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Immunofluorescence Localization of *Schizosaccharomyces pombe cdc103*$^{+}$ Gene Product

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***cdc103*$^{+}$ gene in *Schizosaccharomyces pombe* which is similar to the *CDC3* gene in *Saccharomyces cerevisiae* was cloned and sequenced. Comparision of the predicted amino acid sequences of *cdc103*$^{+}$ and *CDC3* revealed that they share significant similarity (43% identity and 56% identity or similarity) to each other. The gene product of *CDC3* in *S. cerevisiae* is known to be a highly ordered ring of filaments that lies just inside the cytoplasmic membrane in the region of the mother-bud neck. In order to characterize the gene product of *cdc103*$^{+}$ in *Schizosaccharomyces pombe*, fusion proteins were used to generate the polyclonal antibodies specific for the gene product (cdc103p). In immunofluorescence experiments, these antibodies decorate the region of septum formation as a double ring structure late in the cell division cycle.**

Key words: ***Schizosaccharomyces pombe, Saccharomyces cerevisiae, cdc103*$^{+}$, *CDC3***

One of the fundamental problems of cell biology is to understand the mechanism by which cells elicit changes in shape and intracellular spatial organization. The budding yeast *Schizosaccharomyces pombe* and *Saccharomyces cerevisae* provide very useful systems to study morphogenetic processes in cell division because their cell-division cycles involve several morphogenetic events. As in other eukaryotes, cytoskeletal components, including both actin (1, 11, 22) and microtubules (1, 2, 9, 10), are believed to play important roles in these processes.

S. cervisiae also contains another cytoskeletal element, of unknown biochemical nature, which may be involved in cellular morphogenesis. This is a highly ordered array of filaments, ~10-nm in diameter, which lies just inside of the cytoplasmic membrane in the region of the mother-bud neck (2, 3). EM studies suggest that these filaments appear at about the time of bud emergence and disappear just before cytokinesis. Temperature-sensitive mutants defective in any of four distinct cell-division cycle genes (*CDC3, CDC10, CDC11,* and *CDC12*) lack these filaments and display a pleiotropic phenotype when shifted to the restrictive temperature (1, 3, 8, 23). These mutants fail to complete cytokinesis, fail to properly localize chitin to the bases of buds formed at the restrictive temperature, and display hyperpolarized bud growth; DNA synthesis, nuclear division, and budding continues, resulting in the formation of multinucleate, multibudded cells.

CDC3, CDC10, CDC11, and *CDC12* have been cloned and sequenced, revealing that they encode a family of related proteins that are 25~37% identical in amino acid sequence (6). In addition, antibodies specific for the products of *CDC3, CDC10, CDC11,* and *CDC12* have been used to show by immunofluorescence that these proteins localize to the mother-bud neck in the vicinity of the filaments (14, 15, 16). Filament staining disappeared in *cdc3, cdc10, cdc11,* and *cdc12* mutants shifted to the restrictive temperature. These results suggest strongly that *CDC3, CDC10, CDC11,* and *CDC12* encode constituents of the 10-nm filaments; however, the predicted amino acid sequences of these genes lack similarity to other proteins, inculding known filament-forming proteins. Thus, *CDC3, CDC10, CDC11,* and *CDC12* may encode a novel class of filaments-forming proteins.

Despite the accumulated information about the filament proteins, the role of these proteins in morphogenetic processes remains unclear. In order to understand the function of the neck-filament proteins and to determine if they are specific for the budding lifestyle of *S. cervisiae*, we have undertaken a search for homologues

