An Essential Yeast Protein, CBF5p, Binds In Vitro to Centromeres and Microtubules

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Yeast centromere DNA (*CEN*) affinity column chromatography has been used to purify several putative centromere and kinetochore proteins from yeast chromatin extracts. The single yeast gene (*CBF5*) specifying one of the major low-affinity centromere-binding proteins (p64'/CBF5p) has been cloned and shown to be essential for viability of *Saccharomyces cerevisiae*. *CBF5* specifies a 55-kDa highly charged protein that contains a repeating KKD/E sequence domain near the C terminus, similar to known microtubule-binding domains in microtubule-associated proteins 1A and 1B. CBF5p, obtained by overexpression in bacterial cells, binds microtubules in vitro, whereas C-terminal deleted proteins lacking the (KKD/E)_n domain do not. Dividing yeast cells containing a C-terminal truncated *CBF5* gene, producing CBF5p containing only three copies of the KKD/E repeat, delay with replicated genomes at the G₂/M phase of the cell cycle, while depletion of CBF5p arrests most cells in G₁/S. Overproduction of CBF5p in *S. cerevisiae* complements a temperature sensitivity mutation in the gene (*CBF2*) specifying the 110-kDa subunit of the high-affinity *CEN* DNA-binding factor CBF3, suggesting in vivo interaction of CBF5p and CBF3. A second low-affinity centromere-binding factor has been identified as topoisomerase II.

The centromere region of a typical eukaryotic chromosome contains components essential for the accurate segregation of the replicated chromatids in mitosis and meiosis. The kinetochore, a multimolecular structural complex localized at the centromere, provides attachment sites for the spindle microtubules and contains molecular motors that are thought to move the chromosomes on the microtubules during prometaphase and anaphase (reviewed in references 4 and 31). The centromere also maintains attachment of the sister chromatids throughout metaphase and the entire first meiotic division.

An important molecular approach to investigate the mechanism of kinetochore action has been the isolation and characterization of protein components of centromeric heterochromatin or proteins that bind specifically to centromeric DNA. Several centromere DNA-binding proteins have been identified from *Saccharomyces cerevisiae* and higher eukaryotes. A human kinetochore protein, CENP-B, has been shown to bind specifically to a 17-bp sequence (termed the CENP-B box) present in the centromeric alpha-satellite (21, 28). Recently, a protein component (CENP-E) of centromeric chromatin has been shown to contain a consensus kinesin motor domain, including ATP- and microtubule-binding sites (36). In the yeast *S. cerevisiae*, a 125-bp DNA sequence (*CEN*) specifies in *cis* full centromere function and can convert an autonomously replicating plasmid into an artificial chromosome that segregates relatively faithfully through both mitotic and meiotic cell divisions (9; reviewed in reference 8). The *CEN* sequence is organized into three domains, consisting of two highly conserved sequences (termed CDEI [8 bp] and CDEIII [25 bp]) flanking a 78- to 86-bp high-A+T central core (CDEII). Yeast proteins that bind specifically to subregions CDEI and CDEIII have been purified. A protein (CP1 or CBF1) that binds specifically to the CDEI locus has been purified (2, 5, 18). Deletion of the single gene (*CBF1* [also known as *CPF1* or *CEP1*]) specifying

ing this protein results in partial loss of centromere function and conversion to a Met⁻ phenotype (3, 6, 22). The molecular role of CBF1 in centromere function is still unclear, however.

A 240-kDa multisubunit protein complex (CBF3) that binds specifically to the CDEIII region of the yeast centromere has been purified and characterized (20). This protein complex is thought to be absolutely essential for centromere and kinetochore function, since it binds in vitro to the wild-type *CEN* sequence but not to a functionally inactive mutated *CEN* DNA that contains a single-base alteration in CDEIII (20, 24). Affinity-purified CBF3 consists of at least three tightly associated subunits: 110 kDa (CBF3A), 64 kDa (CBF3B), and 58 kDa (CBF3C). Significantly, affinity-purified preparations of CBF3 contain an ATP-dependent motor activity that mediates movement of *CEN* DNA-coated microbeads along microtubules in a plus-to-minus direction (15). Thus, it is likely that CBF3 contains a kinetochore component that brings about attachment and movement of the chromosomes on the spindle microtubules. The gene (*CBF2*) encoding the 110-kDa subunit of this complex was cloned by using tryptic peptide sequences obtained from gel-purified CBF3A as a basis to prepare synthetic oligodeoxyribonucleotide hybridization probes, which were subsequently used to screen a yeast genomic library (17). At the same time, Goh and Kilmartin (12) cloned the identical gene by complementation of a conditional mutation (*ndc10*) leading to a defect in chromosome segregation. The *CBF2/NDCl0* gene product is essential for viability of *S. cerevisiae* and for proper chromosome segregation. Although this protein contains a consensus nucleotide-binding site, no homologies to known molecular motors are apparent.

In this paper, we describe the isolation of two yeast low-affinity centromere-binding proteins, topoisomerase II (Topo II) and CBF5p, and the cloning and sequencing of the *CBF5* gene. The CBF5 protein contains a (KKD/E)_n sequence domain highly homologous to known microtubule-binding domains in microtubule-associated protein 1A (MAP1A) and MAP1B (19, 26). Evidence indicating that

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CBF5p binds bovine microtubules in vitro is provided. Genetic suppression experiments suggest that CBF5p is in some way associated with the CBF3 110-kDa subunit in vivo.

MATERIALS AND METHODS

Strains, media, and microbial techniques. The *S. cerevisiae* strains used are as follows: YPH274 (a/α *ade2-101^{ochre}/ade2-101^{ochre} trp1-Δ1/trp1-Δ1 leu2-Δ1/leu2-Δ1 ura3-52/ura3-52 his3-Δ200/his3-Δ200 lys2-801^{amber}/lys2-801^{amber}*, from P. Hieter), JK418 (a *ura3 leu2-3,112 ndc10-1*) (12), YWJ90 (a/α *leu2-3,112/leu2-Δ1 ade2-101^{ochre}/ADE2 ura3-52/ura3-52 his3/HIS3 gal2/GAL2 pra1/PRA1 prb1/PRB1 prc1/PRC1 cps1/CPS1 cpf1::URA3/cpf1::URA3*) (17), mating type tester strains H272a (a *lys1*) and H272α (α *lys1*) (from C. Wittenberg), and YWJ64MH (a/α *ade2-101^{ochre}/ade2-101^{ochre} trp1-Δ1/trp1-Δ1 leu2-Δ1/leu2-Δ1 ura3-52/ura3-52 his3-Δ200/his3-Δ200 lys2-801^{amber}/lys2-801^{amber} CBF5/cbf5::TRP1*, this work). Strains YWJ64M and YWJ64H are similar to YWJ64MH, except that the *cbf5* gene disruptions were made by *URA3* gene insertions at *MscI* and *HpaI* sites, respectively (see below). Strains YWJ316-13a, YWJ313-13a, YWJ313-15a, YWJ315-13a, and YWJ315-15a are *mata* haploid strains derived from YWJ64-1 with the *cbf5::TRP1* gene disruption but containing plasmids pWJ316-13, pWJ313-13, pWJ313-15, pWJ315-13, and pWJ315-15, respectively. Strain YWJ316-13α has the same genotype as YWJ316-13a except for the α mating type. Diploid strains YWJ313-13, YWJ313-15, YWJ315-13, and YWJ315-15 were constructed by mating strain YWJ316-13α with strains YWJ313-13a, YWJ313-15a, YWJ315-13a, and YWJ315-15a, respectively, and curing the pWJ316-13 plasmid.

