Isolation and Characterization of a Gene (*CBF2*) Specifying a Protein Component of the Budding Yeast Kinetochore

Weidong Jiang, Johannes Lechner and John Carbon

Department of Biological Sciences, University of California, Santa Barbara, California 93106

Abstract. We have cloned and determined the nucleotide sequence of the gene (*CBF2*) specifying the large (110 kD) subunit of the 240-kD multisubunit yeast centromere binding factor CBF3, which binds selectively in vitro to yeast centromere DNA and contains a minus end-directed microtubule motor activity. The deduced amino acid sequence of CBF2p shows no sequence homologies with known molecular motors, although a consensus nucleotide binding site is present. The *CBF2* gene is essential for viability of yeast and is identical to *NDC10*, in which a conditional mutation leads to a defect in chromosome segregation (Goh, P.-Y., and J. V. Kilmartin, in this issue of *The Journal of Cell Biology*). The combined in vitro and in vivo evidence indicate that CBF2p is a key component of the budding yeast kinetochore.

THE accurate segregation of chromosomes during mitosis and meiosis is accomplished by a diversity of microtubule-based movements. Centromeres play an essential role in this process. The centromere of higher eukaryotic chromosomes contains a multilayered protein complex termed the kinetochore. This structure provides an attachment point for the spindle microtubules, and apparently contains molecular motors that move the chromosome along the microtubules during prometaphase and anaphase (Brinkley et al., 1989; Sawin and Scholey, 1991). In addition, the centromere region maintains attachment of the sister chromatids throughout mitotic metaphase and the entire first meiotic division. The presence of microtubule-based molecular motors in the kinetochores of higher eukaryotes has been shown by demonstrating in vitro attachment and ATP-dependent movement of microtubules on the kinetochores of mammalian chromosomes (Hyman and Mitchison, 1991). In addition, antibodies directed against cytoplasmic dynein stain the centromere regions of chromosomes in dividing mammalian (Pfarr et al., 1990) or chicken cells (Steuer et al., 1990). Several proteins have been localized to the centromere and kinetochore regions of higher eukaryotic chromosomes by using autoimmune sera of human patients with the CREST syndrome (Pluta et al., 1990; Saitoh et al., 1992), or with mAbs directed against chromosome scaffold components (Yen et al., 1992). The genes for several of these proteins have been cloned and sequenced (CENP-A, Palmer et al., 1991; CENP-B, Earnshaw et al., 1987; Sullivan and Glass, 1991; CENP-C, Saitoh et al., 1992; CENP-E, Yen et al., 1992). One of these proteins (CENP-E) shows considerable sequence homology with the kinesin family consensus motor domain.

Johannes Lechner's present address is Institut für Biochemie, Genetik und Mikrobiologie, Universität Regensburg, 8400 Regensburg, Germany.

In budding yeast (*Saccharomyces cerevisiae*), the centromeric DNA sequences needed in *cis* to specify accurate mitotic and meiotic chromosome segregation are contained within a 125-bp locus known as *CEN* (reviewed by Carbon and Clarke, 1990; Clarke, 1990). The presence of a *CEN* locus on an autonomously replicating yeast plasmid converts it into an artificial chromosome that segregates faithfully through both mitotic and meiotic cell divisions (Clarke and Carbon, 1980). Thus, the relative simplicity of the yeast centromere region offers a choice experimental system for the study of molecular mechanisms associated with centromere/kinetochore action. The *CEN* sequence is organized into three domains, consisting of two highly conserved protein binding sites (termed CDEI and CDEIII) flanking a 78–86-bp high-(A+T) central sequence (CDEII). Mutational analyses have shown that the 25-bp CDEIII binding site (TGTTT[T/A]TGNTTTCCGAAANNNAAAAA) is absolutely essential for centromere function (McGrew et al., 1986; Ng and Carbon, 1987; Hegemann et al., 1988; Jehn et al., 1991), while mutations in or deletion of CDEI (PuTCACPuTG) impair but do not abolish function (Cumberledge and Carbon, 1987; Gaudet and Fitzgerald-Hayes, 1987; Niedenthal et al., 1991). A protein (CP1 or CBF1) that binds specifically to the CDEI locus has been purified (Baker et al., 1989; Cai and Davis, 1989; Jiang and Philippsen, 1989). Deletion of the single gene (*CBF1/CPF1/CEP1*) specifying this protein results in partial loss of centromere function and to a Met^- phenotype (Baker and Masison, 1990; Cai and Davis, 1990; Mellor et al., 1990). The molecular role of CBF1 in centromere function is still unclear, however.