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of *CDC3*, *CDC10*, *CDC11*, and *CDC12* in the fission yeast *Schizosaccharomyces pombe*. *S. pombe* was selected because it is evolutionarily distant (17) and morphologically distinct from *S. cerevisiae*. *S. pombe* cells are hemispherical-capped cylinders which grow by length extension alone. *S. pombe* divides by binary fission in which a septum is formed across the middle of the cell, resulting in two nearly equal-sized cells; this is very different from the asymmetrical-division process of budding used by *S. cerevisiae*. Moreover, *S. pombe* does not form any structures analogous to the mother-bud neck during the course of its cell-division cycle, nor have any filamentous structures resembling the filaments been described. Thus, identification of homologues of the neck-filament proteins and characterization of their function in *S. pombe* may provide important clues about their possible role in cellular morphogenesis in both yeasts as well as their possible general distribution in other eukaryotes. The *cdc103*$^+$ gene in *S. pombe* which is homologous to the *CDC3* gene in *S. ceresiviae* had been cloned and sequenced (12, 13). Comparision of the predicted amino acid sequences of *cdc103*$^+$ and *CDC3* revealed that they share significant similarity (42% identity and 53% identity or similarity) to each other.

To characterize the *cdc103*$^+$ gene product and to find the role of the gene product, cell biological experiments had been done. Here I report the production of antibodies against its gene product (cdc103p) and the use of these antibodies to localize the cdc103p. The antibodies was produced using gene fusion with *E. coli* gene and the loalization of cdc3p was checked with an immunofluorescence microscope.

Materials and Methods

Reagents

Enzymes were obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN) or BRL Life Technologies (Gaithersburg, MD) and were used according to the supplier's specifications. Nitrocellulose BA85 was obtained from Schleicher and Schuell (Keene, NH), Glusulase from DuPont (Wilmington, DE), Yeast Lytic Enzyme (Lyticase) 70,000 from ICN Biochemicals (Cleveland, OH), and Novozyme 234 from NovoLabs (Wilton, CT). Rhodamine-conjugated goat anti-rabbit-IgG and FITC-conjugated goat anti-rabbit-IgG antibodies were obtained from United States Biochemicals (Cleveland, OH). HRP-conjugated goat anti-rabbit-IgG antibody was obtained from Bio-Rad (Richmond, CA). 4', 6'-diamidino-2-phenylindole (DAPI) from sigma (St. Louis, MO), Calcofluor White M2R New, and rhodamine-phalloidin from Molecular Probes (Eugene, OR). Other reagents were obtained from standard commercial sources.

Protein Isolation, Gel Electrophoresis, and Blotting

Total *E. coli*, *S. cerevisiae*, and *S. pombe* proteins were isolated as described by Haarer and Pringle (5), except that yeast cells were disrupted by vigorous vortexing with 0.5-mm glass beads. Protein concentrations were determined by the method of Lowry *et al.* (19). SDS-polyacrylamide gels and the Laemmli buffer system (18) were used for all protein electrophoresis. When appropriate, gels were stained using 0.5% Coomassie blue R-250 in 45% methanol/10% acetic acid and destained in 20% methanol/10% acetic acid. Proteins were transferred to nitrocellulose (25) with a TE42 electroblotter (Hoefer Scientific Instruments, San Francisco, CA) using a current of 1 A for 2 h. When necessary, the blots were stained using 0.2% Ponceau S in 0.3% trichloroacetic acid for 5 min and destained in PBS (24). Proteins recognized by primary antibodies were detected using HRP-conjugated secondary antibodies as described previously (5).

Preparation of Antigens and Antibodies

The β-galactosidase-cdc103p fusion proteins were prepared from *E. coli* strain BMH71-18 containg fusion plasmid pUR-BI or pUR-BII (see Results) as described previously (14). The anthranilate synthase-cdc103p fusion proteins were prepared from *E. coli* strain HB101 bearing fusion plasmid pATH-AI or pATH-AII (see Results) as described previously (5). For immunization, the four fusion proteins were purified individually from polyacrylamide gels. The two β-galactosidase fusions were then pooled together and injected into rabbits, as were the two anthranilate synthase fusion proteins. cdc103p-specific antibodies were affinity purified from crude antiserum raised against the anthranilate synthase-cdc103p fusion by using the β-galactosidase-cdc103p fusion protein blotted to nitrocellulose, as described by Pringle *et al*(24).

