Immunofluorescence Localization of *Saccharomyces cerevisiae CDC3* Gene Product

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INTRODUCTION

The cell-division cycle of the yeast, *Saccharomyces cervisiae* involves a series of morphogenetic events including highly localized changes in the cell surface (Pringle & Hartwell, 1981; Cabib *et al.*, 1982; Pringle *et al.*, 1986). Cytoskeletal elements, including microtubules and actin (Adams & Pringle, 1984; Kilmartin & Adams, 1984; Novick & Botstein, 1985; Huffaker *et al.*, 1987, 1988; Jacobs *et al.*, 1988) seem to play important roles in these processes. In addition to these cytoskeletal elements, there is a highly ordered array of 10-nm diameter filaments that lies directly beneath the cytoplasmic membrane in the region of the mother-bud neck (Byers & Goetsch, 1976). By EM, these filaments seem to appear at the oneset of bud emergence and to disappear just before cytokinesis. Mutants defective in the *CDC3, CDC10, CDC11,* or *CDC12* genes lack these filaments and display a pleiotropic phenotype. Such mutants are defective in cytokinesis and proper chitin localization at the bases of buds, yet they continue the nuclear cycle and put forth multiple, elongated buds (Hartwell, 1971; Clarke & Carbon, 1980a; Pringle & Hartwell, 1981; Adams, 1984; Adams & Pringle, 1984).

All of four genes are cloned and DNA sequence analysis have now revealed that these four genes encode a family of proteins with similar amino acid sequence. These proteins show no extensive similarities to any known proteins (Haarer *et al.*, 1991).

Among them, *CDC3* gene is fused with *E. coli lacZ* and *trpE* genes and antibodies against the *CDC3* gene product are produced. These antibodies are used to check the localization of this product to the vicinity of the 10-nm filaments in the mother-bud neck.

MATERIALS AND METHODS

Genetic and Recombinant-DNA Manipulations;
Standard procedure were used for recombinant-DNA manipulations (Maniatis *et al.*, 1982), *E. coli* and yeast transformations (Hinnen *et al.*, 1989; Manitias *et al.*, 1989), and yeast genetic manipulations (Sherman *et al.*, 1986).

Plasmids, Strains, and Growth Conditions;
Plasmid pUR290 (Ruther & Muller-Hill, 1983) and *E. coli* strain BMH 71-18 (Messing *et al.*, 1977) were provided by R. Sheckman and were handled essentially as described previously (Ruther & Muller-Hill, 1983). Plasmid pATH2 was provided by T. Koerner and A. Tzagoloff and was propagated in *E. coli* strain RR 1 essentially as described previously (Maniatis *et al.*, 1982). Yeast strains were grown with rotary shaking in the rich-glucose-containing medium YM-P at 23℃. Strain TD4 was propagated in the appropriate selective minimal media either without plasmid or after transformation with appropriate plasmid. Temperature-sensitve cell-division-cycle mutant strains were grown to ~10^7 cells/m*l* at 23℃, than shifited to 36℃ either without dilution or by diluting 6-fold with fresh, prewarmed medium.

Gel Electrophoresis and Protein Blotting;
Sodium dodecyl sulfate polyacrylamide gels and the Laemmli buffer system were used for all protein electrophoresis. Proteins were blotted to nit-

rocellulose paper with a electroblotter with current of 1 A for 2 hrs. Blotted proteins were visualized by staining the nitrocellulose for 10 min in 0.2% Ponceau-S in 0.3% TCA and destained in H_2O.

Preparation of Antigens and Antibodies;

The *lacZ: CDC3* fusion protein was prepared from strain BMH 71-18 harboring fusion plasmid pUR-PH 1 (see results). Cells were grown overnight in LB medium plus ampicillin (50-100 ug/m*l*) at 37℃, then diluted 1:10 in the same medium and grown 1 h at 37℃. Isopropyl -D-thiogalactopyranoside (25 mg/m*l* in H_2O) was added to a final concentration of 100 ug/m*l*, and cultures were grown for two more hours at 37℃. Cells were then harvested, resuspended in loading buffer placed in a boiling water bath for 1 to 2 min, spun for 10 min in a microfuge at room temperature, and the resulting proteins were loaded on a polyacrylamide gel.