Standard media and yeast genetic methods were used (32). Yeast transformations were performed by the lithium method (16).

Gel mobility shift assay and SDS-PAGE. The gel mobility shift assay was carried out as described by Lechner and Carbon (20) and modified by Jiang et al. (17) with a 3% polyacrylamide–0.5% agarose mixing gel. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was run as previously described (17) with 6, 8, or 10% polyacrylamide.

Peptide analysis of the p160 protein (Topo II) and the p64' protein (CBF5p). The p160 protein (Topo II) and the p64' protein (CBF5p) were purified as described for the CBF3 protein complex by Lechner and Carbon (20) and Jiang et al. (17) from a *cbf1*-deficient diploid yeast strain, YWJ90, except that a low concentration of carrier salmon sperm DNA (0.1 mg/ml) was used in the DNA affinity chromatography. About 10 μg of the purified proteins was digested with trypsin after SDS-PAGE and transfer onto polyvinylidene difluoride membranes as described by Mellor et al. (22). Tryptic peptides were purified by high-performance liquid chromatography (HPLC), and partial sequences were obtained for one pure peptide (ELTVTNKPR) and one peptide mixture (GLGTSLAQEVK/EIFHPA??PLYK???AFYNM) from the p160 protein and for four peptides from the p64' protein: a, EYVPLDNAEQSTSSSQE; b, SGHYTPIPAQ; c, ?LENLTGA?F; and d, TIYESNLI.

Cloning of the *CBF5* gene and sequence analysis. Two oligonucleotides were designed from the above partial peptide sequences. Oligonucleotide 64-3, GA(A/G)TA(C/T)GT(T/G)CC(A/T)(C/T)T(A/G)GA(C/T)AA(C/T)GCTGA(A/G)CA (29-mer), and oligonucleotide 64-15, GC(T/G)CC(T/G)

GT(T/G)A(G/A)TT(T/C)TC (17-mer), were designed from tryptic peptides a and c, respectively.

Oligonucleotide labelling and λgt11 library screening were carried out as described by Jiang et al. (17). A positive clone that hybridized to both probes was obtained. A 4-kb *EcoRI* fragment from this lambda clone was subcloned into the Bluescript SK⁺ plasmid (Stratagene, La Jolla, Calif.), resulting in plasmid pWJ64. An exonuclease III deletion series for DNA sequencing was made by using the Erase-a-base System (Promega, Madison, Wis.). Some of these deletion plasmid constructs were also used for functional analysis and overexpression in *Escherichia coli*. Deletion constructs pWJ64-13, pWJ64-15, and pWJ64-17 contain sequences from the left *EcoRI* site (nucleotide 1) to nucleotides 2120, 1915, and 1776 (see Fig. 2B), respectively. DNA sequencing of the alkali-denatured double-stranded DNA was performed by the dideoxy chain termination method with the Sequenase kit (U.S. Biochemicals, Cleveland, Ohio). The sequence of 3 kb from the left *EcoRI* site contains one continuous open reading frame, which includes the amino acid sequences from which the two probes were derived (see Fig. 2B).

DNA sequence data were analyzed by the GCG program (University of Wisconsin Genetics Computer Group) on the VMS/VAX computer. Searches for sequence homology were performed through the GenBank, EMBL, and NBRF/PIR data bases via the FASTA-MAIL program (10).

Disruption of the *CBF5* gene. The 1-kb *MscI*-*HpaI* fragment (see Fig. 2A) containing a 352-amino-acid coding region of the *CBF5* gene (73% of the codons) in pWJ64 was replaced by a 1.4-kb *EcoRI* DNA fragment containing the yeast *TRP1* gene. The cohesive ends of these fragments were filled in by incubation with the DNA polymerase Klenow fragment and deoxynucleoside triphosphates before ligation. The resulting plasmid, pWJ64MH, was digested with *EcoRI*, and the resulting DNA fragment was used to transform the diploid yeast strain YPH274, selecting for tryptophan prototrophs. The transplacement of one copy of the *CBF5* gene in several transformants was verified by mitotic stability of the *TRP1* marker and Southern hybridization of *EcoRI*- or *XbaI*-cut genomic DNA with a 4-kb *EcoRI* fragment containing the *CBF5* gene as a probe. Two of these transformants were sporulated, and 10 tetrads each were dissected. All tetrads gave 2:2 segregation of viable:nonviable spores, and the viable colonies were all Trp⁺.

Two additional gene disruption constructs were made by insertion of a blunt-ended 1.1-kb fragment containing the yeast *URA3* gene into the *MscI* and the *HpaI* sites of pWJ64, resulting in plasmids pWJ64M and pWJ64H, respectively. *EcoRI* fragments derived from these two plasmids were used to transform diploid strain YPH274. The Ura⁺ transformants (YWJ64M and YWJ64H) also gave 2:2 segregation of viable:nonviable spores.

Chromosomal location of the *CBF5* gene. *CBF5* was assigned to chromosome XII by hybridization of a *CBF5* DNA probe to electrophoretically separated yeast chromosomes (7). The *CBF5* gene was localized to a position near *CDC42* proximal to the centromere by hybridization of the *CBF5* probe to dot blots of overlapping yeast DNA clones, kindly provided by L. Riles and M. Olson (Washington University School of Medicine, St. Louis, Mo.).

RNA analysis. Total yeast RNA was isolated as described by Sherman et al. (32). Poly(A) RNA was purified from the total RNA with an oligo(dT) column (Pharmacia, Piscataway, N.J.). Formaldehyde gels were used for fractionation of RNA (32), and a 1-kb *MscI*-*HpaI* internal *CBF5* gene

fragment was used as a hybridization probe to the Northern (RNA) blot (30).

