

Subcellular Localization of a Protein Kinase Required for Cell Cycle Initiation in *Saccharomyces cerevisiae*: Evidence for an Association between the CDC28 Gene Product and the Insoluble Cytoplasmic Matrix

Curt Wittenberg,^{*‡} Stephanie L. Richardson,^{*} and Steven I. Reed^{*‡}

^{*}Biochemistry/Molecular Biology Section, Department of Biological Sciences, University of California, Santa Barbara, California 93106; and [‡]Department of Molecular Biology, Research Institute of Scripps Clinic, La Jolla, California 92037

Abstract. The product of the *Saccharomyces cerevisiae* gene *CDC28*, a protein kinase required for initiation of the cell division cycle, was localized within yeast cells. By using immunofluorescence methods, the *CDC28* product was shown to be primarily cytoplasmic in distribution. The gene product was localized largely to the particulate fraction by differential centrifugation after mechanical disruption in aqueous buffers. The particulate association was not affected by the presence of nonionic detergent. To refine this localization further, a procedure was developed for the preparation of yeast cytoplasmic matrices which resemble the cytoskeletons of vertebrate cells on the basis of methodology, immunochemistry, and gross ultrastructure. A portion of the *CDC28* product was found

to be tightly associated with these detergent-insoluble cytoplasmic matrices by both immunofluorescence and immunoblotting procedures. Although, for technical reasons, precise quantitation was not possible, it is estimated that a minimum of 2–15% of the total *CDC28* product pool is involved in the association with the insoluble matrix. Alcohol dehydrogenase, a soluble cytoplasmic protein, was found not to be associated with the cytoplasmic matrices at any detectable level, whereas, in contrast, ~10–40% of the total cellular actin, a bonafide cytoskeletal protein, was present in these structures. The proportion of *CDC28* gene product associated with the particulate fraction, and perhaps the insoluble matrix, appears to be substantially decreased during the preparation of spheroplasts.

MUTATIONAL analysis of the cell division cycle in *Saccharomyces cerevisiae* has revealed that cell cycle events require the expression of specific genes (12). One cell cycle event of special relevance to the control of division has been designated "start" (12). Both genetic and physiological studies suggest that this event, occurring in G₁, is a primary regulatory event for cell cycle initiation (5, 6, 16, 30). Start mutants, conditionally incapable of cell cycle initiation, have been used to identify initiation-specific genes and, ultimately, their products (30). Using genetic and molecular methods, it has been possible to isolate a number of start genes on recombinant plasmids and subject them and their encoded products to intensive analysis (3, 23, 25, 27, 31–34). The rationale for this approach is predicated upon the assumption that understanding how cell division is controlled will require detailed knowledge of the gene products that function in the process.

Dr. Wittenberg's and Dr. Reed's present address is Department of Molecular Biology, Research Institute of Scripps Clinic, 10666 North Torrey Pines Road, La Jolla, CA 92037; address reprint requests to Dr. Reed. This is Publication No. 4669MB from the Department of Molecular Biology, Research Institute of Scripps Clinic.

Temperature-sensitive mutants in the complementation group *cdc28* are classical start mutants in that, at the permissive temperature, they show normal cell division behavior whereas, at elevated temperatures, they are incapable of initiating new cell cycles. Cells in the process of dividing progress through the cell cycle until they reach start (12, 30). The lesion appears to be cell cycle-specific inasmuch as macromolecular synthesis is unimpeded in arrested cells (16). The *CDC28* gene was isolated from an *S. cerevisiae* recombinant plasmid library by transformational complementation of a *cdc28* mutation (25). Sequence analysis of the cloned gene provided a predicted primary structure for the *CDC28* product and revealed that the latter shared homology with a number of protein kinases (23). Using antisera prepared against the product of a chimeric gene containing part of the *CDC28* coding region, we have demonstrated that the *CDC28* product does in fact possess protein kinase activity (34). This finding suggests that this protein may participate in control of cell division as part of a signaling pathway involving protein phosphorylation events. At this time, the origins and ultimate targets of such a signal are unknown.

The purpose of the work reported here is to establish the

intracellular location of the *CDC28*-encoded protein kinase in the hope of elucidating the molecular components and cellular organization of division-related signaling in yeast. First, we observed that the *CDC28* gene product was localized primarily in the cytoplasm by immunofluorescence experiments and primarily to the particulate fraction by differential centrifugation followed by immunoblotting. Previous studies indicated that, in addition to cell cycle effects, *cdc28* mutants showed aberrations in morphology and in organellar motility and transmission (9, 12, 30; our unpublished observations). These phenotypes are suggestive of functions associated with the cytoskeleton in higher cells (2, 13, 21, 38, 39, 42). We therefore sought to determine whether the *CDC28* product was associated directly with the yeast cytoskeleton. Because the yeast cytoskeleton has not been extensively characterized, we proceeded by adapting methodology developed for cytoskeletal analysis in vertebrate cells (13) for use with *S. cerevisiae*. During the course of our studies we became aware of a similar approach being taken to isolate microtubule associated proteins from mitotic cells of *S. cerevisiae* (28). Although some similarities are clearly apparent, we cannot be certain that the structures we have prepared are precisely analogous to vertebrate cytoskeletons. We, therefore, refer to them as detergent-insoluble cytoplasmic matrices. The preparation of these structures and analysis of association of the *CDC28* gene product with them is described.

Materials and Methods

Strains and Media

All strains used in this study were derivatives of BF264-15D (*MAT α* , *leu2*, *trp1*, *adel*, *his3*) (34). A diploid derivative of BF264-15D, MMY13, and a diploid derivative of BF264-15D carrying the galactose-regulated allele of the *CDC28* gene, MMY53 (described below), were constructed by rendering haploid cells homothallic by transient transformation with a plasmid containing the gene *HO* (15) and allowing the transformant cultures to diploidize. Individual cell clones were then screened for the ability to sporulate to confirm that diploidization had occurred.

Construction of a congeneric diploid strain, MMY53, where the *CDC28* gene was placed under control of the regulatable *GAL1* promoter, is described in Fig. 2 (q.v.). The construction of the plasmid pFT1 was as follows. A *Bam*HI restriction site was introduced by oligonucleotide-directed mutagenesis (44) 10 base pairs upstream from the translation initiation codon of the *CDC28* gene on plasmid YRp7[*CDC28.4*] (33) to form plasmid YRp7[*CDC28.4*(*Bam*HI)]. A 4.4-kb *Xho*I-*Bgl*II fragment of this plasmid containing the *CDC28* coding region and the sequence conferring autonomous replication in yeast (*ARS*) were removed, and the plasmid was reclosed by treatment with DNA polymerase followed by joining of the resulting blunt ends using DNA ligase. This plasmid (YRp7[*CDC28.4*(*Xho*I-*Bgl*II)]) contains 5'-flanking sequences of the *CDC28* coding region now adjacent to the yeast *TRP1* gene. A 4.0-kb *Bam*HI-*Bgl*II fragment of YRp7[*CDC28.4*(*Bam*HI)] was then inserted into plasmid pBM258 (17) placing the *CDC28* gene under control of the yeast *GAL1* (galactokinase) promoter. A 4.1-kb *Eco*RI fragment from this plasmid containing the *GAL1*-*CDC28* hybrid gene was inserted into an *Eco*RI site adjacent to the *TRP1* gene of plasmid YRp7[*CDC28.4*(*Xho*I-*Bgl*II)] to create plasmid pFT1. This plasmid, which contains both 5'- and 3'-*CDC28*-flanking sequences surrounding the *CDC28* coding region under *GAL1* control, served as a source of DNA for transformational gene conversion.

Unless specified, cells were grown in YEPD medium, containing 1% yeast extract/2% Bacto-peptone/2% glucose, supplemented with 50 mg each of adenine and uracil per liter. YEPGal, used when galactose was required as the sole carbon source, was the same as YEPD except that 2% galactose was substituted for glucose. Minimal medium has been described (11).

Antibodies and Affinity Purification of *CDC28* IgG

Anti-yeast alcohol dehydrogenase (ADH)¹ and preimmune serum from the same rabbit were a gift of Dr. Jill Ferguson (8). Anti-yeast actin antiserum was a gift of Drs. Susan Brown (Research Institute of Scripps Clinic) and John Pringle (University of Michigan).