The *trpE: CDC3* fusion protein was prepared from *E. coli* strain HB101 containing plasmid pATH-PH8 (see results). These cells were grown overnight in M9CA medium containing ampicillin and 20 ug of tryptophan per m*l* and then diluted 1:10 in the same medium without tryptophan and grown at 36℃ for 1 hr. Indoleacrylic acid (1 mg/m*l* in ethanol) was added to a final concentration of 5 ug/m*l*, and cultures were frown for 2 more hrs. The fusion proteins were isolated in an insoluble fraction.

For immunization, fusion pretein bands from SDS polyacrylamide gels were collected and minced using a razor blade. About 2 m*l* of minced gel was injected with 2 m*l* of Freund adjuvant (Complete adjuvant for initial injections, incompleted adjuvant for all subsequent injections) and injected subcutaneously into New Zeland White rabbits. Boosters were given 6 to 7 weeks after primary immunization and periodically thereafter. Blood was collected 2 weeks after each booster injection and checked for titer using immunoblot technique.

Antibody Staning of Protein Blots;

Nitrocellulose strips of yeast or *E. coli* proteins were incubated for 30 min in blocking solution (5 % nonfat dry milk in PBS) and washed 5 min in TPBS (0.3% Tween 20 in PBS). They were incubated for 1 hr in primary antibody (1:30 diluted in TPBS) and washed for 10 min in TPBS. After washing, they were incubated for 1 hr in HRP -conjugated secondary antibody (1:200 dilution in TPBS) and washed 10 min in TPBS. To see the color reaction, strips were incubated 5 to 15 min in a solution containing one part 4-chloro-1-napthol (3 mg/m*l* in methanol) plus five parts 0.05% hydrogen peroxide in PBS. Reaction could be stopped by removing the substrate and rinsing the blots with distilled water. All steps were done at 22℃.

Affinity Purification of Antibody;

CDC3-specific antibodies were affinity purified from crude anti-*trpE: CDC3* antiserum using the *lacZ: CDC3* fusion protein blotted to nitrocellulose as described previously (Lillie & Brown, 1987).

Indirect Immunofluoresecence Microscopy;

Yeast cells were grown and fixed in 4% of formaldehyde in 40 mM of phosphate buffer (pH 6.5). They were washed 3 times with 1.2 M sorbitol in phosphate buffer using centrifugation and cell walls were removed by incubation with 1 m*l* of sorbitol/phosphate plus 55 ul of glusalase for 2 hrs at 36℃. After washing once, they were resuspended in sorbitol/phosphate. Fixed cells were attached on the 0.1% poly-L-lysine (size 400,000)-coated multitest slides, and incubated in primary antibody for 2 hrs at 22℃. After washing, cells were incubated for 2 hrs in FITC-conjugated secondary antibody at 22℃. They were checked under a immunofluorescence microscope.

RESULTS

Construction of *lacZ: CDC3* and *trpE: CDC3* Gene Fusions;

To create *lacZ: CDC3* fusion gene, the 1.3 kbp Pvu II-Hind III fragment from YEp-3PS1 was inserted into plasmid PUR 290, to yield plasmid pUR-PH 1 (Fig. 1C). To create a *trpE: CDC3* fusion gene, the same fragment was subcloned into the

Pvu II and Hind III sites of plasmid pATH2, yielding plasmid pATH-PH 8 (Fig. 1C). The DNA sequences of the linker regions of pUR290 and pATH 2 and of the *CDC3* gene (Haarer *et al.*, 1991) predict that these constructions will result in the in-frame fusion of *lacZ* or *trpE* coding sequences to *CDC3* coding sequence. As expected, induction of cells containing plasamid pUR-PH 1 results in the production of an "~160 kD fusion protein (Fig. 2A, lane 1-3). Similary, induction of cells containing plasmid pATH-PH 8 results in the production of an ~90 kD fusion protein (Fig. 2A, lane 4-6) when propagated under inducing conditions.