Recently, a 240-kD multisubunit protein complex (CBF3) that binds specifically to the CDEIII region of the centromere has been purified and characterized (Lechner and Carbon, 1991). This protein complex is thought to be absolutely essential for centromere function, since it binds to a wild-

type *CEN* DNA fragment, but not to functionally inactive mutated *CEN* DNA that contains a single base pair alteration in CDEIII (Ng and Carbon, 1987). Affinity purified CBF3 consists of at least three tightly associated subunits: 110 (CBF3A), 64 (CBF3B), and 58 (CBF3C) kD. Significantly, 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 (Hyman et al., 1992). Thus, it is likely that CBF3 is the key kinetochore component that brings about attachment and movement of the chromosomes on the microtubules. In this paper, we describe a more detailed characterization of the 110-kD subunit of CBF3. We have isolated and determined the nucleotide sequence of the single gene (*CBF2*) specifying this protein, and show that in vivo CBF3A (CBF2p) is essential for the viability of yeast. In an accompanying paper in this issue of *The Journal of Cell Biology*, Goh and Kilmartin (1993) describe a yeast conditional mutant, *ndc10-1*, which displays a chromosome segregation defect at the nonpermissive temperature. The *NDC10* gene is identical to *CBF2*.

Materials and Methods

Strains, Media, and Microbial Techniques

The yeast 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, Johns Hopkins University School of Medicine, Baltimore, MD); 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 cpf::URA3/cpf1::URA3*, derived from a cross of strains YSS92 (*α ura3 leu2-3, -112 his3 gal2 pra1 prb1 prc1 cps1 cpf1::URA3*, from M. Funk, Oxford University, Oxford, England) and YSS90 (*a ura3-52 lys2-801^{amber} ade2-101^{ochre} leu2-1 cpf1::URA3*, Mellor et al., 1990); mating type tester strains H272a (*a lys1*) and H272α (*α lys1*) (from C. Wittenberg, Scripps Research Institute, La Jolla, CA); YWJ110-1 (*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}* CBF2/cbf2::TRP1, this study). Strains YWJ110-2, YWJ110-3, and YWJ110-4 are similar to YWJ110-1, except that the *cbf2* gene disruptions were made by *URA3* gene insertions at SnaI, StuI, and NdeI sites, respectively. Strains YWJ110-313Pα, YWJ110-315Pα, YWJ110-316Bα, and YWJ110-1.2α are haploid α strains derived from YWJ110-1 with the *cbf2::TRP1* gene disruption but containing plasmids pWJ110-313P, pWJ110-315P, pWJ110-316B, and pWJ110-1.2α, respectively.

Standard media and yeast genetic methods were used (Sherman et al., 1986). Yeast transformations were performed using the lithium method (Ito et al., 1983).

Peptide Analysis of CBF3A Protein

The CBF3 protein complex was purified as described by Lechner and Carbon (1991) from a *cbf1*-deficient diploid yeast strain YWJ90. ~10 μg of the 110-kD protein (CBF3A) was digested with trypsin after SDS-PAGE and transfer onto Immobilon polyvinylidene difluoride (PVDF) membranes (Millipore Corp., Bedford, MA) as described by Mellor et al. (1990). Tryptic peptides were purified by HPLC and partial sequences were obtained for four peptides: (*a*) TNFYSLLIERPSQLTFA; (*b*) GDGFPNLNADENGSLLQD; (*c*) DLLVQIFPEI; (*d*) (? × 8)ENSFK.