Preparation of anti-*CDC28* product antiserum (anti-*CDC28*) by immunizing rabbits using an *Escherichia coli* β -galactosidase-*CDC28* product hybrid protein isolated from *E. coli* has been described previously (31). Antibodies with specificity for this fusion protein were affinity purified. The fusion protein was gel purified as had been described (31) by separating crude bacterial lysate on preparative sodium dodecyl sulfate (SDS)-polyacrylamide gels and excising the appropriate band after brief staining with Coomassie Brilliant Blue followed by brief destaining with water. Whole gel slices were then sealed in dialysis tubing filled with Tris-glycinate running buffer without SDS. A number of these sealed gel slices were then submerged simultaneously in a chamber containing the same buffer across which 250 V were applied for 2 h. The polyacrylamide slices were then discarded and the eluted protein was dialyzed against 0.1 M ammonium bicarbonate overnight. The solution was concentrated using a concentration apparatus (Amicon Corp., Danvers, MA) and finally lyophilized. Approximately 400 μ g of protein was dissolved in 2 ml of 0.1 M NaHCO₃, pH 8.3, 0.5 M NaCl by boiling for 2 min followed by centrifugation to remove insoluble debris. This was then reacted with 1 g of cyanogen bromide-activated Sepharose 4B (Sigma Chemical Co., St. Louis, MO) as has been described by March et al. (24), yielding a 3.5-ml bed volume column. The column was washed with 10 vol of each of the following solutions in succession: 4.5 M MgCl₂; 1 M NaCl, 0.1 M acetic acid, pH 4.8; 1 M NaCl, 0.1 M NaHCO₃, pH 7.6; phosphate-buffered saline (PBS), pH 7.6. 3 ml of crude serum was then circulated through the column at 23°C for 2 h. The column was then washed with 10 vol of the following solutions in succession: PBS, pH 7.6; 1 M NaCl, 0.1 M acetic acid, pH 4.8; 1 M NaCl, 0.1 M NaHCO₃, pH 7.6; PBS, pH 7.6. Bound antibodies were eluted by applying 4.5 M MgCl₂. When the high-salt front appeared, two column volumes were collected and dialyzed against 0.14 M NaCl overnight at 4°C. The affinity-purified fraction was then dialyzed against PBS and finally concentrated to ~1 ml using an Amicon concentration apparatus. This gave an IgG solution of ~100 μ g/ml as determined by comparison to a dilution series of normal rabbit IgG on SDS-polyacrylamide gels. Normal rabbit IgG was used at this concentration for control experiments.

Anti-*CDC28* carboxy-terminal antisera (anti-*CDC28*₂₈₁₋₂₉₈) was directed against a synthetic octadecapeptide (provided by Dr. Ralph Arlinghaus, University of Texas, Houston) homologous to the predicted carboxy-terminal amino acid sequence of the *CDC28* gene product (23). This peptide was conjugated to keyhole limpet hemocyanin by glutaraldehyde crosslinking. Briefly, 5 mg of keyhole limpet hemocyanin was mixed with 5 mg of peptide in 0.25 M K₂HPO₄ buffer, pH 7.2, to a total volume of 0.7 ml. 4 μ l of 25% glutaraldehyde was added stepwise with shaking and the mixture was allowed to sit on ice for 30 min. The reaction mixture was then dialyzed overnight against 50 mM sodium phosphate buffer, pH 6.0. The peptide-carrier conjugate (the equivalent of 0.5 mg of peptide) was solubilized by bringing to 1% SDS and heating to 65°C for 5 min and then was diluted 10-fold in PBS. This solution was emulsified with an equal volume of either complete or incomplete Freund's adjuvant for the initial or subsequent injections, respectively, and then used to immunize rabbits. Affinity purification of the anti-*CDC28*₂₈₁₋₂₉₈ IgG was accomplished by the method described above except that the affinity matrix consisted of the carboxy-terminal peptide conjugated to cyanogen bromide-activated Sepharose 4B.

Both of the affinity-purified antisera (anti-*CDC28* and anti-*CDC28*₂₈₁₋₂₉₈) react with a polypeptide of 34 kD on immunoblots (unpublished results). The discrepancy between the published molecular mass of the in vitro translation product observed by immunoprecipitation with the anti-*CDC28* antiserum (31) and that described here can be attributed solely to differences in the molecular mass standards used in these two studies. The anti-*CDC28*₂₈₁₋₂₉₈ antiserum is a higher titer antiserum and gives a substantially stronger signal on immunoblots.

Electrophoresis and Immunoblotting

Samples for electrophoresis were prepared by suspending whole cells or cytoplasmic matrices in an equal volume of twice-concentrated gel sample

1. *Abbreviations used in this paper:* ADH, alcohol dehydrogenase; DAPI, 4',6-diamidino-2-phenylindole.

buffer (20) and vortexing three times for 1 min with an equal volume of 0.45-mm glass beads (B. Braun Instruments, Burlingame, CA). The samples were then boiled for 3 min and the glass beads and cellular debris were removed by centrifugation. The proteins were resolved on SDS polyacrylamide gels (20) and either stained with Coomassie Brilliant Blue R or used for immunoblotting.

Immunoblotting experiments were performed as described (41). Nitrocellulose was blocked overnight with 5% nonfat dry milk (Carnation) and 5% bovine serum albumin (BSA) in Tris-buffered saline (20 mM Tris, pH 7.5, 0.15 M NaCl) after electrophoretic transfer. Nitrocellulose strips were incubated overnight with anti-*CDC28*₂₈₁₋₂₉₈, anti-yeast alcohol dehydrogenase or anti-yeast actin in Tris-buffered saline with 1% BSA followed by ¹²⁵I-Protein A (ICN National Biochemicals, Cleveland, OH) or alkaline phosphatase-coupled goat anti-rabbit IgG (Promega Biotec, Madison, WI) as indicated. Antisera were used at 1:200, 1:5,000, and 1:5,000, respectively. Strips were washed five times for 5 min in Tris-buffered saline after each antiserum incubation. Autoradiography was performed at -70° with Kodak GPB film (Eastman Kodak Co., Rochester, NY) and a Cronex Lightning Plus intensifying screen. (DuPont Co., Wilmington, DE) or color development was performed according to the manufacturer's instructions. Quantitation of signals on immunoblots, where reported, was by densitometry using an Ultrascan XL laser densitometer (LKB Instruments, Inc., Gaithersburg, MD).

Preparation of Cytoplasmic Matrices

Cells were grown to an approximate density of $2-4 \times 10^7$ /ml and harvested by centrifugation. Cells were then resuspended in one-tenth the original culture volume of wall removal buffer: 0.2 M Tris-HCl, pH 9.4, 0.02 M EDTA, 1% 2-mercaptoethanol. After 10 min at 23°C, cells were collected by centrifugation and resuspended in an equivalent volume of YEPD medium stabilized with 1 M sorbitol and adjusted to 0.01 mg/ml chitinase (Sigma Chemical Co.), 0.02 mg/ml zymolyase (Kirin Brewery, Tokyo, Japan) and 1% glucosylase (Endo Laboratories, New York). Incubation was at 30°C for 15–30 min. Wall removal was monitored as a function of susceptibility to lysis in 5% SDS. When cells became uniformly spheroplasted, they were collected by centrifugation at 23°C and washed once in stabilization buffer with 1 M sorbitol. Stabilization buffer is 0.15 M Pipes, pH 6.9, 0.1 mM EGTA, 0.5 mM MgCl₂, 1 mM phenylmethylsulfonyl chloride, containing pepstatin A and leupeptin at 1 µg/ml and aprotinin at 1:2,000 dilution. Preparation of cytoplasmic matrices was essentially as described by Heuser and Kirschner (13). Spheroplasts were resuspended in one tenth the original culture volume of stabilization buffer with 0.2% Triton X-100, pelleted immediately, and resuspended in the same buffer with 0.1 mg/ml bovine pancreatic DNase I for 60 min at 23°C. Cytoplasmic matrices were then washed once by resuspension in stabilization buffer with Triton X-100 followed by recentrifugation, and finally resuspended and washed twice in stabilization buffer without detergent.

Fluorescence Microscopy of Yeast Cells and Cytoplasmic Matrices

Approximately 10^9 log-phase cells were collected by centrifugation and resuspended for 10 min in wall removal buffer (0.2 M Tris-HCl, pH 9.4, 0.02 M EDTA, 1% 2-mercaptoethanol), followed by centrifugation and resuspension in stabilization buffer brought to 3.7% formaldehyde. After fixation at 23°C for 30 min, cells were washed two times by centrifugation through stabilization buffer–1 M sorbitol and then resuspended in 10 ml of stabilization buffer–1 M sorbitol to which were added 150 µg of zymolyase, 50 µg of chitinase and 35 µl of glucosylase. Incubation was for ~30 min at 23°C and permeabilization was monitored by loss of refractility when cells were suspended in an equal volume of 10% SDS. Spheroplasts were washed two times in stabilization buffer–1 M sorbitol and then resuspended in methanol at -20°C and incubated at that temperature for 5 min. After pelleting, the spheroplasts were resuspended in acetone at -20°C and then pelleted immediately. After resuspension in PBS made 1 mg/ml with BSA (Sigma Chemical Co.; Fraction V) (PBS-BSA), fixed spheroplasts were divided among four 1.5-ml microfuge tubes for reaction with antisera. Each tube was used for a staining experiment. Pellets were resuspended in 20 µl of 1:4 affinity-purified anti-*CDC28*_r product IgG or 1:150 anti-ADH serum. All dilutions were with PBS-BSA. Reaction was for 1–2 h at 23°C. Five washes were performed by resuspension of the cells in 1 ml of PBS-BSA followed by centrifugation for 1 s using an Eppendorf microfuge (Brinkmann Instruments Co., Westbury, NY). Pellets were then resuspended in 20 µl

of 1:16 affinity-purified fluorescein-conjugated goat anti-rabbit IgG (Miles Laboratories, Inc., Naperville, IL). Incubation was for 1 h in the dark followed by five washes in PBS-BSA. Pellets were resuspended in 97% glycerol/3% PBS containing the antibleaching agent *p*-phenylenediamine as described by Adams and Pringle (1). Samples at this point could be stored at -20°C. Samples were mounted and then observed and photographed using a Zeiss Photomicroscope II fitted with epifluorescence and a ×100 Planapo oil immersion objective. Tri-X Pan film was developed using Diafine (Eastman Kodak Co.). Photomicrographs are shown at a magnification of ~1,500.