Preparation of *CDC3*-Specific Antibodies and Identification of the *CDC3* Protein;

An affinity-purified fraction was prepared from serum raised against the *trpE: CDC3* fusion protein (see Methods). Immunoblotting showed that this preparation was able to reconize the *lacZ: CDC3* and *trpE: CDC3* fusion proteins prepared from *E. coli* strains harboring the appropriate fusion plasmids (data not shown). Thus, the purified preparations appear to contain antibodies that react with the *CDC3* determinants common to both fusion proteins.

The *CDC3*-specific antibodies were then used to identify the *CDC3* protein in blots of yeast proteins. These antibodies reconized an 62 kD protein in blots of total protein from *CDC3* cells containing no plasmid (data no shown) or plasmid YEp 102 (Fig. 2B, lane 1). A stronger reaction at the same position was observed in extracts of the same strain harboring a high-copy-number plasmid containing the entire *CDC3* coding region (Fig. 2C, lane 3), suggesting that the 62 kD protein is the authentic *CDC3* gene product. In addition, a plasmid containing a truncated CDC3 coding region in the same strain directs synthesis of a 50 kD protein (Fig. 2B, lane 2).

Immunofluorescence Localization of the *CDC3* Gene Product

Use of affinity-purified anti-*trpE:CDC3* (Fig. 3A) or anti-*lacZ:CDC3* (data not shown) antibodies to stain wild-type yeast cells revealed that *CDC3* gene product is localized in the vicinity of the 10-nm filaments in the mother-bud neck. In cells with small buds, the *CDC3* gene product appeared in a ring at the base of the bud (Fig. 3A, cells a-c). In cells with medium or large buds, the *CDC 3* gene product appeared as a pair of rings flan-

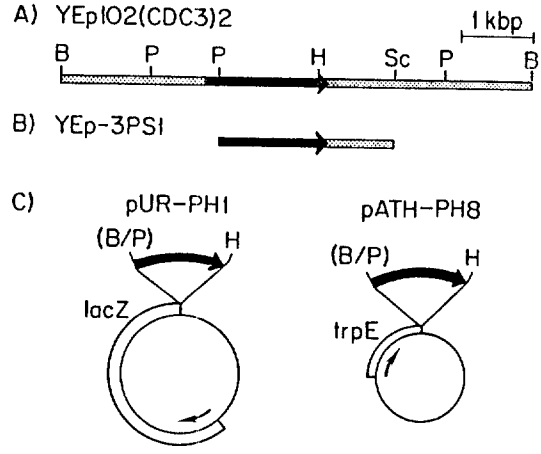

Fig. 1. Structures of *CDC3*-containing plasmid inserts and of *lacZ:CDC3* and *trpE:CDC3* gene-fusion plasmids. (A) A 6.7 kbp *Bam*HI fragment was subcloned from plasmid YEp13(CDC3) 1 into the pBR322-deriver *Bam*HI site of the yeast-*E. coli* shuttle vector YEp102 to produce the *CDC3*-complementing plasmid YEp102(CDC3) 2. (B) A 2.4 kbp *PvuII-Sca*I fragment was subcloned from YEp13(CDC3) 1 into the pBR322-derived *PvuII* site of YEp102 to produce the *CDC3*-complementing plasmid YEp-3PS1. (C) The 1.3 kbp *Pvu*II-*Hind*III fragment from YEp-3PS1 was inserted into the fusion vectors pUR 290 and pATH2 to produce plasmids pUR-PH1 and pATH-PH8, respectively. In each case, the vector was linearized with *Bam*HI, treated with the Klenow fragment of DNA polymerase to produce blunt ends, digested with *Hind*III, and then ligated to the gel-purified *Pvu*II-*Hind*III fragment. Thick arrows represent the *CDC3* coding region as inferred from DNA sequence analysis, and stippled boxes represent adjacent yeast DNA. Open boxes represent the *lacZ* and *trpE* coding regions and thin lines represent other *E. coli* DNA. Thin arrows indicate the directions of transcription for the fusion genes. Restriction sites are indicated: *B. Bam*HI; H, *HindIII; P, Pvu*II; Sc, *Sca*I; [B/P], *Bam*HI-*Pvu*II junction.

