305-bud5 protein C AatIIΔ              | This study |
| pPKY539 | pRS316-GAL1-GST                           | (7)    |
| pPKY540 | TRP1 (integrative)                        | (22)   |
| pPKY541 | LEU2 (integrative)                        | (22)   |
| pPKY542 | CEN6 ARS4 URA3 (low copy)                 | (22)   |

* The original plasmid carrying bud5-4 generated from YJZ219 (12) by PCR (see “Experimental Procedure”).

RESULTS

Determination of the Start Codon of BUD5—The BUD5 ORF predicted from the Saccharomyces Genome Data Base (www.yeastgenome.org/) extends 312 bp upstream of the start codon originally reported by Chant et al. (6) and Powers et al. (11). We confirmed that the BUD5 sequence in the Saccharomyces Genome Data Base is correct by sequencing the BUD5 clone used in the original report by Chant et al. (6). Thus, the discrepances of the sequences are unlikely to be due to polymorphisms in the DNAs sequenced but to sequencing errors. There are two additional ATGs within the 312-bp region. The BUD5 locus partially overlaps with the MAT locus; the stop codon of MATα2 overlaps with the first ATG of the BUD5 locus (Fig. 1A) (15).

To determine which ATG is used as an actual start codon of BUD5, a frameshift mutation at the BanII site (which is located between the first and the second ATGs; Fig. 1A) was introduced to BUD5 on a plasmid (see supplemental Methods). This plasmid fully complemented the random budding pattern of bud5Δ mutants (reverted to 88% axial budding with the plasmid; n = 200), indicating that the wild type BUD5 is expressed. An immunoblot analysis indicated that Bud5p was still expressed from the BanII site mutant and was indistinguishable from the wild type Bud5p (Fig. 1B, compare lanes 1 and 2). Thus, it is unlikely that the first ATG is used as a start codon. Next, a frameshift mutation at the AatII site that is located between the second and third ATGs (Fig. 1A) was introduced. This mutant failed to complement the bud site selection defect of bud5Δ (95% random budding; n = 100). An immunoblot analysis confirmed that Bud5p was not expressed from this mutant (Fig. 1B, lane 8), suggesting that the second ATG is likely to be used as a start codon of BUD5. To confirm that the second ATG is indeed used as a start site (to rule out the possibility that the AatII mutation simply caused decreased expression of BUD5), we introduced six copies of an HA epitope-tagged Bud5p (Bud5p-HA) and the protein C epitope-tagged Bud5p (Bud5p-C) (10) to confirm that the second ATG is likely to be the start codon of BUD5. Thus, the newly defined BUD5 ORF extends 210 bp upstream of the start codon predicted from the previous reports (6, 11), encoding a polypeptide of 608 amino acids.

To determine whether the additional N-terminal region is important for interaction with a potential spatial landmark, we expressed the full-length Bud5p or a truncated Bud5p lacking the N-terminal 70 amino acids as glutathione S-transferase fusion proteins in yeast, which also expressed Bud8p, a potential bipolar landmark, as an HA epitope-tagged protein (HA-Bud8p). Interestingly, we found that HA-Bud8p was co-purified with the GST-Bud5p (full length) but not with the GST-
Bud5p that lacks the N-terminal 70 residues (GST-Bud5-N) that the N-terminal region is necessary for both budding patterns. Our results also suggest that the N-terminal region is necessary for both budding patterns and residues near the N terminus of Bud5p are involved in the specificity of Bud5p interactions (see “Experimental Procedures”). The bud5-4 mutant carries a single nucleotide change from G to A, resulting in A596T, which falls within the region deleted in CΔ1. These and other data discussed below suggest that both the C terminus and residues near the N terminus of Bud5p are involved in the bipolar budding pattern.