Cloning of the CBF2 Gene and Sequence Analysis

Three oligonucleotides were designed from the above partial peptide sequences. Oligo p110-21-a, GCGAAGGTIA(G/A)TTGIGAIGGTC(T/G)TTCIATTA(A/G)TA(A/G)GGAGTAGAAGTTGGT(50-mer); and oligo p110-21-b, AT(C/T/A)GA(A/G)(C/A)GICCI(T/A)(C/G)ICA(A/G)(C/T)TIACITT(C/T)GC (29-mer) were designed from tryptic peptide a; oligo p110-25, TT(C/T)CC(G/A/T/C)AA(C/TC/T)T(G/A/T/C)AA(C/T)GC(G/A/T/C)GA(C/T)GA(A/G)AA(C/T)GG (29-mer), was designed from tryptic peptide b.

Probes were end labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP and used to screen a yeast genomic λgt11 library (Clontech Lab. Inc., Palo Alto, CA) according to Sambrook et al. (1989). A positive clone was obtained that hybridized to all three probes. Two EcoRI fragments (1.0 and 1.3 kb) from this lambda clone were cloned into the Bluescript SK plasmid (Stratagene, La Jolla, CA), resulting in plasmids pWJ110-1R and pWJ110-1.3R, respectively. An exonuclease III deletion series was made using the Erase-a-base System (Promega Corp., Madison, WI). DNA sequencing of the alkali-denatured double-stranded DNA was performed by the dideoxy chain termination method using the Sequenase Kit (US Biochemicals, Cleveland, OH). The sequences of both EcoRI fragments were shown to contain one continuous open reading frame, which contained the amino acid sequences from which the three probes were derived. These two EcoRI fragments were then used as hybridization probes to screen a yeast genomic plasmid library containing average inserts of 10 kb (obtained from A. Sperry and P. Hieter, both from Johns Hopkins School of Medicine). Several strongly hybridizing plasmid clones were obtained; the *CBF2* gene was finally cloned from one of these plasmid clones on a 6.5-kb BamHI fragment. This fragment was subsequently cloned in a Bluescript SK vector, resulting in plasmid pWJ110-6.5B. The nucleotide sequence of ~4.1 kb of this DNA was obtained (shown in Fig. 2).

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

Disruption of the CBF2 Gene

A 2.1-kb NdeI fragment containing more than two thirds of the *CBF2* gene (including the NH_2-terminal 705 amino acids of the CBF2 protein) in pWJ110-6.5B was replaced by an 852-bp EcoRI–BglII DNA fragment containing the *TRP1* gene. The cohesive ends of these fragments were filled in with Klenow and deoxynucleotide triphosphates before ligation. The resulting plasmid, pWJ110-T, was digested with BamHI and the resulting DNA fragment used to transform the diploid yeast strain YPH274, selecting for tryptophan phototrophs. The transplacement of one copy of the *CBF2* gene in these transformants was verified by Southern hybridization of HindIII- or XbaI-cut genomic DNA with the *CBF2* internal 1.3-kb EcoRI fragment. Two transformants were sporulated, and tetrads were dissected. Both gave 2:2 segregation of viable/nonviable spores, and the viable colonies were all Trp^-.

Three additional gene disruption constructs were made by insertion of a blunt-ended 1.1-kb fragment containing the *URA3* gene into the SnaI site of pWJ110-1R and into the StuI and NdeI sites of pWJ110-1.3R. EcoRI fragments containing these constructs were used to transform strain YPH274. The transformants also gave 2:2 segregation of viable/nonviable spores.

Chromosomal Location of the CBF2 Gene

CBF2 was assigned to chromosome VII by hybridization of a *CBF2* probe to electrophoretically separated yeast chromosomes (Carle and Olson, 1985). The *CBF2* gene was localized to a position near *SUF4* on the right arm of chromosome VII by hybridization of the *CBF2* 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).

Construction of Expression Vectors and Plasmids

A 1.3-kb EcoRI fragment from within the *CBF2* structural gene was ligated into the BamHI site of the *E. coli* expression vector pET3c (Novagen, Madison, WI; a T7 RNA polymerase-based vector system) after blunt ending with Klenow and deoxynucleotide triphosphates. This plasmid (pWJ110-1.3c) should express a fusion protein with 13 amino acids from the T7 gene 10 leader. The nucleotide sequence of the fusion site was verified by double-strand sequencing as described above, except that a specific oligonucleotide was used as a primer. Three nucleotides at the BamHI site were deleted during the construction, resulting in an in-frame fusion, but with one less amino acid. A 3.3-kb ClaI–AvaI fragment of the *CBF2* gene containing the full length of the open reading frame except for the first four amino acids was also ligated into the BamHI site of pET3b, resulting in plasmid pWJ110-3b.