Cytoplasmic matrices prepared as described were resuspended in methanol at -20°C and incubated for 5 min. After pelleting and resuspension in PBS-BSA, they were stained with antibodies as described for whole cells. Antibody-stained cytoskeletons were then fixed by resuspension in stabilization buffer brought to 3.7% formaldehyde and incubation for 30 min at 23°C. Alternatively, cytoplasmic matrices without methanol extraction were stained for actin using rhodamine-phalloidin (Molecular Probes, Eugene, OR; 25 U/ml in PBS for 1 h) or for DNA using 4',6-diamidino-2-phenylindole (DAPI) (Sigma; 10 µg/ml for 30 min). For staining with DAPI, matrices were prefixed in 3.7% formaldehyde for 15 min before addition of the dye. In all cases, several washes in PBS-BSA were performed before mounting. Mounting, observation, and photography were as described for whole, fixed cells except that for phalloidin staining, *n*-propyl gallate at 0.2% was used in the mounting solution to retard photobleaching.

Electron Microscopy of Critical Point-dried Cytoplasmic Matrices

Preparation of cytoplasmic matrices for electron microscopy (EM) was by procedures adapted from those developed for visualization of the three-dimensional structure of animal cell cytoskeletons (18, 43). Cytoplasmic matrices prepared as described above were fixed by resuspension in stabilization buffer containing 3% EM grade glutaraldehyde and then washed two times by centrifugation through Sorenson's phosphate buffer (pH 6.8). Fixed matrices were then resuspended in the same buffer containing 1% OsO₄ for 1 h followed by two washes in buffer without OsO₄ and one wash with water. The matrices were then placed on Formvar-coated grids which had been previously coated with 0.1% poly-L-lysine and allowed to settle by gravity. The grids were then dehydrated by submersion in water followed by a graded ethanol series (30%, 50%, 70%, 90%, and three changes of 100%) for 5 min each. It is critical that the cytoplasmic matrices not dry at any point during the procedure as this results in a collapse of the three-dimensional structure. Staining of the matrices was achieved by including 1% uranyl acetate in the 70% ethanol used in the graded ethanol series. The three 100% ethanol washes were followed by critical point drying in a Samdra-78 dryer (Tousimis Research Corp., Rockville, MD). The resulting critical-point-dried cytoplasmic matrices were photographed by transmission EM at the magnifications indicated in the figure legends.

Results

Localization of *CDC28* Product in Whole Cells

We first sought to determine the location of the *CDC28* product by immunofluorescence in whole, fixed yeast cells. A diploid strain was chosen for these experiments rather than a haploid strain in order to exploit the larger cell size for resolution of detail. Fig. 1, *a* and *b* shows fluorescence micrographs of diploid cells stained with anti-*CDC28*_r product IgG and an equivalent concentration of normal rabbit IgG, respectively. A general cytoplasmic staining pattern was observed using anti-*CDC28*_r product IgG (*a*) but not in the control panel (*b*). To insure that the IgG was reactive specifically with the *CDC28* gene product and not another immunologically cross-reactive species, two additional control experiments were performed. A congenic diploid strain where the *CDC28* gene had been placed under control of the inducible yeast *GAL1* promoter was used. The methodology used to construct this strain is presented in Fig. 2 (see

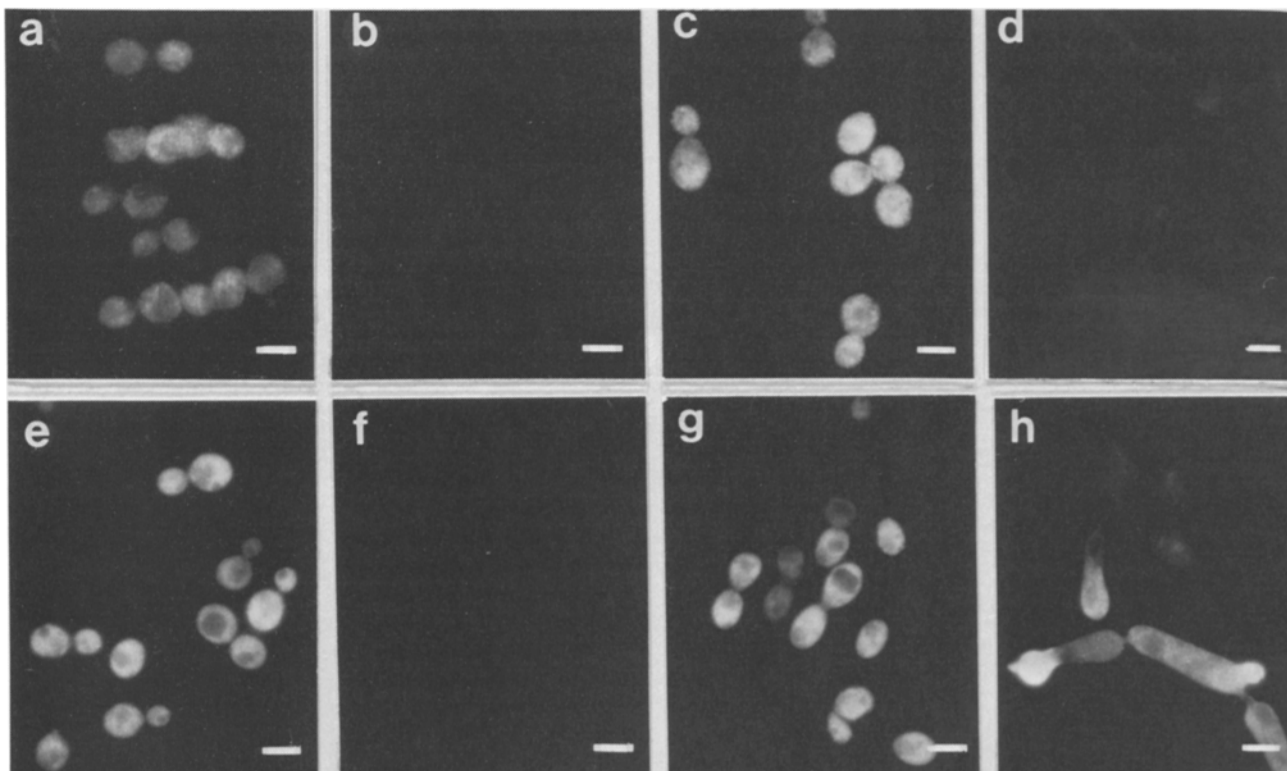


Figure 1. Immunofluorescence staining of fixed yeast cells. (a) Normal diploid cells (MMY13) stained with affinity-purified anti-*CDC28* product IgG. (b) Same cells stained with equivalent concentration of normal rabbit IgG. (c) Diploid cells with *CDC28* under *GAL1* promoter control (MMY53) grown in galactose medium and stained with anti-*CDC28* product IgG. (d) Same cells shifted to glucose medium for 36 h prior to staining with anti-*CDC28* product IgG. (e) Normal diploid cells stained with anti-yeast alcohol dehydrogenase serum. (f) Same cells stained with preimmune serum. (g) MMY53 (galactose-regulated *CDC28* allele) grown in galactose medium stained with anti-ADH serum. (h) MMY53 shifted to glucose medium for 36 h prior to staining with anti-ADH serum. For both the *CDC28* product and ADH series, the exposure time was automatically set for normal diploid cells stained with immune sera. All other exposures in each series were manually normalized to this time. The second antibody in each case was fluorescein-conjugated (FITC) affinity-purified goat anti-rabbit IgG. Bars, 10 μ m.

Materials and Methods. When these cells are grown with galactose as the sole carbon source, they contain ~ 100 times the wild-type level of *CDC28* product based on both densitometry of immunoblots and biological activity (the ability to support cell division). These results are predicted by a comparison of the estimated efficiencies of the *CDC28* and *GAL1* promoters (31, 40). When cells are shifted to medium containing glucose as the sole carbon source, synthesis of *CDC28* mRNA is terminated, and the preexisting protein is expected to be lost by turnover and dilution. The latter is confirmed because the cells cease dividing and become extremely morphologically aberrant after approximately seven generations of growth on glucose (data not shown). Anti-*CDC28*_r product staining of cells in the overexpression and "depleted" states described above is compared to staining of normal wild-type cells in Fig. 1, c and d, respectively. As can be seen, galactose-grown cells stain much more intensely than the normal diploid whereas glucose-shifted cells, although at least 10 times larger than normal cells, show little staining at all. To substantiate that the differences observed were not a staining artifact attributable to diverse metabolic states of the cells under the various growth conditions, preparations of the same cells shown in Fig. 1, a-d were stained with anti-yeast ADH antiserum (Fig. 1, e, g, and h) and preimmune serum (Fig. 1). It is not anticipated

that levels of ADH should change substantially and this is confirmed. In the case of glucose-shifted *CDC28*-depleted cultures most of the alcohol dehydrogenase staining appears to be localized near the ends of the rod-shaped cells. Although the reason for this is not known, it is conceivable that the rest of the cell in this terminal state consists mostly of endolytic vacuoles. The fact that the increase in anti-*CDC28* product immunofluorescence signal is not proportional to the extent of increase in *CDC28* gene expression observed in the presence of galactose can be explained by the semiquantitative nature of immunofluorescence. The immunofluorescence signal is not expected to increase in a linear fashion over a 100-fold range of antigen concentration. Based on these observations, we conclude that the *CDC28* product is primarily cytoplasmic in its distribution.