Immunofluorescence and Other Microscopy

For immunofluorescence or staining of actin with rhodamine-phalloidin, *S. pombe* cells were grown overnight at 30°C with agitation in YEPD medium to a cell density of 6~10×10^6 cells/ml. To avoid disrupting the chains of mutant cells (see Results), only gentle agitation was used with all cultures. For staining with cdc103p-specific antibodies, cells were prepared essentially as described by Hagan and Hyams (7), except that after the post-fixation washes in PEM (100 mM Pipes, 1 mM EGTA, 1 mM $MgSO_4$) and resuspension in PEMS (PEM containing 1.2 M Sorbitol), the cells were treated with 0.1 vol of a 1 : 1 mixture of 10 mg/ml Lyticase 70,000 and 50 mg/ml

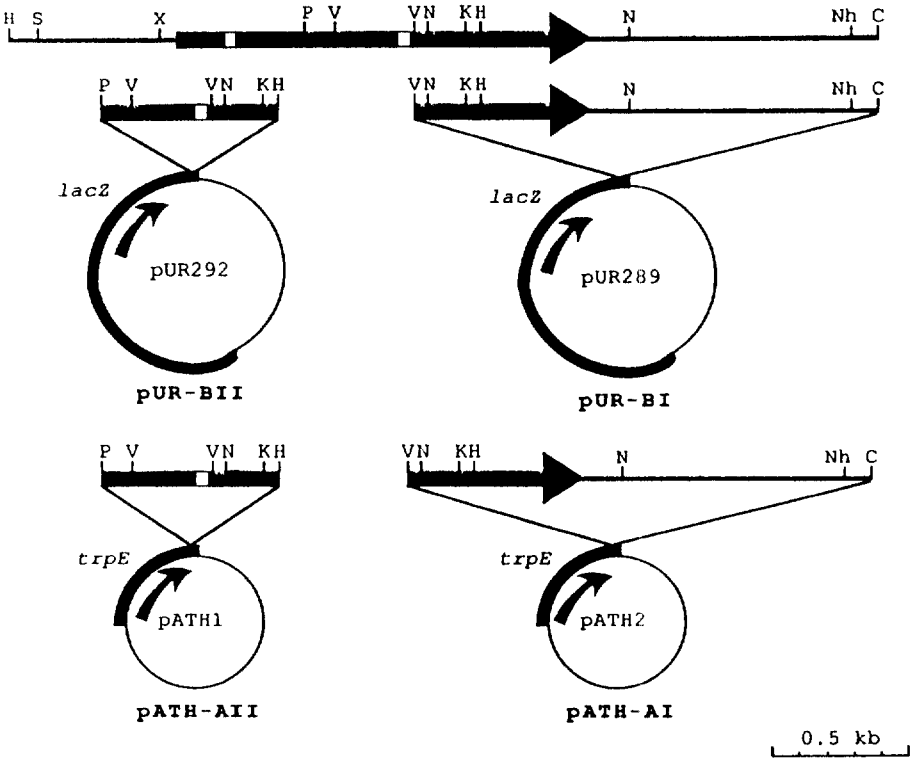

Fig. 1. Construction of *lacZ*: *cdc103*$^+$ and *trpE*: *cdc103*$^+$ gene fusions. See text for details. The coding region of *cdc103*$^+$ is indicated by the heavy arrow. The fragments subcloned are shown above each plasmid. In plasmids pUR289 and pUR292, the open box represents the *E. coli lacZ* gene; in plasmid pATH1 and pATH2, it represents the *E. coli trpE* gene. The direction of transcription is indicated by the curved arrow.