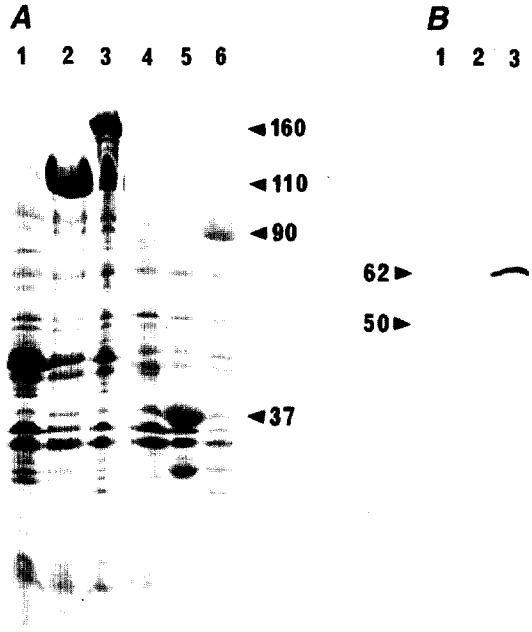

Fig. 2. Identification of *lacZ:CDC3* fusion proteins by SDS/polyacrylamide gel electrophoresis. Lanes 1-3: total proteins prepared from *E. coli* strain BMH 71-18 containing (lane 1) no plasmid; (lane 2) plasmid pUR290; or (lane 3) plasmid pUR-PH1. Lanes 4-6: total proteins prepared from *E. coli* strain HB101 containing (lane 4) no plasmid; (lane 5) plasmid pATH2; or (lane 6) plasmid pATH-PH8. In all cases, strains were propagated under inducing conditions as described in Materials and Methods. The approximate sizes of the truncated *lacZ* and *trpE* proteins encoded by the vectors and of the fusion proteins are given in kD. (B) Identification of the *CDC3* product in blots of yeast proteins using affinity-purified antibodies. Lanes 1-3: total cellular proteins were isolated from strain TD4 containing (lane 1) plasmid YEp102; (lane 2) plasmid YEp-3PS1; or (lane 3) plasmid YEp102(CDC3) 2. Primary antibody used for all lanes was a 1:50 dilution of affinity-purified anti-*trpE*: *CDC3*. Secondary antibody used was a 1:100 dilution of horseradish peroxidase-conjugated goat anti-rabbit IgG. The position of the putative wild-type *CDC3* protein at ~62 kD is indicated, as is the truncated product (at ~50 kD) encoded by plasmid YEp-3PS1. The Faint bands in lane 3 are presumed to be breakdown products of the overproduced 62 kD protein.

king the constriction of the mother-bud neck (Fig. 3A, cells d-f). This appearance is that expected for an antigen present in a structure, such as the ring of 10-nm filaments, that follows the contour of the cell membrane through the neck region; thus, densest packing (in a two-dimensional projection), and therefore brightest fluorescence, would occur on the slopes of the mother and bud portions of the cell flanking the neck constriction. Finally, many unbudded cells also appeared to possess rings of *CDC3* antigen (Fig. 3A, cells g-l), as considered further below.