To determine whether the *CDC28* gene product is in a particulate or a soluble form, differential centrifugation of whole-cell lysates was performed. Cells were mechanically disrupted by vortexing with glass beads in 50 mM Tris, pH 7.0. Cell lysis was determined to be $>99\%$ by microscopic examination. Particulate fractions were prepared by sedimentation at 5,000, 20,000, or 100,000 g. These fractions, as well as the soluble supernatant fraction, were examined by immunoblotting for the presence of the *CDC28* gene product (Fig. 3, top panel, lanes 1-4, respectively). The

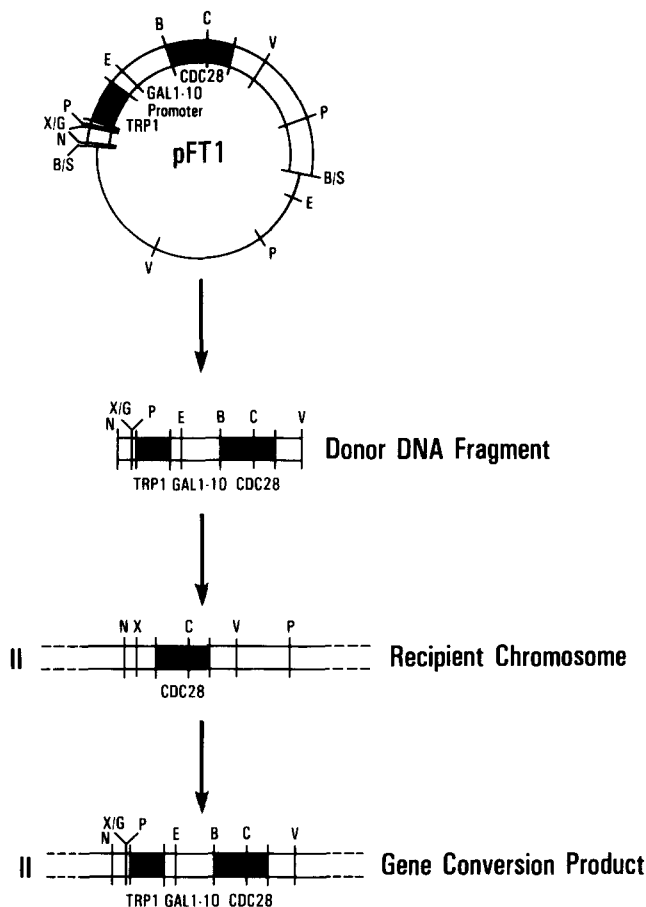


Figure 2. Construction of a yeast strain in which *CDC28* expression is regulated in response to carbon source. The construction of the plasmid pFT1, carrying the *CDC28* gene under control of the yeast galactose promoter, is described in Materials and Methods. The galactose inducible allele of *CDC28* was then inserted into haploid yeast strain BF264-15D (33) in place of the endogenous *CDC28* gene by fragment-mediated transformation (35) with a 3.1-kb *NcoI*-*PvuII* fragment from the plasmid, pFT1. The 3.1 kb *NcoI*-*PvuII* fragment from plasmid pFT1 carries the 5' flanking region of *CDC28*, the yeast *TRP1* gene capable of complementing a *trp1* mutation, the *CDC28* coding region under control of the *GAL1* promoter and the 3' flanking region of *CDC28*. Transformants were selected on the basis of the conversion of the tryptophan auxotroph BF264-15D to prototrophy (37). The structure of the wild-type *CDC28* locus is depicted as well as the structure of the chromosomal locus after gene conversion by the galactose-inducible *CDC28* fragment. The diploid derivative of the resulting strain (MMY53) was constructed as described in Materials and Methods. The solid single lines represent pBR322 DNA sequences, double lines represent yeast DNA sequences, and bold lines represent yeast protein-coding regions. The dashed lines represent chromosomal DNA sequences. Restriction endonuclease cleavage sites are denoted as follows: B, *Bam*HI, C, *Sac*I, E, *Eco*RI, G, *Bgl*II; N, *Nco*I; P, *Pst*I; S, *Sau*3A; V, *Pvu*II; X, *Xho*I. Standard procedures were employed for manipulation of DNA.

presence of alcohol dehydrogenase was also monitored as a control for efficient lysis and washing (bottom panel, lanes 1–4, respectively). Greater than 70% of the gene product sediments in the 5,000- and 20,000-g fractions, with <20% in either the 100,000-g pellet or the soluble supernatant. In contrast, the pellet fractions are devoid of detectable soluble

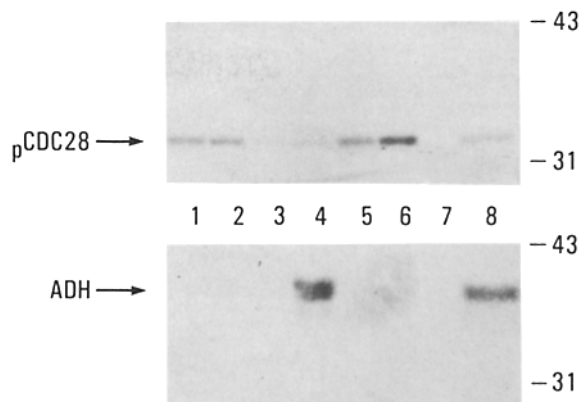


Figure 3. Analysis of *CDC28* gene product associated with particulate and soluble fractions of mechanically disrupted cells. Cells were disrupted by vortexing with glass beads either in 50 mM Tris, pH 7.0, on ice (lanes 1–4) or in stabilization buffer containing 0.2% Triton X-100 at 23°C (lanes 5–8). Each cell lysate was then fractionated by consecutive centrifugations at 5,000 g for 7 min (lanes 1 and 5), 20,000 g for 30 min (lanes 2 and 6), and 100,000 g for 90 min (lanes 3 and 7). Each pellet was washed once by resuspension in the same buffer followed by recentrifugation at the same velocity. The proteins in the pellet fractions and the remaining supernatant fractions (lanes 4 and 8) were solubilized in SDS-polyacrylamide gel electrophoresis sample buffer and separated by electrophoresis through a 4%/8.5% discontinuous SDS-polyacrylamide gel followed by immunoblotting using anti-*CDC28*_{281–298} antiserum (upper panel) or anti-ADH antiserum (lower panel). All lanes represent the material from 2×10^7 cells. Molecular weight markers are actin and carbonic anhydrase. Molecular weights given are $\times 10^{-3}$.

protein as judged by the absence of ADH. This confirms that these fractions do not contain an appreciable number of unlysed cells or trapped soluble protein. Furthermore, the *CDC28* gene product associated with the 5,000- and 20,000-g pellets is not released when the pellet is washed with 1.0% Triton X-100 (not shown), suggesting that it is not associated with membrane. In fact, when the same fractionation scheme is performed using a buffer commonly used for the preparation of vertebrate cytoskeletons (discussed below), containing 0.2% Triton X-100 for the disruption of cellular and organellar membranes, a similar result is observed (Fig. 3, lanes 5–8). The only qualitative difference in these results is the absence of the *CDC28* product in the 100,000-g fraction (lane 7) and the possibility of a slight amount of contamination with soluble protein in the 20,000-g fraction (lane 6). These same results are observed when Triton-X100 is absent from the buffer (data not shown). We conclude that under each of these conditions the *CDC28* gene product is associated with particulate, nonmembranous structures. In addition, because most of the ribosomes sediment in the 100,000-g fraction (36), it is doubtful that a significant portion of this particulate *CDC28* gene product is ribosome-associated.

Preparation of Detergent-insoluble Cytoplasmic Matrices

Considering the general cytoplasmic staining observed by immunofluorescence experiments along with localization to