Novozym 234 for 30 min at 37°C to digest the cell walls. After washing in PEMBAL (PEM containing 1.0% BSA, 0.1% sodium azide, and 100 mM lysine hydrochloride), 30 μl of cell suspension was mixed with 10 μl of undiluted, affinity-purified cdc103p-specific antibodies and incubated overnight at room temperature. The cells were then washed three times with 0.5 ml of PEMBAL, resuspended in 40 μl of PEMBAL, and treated with 10 μl of 1 : 10 diluted rhodamine-conjugated goat anti-rabbit-IgG antibody, which was preabsorbed in 1% BSA and 5% fetal calf serum, for 4 h at room temperature in the dark. For staining with actin-specific antibodies, cells were fixed by adding formaldehyde directly to the culture medium (final concentration, 3.7%) and prepared for immunofluorescence essentially as described for *S. cerevisiae* (24), except that the cell walls were removed by treating the cells with 5 mg/ml Novozym 234 prepared in solution A for 1 h at 37°C. The primary antibody, raised against *S. cerevisiae* actin, was diluted 1 : 20; the secondary antibody, FITC-conjugated goat anti-rabbit-IgG, was diluted 1 : 40. Cells were stained with rhodamine-phalloidin as described previously (20). After staining, cells were mounted on poly-L-lysine coated slides using DAPI-containing mounting medium (24), and were then viewed and photographed using an fluorescence microscope.

For phase-contrast microscopy and fluorescence microscopy of cells stained with Calcofluor and/or DAPI, *S. pombe* cells were collected with a toothpick from the leading edge of a patch growing on YEPD or EMM3 solid medium and suspended in distilled water. Staining and viewing of Calcofluor- and DAPI-stained cells was as described for *S. cerevisiae*(24).

Results

Generation of cdc103p-specific Antibodies

To obtain proteins suitable for the elicitation and affinity purification of polyclonal antibodies, DNA fragments derived from the two largest exons of cdc103+ were fused in-frame with the *E. coli lacZ* and *trpE* genes. Two *lacZ*: *cdc103*$^+$ fusion plasmids were constructed (Fig. 1). pUR-BII was made by inserting the 0.7 kb *PstI-HindIII* fragment of *cdc103*$^+$ into *PstI/HindIII*-cut pUR 292. pUR-BI was made by inserting the 1.6 kb *EcoRV-ClaI* fragment of *cdc103*$^+$ into *HindIII/BamHI*-cut pUR289 after filling in all of the 5'-overhanging ends with DNA polymerase Klenow fragment. As predicted, pUR-BII and pUR-BI directed the synthesis of fusion proteins of

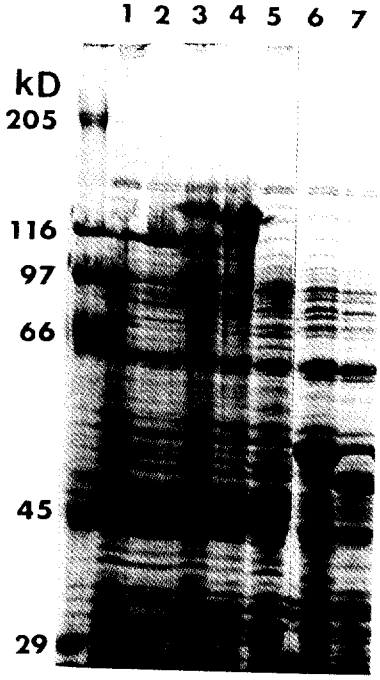

Fig. 2. Identification of β-galactosidase-cdc103p and anthranilate from *E. coli* strain BMH71-18 containing no plasmid (lane 1), plasmid pUR289 (lane 2), plasmid pUR-BI (lane 3), or plamid pUR-BII (lane 4). Lanes 5, 6, and 7: total proteins prepared from *E. coli* strain HB101 containing no plasmid (lane 5), plasmid pATH-AI (lane 6), or plasmid pATH-AII (lane 7). In all cases, strains were propagated under inducing conditions (see Materials and Methods). The approximate sizes of the fusion proteins are given in kilodaltons.