Plasmid constructions for expression in *S. cerevisiae* and in *E. coli*. *Apa*I-*Sac*I fragments containing the *CBF5* gene from plasmids pWJ64-13, pWJ64-15, and pWJ64-17 were further subcloned into the *Apa*I-*Sac*I sites of CEN plasmids pRS313 (*URA3*) and pRS315 (*LEU2*) (33), resulting in plasmids pWJ313-13, pWJ313-15, and pWJ313-17 and pWJ315-13, pWJ315-15, and pWJ315-17, respectively. The *Eco*RI-*Kpn*I fragment containing the *CBF5* gene from plasmid pWJ64-13 was subcloned into the *Sma*I site of pRS316 after being blunt ended with T4 DNA polymerase and deoxyribonucleoside triphosphates, resulting in plasmid pWJ316-13. A *Sma*I-*Kpn*I fragment containing *CBF5* from pWJ64-13 was ligated into the *Sma*I site of pRS315 after being blunt ended with T4 DNA polymerase, and the *Eco*RI fragment containing the *CBF5* gene from the resulting plasmid was further cloned into the *Eco*RI site of pSEY18-GAL (*URA3*, 2 μ m vector, from S. D. Emr), resulting in plasmid pSEY64 (with *CBF5* gene transcription oriented toward the *URA3* marker).

To get expression of the full-length *CBF5* gene in both *E. coli* and *S. cerevisiae*, a DNA fragment containing a convenient *Eco*RI site at the N terminus of the *CBF5* open reading frame was obtained by standard polymerase chain reaction (PCR) amplification with, as primers, a synthetic oligonucleotide, ACGAATTCATATGTCAAAGGAGGATTTCG (Operon, Alameda, Calif.), and a commercial M13 (−40) primer (U.S. Biochemicals) to amplify from plasmid pWJ64-13. After treatment with T4 DNA polymerase, this PCR product was ligated into the *Sma*I site of the Bluescript vector pSK⁺, resulting in plasmid pWJ64PCR, which has one *Eco*RI site immediately upstream of the ATG start codon (see above oligonucleotide sequence) and the other within the vector. This *Eco*RI fragment was then cloned into the *Eco*RI sites of yeast expression vector pYES1.2 (In Vitrogen, San Diego, Calif.) and *E. coli* fusion protein expression vector pGEX-KG (13), resulting in plasmids pWJ64-1.2 and pKG64, respectively. Clones containing the correct orientation of the DNA inserts were verified by restriction mapping.

To produce glutathione *S*-transferase (GST) fusions, *Xba*I-*Pvu*II fragments containing the *CBF5* gene from pWJ64-13, pWJ64-15, and pWJ64-17 were ligated into the pGEX-KG plasmid (13) linearized with *Xba*I and *Hind*III. The ligation mixtures were further treated with Klenow fragment and deoxynucleoside triphosphates and religated. The resulting plasmids pKG13, pKG15, and pKG17 produce fusion proteins of GST and N-terminal truncated CBF5p in *E. coli*. The structures of plasmids pKG64, pKG13, pKG15, and pKG17 were verified by Western blot (immunoblot) analysis of proteins overproduced from *E. coli*.

Protein overexpression and purification from *E. coli*. Purification of GST fusion proteins from *E. coli* strains (DH5 α , GIBCO/BRL) bearing plasmids pKG13, pKG15, and pKG17 was carried out as described by Guan and Dixon (13) with the following modifications. Cultures (500 ml) were grown at room temperature to log phase, and isopropyl- β -D-thiogalactopyranoside (IPTG) was added to 0.2 mM. After continued incubation with shaking for another 12 h, cells were spun down, washed once with phosphate-buffered saline (PBS), and stored at −70°C. Cells were thawed, sonicated in PBS–1% Triton X-100, and centrifuged at 15,000 rpm (Sorvall SS-34 rotor) to obtain a clear supernatant. The supernatants were incubated with glutathione-agarose (Sigma) at 4°C for 1 h, and fusion proteins were eluted with 10 mM

glutathione in 10 mM TrisCl (pH 8.0) after being washed with PBS–1% Triton X-100.

Antibody preparation and Western blot analysis. An amino acid sequence (peptide a) obtained from the tryptic peptide sequences was used to design a peptide antigen for antibody production. The peptide containing 13 amino acids (EYVPLDNAEQSTS) was synthesized, conjugated to keyhole limpet hemocyanin with maleimidobenzoyl-*n*-hydroxy-succinimide ester, and used in a series of injections to obtain antisera in two rabbits (Bio-synthesis Inc., Denton, Tex.). Another 20 mg of the same peptide was coupled to Affi-gel 10/15 mix (1:1; Bio-Rad, Richmond, Calif.) to make a peptide affinity column. Antiserum (25 ml) was diluted with 25 ml of PBS, incubated with 2 ml of peptide–Affi-gel matrix at 4°C overnight, and then packed into a column. The column was washed with 60 ml of PBS–0.5% Tween 20 and 60 ml of PBS–500 mM NaCl. The specific antibody was eluted with 9 ml of 100 mM glycine (pH 2.5), neutralized with 1 ml of 1 M TrisCl (pH 8.0), and stored at 4°C.

For Western blot analysis, proteins were fractionated by SDS-PAGE and transferred onto either polyvinylidene difluoride or nitrocellulose membranes. After preincubation of the membrane with 6% nonfat milk in Tris-buffered saline (TBS)–0.5% Tween 20, the above antibody, diluted 1:5,000 in TBS–0.5% Tween 20, was added and incubated for 1 to 2 h. The membrane was then quickly rinsed with distilled water and washed twice for 5-min intervals with TBS–0.5% Tween 20. Alkaline phosphatase-conjugated goat antirabbit immunoglobulin G secondary antibody (1:2,000, Fisher) was used to detect specific signals.

Microscopic and flow cytometric analysis of cells. To examine cell morphologies, cultured yeast cells were fixed in 75% ethanol and stained for DNA either with 4',6-diamidino-2-phenylindole (DAPI) (32) or with acriflavine (27). DAPI-stained cells were directly used for photomicroscopy. Acriflavine-stained cells were checked for staining under the microscope and further analyzed with a Coulter EPICS 541 flow cytometer as described previously (27).