Mutants defective in *CDC3* , *CDC10, CDC11,* or *CDC12* are unable to form or (at least for some alleles of *CDC10* and *CDC12*) maintain rings of 10-nm filaments at restrictive temperature (Byers & Goetsch, 1976; Adams, 1984). The pattern of *CDC3* product localization was examined in restrictive mutant strains. At permissive temperature, all four mutants displayed normal localization of the *CDC3* product (Fig. 3B, D, F), although the staining was somewhat diminished in *cdc3* (Fig. 3 B) and *cdc11* (strain JPT194-HO1; data not shown) cells. After 30 min at restrictive temperature, staining of *CDC3* product was not detectable in *cdc10* and *cdc12* cells (Fig. 3E, G) and was greatly diminished or absent in *cdc3* cells (Fig. 3C). *cdc11* cells showed only a minor reduction in staining intensity relative to the patern observed at permissive temperature (data not shown). Moreover, a *cdc4* mutant strain (which also forms multiple, abnormally elongated buds at restrictive temperature, but has normal rings of 10-nm filaments) displayed apparently normol localization of CDC3 product to the mother-bud necks even after several hours at restrictive temperature (Fig. 3H). Thus, the results obtained with the mutant strains are also consistent with the hypothesis that the *CDC3* product is a constituent of the ring of 10-nm filaments.

Electron Microscopic observations have suggested that the ring of 10-nm filaments appears coincident with bud emergence and disappears just before cytokinesis. To begin exploring the behavior of the filament-associated polypeptides at the beginning and end of the budding cycle, we examined in detail the patterns of staining observed