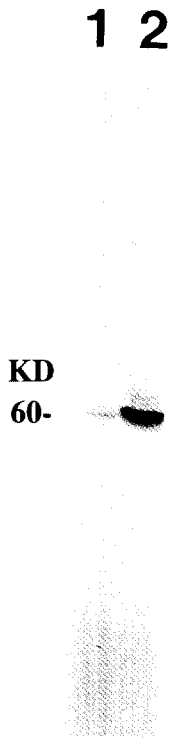

Fig. 3. Recognition of cdc103p by affinity-purified antibodies raised against the anthranilate synthase-cdc103p fusion proteins. Total cellular proteins were prepared from wild types (lane 1) and wild types containing a multicopy plasmid which had the *cdc103*$^{+}$ gene (lane 2). The primary antibody was used at a 1 : 50 dilution; the secondary antibody was a 1 : 100 dilution of HRP-conjugated goat anti-rabbit-IgG. The position of cdc103p at ~60 kD is indicated.

130 and 140 kD, respectively (Fig.2, lanes 1-4). Similarly, two *trpE : cdc103*$^{+}$ fusion plasmids were constructed (Fig.1). pATH-AII was made by inserting the 0.7 kb *BamHI-HindIII* fragment from pUR-BII, which contains the 0.7 kb *PstI-HindIII* fragment of *cdc103*$^{+}$ (the *BamHI* site was donated by the multiple cloning site of pUR-BII), into *BamHI/HindIII*-cut pATH1. pATH-AI was made by inserting the 1.6 kb *EcoRV-ClaI* fragment of *cdc103*$^{+}$ into *SmaI/ClaI*-cut pATH2. As predicted, pATH-AII and pATH-AI directed the synthesis of fusion proteins of 50 and 60 kD, respectively (Fig. 2, lanes 5-7).

The β-galactosidase-cdc103p and anthranilate synthase-cdc103p fusion proteins were used to prepare affinity-purified cdc103p-specific antibodies as described in Materials and Methods. To test the specificity of these antibodies, they were used to probe blots of separated proteins derived from *S. pombe*. In extracts from wild-type cells, a single protein of ~60 kD was recognized (Fig. 3, lane 1). A protein of ~60 kD was again detected at higher levels than in wild-type cells, when extracts from wild-type cells containing a multy copy plasmid which had *cdc103*$^{+}$ gene (Fig. 3, lane 2). These results demonstrated that the antibodies were indeed specific for cdc103p.

Immunofluorescence Localization of cdc103p

The intracellular localization of cdc103p was investigated by indirect immunofluorescence; cells were also labeled with DAPI to provide an indicator of their position in the cell-division cycle. Most cells in an asynchronous population showed no detectable straining with the cdc103p-specific antibodies (Fig. 4A and B, cells a and b). However, cells that were very late in the cell cycle (as judged by their length and the presence of two well separated regions of nuclear DNA) showed a double ring structure in the middle of the cell, presumably in the region of septum formation (Fig. 4A and B, cells c,d, e and f). In addition, some cells that were early in the cell cycle (as judged by their shortness and the presence of a single region of nuclear DNA) showed a ring of staining at one end (Fig 4A and B, cell g); the stained ends invariably had a "squared-off" appearance, suggesting that these cells had actually still been attached to their sisters at these ends prior to fixation and removal of the