In vitro microtubule-binding assay. Perfusion chambers and fluorescence-labelled bovine microtubules were prepared as previously described (15), except that microtubules were not polarity marked. Proteins (3 μ l of a 1.3- to 3-mg/ml solution) purified by glutathione column chromatography (see above) were introduced into the perfusion chamber and incubated at room temperature for 10 min. Three microliters of casein (5 mg/ml in 50 mM Tris-Cl, pH 8.0) was perfused in as a blocking agent, and the chamber was incubated at room temperature for an additional 10 min. Three microliters of microtubules in antifade buffer [0.02 mg of glucose oxidase per ml, 0.05 mg of catalase per ml, 10 μ M taxol, 5 mM glucose, 0.05% β -mercaptoethanol, 40 mM piperazine-*N,N'*-bis(2-ethanesulfonic acid) (PIPES; pH 6.8), 0.5 mM ethylene glycol-bis(β -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA), 0.5 mM MgCl₂] was introduced into the chamber and incubated at room temperature for 10 min. After this time, any unbound microtubules were washed from the chamber with 6 μ l of antifade buffer. The microtubules bound per μ g of GST-CBF5p fusion protein (estimated from Bradford protein assays and Western blot analysis) were determined by counting the number of microtubules bound per field (at least seven fields per experiment were counted) and by normalizing to 1 μ g of protein. The microtubules were observed by epifluorescence with a Nikon Microphot-SA microscope.

Nucleotide sequence accession number. The GenBank accession number for the sequence of *CBF5* is L12351.

FIG. 1. Proteins binding to *CEN* DNA at high- and low-stringency conditions. (A) Gel mobility shift assay of a *CEN3* DNA fragment by partially purified CBF3 (Sephacryl S-300 fraction [20]) in the presence of various concentrations of calf thymus competitor DNA. Lanes 1, 2, and 3, 0.5, 1.0, and 5.0 μ g, respectively, of competitor DNA per reaction; lane 4, *CEN3* DNA only without CBF3. F, free *CEN3* probe DNA; B and C, *CEN3*-CBF3 complexes B and C, respectively (20). (B and C) Proteins purified from a *CEN3* DNA affinity column under high- and low-stringency conditions (0.3 and 0.1 mg of calf thymus DNA per ml, respectively). Lanes 1, molecular weight markers (Bio-Rad); lanes 2, proteins eluted from DNA affinity column; 10% (B) and 6% (A) polyacrylamide-SDS gels were used. Gels were stained with silver (18) (B) or with Coomassie blue R-250 (C). Arrowheads indicate the CBF3 core subunits (CBF3A, 110 kDa; CBF3B, 64 kDa; and CBF3C, 58 kDa). Two major protein components from the DNA affinity column run at low stringency, Topo II and p64', are indicated.

RESULTS

High- and low-stringency conditions for binding of proteins to *CEN* DNA give different gel shift and protein composition patterns. The protein complex (CBF3) that binds to the CDEIII region of the yeast centromere DNA contains at least three major subunits when purified by *CEN* DNA affinity chromatography under stringent conditions (0.3 mg of competitor salmon sperm DNA per ml in the loading buffer) (17, 20). The tightly bound major CBF3 components are CBF3A (110 kDa), CBF3B (64 kDa), and CBF3C (58 kDa) plus, depending upon the column conditions, variable quantities of a few relatively minor protein bands (Fig. 1B, lane 2). Binding of subunits A, B, and C is specific for wild-type *CEN* DNA, whereas many of the minor components appear to bind to functionally inactive mutated *CEN* DNA containing a single-base change in the central CCG of CDEIII (20). CBF3 binding to a wild-type *CEN* DNA fragment results in the appearance of two shifted bands of reduced electrophoretic mobility, termed bands B and C in a previous work (20) (Fig. 1A). Under low-stringency conditions (0.1 mg of competitor DNA per ml), both complexes are formed; however, at higher concentrations of competitor DNA, only complex B is seen (Fig. 1A), indicating that the larger complexes are more sensitive to competition by the foreign DNA.

It is possible that proteins binding with lower affinity than the three CBF3 subunits to the *CEN* DNA locus or the CBF3-*CEN* complex could also be involved in centromere function. We therefore made preparations of CBF3 under low-stringency conditions (0.1 mg of competitor DNA per

ml) in the final *CEN* DNA affinity column step. One of the major proteins (160 kDa) eluted from the column under these conditions (Fig. 1C, lane 2) has now been shown to be identical to DNA Topo II (11) by comparing four tryptic peptide sequences with the protein sequence data bank by using the GCG program (amino acids 578 to 582, 605 to 615, 794 to 806, and 1038 to 1046 in reference 11). Another major protein appears as an SDS-PAGE band with mobility slightly faster than that of the 64-kDa subunit of CBF3 (Fig. 1C, lane 2). We have now characterized this protein (termed p64') by cloning and sequencing its corresponding gene (*CBF5*).

Cloning and sequencing of the *CBF5* gene reveals a protein containing a repeating KKD/E domain homologous to microtubule-binding domains in MAP1A and MAP1B. The same procedure used to clone the *CBF2* gene encoding the 110-kDa subunit of CBF3 (17) was applied to purified p64', excised as a band from SDS-polyacrylamide gels carried out on the affinity-purified material (Fig. 1C). Tryptic peptides of p64' were fractionated by HPLC, and partial sequences were obtained for four peptides. Two oligodeoxyribonucleotides with degenerate sequences based upon these peptides were synthesized and used to screen a yeast genomic λ gt11 library (Clontech, Palo Alto, Calif.). A positive clone containing a 4-kb *EcoRI* fragment was found to hybridize to both of these mixed probes. This DNA fragment was subcloned into a Bluescript plasmid vector, and the nucleotide sequence of a 3.2-kb region was determined (Fig. 2). A single open reading frame of 483 amino acids was found in this sequence. All four tryptic peptide sequences previously determined, including those used to design the probes, were found in this open reading frame (underlined in Fig. 2B). The predicted polypeptide is basic and highly charged (net overall positive charge, +13; pI = 9.5) and contains 16% acidic (D+E), 19% basic (K+R), and 12% hydroxy (S+T) amino acids. The calculated molecular mass of 54.7 kDa is smaller than the 64-kDa molecular mass estimated from the electrophoretic mobility in SDS gels, probably because of the high content of charged amino acids and/or possible secondary modifications. The protein has 6 putative phosphorylation sites for protein kinase C and 12 for casein kinase II. A putative mRNA initiation signal (TATAAT) occurs -106 bases upstream of the ATG start codon (Fig. 2B, double underline). Northern blot analysis of poly(A) RNA from *S. cerevisiae*, with an internal gene fragment as hybridization probe, detected a single 1.7-kb transcript (data not shown).