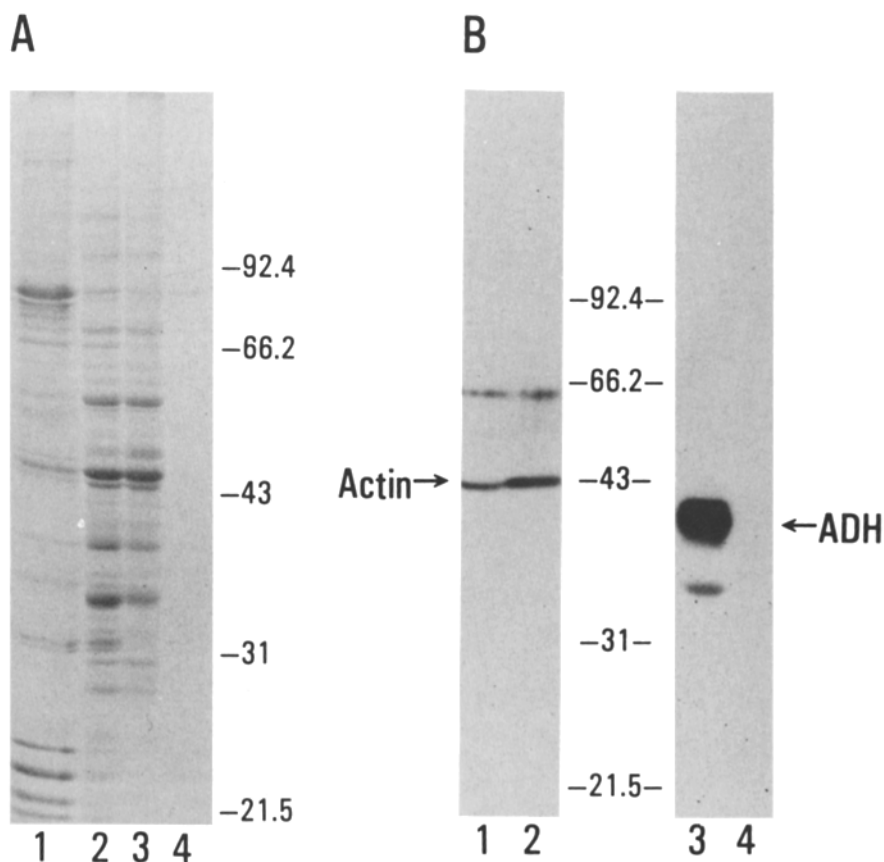


Figure 4. (A) Analysis of proteins in cytoplasmic matrices. Cells were spheroplasted and extracted with detergent as described. Cytoplasmic matrix, whole spheroplast, and extraction supernatant fractions were homogenized by vortexing with glass beads and boiled in SDS-polyacrylamide gel sample buffer prior to electrophoresis through a 4%/8.5% discontinuous SDS-polyacrylamide gel. The gel was then stained with Coomassie Brilliant Blue R, destained, and photographed. Lanes 2, 3, and 4 correspond to cell number equivalents (approximately 1×10^7 cells/sample) of spheroplast, extraction supernatant, and matrix fractions, respectively. Lane 1 corresponds to matrix fraction proteins from 10 times as many cells as the other lanes. (B) Analysis of cytoplasmic matrix components by immunoblotting. Sample preparation, electrophoresis, and immunoblotting were as described. Whole cell and cytoplasmic matrix samples (2×10^7 and 6×10^7 cells/lane, respectively) were stained with anti-yeast actin (lanes 1 and 2, respectively) or anti-yeast ADH (lanes 3 and 4, respectively). The ADH signal was intentionally overexposed to emphasize the absence of ADH in the cytoplasmic matrices. Molecular weight standards are phosphorylase *b*, BSA, actin, carbonic anhydrase, and soybean trypsin inhibitor. Values given are $\times 10^{-3}$.

the particulate fraction in cell lysates, we chose to examine the yeast cytoplasmic matrix as a potential site of association of the *CDC28* gene product. In vertebrate cells, the cytoplasmic matrix or cytoskeleton is defined as the residual structure after detergent extraction of cells (4, 13, 22, 43). Adaptation of the methods used with vertebrate cells for use with yeast required several important modifications. First, because intact yeast cells are resistant to detergent extractions as a result of a dense protective cell wall, enzymatic pretreatment is required. We found that after a short treatment with a mixture of several hydrolytic enzymes, the cells, presumably now spheroplasts, became accessible to extraction with detergent. This was the case even though, based on both light and electron microscopy, the cells were still mostly encased in a matrix of, now presumably porous, cell wall material. Next, because yeast cells do not attach to a substratum, as do most vertebrate cells used in cytoskeletal studies, a principal modification involved the use of centrifugation to perform detergent washes and other extractions. Here, the remaining cell wall matrix proved to be useful in that it protected the material inside from the mechanical shock associated with repeated centrifugation, although it appeared to present no barrier to the extraction of detergent-soluble proteins. This was crucial to the procedure as demonstrated by the fact that, when the same extraction procedures described below are applied to true protoplasts (devoid of all cell wall material), the remaining material becomes condensed and intractable to further manipulation or analysis.

Accordingly, spheroplasts were prepared and extracted with 0.2% Triton X-100 according to a modification of the procedure of Heuser and Kirschner (13) followed by treatment with DNase I as described in Materials and Methods. Such structures rapidly lose most of their protein content (Fig. 4). Fig. 4A summarizes the gross biochemical results of detergent extraction of spheroplasts. Proteins from whole yeast cells, Triton X-100 extraction supernatant, and extracted cytoplasmic matrices were analyzed by SDS-polyacrylamide gel electrophoresis. Lanes 2 and 3, equal cell equivalents of whole-cell lysate and detergent supernatant, respectively, indicate that all of the most abundant cellular proteins are accounted for quantitatively in the extraction supernatant. Lane 4 contains an equal cell equivalent of detergent-insoluble cytoplasmic matrices and serves to demonstrate that most of the cellular protein has been removed in the process. Lane 1 displays an approximate protein equivalent of the matrix sample. It is obvious that the cytoplasmic matrix is composed of a small number of major polypeptides and a larger number of minor species. Furthermore, several of the proteins enriched in the matrix fraction (lane 1) are visibly depleted in the supernatant fraction (lane 3). The polypeptide composition of the matrix bears no resemblance to that seen for whole cells and close scrutiny of individual bands suggests that most of the principal matrix species are, at best, minor species in the cell, but are highly enriched in this structure. The identities of the majority of polypeptides present in the cytoplasmic matrix fraction are

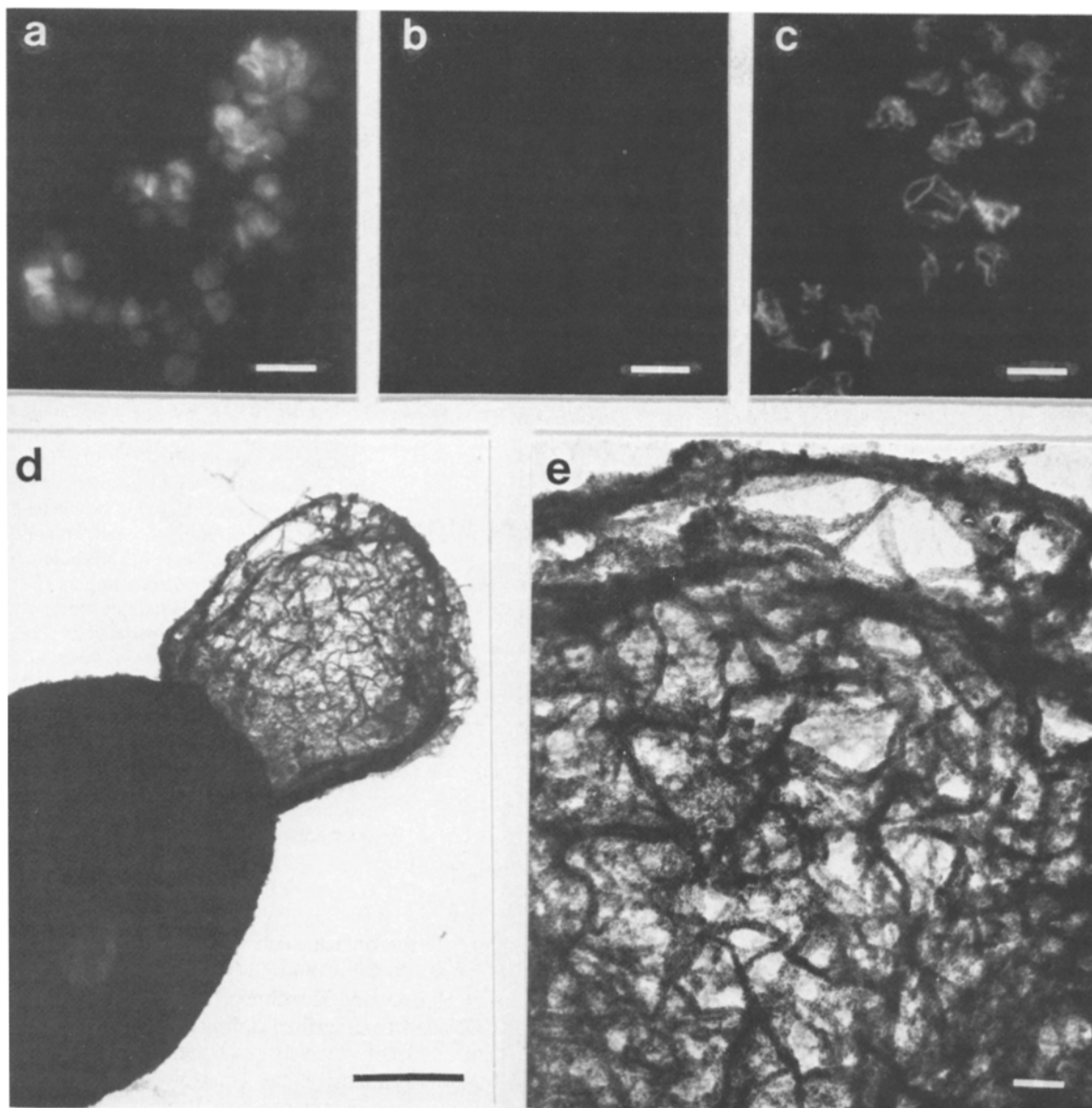


Figure 5. Characterization of yeast cytoplasmic matrices. (a) Detergent-extracted spheroplasts prior to DNase I digestion stained with DAPI. Chromatin is seen as a diffusely stained area in cytoplasmic matrices. (b) Detergent-extracted, DNase I-treated cytoplasmic matrices stained with DAPI. (c) Detergent-extracted, DNase I-treated cytoplasmic matrices stained with rhodamine-phalloidin. (d and e) Electron micrographs of whole mounts of critical-point-dried cytoplasmic matrices. Bars: (a–c) 5 μ m; (d and e) 1 and 0.1 μ m.

unknown. The majority of tubulin is lost during the preparation of these structures (de Barros Lopes, M., C. Wittenberg, and S. Reed, unpublished observations). Fluorescence microscopy using the DNA-specific dye DAPI indicates that a significant portion, if not all of, the nuclear DNA remains in the structures prior to treatment with DNase I (Fig. 5 a). Treatment with DNase I, a procedure routinely incorporated into our preparation of insoluble cytoplasmic matrices, removes the DNA (Fig. 5 b), thus averting the concern that chromatin in the structures might contribute to nonspecific adhesion of soluble proteins.