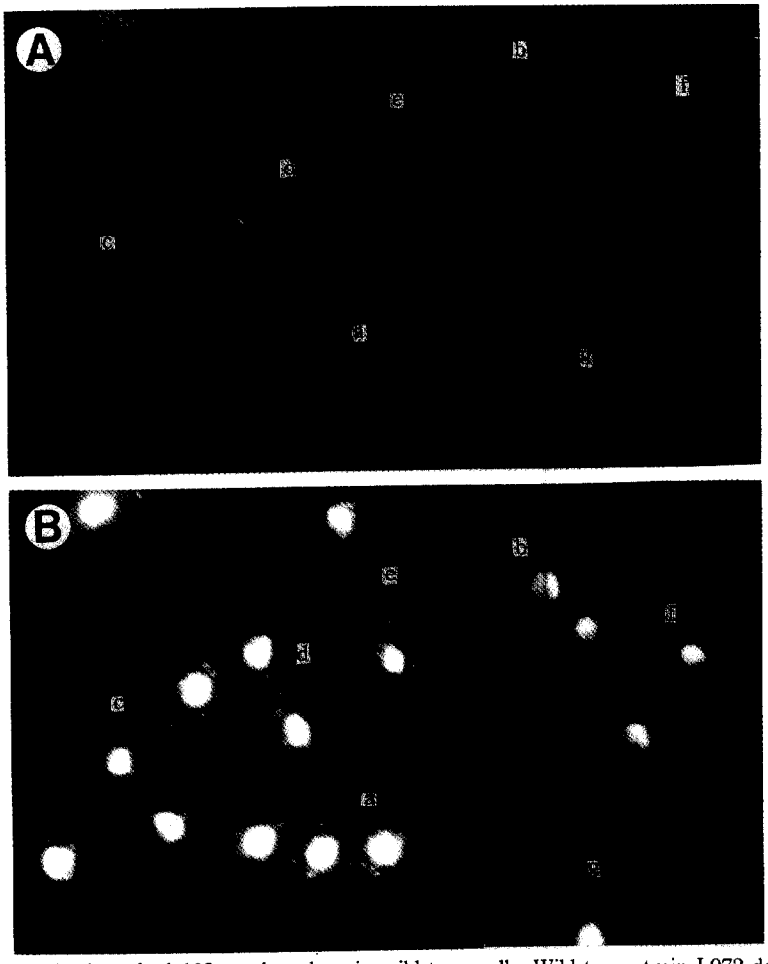

Fig. 4. Immunofluorescence localization of cdc103p and nucleus in wild-type cells. Wild-type strain L972 double-stained with anthranilate synthase-cdc103p-specific antibodies (A) and DAPI (B). See Materials and Methods for details of staining. Individual cells are labeled for reference in the text.

cell wall.

The localization of cdc103p resembled that of actin (20), except that actin appeared to localize to the middle of cell at an earlier stage in the cell cycle. Both actin and cdc103p appeared to linger at the septal region after cytokinesis (20), as judged by the staining of the squared-off ends of (presumably) newborn cells (Fig. 4A and B, cell g).

Discussion

To investigate the intracellular localization and function of cdc103p in the *S. pombe* cell-division cycle, β-galactosidase-cdc103p and anthranilate synthase-cdc103p fusion proteins were constructed and used to generate and affinity purify antibodies specific for cdc103p. Immunoblot analysis using the purified antibodies revealed a ~60 kD band in extracts of total *S. pombe* proteins; this band was detected at much higher levels in extracts prepared from a strain transformed with a multicopy plasmid carrying *cdc103*$^+$. These data demonstrated that the antibodies used were specific for cdc103p and that the ~60 kD band detected on immunoblots represents the genuine *cdc103*$^+$ gene product. The observed molecular weight of ~60 kD is somewhat greater than the 53.7 kD calculated from the predicted amino acid sequence. The reason for this discrepancy is not understood, but it is not unprecedented. The *S. cerevisiae CDC11* gene product has a calculated molecular weight of 47.7 kD, but migrates as a ~55 kD protein(4).

Immunofluorescence localization of cdc103p in wild-type cells revealed that it localized to the middle of the cell in the region of septum formation as a double ring structure. cdc103p localized to this region very late in the cell-division cycle: ring structures were only seen in cells in which the dividing nuclei were clearly separated and were approaching the ends of the dividing cells. Actin also localizes transiently to the region of septum formation during the cell cycle (20, 21).