The deduced amino acid sequence of CBF5p contains 10 tandemly repeated KKE and KKD sequences near the C terminus (Fig. 2B, boldface letters). The CBF5 sequence was also used to search the GenBank and EMBL data bases for homologies by using the BLAST program. A glutamic acid-rich protein (GARP) of unknown function from *Plasmodium falciparum* contains 15 tandem KKD/E repeats (Fig. 3). Two microtubule-binding proteins, MAP1A and MAP1B, contain a similar domain (KKD/EX repeats; Fig. 3), which, in the case of MAP1B, has been shown to be responsible for the interaction with microtubules both in vitro and in vivo (19, 26). A similar sequence domain has been found in cDNA clones of possible vesicle-associated proteins (termed Torp1) from *Torpedo californica* (25).

***CBF5* is an essential gene on chromosome XII.** Several gene disruption mutants were constructed to determine whether *CBF5* is essential for yeast cell viability. A 1.1-kb DNA fragment containing the yeast *URA3* gene was inserted into the *MscI* or *HpaI* site within the *CBF5* coding region, leading to gene disruptions at amino acids 22 and 374, respectively (Fig. 2A). The 1.4-kb *TRP1-ARS1* DNA fragment was used

FIG. 2. Restriction map and nucleotide and amino acid sequences of the yeast *CBF5* gene. (A) Restriction map of the *CBF5* gene. A 4-kb *EcoRI* fragment containing *CBF5* was isolated from a λ gt11 genomic library. A 3.2-kb fragment containing the open reading frame (shown by thick arrow) of the *CBF5* gene was sequenced. Some useful restriction sites for gene disruption and gene expression experiments are shown. (B) Nucleotide and amino acid sequences of *CBF5*. A putative mRNA initiation signal (TATAAT) is double underlined, and the four tryptic peptide sequences obtained from purified CBF5p are underlined. The KKE/D repeat is indicated by boldface letters.

to replace the 1-kb *MscI*-*HpaI* region of *CBF5*, thus removing codons 23 to 374. All three constructs were transplanted into the yeast genome by transformation of the diploid strain YPH274 with a linear DNA fragment homologous at both ends with the genomic *CBF5* region. Eight *Ura*⁺ or *Trp*⁺

FIG. 3. Sequence homologies of CBF5p with other known proteins. Conserved KKE/D repeats are enclosed in boxes. Sequences are taken from the work of Triglia et al. (35) for glutamic acid-rich protein (GARP) from *P. falciparum*, from the work of Noble et al. (26) for MAP1B from mouse, from the work of Langkopf et al. (19) for MAP1A from rat, and from the work of Ngsee and Scheller (25) for Torp1 from *T. californica*.

transformants of each were checked for the correct gene replacement by Southern analysis (data not shown). Sporulation of the gene-disrupted strains gave rise to tetrads containing two viable spores with the *Ura*⁻ or *Trp*⁻ phenotype plus two spores which germinated to form microcolonies that stopped growing at the 4- to 20-cell stage, indicating that the *CBF5* gene product is essential for cell growth. In addition, the lethality of the gene interruption at amino acid 374 indicates that the C-terminal 109 amino acids are necessary for CBF5p function.

Data from hybridization of *CBF5* DNA to yeast chromosomes separated by pulsed-field gel electrophoresis indicated that *CBF5* is located on chromosome XII (not shown). Hybridization of the same probe to a set of contiguous overlapping lambda clones, obtained from Maynard Olson's laboratory, localized the *CBF5* gene adjacent to and centro-

FIG. 4. Suppression of an *ndc10/cbf2* temperature sensitivity mutation by overexpression of the *CBF5* gene. Plasmids pRS316 (*CEN6 URA3* vector) (33), pWJ110-316B (pRS316 plus *CBF2*/p110) (17), pWJ316-13 (pRS316 plus *CBF5*/p64'), and pSEY64 (2μm *URA3* vector plus *CBF5*/p64') were transformed into *cbf2/ndc10* temperature-sensitive mutant strain, JK418 (12), and *Ura*⁺ transformants were restreaked on salts-dextrose minimal medium plates without uracil and incubated at 25, 34, or 37°C.

were proximal from *CDC42* on chromosome XII (data not shown).

Overexpression of *CBF5* partially suppresses a conditional mutation in the gene (*CBF2/ND10*) specifying the 110-kDa subunit of CBF3. Because CBF5p (p64') was isolated from a *CEN* DNA affinity column under low-stringency conditions, it is possible that binding of this protein to the column is nonspecific. Thus, the *in vivo* role of CBF5p could be unrelated to centromere function. However, if CBF5p does interact in some way *in vivo* with any of the known CBF3 subunits, abnormally high intracellular levels of CBF5p could suppress temperature sensitivity or otherwise conditional mutations in any of the CBF3 protein genes. To test that possibility, we introduced the *CBF5* gene on either a *CEN*-based plasmid vector (single copy) or on a vector containing the 2μm plasmid replication origin (high-copy-number vector) into a yeast strain bearing a temperature sensitivity allele of *cbf2(ndc10)* (12) and scored the transformants for growth at the permissive and restrictive temperatures. Overexpression of *CBF5* on a high-copy-number vector suppressed the *cbf2/ndc10* mutation at 34 but not at 37°C, while a wild-type copy of *CBF2* complemented the same temperature sensitivity mutation at both temperatures (Fig. 4). Surprisingly, the expression of *CBF5* from a *CEN* plasmid vector also partially suppressed the mutant at 34°C, although suppression by the low-copy-number plasmid was marginal (Fig. 4). Overexpression of *CBF5* had no effect on the growth rate of *ND10* wild-type yeast cells. This suppression experiment provides genetic evidence suggesting that CBF5p interacts either directly or indirectly with the CBF3 110-kDa subunit *in vivo*.

Depletion of CBF5p arrests cell division at G₁/S. A depletion experiment was carried out to study the function of CBF5p in mitotically dividing cells. The *CBF5* gene, under control of the *GAL1* promoter in plasmid pWJ64-1.2 (see

FIG. 5. Depletion of CBF5p arrests cell division mainly at G₁/S. Yeast haploid strain YWJ64-1.2α contains plasmid pWJ64-1.2 (*CBF5* gene under *GAL1* control in a 2μm *URA3* vector) in a *cbf5* null background. Cells grow normally in galactose medium (left panels) and arrest after shifting to glucose medium (right panels). Log-phase cells from galactose medium and arrested cells from glucose medium at a low optical density (*A*₆₆₀ = 0.1) were taken for Normarski microscopy (upper panels) and for flow cytometry analysis after acriflavine staining (lower panels). Arrows indicate 1N (unreplicated) or 2N (replicated) DNA content.