The presence of actin in these cytoplasmic matrices is demonstrated both by fluorescence microscopy using rhodamine-tagged phalloidin (Fig. 5 c) and by immunoblotting

(discussed below). An organized network of detergent-resistant actin bundles is observed. Cortical actin spots, seen when whole, fixed cells are stained (1, 19, 26, 32), are not seen in cytoplasmic matrices. The reason for this selective extraction is not known. The presence of actin is demonstrated on a biochemical level by immunoblots of whole-cell and cytoplasmic matrix polypeptides reacted with anti-yeast actin antiserum. Such an experiment is shown in Fig. 4 B (lanes 1 and 2). A strongly reacting band is observed in both whole-cell and cytoplasmic matrix preparations which comigrates with vertebrate actin as previously demonstrated for authentic yeast actin by Greer and Scheckman (10). This result corroborates those observed with rhodamine-labeled phalloidin. DNase I treatment has no significant effect on the

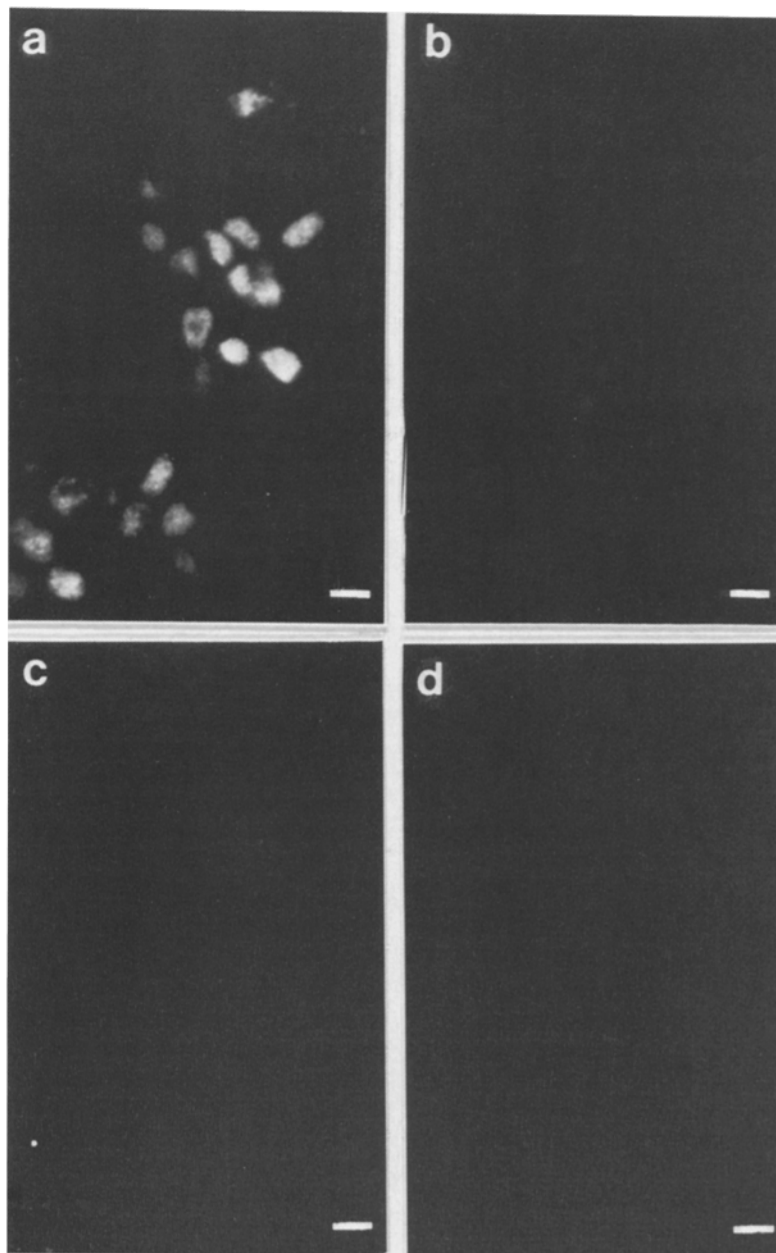


Figure 6. Immunofluorescence analysis of yeast cytoplasmic matrices. (a) Cytoplasmic matrix preparation stained with affinity-purified anti-*CDC28* product IgG. (b) Cytoplasmic matrix preparation stained with normal rabbit IgG. (c) Cytoplasmic matrix preparation stained with anti-yeast ADH serum. (d) Cytoplasmic matrix preparation stained with anti-ADH preimmune serum. Second antibody in all cases is fluorescein-conjugated (FITC) affinity-purified goat anti-rabbit IgG. Bars, 10 μ m.

actin signal on immunoblots or on the structure observed by fluorescence microscopy with rhodamine-tagged phalloidin (see Discussion).

To confirm that the cytoplasmic matrices are devoid of soluble proteins, and that a protein known to be soluble does not form a stable association with them, matrices were examined for the presence of ADH by immunoblotting. The results of an experiment comparing the level of ADH in whole-cell and cytoplasmic matrix extracts is shown in Fig. 4 *B*, lanes 3 and 4. Proteins extracted from whole cells (lane 3) react strongly with the anti-ADH antisera while there is no detectable ADH signal in cytoplasmic matrices from three times as many cells (lane 4). We estimate that <0.1% of the amount of ADH present in whole cells could have been detected by this analysis.

The structure of these cytoplasmic matrices is revealed most clearly by preparing whole mounts after critical point drying and observing by EM (Fig. 5, *d* and *e*). This procedure is considered to conserve the three-dimensional structure of vertebrate cytoskeletons and to allow for clear visualization of spatial relationships (43). A cytoplasmic matrix visualized in this manner is shown in Fig. 5, *d* and *e*. One observes a complex network of filamentous material revealed by the complete removal of the cell wall surrounding the new cell bud. Cell wall material obscures the majority of the cell depicted in Fig. 5 *d*. Most of the cells in these preparations display only occasional gaps revealing the internal structure. Further ultrastructural and biochemical analysis of this structure will be required to determine the organization and composition of these matrices.

Localization of CDC28 Product in Cytoplasmic Matrices

To determine whether the *CDC28* gene product is associated with detergent extracted cytoplasmic matrices, these structures were prepared, reacted with affinity-purified anti-*CDC28*_r product IgG and subjected to indirect immunofluorescence microscopy. Cytoplasmic matrices were also reacted with normal rabbit IgG and anti-ADH serum. The concentrations and reaction times for all immunologic reagents were identical to those used in experiments with whole fixed cells. Fig. 6, *a* and *b*, respectively, shows cytoplasmic matrices stained with anti-*CDC28*_r product IgG and the normal IgG control. The matrices display a staining pattern similar to that seen for whole cells. For reasons to be discussed below, we do not feel that quantitative conclusions are justified from this experiment although the level of staining appears to be roughly comparable. Cytoplasmic matrices reacted with anti-ADH and corresponding preimmune sera show no staining (Fig. 6, *c* and *d*, respectively). This lack of staining is consistent with the observation by immunoblotting that ADH, being a soluble cytoplasmic protein, does not have a strong affinity for insoluble cytoplasmic elements. On the other hand, one may conclude that at least a fraction of the *CDC28* product is tightly associated with the cytoplasmic matrix and that this association is resistant to repeated and lengthy detergent extractions.

In order to assess the association of the *CDC28* product with the cytoplasmic matrix using a different criterion, immunoblotting experiments were performed using anti-*CDC28*₂₈₁₋₂₉₈ product IgG. This antiserum was of higher titer than the anti-*CDC28*_r sera and could be used in immunoblotting experiments at a lower concentration without loss of signal. This is an advantage in that nonspecific background is decreased at lower antisera concentrations. Cytoplasmic matrices were prepared as described. Immunoblots were prepared from SDS-polyacrylamide gels of whole-cell and cytoplasmic matrix polypeptides using antisera directed against the *CDC28* gene product (anti-*CDC28*₂₈₁₋₂₉₈) either alone or preincubated with the peptide antigen as a blocking agent. The result of such an experiment is shown in Fig. 7 (lanes 1-4). The presence of the 34-kD *CDC28* gene product in the cytoplasmic matrices is indicated by the comigration of the band in both whole cells and cytoskeletal matrices (lanes 1 and 2, respectively). The 34-kD band in both lanes is blocked by the peptide antigen, confirming that the signal is due to the specific interaction between the antiserum and the polypeptide. The whole-cell lane contains a second unidentified band which is also blocked by the peptide antigen. The intensity of this band varies between preparations. This could represent a polypeptide related to the *CDC28* gene product at the carboxy terminus, a modified form of the *CDC28* product or the *CDC28* polypeptide in complex with other molecules. The only modified forms of the *CDC28* that we have observed are phosphorylated forms which comigrate with the unphosphorylated form on SDS-polyacrylamide gels (Hadwiger, J., and S. Reed, unpublished observations).