The intracellular localization of cdc103p to the septral region was not unexpected considering that each of the neck-filament proteins localize to the region of septum formation (the mother-bud neck) in *S. cerevisiae* (4, 5, 14). However, the transient pattern of cdc103p localization in *S. pombe* is very different from the pattern of Cdc3p localization in *S. cerevisiae*. Cdc3p localizes to the site of bud emergence prior to the appearance of the bud, remains at the budding site throughout the cell-division cycle, and lingers there after completion of cytokinesis and cell separation (14). This difference in the patterns of localization of these homologous proteins probably reflects the fundamental mechanistic differences between the cell-division processes of budding and fission. The localization pattern of Cdc3p suggests that it may be involved in two distinct functions: establishment and maintenance of the budding site and completion of cell division (i. e., cytokinesis, septum formation, and cell separation). Since *S. pombe* does not possess a structure similar to the site of bud emergence or the mother-bud neck, it is possible that cdc103p is involved only in completion of cell division.

The results presented here demonstrate that *S. pombe* contains a homologue of one of the neck-filament proteins of *S. cerevisiae* and that these homologous proteins seem to be involved, at least in part, in similar functions (i. e., completion of septum formation and cell separation) in these diverse yeasts. However, the molecular basis of the function of these proteins remains unclear in both species. It is not known if cdc103p is a constituent of filaments, nor is it known if cdc103p (and Cdc3p) must form filaments to be functional. No filamentous structures resembling the 10-nm filaments have been described in EM studies of *S. pombe*, although the septal region is crowded with vescicles. Thus, additional investigation into the function of cdc103' and the other neck-filament protein homologues in *S. pombe* is required to better understand the function of this potentially ubiquitous family of closely related proteins in cellular morphogenesis.

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References

1. **Adams, A.E.M. and J.R. Pringle,** 1984. Relation of actin and tubulin distribution to bud growth in wild type and morphogenetic mutant *Saccharomyces cerevisiae*. *J. Cell Biol.* **98**, 934-945.
2. **Byers. B.,** 1981. Cytology of the yeast life cycle. p.59-66. J. N. Strathern E.W. Jones, and J.R. Broach (ed.). *In* The molecular biology of the yeast *Saccharomyces*: Life cycle and inheritance. Cold Spring Harbor Lab. Cold Spring Harbor, N.Y.
3. **Byers, B. and L. Goetsch,** 1976. Loss of the filamentous ring in cytokinesis defective mutants of budding yeast. *J. Cell Biol.* **70**, 35a.
4. **Ford, S.K. and J.R. Pringle,** 1991. Cellular morphogenesis in the *Saccharomyces cerevisiae* cell cycle: localization of the *CDC11* gene product and the timing of events at the budding site. *Devel. Genet.* **12**, 282-292.
5. **Haarer, B.K. and J.R. Pringle,** 1987. Immunofluorescence localization of the *Saccharomyces cerevisiae CDC12* gene product to the vicinity of the 10-nm filaments in the mother-bud neck. *Mol. Cell. Biol.* **7**, 3678-3687.
6. **Haarer, B.K., S.R. Ketcham, S.K. Ford, D.J. Ashcroft, and J.R. Pringle,** 1996. The *Saccharomyces cerevisiae CDC3, CDC10, CDC11, CDC12* genes encode a family of similar proteins. *Genetics* in press.
7. **Hagan, I.M. and J.S. Hyams,** 1988. The use of cell division cycle mutants to investigate the control of microtubule distribution in the fission yeast *Schizosaccharomyces pombe*. *J. Cell Sci.* **89**, 343-357.
8. **Hartwell, L.H.,** 1971. Genetic control of the cell division cycle in yeast. Genes controlling bud emergence and cytokinesis. *Exp. Cell. Res.* **69**, 265-276.
9. **Huffaker, T.C., J.H. Thomas, and D. Botstein,** 1988. Diverse effects of -tubulin mutations on microtubule formation and function. *J. Cell Biol.* **106**, 1997-2009.
10. **Jacobs, C.W., A.E.M. Adams, P.J. Szaniszio, and J.R. Pringle,** 1988. Functions of microtubules in the *Saccharomyces cerevisiae* cell cycle. *J. Cell Biol.* **107**, 1409-1426.
11. **Kilmartin, J.V. and A.E.M. Adams,** 1984. Structural rearrangements of tubulin and actin during the cell cycle of the yeast *Saccharomyces*. *J. Cell Biol.* **98**, 922-933.
12. **Kim, H.B.,** 1993. Molecular cloning of the gene in *Schizosaccharomyces pombe* related to the *CDC3* gene in the *Saccahromyces cerevisiae*. *Kor. J. Microbiol.* **31**, 197-202.
13. **Kim, H.B.,** 1995. Sequence analysis of the *Schizosaccharomyces pombe* homologue of the *CDC3* gene in *Saccharomyces cerevisiae*. *Jour. Microbiol.* **33**, 350-354.
14. **Kim, H.B., B.K. Haarer, and J.R. Pringle,** 1991. Cellular morphogenesis in the *Saccharomyces cerevisiae* cell cycle. Localization of the *CDC3* gene product and the timing of events at the budding site. *J. Cell Biol.* **112**, 535-544.
15. **Kim, H.B., S.R. Ketcham, B.K. Haarer, and J.R. Pringle,** 1996. Establishment of cell polarity in yeast : Early events in the organization of the budding yeast. *Mol. Cell*