A 6.5-kb BamHI DNA fragment from pWJ110-6.5B was cloned into the BamHI sites of pRS316 and YEp13, resulting in pWJ110-316B and pWJ110-13B, respectively. A 4.2-kb PvuII DNA fragment from pWJ110-6.5B was subcloned into the SmaI sites of plasmid Bluescript SK, pRS313, pRS315, and pRS316 (Sikorski and Hieter, 1989), resulting in pWJ110-4.2P, pWJ110-313P, pWJ110-315P, and pWJ110-316P, respectively.

Expression in E. coli *and Antibody Preparation*

The *E. coli* expression vectors described above were introduced into *E. coli* strain BL21 (DE3) (Novagen), which contains an integrated copy of the T7 RNA polymerase gene under the control of the *lacUV5* promoter. Bacterial cultures were grown at 37°C in LB containing 100 μg/ml ampicillin. Overexpression of proteins was initiated by induction with 0.8 mM IPTG for 7 h. Cells were harvested by centrifugation and stored at −80°C or directly sonicated in STE buffer (10 mM Tris-Cl, pH 7.5, 1 mM EDTA, 100 mM NaCl) containing 1% of NP-40. Analysis by SDS-PAGE revealed the expressed protein to be in the insoluble pellet. After two sonications the pellets were either kept for injection or subjected to SDS-PAGE for excision of gel bands containing the expressed proteins. Both the pellet and the gel were used to produce antibodies in rabbits (Babco, Richmond, CA). The antibody against a polypeptide (amino acid 453–898) of the CBF2 protein derived from the 1.3-kb EcoRI fragment was purified from antiserum by affinity chromatography. Either *E. coli* crude lysate or yeast proteins overexpressed in *E. coli* were coupled to Affi-gel 10/15 mixture (Bio Rad Labs, Hercules, CA) in SDS-MES buffer, pH 6.0, as described by the manufacturer. The antiserum was first incubated with Affi-gel coupled with *E. coli* proteins overnight at 4°C, and the flow-through of this column was incubated with Affi-gel coupled with this specific polypeptide at 4°C overnight and washed with PBS buffer. The specific antibody was eluted with 0.1 M glycine, pH 2.8, and neutralized by adding one-tenth volume of 1 M Tris-Cl, pH 8.0.

Gel Mobility Shift Assay

The gel mobility shift assay was carried out as described by Lechner and Carbon (1991), except that a 3% polyacrylamide/0.5% agarose mixing gel was used, and 1 μl of the flow-through fraction from the DNA affinity column was used as chaperone instead of casein. Gel shift interference by antibody against the CBF2 protein was performed by adding antibody to the CBF3 proteins before mixing with the *CEN3* DNA probe. CBF1 (CPF1) protein was partially purified by heat treatment and Sephacryl S-200 chromatography basically as described by Jiang and Philippsen (1989).

Results

Cloning and Identification of the CBF2 *Gene Encoding the 110-kD Subunit (CBF3A) of a Centromere DNA-binding Protein Complex*

A multisubunit protein complex (CBF3) binding to CDEIII DNA sequences was isolated from *S. cerevisiae* as previously described (Lechner and Carbon, 1991). The *CEN* DNA affinity-purified material contains three major subunits of 110 (CBF3A), 64 (CBF3B), and 58 (CBF3C) kD. ~10 μg of the 110-kD protein, purified by SDS-PAGE, was subjected to tryptic digestion. Tryptic peptides were fractionated by HPLC and partial sequences were obtained for four peptides. Three degenerate oligonucleotides with sequences based upon these peptides were synthesized and subsequently used to screen a yeast genomic λgt11 library. A positive clone containing 1- and 1.3-kb EcoRI DNA fragments was found to hybridize to two of the oligonucleotide probes. These EcoRI DNA fragments were individually subcloned into a Bluescript plasmid vector and sequenced. The exact peptide sequences used to design the probes were found in a large open reading frame within these sequences. Since both EcoRI fragments contained only one continuous open reading frame without a stop codon, and the gene (*CBF2*) encoding the 110-kD protein would be predicted to be larger, these two DNA fragments were then used as probes to screen a genomic plasmid library, containing average inserts of 10 kb. The *CBF2* gene was finally localized to a 6.5-kb genomic BamHI fragment, shown in Fig. 1.