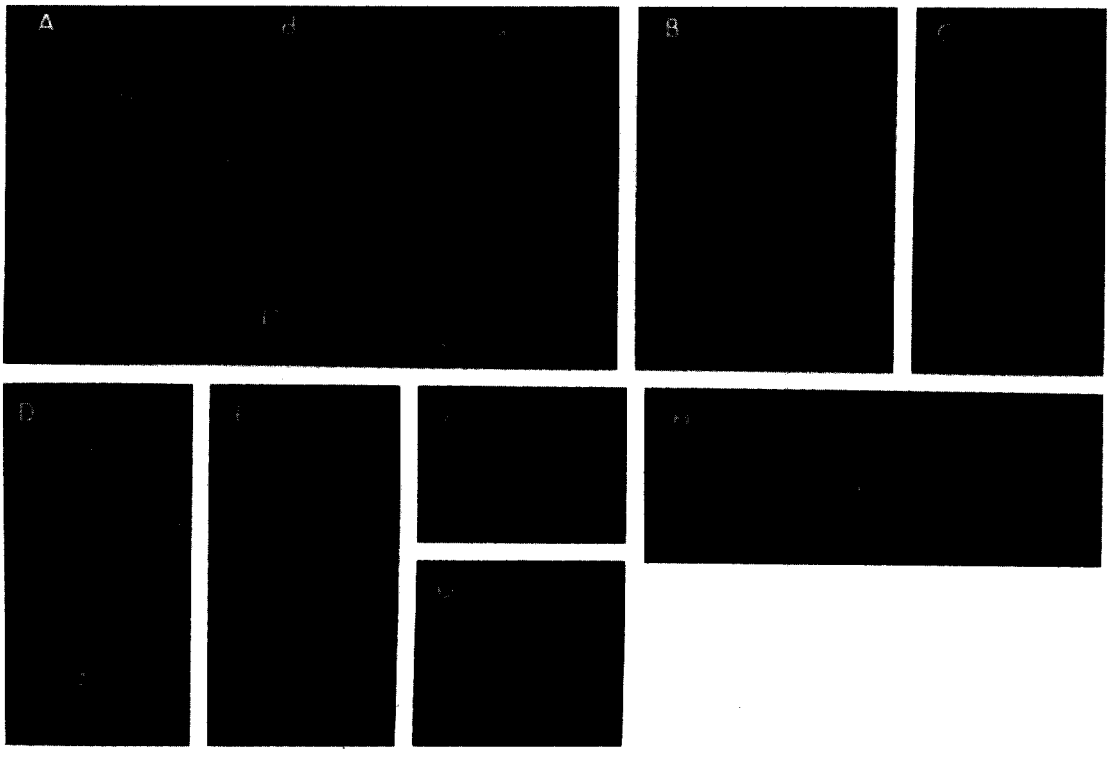

Fig. 3. Immunofluorescence localization of the *CDC3* gene product in wild-type and mutant cells. (A) C276 (wild-type) cells stained with anti-*trpE*:*CDC3* antibodies. Individual cells are labeled for reference in the text. (B, C) LH104-HO1 (*cdc3*) cells grown at ~23℃ (B) or at 36℃ for 30 min (C) and stained with anti-*trpE*:*CDC3* antibodies. (D, E) LH1701-HO1 (*cdc10*) cells grown at ~23℃ (D) or at 36℃ for 30 min (E) and stained with anti-*trpE*:*CDC3* antibodies. (F, G) JPTA1493-HO1 (*cdc12*) cells grown at ~23℃ (F) or at 36℃ for 30 min (G) and stained with anti-*trpE*:*CDC3* antibodies. (H) 314D5 (*cdc4*) cells stained with anti-*trpE*:*CDC3* antibodies after growth at 36℃ for 4 h. Affinity-purified rabbit anti-*trpE*:*CDC3* antibodies were used at 1:3 dilution; FITC-conjugated goat anti-rabbi IgG secondary antibody was used at 1:40 dilution. Cells were photographed and printed at somewhat different sizes to show optimum detail; magnifications are approximately 1300 to 2000 fold.

with *CDC3*-specific antibody in exponentially growing wild-type cells. In a sample of strain C276, 62% of the cells were budded as judged by phase-contrast microscopy. Immunofluorescence revealed detectable rings of *CDC3* antigen in 96% of the total population. When we attempted to score the unbudded cells alone, we detected rings of *CDC3* product in 93% of the cells. However, it should be noted that the latter counts are somewhat uncertain because small buds are difficult to see under the conditions used for immunofluorescence; thus, cells with small buds may be inadvertently included in the counts, with the effect of inflating the apparent proportions of unbudded cells that possess rings of *CDC3* antigen. Nonetheless, it seems clear that the *CDC3* product must (a) be organized into a ring structure before bud emergence; (b) linger in a ring structure after cytokinesis; or (c) both. Support for possibility (*c*) is provided by the observation that the rings of antigen observed in unbudded cells appear to be of two types: first, a relatively narrow-diameter, generally brightly staining structure [Fig. 3A, cells g, h, k(right-hand ring), and l (left-hand ring)], such as would be expected if the structures observed at the bases of small buds (Fig. 3A, cells a-c) actually appeared in advance of bud emergence; and second, a relatively broad-diameter, generally less

brightly staining structure [Fig. 3A, cells i, j, k (left-hand ring), and l (right-hand ring)], such as would be expected if the structures of cells e and f, Fig. 3A, were retained on mother and daughter cells after cytokinesis. The narrow and broad rings were present in ~35% and ~58%, respectively, of the apparently unbudded cells stained with *CDC3*-specific antibodies. In addition, a small number of apparently unbudded cells (and even some cells with small buds) displayed coincidently both a narrow ring and a broad ring when stained with *CDC3*-specific antibodies (Fig. 3A, cells a, c, k, l), providing further support for possibility (c), above.

DISCUSSION

In order to study the intracellular organization and function of the yeast *CDC3* gene product, we raised antibodies against the fusion proteins encoded by in-frame fusions of *CDC3* to the *E. coli lacZ* and *trpE* gene. Antisera raised against each fusion protein were able to recognize both fusion proteins; this both demonstrated the presence of *CDC3*-specific antibodies and facilitated their effective affinity purification. Immunoblotting experiments using the purified antibodies revealed a single polypeptide of ~62 kD in extracts from normal yeast cells and a more abundant polypeptide of the same mobility in extracts from cells harboring a high-copy-number plasmid that contains the complete *CDC3* coding region (Haarer *et al.*, 1991; Lillie *et al.*, 1991). The size of the presumed *CDC3* product agrees well with the 60 kD predicted from the sequence. In addition, the antibodies revealed an abundant polypeptide of ~50 kD in extracts from cells harboring a plasmid that appears to contain a *CDC3* gene that is truncated at its 5' end (Haarer *et al.*, 1991). The size of this polypeptide is consistent with the hypothesis that its translation begins at the first available ATG (codon 70 of the normal *CDC3* coding region). The retention of *CDC3*-complementing activity by this plasmid (Lillie *et al.*, 1991) suggests that the amino-terminal segment of the *CDC3* protein is nonessential.