Quantitation of the proportion of the *CDC28* gene product in the cytoplasmic matrix is complicated by a number of factors, the most substantial of which are the difficulties in determining the yield of matrices and in controlling proteoly-

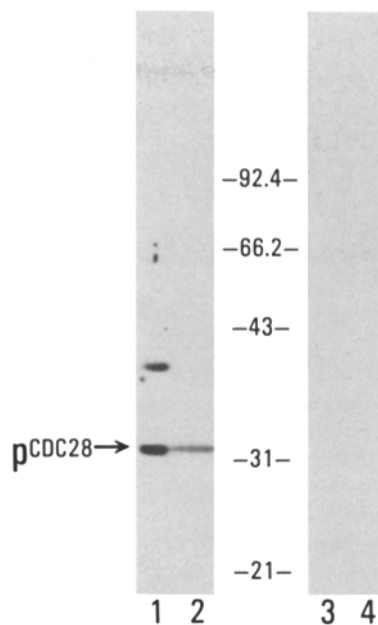


Figure 7. Immunoblotting analysis of *CDC28* product in cytoplasmic matrices. Sample preparation, electrophoresis, and immunoblotting were as described. Whole-cell and cytoplasmic matrix samples (2×10^7 and 6×10^7 cells/lane, respectively) were stained with affinity purified anti-*CDC28*₂₈₁₋₂₉₈ IgG (lanes 1 and 2, respectively) or with the same antibody blocked by preincubation with 0.5 μ g/ml of the peptide antigen (lanes 3 and 4, respectively). The position of the *CDC28* gene product on the gel is denoted (p^{CDC28}). Molecular weight standards were the same as those given in Fig. 4. Values given are $\times 10^{-3}$.

sis. There is variability between preparations of cytoplasmic matrices in the yield of *CDC28* gene product as well as the ratio of actin to *CDC28* gene product. With consideration for these limitations, the upper and lower limits for the proportion of *CDC28* gene product associated with the cytoplasmic matrix when prepared in the manner described are 15% and 2%, respectively. A portion of the remaining *CDC28* gene product can be detected in the dialyzed supernatant of the first Triton X-100 extraction by immunoblotting (data not shown). Actin association with the cytoplasmic matrix ranges from ~10% to 40% of the whole cell level. Presumably, a portion of the actin associated with the "cortical dots" (1, 19) as well as any soluble actin is lost during the preparation of the cytoplasmic matrices. Neither the *CDC28* gene product nor actin is affected by the DNase I digestion used to prepare the insoluble cytoplasmic matrices (data not shown).

It is notable that when the insoluble fraction is isolated by centrifugation of lysates from yeast spheroplasts mechanically disrupted in buffer with or without detergent, approximately the same proportion of the *CDC28* gene product is recovered as in cytoplasmic matrices (data not shown). The portion remaining in the supernatant is not sedimented by centrifugation at 100,000 *g* for 90 min and can, therefore, be considered truly soluble. This is in strong contrast to the relatively large proportion of the gene product found in the particulate fraction when whole non-pretreated cells are similarly disrupted (Fig. 3). This argues that the association of the *CDC28* gene product with the particulate fraction is

strongly influenced by some aspect of the spheroplasting process. The finding that approximately the same proportion of the gene product is associated with the particulate fraction in the presence or absence of detergent further supports the argument that this association is not due to a surface artifact.

Discussion

These results indicate that the majority of the *CDC28* protein kinase of *S. cerevisiae* resides in the cytoplasmic compartment and that at least a portion of it is tightly associated with the insoluble cytoplasmic matrix. Cytoplasmic localization was demonstrated by indirect-immunofluorescence studies. Due to the small size of yeast cells and nuclei, in particular, we cannot eliminate the possibility that this protein is present in the nucleus as well. Such resolution must await immunocytochemical analysis at the electron microscopic level.

The localization of the *CDC28* gene product within the cytoplasmic compartment has been examined using several approaches. First, the distribution of the gene product within particulate and soluble fractions of mechanically disrupted whole cells was determined by immunoblotting. The material in the particulate fraction is unlikely to be membrane associated in that it is not extractable by concentration of Triton X-100 as high as 2%.

To refine the localization of this particulate fraction further within the cytoplasm, a detergent-resistant fraction of yeast spheroplasts was prepared. We refer to this fraction as the insoluble cytoplasmic matrix. Based on immunofluorescence, it would appear that the majority of the *CDC28* product is matrix bound. However, it is conceivable that matrices, which are of low density and are not aldehyde fixed in our procedure, are more accessible and reactive with antibodies than are prefixed, whole yeast cells. As a result, quantitative assessments are probably not justified. On the other hand, based on immunoblotting experiments, it appears that only a fraction (2–15%) of the *CDC28* product is in the detergent-resistant fraction of spheroplasts.

We cannot be certain that our recovery of cytoplasmic matrices is quantitative and to what extent they have been damaged. Rhodamine-phalloidin staining and immunoblotting of protein extracts of whole cells versus cytoskeletons suggests that a considerable portion of actin is lost during our extraction procedure (as much as 90%). It is possible that extractable and nonextractable actin represent biochemically distinct populations. This hypothesis is supported by our observation that so-called cortical actin spots seen in stained whole cells (1, 19) are not seen in stained cytoskeletons whereas filamentous actin appears to be conserved (Fig. 5 c). It is also likely, however, that there has been a systematic loss of cytoskeletal proteins during the extraction procedure. With respect to this point, we have observed that the variability in the extent of association between the *CDC28* gene product and the detergent-resistant matrix correlates well with the extent to which those matrices have become proteolyzed during their preparation. Proteolysis varies between preparations as evidenced by a relatively low yield of high molecular weight polypeptides in some preparations based on analysis by SDS-polyacrylamide gel electrophoresis. Although precautions have been taken, the sensitivity of cytoplasmic matrices to proteolysis and the abundance of

proteases in yeast pose a formidable barrier to precise quantitation (29).

Finally, the *CDC28* product may be largely matrix-associated in a manner which is affected by the spheroplasting process. The presence of >70% of the gene product in the particulate fraction when whole cells are disrupted under buffer and detergent conditions similar to those used for the preparation of insoluble cytoplasmic matrices and the differences observed between spheroplasted and nonspheroplasted cells (discussed further below) suggest that a larger portion of the gene product may, in fact, be associated with the matrix in living cells. The nature of this effect is unclear at present. The loss observed during the preparation of cytoplasmic matrices cannot be explained simply by association with cell wall debris because the spheroplasts retain a significant proportion of their cell wall (Fig. 5 d). Considering these complications, it is clear that absolute quantitation of matrix association of the *CDC28* product must await further investigation.

Treatment of detergent-extracted spheroplasts with DNase I has not been observed to affect the proportion of actin or *CDC28* gene product with the insoluble material. The absence of an effect on actin content and structure, at least so far as can be discerned by rhodamine-phalloidin staining, is surprising in light of the ability of actin to specifically bind to DNase I (10) and the observation that DNase I can induce depolymerization of F-actin under some conditions (14). Clearly, actin, in the state present in these matrices, either does not interact with DNase I or, at least, is not affected by such interaction. Similar observations have been made concerning DNase I treatment of vertebrate cytoskeletal preparations (7).

It is not clear at the moment whether association of the *CDC28* product with the cytoplasmic matrix has any quantitative meaning or biological significance. A cytoskeleton-related function for the *CDC28* protein kinase is consistent with a number of experimental observations. G1 arrest conferred by *cdc28* mutations is accompanied by a number of gross morphologic changes. Most notably, cell growth is asymmetric in the absence of budding, causing a loss of the normal spheroid shape and a transition to large amorphous cells often called "shmoo" (9, 30). Such extreme changes of form must involve cytoskeletal interactions at some level. This is confirmed by observing the structure of actin in *cdc28* mutants. Shortly after a shift of temperature sensitive *cdc28* mutants to the restrictive temperature, we have observed the disintegration of actin fibers and the concomitant appearance of apparently disorganized actin foci (32). We, of course, do not know whether this effect of *cdc28* dysfunction on actin structure is direct, but it serves to underscore an inherent relationship of the *cdc28* phenotype to cytoskeletal components. Similar alterations in actin structure are observed in temperature-sensitive actin mutants at the restrictive temperature (26). Another phenotype conferred by *cdc28* mutants is a defect in nuclear fusion (karyogamy) during conjugation (9). Initial reports described a unilateral *kar* defect (9), meaning that only one parent need be mutant, to observe the effect on conjugation for *cdc28* mutants. We have recently found, however, that in our strains, both conjugants need to be *cdc28* mutants (bilateral *kar* defect; unpublished observations). Mutant zygotes stained using DAPI in order to visual-

ize nuclei indicate a defect in nuclear migration (unpublished observations). It is expected that organelle motility involves cytoskeletal function (42).

We report the preparation of detergent-resistant cytoplasmic matrices from yeast spheroplasts by a procedure adapted from a regime developed for the preparation of cytoskeletons from vertebrate cells (13). A preliminary ultrastructural and biochemical analysis of the resulting structures is presented. If the complete extraction of alcohol dehydrogenase is an accurate criterion, these structures are devoid of soluble proteins. The procedure results in a complex three-dimensional structure which conserves the structure of the actin "cage" revealed by rhodamine-phalloidin staining of whole cells. As is the case with vertebrate cells, only a limited subset of proteins is retained during the course of the extraction procedure. Evidence is presented that one of these is actin. Filamentous structures of several different forms are recognizable in electron micrographs. One of these is likely to be actin filaments. Another form appears to have a tubular structure similar to known vertebrate cytoskeletal elements such as microtubules and intermediate filaments. At this time, there is no strong evidence for the existence in yeast of intermediate filaments, a major constituent of vertebrate cytoskeletons (21). Our structures do not contain significant amounts of tubulin, as is expected because extractions were not carried out under conditions necessary to stabilize microtubules. Similar procedures which preserve microtubule structure have been adapted for use with *S. cerevisiae* (28). The procedure we have developed makes use of the residual cell wall to protect the cytoplasmic matrices from mechanical shock associated with centrifugal washes. It was found that cytoplasmic matrices rapidly disintegrate if cell walls are removed completely. It is conceivable, therefore, that some proteins retained during our extraction procedure are wall-associated rather than matrix associated.