Biol. in press

16. **Kim, S.C., J.W. Jung, and H.B. Kim,** 1992. Studies on the organization of 10-nm filament ring in *Saccharomyces cerevisiae*. *Kor. J. Microbiol.* **30**, 333-338.
17. **Kurtzman, C.P. and C.J. Robnett,** 1991. Phylogenetic relationships among species of *Saccharomyces, Schizosaccharomyces, Debaromyces,* and *Schwanniomyces* determined from partial ribosomal RNA sequences. *Yeast* **7**, 61-72.
18. **Laemmli, U.K.,** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.
19. **Lowry, O.H., N.J. Rosebrough, A.L. Farr, and R.J. Randall,** 1951. Protein measurement with the folin phenol reagent. *J. Biol. Chem.* **193**, 265-275.
20. **Marks, J. and J.S. Hyams,** 1985. Localization of F-actin through the cell division cycle of *Schizosaccharomyces pombe*. *Eur. J. Cell Biol.* **39**, 27-32.
21. **Marks, J., I.M. Hagan, and J.S. Hyams,** 1986. Growth polarity and cytokinesis in fission yeast : the role of the cytoskeleton. *J. Cell Sci.* (Suppl.) **5**, 229-241.
22. **Novick, P. and D. Botstein,** 1985. Phenotypic analysis of temperature sensitive yeast actin mutants. *Cell* **40**, 405-416.
23. **Pringle, J.R. and L.H. Hartwell,** 1981. The *Saccharomyces cerevisiae* cell cycle. p. 97-142 J.N. Strathern, E. W. Jones, and J.R. Broach (ed.), *In* The molecular biology of the yeast *Saccharomyces* : Life cycle and inheritance. J.N. Strathern, E.W. Jones, and J.R. Broach, Cold Spring Harbor Lab. Cold Spring Harbor, N.Y.
24. **Pringle, J.R., R.A. Preston, A.E.M. Adams, T. Sterns, D.G. Drubin, B.K. Haarer, and E.W. Jones,** 1989. Fluorescence microscopy methods for yeast. *Meth. Cell Biol.* **31**, 357-435.
25. **Towbin, H., T. Staehelin, and J. Gordon,** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets : procedures and some applications. *Proc. Natl. Acad. Sci. USA* **76**, 4350-4354.