In view of the similarities in predicted amino acid sequence among the *CDC3*, *CDC10*, *CDC11*, and *CDC12* products (Haarer *et al.*, 1991), it is noteworthy that the *CDC3*-specific antibodies, showed no apparent cross-reaction with the other three gene products. Although it is possible that further testing may reveal weak cross-rections, it seems likely that our immunofluorescence experiments have indeed revealed the localization of the *CDC3* product and not of a weakly cross-reacting species. As in previous studies (Lillie & Brown, 1987), affinity purification of the *CDC3*-specific antibodies was essential to avoid misleading immunofluorescence results due to other antibodies, present in all rabbit sera tested, that were reactive with yeast cell constituents.

Our result suggest that *CDC3* gene product is associated with the ring of 10-nm filaments in the mother-bud neck. This hypothesis is supported by the immunofluorescence localization of *CDC3* product in mutant strains. Localization of the *CDC3* product to the neck region was lost rapidly after a shift to restrictive temperature in *cdc10* or *cdc12* mutants, but not in *cdc11* or *cdc4* mutants, consistent with electron microscopic observations on the behavior of the 10-nm filaments in these strains (Byers and Goetsch, 1976; Adams, 1984). Results with the *cdc3* mutant were more difficult to interpret; staining of the neck region with *CDC3*-specific antibodies was lost rapidly after the shift to restrictive temperature, although the electron microscopic observations suggest that the 10-nm filaments should remain in place until completion of the cell cycle in progress at the time of the temperature shift. Presumably, either the mutant *CDC3* product remains in the filaments but becomes unreactive to the antibodies after the temperature shift, or it dissociates from the filaments without destroying their higher-order structure.

The most economical interpretation of the available data is that the *CDC3*, *CDC10*, *CDC11*, and *CDC12* gene products are the primary constituents of the 10-nm filaments. As the predicted amino-acid sequences of these polypeptides show

no extensive similarities to those of known filament-forming proteins, the yeast 10-nm filaments would thus represent a novel type of eukaryotic cytoskeletal element. However, it also remains possible that the *CDC3*, *CDC10*, *CDC11*, and *CDC12* products are all acessory proteins that associate with a filamentous structure composed primarily of some as yet unidentified, but more conventional, filament-forming protein.

The immunofluorescence observations appear to clarify the possible role of the 10-nm filaments in formation of the chitin ring. Such a role had been suggested by the evidence that the *cdc3*, *cdc 10*, *cdc11*, and *cdc12* mutants, which lack the 10-nm filaments, also fail to form normal chitin rings, whereas other *cdc* mutants such as *cdc4*, which possess apparently normal 10-nm filaments, also form normal-looking chitin rings (Fig. 3C, E, G, H). This correlation might mean either that chitin-ring formation is dependent on the 10-nm filaments or the reverse, but support for the former interpretation was provided by the observation (a) that the *CDC3* gene product localize to the vicinity of the 10-nm filaments, and (b) that the *cdc12* mutants lose preexisting filaments after a shift to restrictive temperature; taken together, these data suggest strongly that the primary lesions in these mutants involve formation of the 10-nm filaments, Moreover, a *chs1*: *URA3 chs2-1* mutant strain, which produces little or no chitin, nonetheless shows apparently normal localization of the *CDC3* product (our unpublished results), suggesting that filament formation is not dependent on the presence of a chitin ring. However, the putative role for the 10-nm filaments in formation of the chitin ring was difficult to reconcile with the electron microscopic evidence that the filaments only form as the bud is emerging, whereas the chitin ring is well developed by that time (our unpublished results). From our immunofluorescence studies, it now seems clear that at least some of the filament-associated polypeptides, if not actually the filaments themselves, are assembled at the budding site in time to play a role in chitin-ring formation.

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