We acknowledge Steven Gould and Dr. Michael Mendenhall for help in the construction of strains MMY13 and MMY53 and Dr. Kevin Sullivan for his careful reading of the manuscript.

This research was supported by grants GM-28005 and DCB84-02344 from the National Institutes of Health and the National Science Foundation, respectively, to Dr. Reed, was also supported in part by American Cancer Society Faculty Research Award FRA-248.

Received for publication 15 December 1986, and in revised form 11 May 1987.

References

- Adams, A. E. M., and J. R. Pringle. 1984. Relationship of actin and tubulin distribution to bud growth in wild-type and morphogenetic-mutant *Saccharomyces cerevisiae*. *J. Cell Biol.* 98:934-945.
- Bravo, R., J. V. Small, S. J. Fey, P. M. Larsen, and J. E. Celis. 1982. Architecture and polypeptide composition of HeLa cytoskeletons: modification of cytoarchitectural polypeptides during mitosis. *J. Mol. Biol.* 154:121-143.
- Breter, H.-J., J. Ferguson, T. A. Peterson, and S. I. Reed. 1983. The isolation and transcriptional characterization of three genes which function at start, the controlling event of the *S. cerevisiae* cell division cycle: *CDC36*, *CDC37* and *CDC39*. *Mol. Cell. Biol.* 3:881-891.
- Brown, S., W. Levinson, and J. A. Spudich. 1976. Cytoskeletal elements of duck embryo fibroblasts revealed by detergent extraction. *J. Supramol. Struct.* 5:119-130.
- Byers, B., and L. Goetsch. 1974. Duplication of spindle plaques and integration of the yeast cell cycle. *Cold Spring Harbor Symp. Quant. Biol.* 38:123-131.
- Bücking-Throm, E., W. Duntze, L. H. Hartwell, and T. R. Manney. 1973. Reversible arrest of haploid yeast cells at the initiation of DNA synthesis by a diffusible sex factor. *Exp. Cell Res.* 76:99-110.
- Capco, D. G., K. M. Wan, and S. Penman. 1982. The nuclear matrix: three-dimensional architecture and protein composition. *Cell.* 29:847-858.
- Denis, C. L., J. Ferguson, and E. T. Young. 1983. mRNA levels for the fermentative alcohol dehydrogenase decrease upon growth on a nonfermentable carbon source. *J. Biol. Chem.* 258:1165-1171.
- Dutcher, S. K., and L. H. Hartwell. 1982. The role of *S. cerevisiae* cell division cycle genes in nuclear fusion. *Genetics.* 100:175-184.
- Greer, C., and R. Scheckman. 1982. Actin from *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* 2:1270-1278.
- Hartwell, L. H. 1967. Macromolecule synthesis in temperature-sensitive mutants of yeast. *J. Bacteriol.* 93:1662-1670.
- Hartwell, L. H., J. Culotti, J. R. Pringle, and B. J. Reid. 1974. Genetic control of the cell division cycle in yeast. *Science (Wash. D.C.)*. 183:46-51.
- Heuser, J. E., and M. W. Kirschner. 1980. Filament organization revealed in platinum replicas of freeze-dried cytoskeletons. *J. Cell Biol.* 86:212-234.
- Hitchcock, S. E., L. Carlsson, and U. Lindberg. 1976. Depolymerization of F-actin by deoxyribonuclease 1. *Cell.* 7:531-542.
- Jensen, R., G. F. Sprague, and I. Herskowitz. 1983. Regulation of yeast mating type inter-conversion: feedback control of *HO* gene expression by the mating type locus. *Proc. Natl. Acad. Sci. USA.* 80:3035-3039.
- Johnston, G. C., J. R. Pringle, and L. H. Hartwell. 1977. Coordination of growth with cell division in the yeast *Saccharomyces cerevisiae*. *Exp. Cell Res.* 105:79-88.
- Johnston, M., and R. W. Davis. 1984. Sequences that regulate the divergent *GALI-GAL10* promoter in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* 4:1440-1448.
- Katsumoto, T., and T. Inoue. 1985. Simultaneous stereoscopic observation of the cytoskeleton and the membrane organization of saponin-extracted and critical-point-dried culture cells. *J. Electron Microsc. Tech.* 2:517-518.
- Kilmartin, J. V., and A. E. M. Adams. 1984. Relationship of actin and tubulin distribution to bud growth in wild-type and morphogenetic-mutant *Saccharomyces cerevisiae*. *J. Cell Biol.* 98:934-945.
- Laemmli, U. K. 1970. Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature (Lond.)*. 227:680-685.
- Lazarides, E. 1980. Intermediate filaments as mechanical integrators of cellular space. *Nature (Lond.)*. 285:249-255.
- Lenk, R., L. Ransom, Y. Kaufman, and S. Penman. 1977. A cytoskeletal structure with associated polyribosomes obtained from HeLa cells. *Cell.* 10:67-78.
- Lorincz, A. T., and S. I. Reed. 1984. Primary structure homology between the product of the yeast cell division control gene *CDC28* and vertebrate oncogenes. *Nature (Lond.)*. 307:183-185.
- March, S. C., I. Parikh, and P. Cuatrecasas. 1974. A simplified method for cyanogen bromide activation of agarose for affinity chromatography. *Anal. Biochem.* 60:149-152.
- Nasmyth, K. A., and S. I. Reed. 1980. The isolation of genes by complementation in yeast: the molecular cloning of a cell cycle gene. *Proc. Natl. Acad. Sci. USA.* 77:2119-2123.
- Novick, P., and D. Botstein. 1985. Phenotypic analysis of temperature-sensitive yeast actin mutants. *Cell.* 40:405-416.
- Peterson, T. A., J. Yochem, B. Byers, M. F. Nunn, P. H. Duesberg, R. F. Doolittle, and S. I. Reed. 1984. Relationship between the yeast cell cycle genes *CDC4* and *CDC36* and the *ets* sequence of oncogenic virus E26. *Nature (Lond.)*. 309:556-558.
- Pillus, L., and F. Solomon. 1986. Components of microtubular structures in *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci. USA.* 83:2468-2472.
- Pringle, J. R. 1975. Methods for avoiding proteolytic artefacts in studies of enzymes and other proteins from yeasts. *Methods Cell Biol.* 12:149-185.
- Reed, S. I. 1980. The selection of *S. cerevisiae* mutants defective in the start event of cell division. *Genetics.* 95:561-577.
- Reed, S. I. 1982. Preparation of product-specific antisera by gene fusion: antibodies specific for the product of the yeast cell cycle gene *CDC28*. *Gene.* 20:253-263.
- Reed, S. I., M. A. de Barros Lopes, J. Ferguson, J. A. Hadwiger, J.-Y. Ho, R. Horwitz, C. A. Jones, A. T. Lorincz, M. D. Mendenhall, T. A. Peterson, S. L. Richardson, and C. Wittenberg. 1985. Genetic and molecular analysis of division control in yeast. *Cold Spring Harbor Symp. Quant. Biol.* 50:627-634.
- Reed, S. J., J. Ferguson, and J. C. Groppe. 1982. A preliminary characterization of the transcriptional and translational products of the yeast cell division cycle gene *CDC28*. *Mol. Cell. Biol.* 2:412-425.
- Reed, S. I., J. A. Hadwiger, and A. T. Lorincz. 1985. Protein kinase activity associated with the product of the yeast cell division cycle gene *CDC28*. *Proc. Natl. Acad. Sci. USA.* 82:4055-4059.
- Rothstein, R. J. 1983. One-step gene disruption in yeast. *Methods Enzymol.* 101:202-211.
- Rubin, G. M. 1975. Preparation of RNA and ribosomes. *Methods. Cell Biol.* 12:45-64.
- Scherer, S., and R. W. Davis. 1979. Replacement of chromosome seg-

- ments with altered sequences constructed in vitro. *Proc. Natl. Acad. Sci. USA.* 76:4951-4955.
38. Schliwa, M. 1982. Action of cytochalasin D on cytoskeletal networks. *J. Cell Biol.* 92:79-91.
 39. Schliwa, M., and J. van Blerkom. 1981. Structural interaction of cytoskeletal components. *J. Cell Biol.* 90:222-235.
 40. St. John, T. P., and R. W. Davis. 1981. The organization and transcription of the galactose gene cluster of *Saccharomyces*. *J. Mol. Biol.* 131:41-53.
 41. Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA.* 76:4350-4354.
 42. Vale, R. D., B. J. Schnapp, T. S. Reese, and M. P. Sheetz. 1985. Movement of organelles along filaments dissociated from the axoplasm of the squid giant axon. *Cell.* 40:449-454.
 43. Webster, R. E., E. Henderson, M. Osborn, and K. Weber. 1978. Three-dimensional electron microscopical visualization of the cytoskeleton of animal cells: immunoferritin identification of actin- and tubulin-containing structures. *Proc. Natl. Acad. Sci. USA.* 75:5511-5515.
 44. Zoller, M. J., and M. Smith. 1983. Oligonucleotide-directed mutagenesis of DNA fragments cloned in M13 vectors. *Methods Enzymol.* 100:468-500.