Materials and Methods), was introduced into a *CBF5* gene-disrupted strain bearing a wild-type copy of the *CBF5* gene on a *CEN* plasmid (pWJ315-13). The resulting strain was cured of the *CEN* plasmid to give strain YWJ64-1.2α, which contains plasmid pWJ64-1.2 in a *cbf5*-null background. The YWJ64-1.2α cells grow normally in galactose medium but, when *CBF5* gene expression is repressed by shifting the cells to glucose, cell division slows and finally stops at a low cell density after 24 h. The relatively long time needed to completely shut down cell division in the repressed cultures probably is due to overexpression of *CBF5* in galactose, thus leading to a high residual level of the protein, which requires several generations to be depleted. A similar phenomenon is seen in germinating *cbf5* null spores, which go through two to five cell divisions before growth is completely arrested. The CBF5p-depleted YWJ64-1.2α cells are arrested mainly in the small-bud stage of the cell cycle (Fig. 5, upper panels). Flow cytometry analysis indicated that most (more than 70%) of these cells have unreplicated genomes, whereas at least 50% of actively dividing wild-type *CBF5* cells have a 2N DNA content (Fig. 5, lower panels). Thus, cells completely deprived of CBF5p appear to arrest mainly at the G₁/S boundary.

The (KKD/E)_n domain of CBF5p is necessary for timely progression through the cell cycle and for cell viability. To determine the importance of the (KKD/E)_n domain in CBF5p, various C-terminal deletions were constructed (see Materials and Methods) and examined for the ability to complement a *cbf5* null yeast strain (Fig. 6). Deletion p64'-15 terminates at amino acid 443, leaving only 3 of 10 copies of the KKD/E repeat, whereas p64'-17 terminates at amino acid 398, removing all of the KKD/E domain. These *CBF5* constructs were cloned in *CEN* plasmid vectors pRS313 (*HIS3* selection) and pRS315 (*LEU2* selection) and finally introduced into haploid strain YWJ316-13a, which contains a

FIG. 6. Functional analysis of C-terminal truncations of the *CBF5* gene. DNA fragments containing various *CBF5* deletions from sequencing plasmids pWJ64-13, pWJ64-15, and pWJ17 were cloned into *CEN* plasmids pRS313 and pRS315 and then introduced into a haploid strain, YWJ316-13a, which contains plasmid pWJ316-13 bearing a wild-type *CBF5* gene over a *cbf5* null genomic background. Transformants were tested for the ability to grow on 5-fluoroorotic acid medium to determine whether cells lacking pWJ316-13 were viable. Plasmid p64'-13 contains full-length *CBF5*p. p64'-15 retains only three repeats of KKD/E at its C terminus, and p64'-17 has no KKD/E repeat. Identical results were obtained with both pRS313- and pRS315-based constructs. The p64'-H result is derived from a gene disruption experiment, in which the *URA3* gene insertion at a *HpaI* site leads to nonviable spores.

URA3-CBF5 plasmid (pWJ316-13) over a *cbf5* null background. These transformants were screened on a 5-fluoroorotic acid plus uracil medium to determine if segregants lacking the pWJ316-13 plasmid (containing the wild-type *CBF5* gene) were viable. The results (Fig. 6) indicated that only the p64'-13 (full-length KKD/E) and p64'-15 (three copies of KKD/E) constructs would functionally complement the *cbf5* null mutation. A strain carrying deletion p64'-17, completely lacking the KKD/E domain, could not be recovered in the *cbf5* null background. The diploid strain homozygous for the *CBF5* gene disruption bearing deletion p64'-15 (three copies of KKD/E) grew somewhat slower than

p64'-13, with a doubling time of 4.7 h compared with 3.7 h for p64'-13. The overall results were identical with the *CBF5* deletions cloned into either the pRS313 (*HIS3*) or pRS315 (*LEU2*) vectors. Gene disruption at the *HpaI* site within the *CBF5* gene (p64'-H, leaving amino acids 1 to 374 without any KKD/E repeat) also leads to nonviable spores (Fig. 6).

The morphology and DNA content of log-phase cells containing the p64'-15 *CBF5*p construct (three copies of KKD/E) were further examined to determine if cell division was delayed at a specific stage of the cell cycle. Diploid strains homozygous for the *CBF5* gene disruption and containing a *CEN* plasmid with either the p64'-13 control *CBF5* gene (full-length KKD/E) or the deletion p64'-15 (three copies of KKD/E) were compared in this experiment. The log-phase cultures of p64'-15 contained mainly large-bud cells, and flow cytometry analysis indicated that most of the p64'-15 cells were at the tetraploid level, whereas the control cultures showed a more equal distribution of tetraploid versus diploid cells in the population (Fig. 7A). DAPI staining of the p64'-15 cells revealed most of the DNA to be in the mother cell at the bud junction (Fig. 7B). We found that about 40% ($n > 200$) of the log-phase p64'-15 cells had large buds and a single, undivided nucleus, whereas only 15% of the control p64'-13 cells were large budded. These results indicate that the cells containing *CBF5*p with only three copies of KKD/E are delaying at G_2/M . It is noteworthy that depletion of *CBF5*p causes most cells to arrest at G_1/S , whereas truncation of the KKD/E region results in a delay in mitosis. This cell cycle delay could also be observed in haploid cells containing the p64'-15 (three copies of KKD/E) construct, although the phenotype was less dramatic.

To determine if the above-described G_2/M delay in cells containing the p64'-15 deletion could be due to protein instability, a Western blot analysis, with polyclonal antisera

FIG. 7. Effects of partial deletion of the KKD/E repeat on cell growth. (A) A C-terminal deletion in *CBF5* (10 x KKE) to leave three copies of KKD/E (3 x KKE) results in a delay at G_2/M . Yeast diploid strains YWJ313-13 (left panels) and YWJ313-15 (right panels) homozygous for a *cbf5::TRP1* gene disruption contain plasmids pWJ313-13 and pWJ313-15, respectively. Log-phase cells were taken for Normarski microscopy (upper panels) and for flow cytometry analysis (lower panels). Arrows indicate 2N (unreplicated) and 4N (replicated) DNA content. (B) DNA localization in the YWJ313-15 cells. Log-phase cells were visualized by DAPI staining. (C) Western blot analysis of crude nuclear extracts from yeast YWJ313-13 (lanes W, wild-type *CBF5*p) and YWJ313-15 cells (lane C, C-terminal truncated *CBF5*p). Aliquots containing the same amount of total protein were fractionated on a 10% polyacrylamide-SDS gel, blotted onto a nitrocellulose membrane, and hybridized with an antipeptide antibody (α -p64-3).

directed against an internal peptide in CBF5p (see Materials and Methods), was carried out on total nuclear extracts from *cbf5* null cells bearing either the p64'-13 or the p64'-15 constructs. The Western blot (Fig. 7C) shows clearly that, as expected, p64'-15 is smaller than p64'-13, but the proteins are present in roughly equal quantities. Thus, it is unlikely that the cell cycle delay seen in p64'-15 cells is due to instability of the truncated CBF5p. Cells containing the p64'-13 or p64'-15 constructs were also checked for spindle structures by immunostaining with anti-tubulin antibody. Both types of cells showed normal spindle structures through the cell cycle (data not shown).