Site-directed Mutagenesis Identifies Additional Bipolar-specific Alleles of BUD5 and Other Alleles with Defects in Both Budding Patterns—Although a deletion of the N-terminal 70 residues caused random budding in both a and a/o cells (Fig. 2B), four specific point mutations on the charged residues within the region did not result in any defect in either cell type (data not shown). Thus, even though they were necessary for an interaction between Bud5p and Bud8p (Fig. 1C), the N-terminal 70 residues may not be directly involved in interaction with a spatial landmark. Instead, deletion of the region may have perturbed the global structure of Bud5p. To avoid potential problems caused by deletion, such as instability or misfolding of a protein, we carried out site-directed mutagenesis of BUD5.

### Table II

| Strain          | Relevant genotype          | Source          |
|-----------------|----------------------------|-----------------|
| HPY49           | MATα bud5Δ::URA3            | This study      |
| HPY364          | MATα bud5Δ::URA3            | This study      |
| HPY377          | MATα bud5Δ::URA3            | This study      |
| HPY378          | MATα bud5Δ::URA3            | This study      |
| HPY380          | MATα bud5Δ::URA3            | This study      |
| HPY381          | MATα bud5Δ::URA3            | This study      |
| HPY382          | MATα bud5Δ::URA3            | This study      |
| HPY383          | MATα bud5Δ::URA3            | This study      |
| HPY384          | MATα bud5Δ::URA3            | This study      |
| HPY385          | MATα bud5Δ::URA3            | This study      |
| HPY386          | MATα bud5Δ::URA3            | This study      |
| HPY387          | MATα bud5Δ::URA3            | This study      |
| HPY388          | MATα bud5Δ::URA3            | This study      |
| HPY389          | MATα bud5Δ::URA3            | This study      |
| HPY390          | MATα bud5Δ::URA3            | This study      |
| HPY391          | MATα bud5Δ::URA3            | This study      |
| HPY392          | MATα bud5Δ::URA3            | This study      |
| HPY393          | MATα bud5Δ::URA3            | This study      |
| HPY394          | MATα bud5Δ::URA3            | This study      |
| HPY395          | MATα bud5Δ::URA3            | This study      |
| HPY396          | MATα bud5Δ::URA3            | This study      |
| HPY397          | MATα bud5Δ::URA3            | This study      |
| HPY398          | MATα bud5Δ::URA3            | This study      |
| HPY399          | MATα bud5Δ::URA3            | This study      |
| HPY400          | MATα bud5Δ::URA3            | This study      |
| HPY401          | MATα bud5Δ::URA3            | This study      |
| HPY402          | MATα bud5Δ::URA3            | This study      |
| HPY403          | MATα bud5Δ::URA3            | This study      |
| HPY404          | MATα bud5Δ::URA3            | This study      |
| HPY405          | MATα bud5Δ::URA3            | This study      |
| HPY406          | MATα bud5Δ::URA3            | This study      |
| HPY407          | MATα bud5Δ::URA3            | This study      |
| HPY408          | MATα bud5Δ::URA3            | This study      |
| HPY409          | MATα bud5Δ::URA3            | This study      |
| HPY410          | MATα bud5Δ::URA3            | This study      |
| HPY411          | MATα bud5Δ::URA3            | This study      |
| HPY412          | MATα bud5Δ::URA3            | This study      |
| HPY413          | MATα bud5Δ::URA3            | This study      |
| HPY414          | MATα bud5Δ::URA3            | This study      |
| HPY415          | MATα bud5Δ::URA3            | This study      |
| HPY416          | MATα bud5Δ::URA3            | This study      |
| HPY417          | MATα bud5Δ::URA3            | This study      |
| YZ221           | MATα bud5Δ::URA3            | This study      |

Bud5p lacks the N-terminal 70 residues (GST-Bud5-N) or GST-Bud5 control (Fig. 1C). Taken together, these data suggest that Bud5p interacts with Bud8p and that the N-terminal region of Bud5p is necessary for their interaction.