Nucleotide sequence analysis of the 4.1-kb DNA fragment shown in Fig. 1 revealed an uninterrupted open reading frame (ORF)[1] of 956 amino acids (Fig. 2), encoding a protein with a calculated molecular mass of 112 kD, agreeing quite well with the 110-kD molecular mass estimation from SDS gels. All four tryptic peptide sequences determined on the purified 110-kD protein could be found in this ORF. The deduced amino acid sequence indicates the CBF2 protein to be substantially hydrophilic with high amounts of acidic (15%), basic (13%) and hydroxyl (14%) residues. The net overall negative charge is −20 with a calculated isoelectric point of 6.05.

The deduced amino acid sequence of the CBF2 protein (CBF3A) (Fig. 2) was used to search the Genbank, EMBL, and NBRF/PIR data base for homology, using the FASTA program of Pearson and Lipman (1988). No significant homology could be found, except for a small COOH-terminal domain, which has a weak homology to the coiled-coil domain of myosin. The sequence was extensively compared with microtubule-based motor proteins, such as kinesin and dynein; no homology was detected. However, when we compared the sequence carefully with the consensus GTP-binding domain of most G-proteins (Bourne et al., 1991), a partial homology motif was found (Fig. 3). There are two

1. *Abbreviation used in this paper*: ORF, open reading frame.

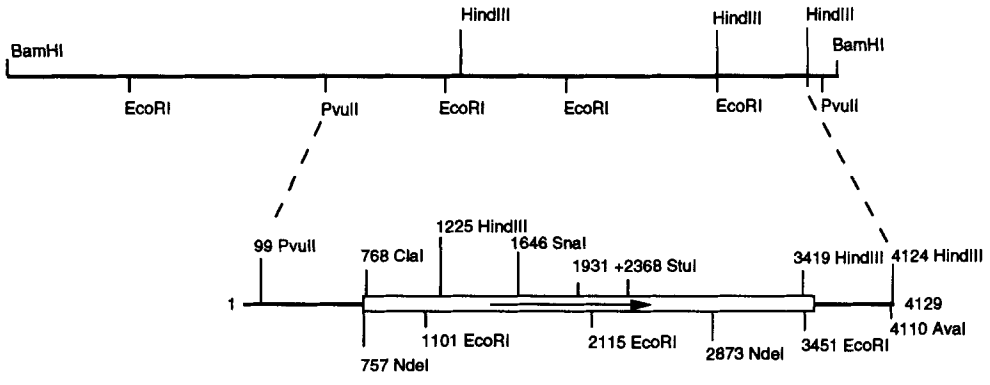

Figure 1. Restriction map of the yeast *CBF2* gene and its flanking regions. (*Upper panel*) A 6.5-kb BamHI DNA fragment containing the *CBF2* gene obtained from a *CEN4-LEU2* genomic library. (*Lower panel*) The 4,129-bp DNA fragment that was sequenced, showing restriction sites used for construction of *E. coli* and yeast overexpression vectors and for gene disruptions. The open bar and arrow indicate the open reading frame of the *CBF2* gene and the direction of translation, respectively.