CBF5p binding to microtubules in vitro is dependent upon an intact (KKD/E)_n domain. It has previously been shown that the (KKEX)_n domain in MAP1B is responsible for binding of the protein to microtubules in vitro (26). Since CBF5p contains a similar domain (Fig. 3), we assayed the microtubule-binding activity of CBF5p. We first tried a standard microtubule cosedimentation experiment to look for binding of in vitro-translated or *E. coli*-overproduced CBF5p to taxol-stabilized bovine microtubules. Reproducibly significant levels of binding of CBF5p to microtubules could not be obtained in these experiments, however. We then assayed CBF5p for the ability to bind microtubules to precoated glass surfaces in vitro. This assay (see Materials and Methods) is similar to the usual microtubule gliding assay for motor activity (1) and is considerably more sensitive than the cosedimentation assay. Glass coverslips precoated with the test protein are exposed to taxol-stabilized fluorescent microtubules in a suitable binding buffer, and the number of microtubules adhering to the glass surface was determined in the fluorescence microscope. N-terminal GST fusion proteins (13) with different C-terminal truncations of CBF5p (Fig. 6) were overproduced in *E. coli* and affinity purified on a glutathione-agarose column. Western blot analysis showed that the C-terminal truncated forms of CBF5 are synthesized to somewhat higher levels than full-length CBF5p in *E. coli*, and the proteins are reasonably stable in the extracts (Fig. 8). Both the full-length CBF5p fusion protein (KG64) and an N-terminal truncated version (KG13, lacking 63 amino acids from the N terminus) could bring about binding of microtubules to the glass surface (Table 1). However, the C-terminal deleted proteins containing only three tandem copies of KKD/E (KG15) or completely lacking the (KKD/E)_n region (KG17) were ineffective in binding microtubules. The GST protein alone (KG0) also had no microtubule-binding activity in this assay. These results indicate that CBF5p does bind to microtubules, and the findings are consistent with the idea that the C-terminal (KKD/E)_n region is responsible for this binding. It is possible that KG15, which can complement a null allele of *cbf5*, does bind microtubules weakly in vivo but is ineffective in our in vitro assay. However, we have no direct evidence to indicate that CBF5p interacts with microtubules in vivo.

DISCUSSION

The *CBF5* gene encodes a protein that binds in vitro to the *CEN* DNA-CBF3 complex. In previous work, we have characterized a multisubunit protein complex (CBF3) that binds with high specificity to the CDEIII consensus sequence within the yeast *CEN* locus (20). The CBF3 core complex obtained by *CEN* DNA affinity chromatography in the presence of a large excess of nonspecific carrier DNA consists of three major subunits: 110 kDa (CBF3A), 64 kDa (CBF3B), and 58 kDa (CBF3C). However, under low-strin-

FIG. 8. Western blot analysis of the *E. coli* overproduced GST-CBF5p fusion proteins. Proteins purified from a glutathione-agarose column (lanes 1 to 5, 0.11, 0.12, 0.04, 0.06, and 0.3 μ g, respectively, of total proteins) and crude nuclear extract (CNE) from a wild-type yeast strain (YPH274) (lane 6, 100 μ g) were fractionated on a 10% polyacrylamide-SDS gel, blotted onto a nitrocellulose membrane, and hybridized with anti-CBF5p peptide antibody (α -p64-3). Lane 1, GST plus full-length CBF5p; lanes 2, 3, and 4, GST plus N-terminal truncation of CBF5p (lacking the N-terminal 63 amino acids of CBF5p) and various C-terminal truncations of CBF5p (KG13, full length, amino acids 1 to 483; KG15, amino acids 64 to 443; KG17, amino acids 64 to 398 [Fig. 6]); lane 5, GST protein alone.

gency conditions (about one-third-less competitor DNA), several additional proteins bind either to the DNA or to the *CEN* DNA-CBF3 core complex. Although it is possible that the binding of these proteins to the centromere complex is an in vitro artifact, we feel that it is necessary to investigate these proteins on the chance that the observed centromere binding is biologically relevant. One of the major reduced affinity proteins (p160) has a molecular weight of approximately 160,000, and the other (p64') migrates slightly faster than CBF3B on SDS gels. In this work, we have purified these two proteins and analyzed several tryptic peptides.

TABLE 1. Microtubule binding to GST-CBF5p fusion proteins containing various C-terminal deletions^a

GST-fusion protein ^b	Avg no. of MTs/field	% MTs bound/ μ g of protein ^c	Relative sp act
KG64	27 $\pm$ 2.2	5.00	100
KG13	21 $\pm$ 3.3	4.00	80
KG15	0.7 $\pm$ 0.7	0.04	0.8
KG17	1.1 $\pm$ 0.9	0.10	2.0
KG0	1.0 $\pm$ 1.0		

^a The data given for each protein sample are compiled from at least four separate experiments. In each experiment, at least seven fields were examined per sample. MT, microtubule.

^b KG64 has full-length CBF5p fused to GST; KG0 contains only GST; KG13, KG15, and KG17 are GST fusions with the N-terminal 63 amino acids truncated at the *Xba*I site and C-terminal truncations of different length as described in the legend to Fig. 6.

^c Data in this column refer to microtubule binding per microgram of GST-p64' fusion protein. This protein concentration was estimated by Western blot quantitation (Fig. 8). Approximately 6×10^8 microtubules were used per experiment.

The p160 protein has proven to be DNA Topo II, which is known to be essential for proper chromosome segregation in both mitosis and meiosis (14, 29). It is possible that Topo II is directly involved in centromere function; however, we cannot exclude the possibility that binding of Topo II to the *CEN* DNA affinity column is artifactual. The p64' protein is shown to be encoded by a new gene (designated *CBF5*). The relevance of p64' (*CBF5p*) to *CBF3* function is suggested by a genetic suppression experiment, which showed that overexpression of *CBF5* in *S. cerevisiae* can partially suppress a temperature sensitivity mutation in the gene (*cbf2/ndc10*) specifying the 110-kDa subunit of *CBF3*. This finding provides support for the idea that p64' interacts either directly or indirectly with *CBF3A* in vivo. In addition, the gene disruption experiments show that *CBF5p* is essential for cell viability.