Deletion Mutagenesis and Rescue of a Bipolar-specific Allele of BUD5—Previous studies suggested that Bud5p interacts with an axial or bipolar landmark in haploid or diploid cells, respectively (10, 16). In fact, Bud5p has been shown to interact with Axl2p, a potential axial landmark (10). We hypothesized that a specific allele of BUD5, unlike the bud5 null mutant, may be defective in interaction with either an axial or bipolar (but not both) landmark. To determine functional domains of Bud5p that are involved in the cell type-specific budding pattern, we generated both deletion and point mutants of BUD5. Because the internal region of BUD5 contains homologies to the Ras GEF domain and the conserved domain present in the N-terminal region of the Ras GEF family of proteins (Ras-GEF-N) (Fig. 2A), which are likely to be important for both axial and bipolar budding, we focused on the N- and C-terminal regions of Bud5p for mutagenesis. We initially generated small deletions from either the N or C terminus of Bud5p. The bud5Δ cells carrying each deletion on a plasmid showed random budding in all cell types, except that a small deletion (CA1) from the C terminus resulted in a specific defect in bipolar budding (Fig. 2B), suggesting that the C terminus is important for bipolar budding. Deletion of the N-terminal 70 residues caused random budding in both a and a/o cells (Fig. 2B), suggesting that the N-terminal region is necessary for both budding patterns (see below).

We also rescued a bipolar allele of BUD5 (bud5-4), which was originally isolated by Zahner et al. (12), and mapped the mutation (see “Experimental Procedures”). The bud5-4 mutant carries a single nucleotide change from G to A, resulting in A596T, which falls within the region deleted in CΔ1. These and other data discussed below suggest that both the C terminus and residues near the N terminus of Bud5p are involved in the bipolar budding pattern.
budding. Thus, Arg97, Glu88, Lys93, Asp108, and Arg111 are likely to be important only for bipolar budding. The second group includes bud5-m9R102A, bud5-m10R104A, bud5-b2R97A,R99A,R103A, and bud5-4 (12), which were mildly defective in the axial pattern but severely defective in the bipolar budding pattern. Interestingly, most haploid cells of this group exhibited the axial budding pattern, while a small percentage of cells showed disrupted axial budding in a unique way (denoted as an asterisk for mixed budding patterns in Fig. 3B); some cells showed chains of bud scars that were disconnected (see arrow in Fig. 3C for a bud5-m9). For example, cells of a bud5-m9 exhibited mixed budding patterns that included cells with disconnected chains of bud scars (20%) and randomly distributed bud scars (7%). Thus, Asp97, Lys99, Arg100, Lys103, Arg104, and Ala506 are also likely to be important for bipolar budding, although they also appear to be necessary for high fidelity of the axial budding pattern. The third group includes bud5-b3R102A,K103A,R104A, bud5-b6R97A,R99A,R103A, bud5-m9R102A, and bud5-m12R94A, which exhibited defects in both budding patterns (Fig. 3, B and C). Among those mutants, bud5-b3 and bud5-b6 produced an unstable Bud5p based on immunoblot analyses (data not shown). However, bud5-m8 and bud5-m12 produced a quite stable Bud5p (data not shown), which failed to localize to the proper sites (see below), thus Arg102 and Arg104 are likely to be involved in both budding patterns. Finally, the fourth group includes bud5-b5D115A,D119A and bud5-b7R97A,R99A, which exhibited little defect in either axial or bipolar budding, although bud5-b7 exhibited a slight increase in mixed budding patterns (Fig. 3B). Taken together, these results identified additional residues of Bud5p involved only in the bipolar budding pattern and other residues necessary for both axial and bipolar budding patterns.

Localization of Bud5p Expressed in the bud5 Mutants—Our previous study (10) showed that Bud5p localizes to distinct sites in haploid and diploid cells (see top panel in Fig. 4) and that the localization is defective in mutants lacking potential cell type-specific landmarks in each cell type. Thus, we examined the localization of Bud5p-GFP expressed in the bud5 mutants with a cell type-specific defect (Fig. 4). Bud5p-GFP in bud5-b1, which is specifically defective in the bipolar budding pattern, failed to localize properly in a/a cells but localized properly in a cells. Bud5p-GFP in bud5-4 or bud5-m9, which showed severe defects in the bipolar budding of diploid cells but a slight increase in mixed budding in haploid cells, localized similarly to that in bud5-b1. Although a/a cells carrying the bud5-4 or bud5-b1 mutation showed strong Bud5p-GFP signals in the small buds, the Bud5p-GFP signals were not detected in cells with medium- or large-sized buds. A small percentage of a/a cells carrying the bud5-m9 mutation showed a faint Bud5p-GFP signal at the bud tip or mother-bud neck, which was much weaker than that observed in the wild type cells. In contrast, Bud5p-GFP from bud5-m8, which is defective in both axial and bipolar budding patterns, failed to localize in both a and a/a cells, suggesting that Arg102 is likely to be involved in interaction with both axial and bipolar landmarks. Taken together, these data support the idea that specific residues of Bud5p are involved in interaction with bipolar landmarks that recruit Bud5p to either pole of diploid cells, while some other residues are involved in interaction with both axial and bipolar landmarks.