The deduced amino acid sequence of the *CBF5* open reading frame contains 483 amino acids with a calculated molecular mass of 54.7 kDa, smaller than the 64 kDa estimated from SDS gels. This discrepancy could be due to the high content of charged amino acids (overall positive charge, +13, with 35% acidic plus basic amino acids) and to secondary modifications (multiple putative phosphorylation sites are present). The presence of several modified forms of *CBF5p* in vivo is supported by the observation that multiple closely running cross-reacting bands are frequently observed on Western blots stained with antisera raised against *CBF5p* (data not shown).

It is uncertain whether *CBF5p* binds directly to *CEN* DNA on the affinity column or to one or more of the *CBF3* subunits bound to the DNA. *CBF5p* prepared by overexpression in *E. coli* does not bind to *CEN* DNA, as determined by the absence of a *CEN* fragment mobility shift in the usual assay. However, a lack of posttranslational modifications could have prevented DNA binding in those experiments. In addition, *CBF5p* is apparently not a component of the *CBF3-CEN* DNA complex as visualized in the standard *CEN* DNA fragment mobility shift assay (20) (Fig. 1A), since identical shifted band patterns are given by *CBF3* plus *CEN* DNA in the presence or absence of *CBF5p*. Furthermore, the addition of antisera directed against *CBF5p* has no effect on the mobility of the *CBF3-CEN* DNA complexes. Thus, the biological relevance of the low-affinity binding of *CBF5p* to the *CEN* DNA affinity column remains unclear.

A C-terminal domain containing a (KKD/E)_n repeat within *CBF5p* is necessary for microtubule-binding activity in vitro. A (KKD/E)_n tandem repeat sequence was found near the C terminus of the *CBF5* protein. A very similar domain, a KKD/EX repeat, has also been found in two microtubule-binding proteins, MAP1A and MAP1B (19, 26). In MAP1B, it has been shown that this highly charged domain is responsible for the interaction with microtubules both in vitro and in vivo (26). Therefore, we assayed the microtubule-binding activity of *CBF5p* in vitro.

GST-*CBF5p* fusion proteins containing various C-terminal deletions in *CBF5p* were overproduced in *E. coli* and purified by glutathione affinity chromatography. The fusion protein containing the full-length KKD/E repeat domain does mediate binding of taxol-stabilized bovine microtubules to glass surfaces in vitro. Deletion of the KKD/E repeats leads to loss of this binding activity. At present, we have no direct evidence indicating that *CBF5p* is associated with tubulin or microtubules in vivo; however, *CBF5p* containing a C-terminal deletion in which three copies of the KKD/E repeat remain intact does partially complement a *cbf5* null mutation, whereas a deletion that completely removes the

KKD/E region inactivates the protein in vivo. The latter deletion also removes several amino acids internal to the KKD/E domain, however, and thus we cannot conclude from this experiment that the KKD/E repeat is absolutely essential for *CBF5p* activity in vivo. Truncation of the KKD/E region to leave only three copies of the repeat does result in dramatic effects upon cell growth (see below), suggesting that this region is important for the biological function of *CBF5p*. It should be noted, however, that the microtubule-binding and motor activity characteristics of affinity-purified *CBF3* preparations (15) are not dependent upon the presence of *CBF5p*, since *CBF3* purified under stringent conditions and containing no detectable *CBF5p* (by Western analysis) retains microtubule binding and motor activity. Moreover, addition of ATP has no effect on the *CBF5p*-microtubule complex. Thus, at this point, it appears unlikely that *CBF5p* is an essential component of the *CBF3*-based motor. Immunostaining of yeast cells with antisera directed against *CBF5p* shows strong nuclear staining, with no apparent colocalization of the antigen with the spindle pole bodies (data not shown).

Proteins containing a repeating KKD/E domain appear to be highly conserved in nature. In addition to MAP1A, MAP1B, and Torp 1, all of which contain very similar repeat domains, a protein of unknown function from *P. falciparum* contains a direct repeat of KKD/E units nearly identical to that in *CBF5p* (Fig. 3). In addition, using as probe antiserum directed against a synthetic peptide with the same sequence as the (KKD/E)_n domain in *CBF5p*, we have found cross-reacting proteins in extracts from several organisms, including *Schizosaccharomyces pombe*, *Drosophila melanogaster*, PC12 cells, and HeLa cells (16a). This antiserum reacts with a 70-kDa antigen in an enriched MAP fraction that had been through three cycles of microtubule polymerization-depolymerization from bovine brain tissue extracts and from HeLa cell extracts.

Partial inactivation or depletion of *CBF5p* results in delay or arrest of the yeast cell cycle. Depletion of *CBF5p* in *S. cerevisiae* by glucose-mediated repression of a *CBF5* gene under control of the *GAL1* promoter in a *cbf5* null background results in a gradual arrest of cell division, producing a population of small-bud cells with unreplicated DNA, presumably blocked at the G₁/S boundary. However, *cbf5* null cells containing a plasmid-borne copy of a gene producing a C-terminal truncated version of *CBF5p* (leaving only three copies of the KKD/E repeat) divide more slowly than *CBF5* wild-type cells, and the population consists principally of large-bud cells with the DNA at the bud junction or in the neck. The majority of these dividing cells have replicated their DNA; thus, it appears that cell division is delayed at some point after S phase, presumably during mitosis. The latter finding implies that *CBF5p* function is either directly or indirectly involved in mitosis, since reliance on a partially defective version of the protein causes cells to delay the division cycle during DNA transfer. We also have observed a very similar G₂/M delay in cells containing a temperature sensitivity *cbf5* mutation when grown under semipermissive conditions; however, a chromosome missegregation phenotype is not evident in these cells (16a). Similar G₂/M delay phenotypes have also been observed with yeast cells containing centromeric DNA mutations (34) and with those containing dicentric chromosomes (23). Thus, several lines of evidence point to involvement of *CBF5p* in mitotic chromosome segregation: (i) the protein was isolated as a centromere DNA-*CBF3*-binding factor, (ii) overexpression of *CBF5p* suppresses a conditional mutation (*ndc10*) in the

gene specifying a known *CEN* DNA-binding factor (CBF3A), (iii) the protein binds microtubules in vitro and contains significant sequence homology to a known microtubule-binding domain in MAP1A and MAP1B, and (iv) complementation of a *cbf5* null allele by a partially defective CBF5p causes cells to delay in the process of DNA transfer. In addition, CBF5p appears also to be required for the G₁/S transition, since complete depletion of CBF5p blocks division at the small-bud stage before DNA replication occurs.

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