**DISCUSSION**

Our previous study indicated that Bud5p plays a key role in linking spatial signals to polarity establishment (10). In this report, we determined whether specific domains/residues of Bud5p are involved in establishment of cell type-specific budding patterns. We identified, through deletion and site-specific mutagenesis, specific residues of Bud5p involved only in bipolar budding and other residues involved in both axial and bipolar budding. Since the bipolar-specific mutations do not affect axial budding, it is unlikely that these mutations perturb the global structure of Bud5p. Instead, the residues mutated in the bipolar-specific mutants are defective in interaction only with a bipolar landmark. Among the mutants defective in both budding patterns, the bud5-m12 and bud5-m8 mutations, unlike bud5-b3 and bud5-b6, had little effect on the stability of Bud5p. Thus, the residues Arg94 and Arg102 are likely to be involved in interaction with both axial and bipolar landmarks. However, we cannot rule out the possibility that these mutations perturb the global structure of Bud5p, rather than being directly involved in the recognition of spatial landmarks.

We were unable to identify any bud5 mutations with specific defects only in the axial budding of haploid cells. Because our mutagenesis was not saturated, we cannot completely rule out the possibility that a specific residue of Bud5p is important only for axial budding. Nonetheless, our results suggest that the domains of Bud5p involved in each budding pattern are not completely separable. On the other hand, the large spectrum of bud site selection defects of bud5 mutants reported here suggests that interactions between specific residues of Bud5p and cell type-specific landmarks are likely to be distinct in each cell type. Localization of Bud5p in haploid a and diploid a/a cells carrying these bud5 mutations also supports this view.

Our data indicate that the BUD5 ORF encodes a polypeptide...
of 608 amino acid residues, which contains 70 more residues at the N terminus than reported previously (6, 11). We also showed that this additional N-terminal region of Bud5p was necessary for interaction with Bud8p, a potential bipolar landmark. However, the deletion of the region disrupted the budding patterns in both a and α/α cells, suggesting that the region is necessary for both budding patterns.

Previous studies suggest that Bud8p and Bud9p function as a distal and a proximal marker of diploid cells, respectively; the bud8Δ mutants bud predominantly at the proximal pole, while the bud9Δ mutants bud predominantly at the distal pole. In addition, Bud8p localizes to the distal pole of unbudded cells and the tip of growing buds, whereas Bud9p localizes to the bud side of the neck in large budded cells and to the proximal poles of daughter cells (12, 18–20). The cytoplasmic domains of Bud8p and Bud9p are very similar to each other, unlike the extracellular domains of Bud8p and Bud9p. This similarity may allow the general bud site selection machinery, which includes Rsr1p/Bud1p, Bud2p, and Bud5p, to recognize essentially the same signal at the two poles of a diploid cell (18). Consistent with this idea, all bipolar-specific bud5 mutants isolated in this study as well as bud5-4 (12) budded randomly in diploid cells, rather than either at the proximal or at the distal pole. Thus, these mutants are likely to be defective in interacting with both proximal and distal pole markers of diploid cells. Localization of Bud5p in α/α cells carrying these bipolar-specific alleles also supports this view. However, it remains to be determined whether Bud5p directly interacts with Bud8p and Bud9p and whether these bipolar-specific bud5 mutations disrupt such interactions.

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