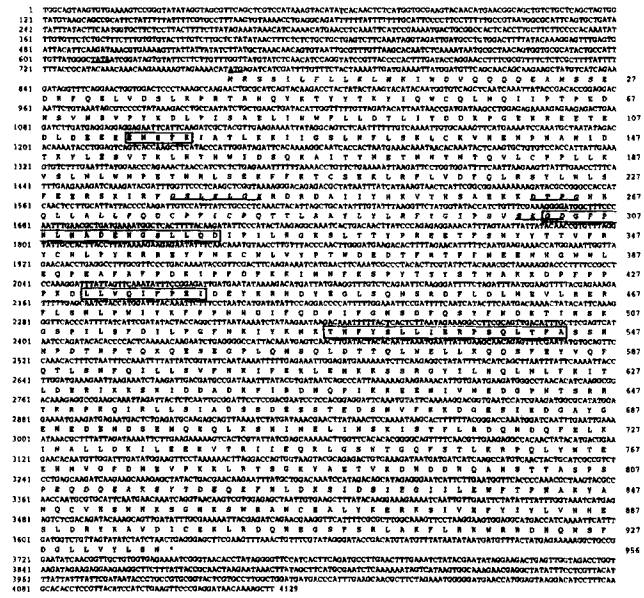

Figure 2. Nucleotide and amino acid sequences of *CBF2*. A TATA-like sequence and the most possible initiation ATG codon are underlined. The four tryptic peptide sequences obtained from purified CBF3A are enclosed in boxes. A possible nucleotide-binding site is underlined and italicized (see Fig. 3). These sequence data are available from EMBL under accession number Z21627.

differences between the putative nucleotide-binding domain of the CBF2 protein and the G-protein consensus, however; there is no Ser or Thr directly after the GX_4GK domain; and the distance between the GX_4GK and DXXG domains is appreciably less in the CBF2 protein than in the consensus sequence.

CBF2 *Is an Essential Gene on Chromosome VII*

To generate a null mutation in the *CBF2* gene, the 2016 bp NdeI fragment of the cloned gene (from pWJ110-6.5B, Fig. 1), which contains the NH_2-terminal 705 amino acids, was replaced by an EcoRI–BglII fragment containing the *TRP1* gene. A BamHI fragment containing this replacement construct was used to transform diploid yeast strain YPH274, selecting Trp^+ transformants. The expected replacement of one copy of the wild-type *CBF2* gene with the disrupted copy was confirmed by Southern analysis (data not shown). Sporulation of this gene-disrupted strain (YWJ110-1) gave rise to two viable spores with Trp^- phenotype and two nonviable spores which germinate to form only one to three small undivided buds, indicating that the *CBF2* gene is essential for cell growth.

Additional gene disruption mutations were made at a SnaI site (amino acid 296), the second StuI site (amino acid 537) and the second NdeI (amino acid 705) site of the ORF by insertion of a *URA3* gene into one copy of the wild-type *CBF2* gene. Sporulation of these three strains (YWJ110-2, YWJ110-3, and YWJ110-4) also gave 2:2 segregation of viable and nonviable spores, indicating that the amino acid sequence from 706 to the COOH terminus is important to maintain CBF2 protein function in vivo.

Data from hybridization of *CBF2* DNA to yeast chromosomes separated by pulsed field electrophoresis suggested that the *CBF2* gene is located on chromosome VII. Hybridization of the probe to a set of contiguous overlapping lambda clones, obtained from Maynard Olson's laboratory, localized the *CBF2* gene between the *spt6* and *rsr1* genes, closely linked to *SUF4* on the right arm of chromosome VII.

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G-Protein          GxxxxGKS------(40-130)----DxxG--(35-70)--NKxD
Consensus:              T                          T

Dynamin D100:    VVGGQSAGKSSVL-----(85)----LVDLPGM---(63)--ITKLDL
Yeast   MGM1:    VIGSQSSGKSSVL-----(87)----LVDLPGY---(62)--ITKLDL
Yeast   SPO15:   VVGSQSSGKSSVL-----(129)---LVDLPGL---(63)--LTKVDL
Mouse     Mx:    VIGDQSSGKSSVL-----(89)----LIDLPGI---(63)--LTKPDL

E. coli  ERA:    IVGRPNVGKSTLL-----(34)----YVDTPGG---(50)--VNKVD
E. coli  IF2:    IMGHVDHGKTSLL-----(33)----FLDTPGH---(49)--VNKID

Yeast   CBF2: 235RFGSLKLGKRDRD-----(12)----KKDTPGH---(36)--VSKGDG305
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Figure 3. Amino acid sequence similarities between CBF2p and some GTP-binding proteins. The GTP-binding consensus sequences are indicated by bold letters (reviewed by Bourne et al., 1991). References are taken from Jones and Fangman (1992) (for dynamin D100, MGM1, SPO15/VPS, Mx) and from Bourne et al. (1991) (for ERA and IF2).

The CBF2 *Gene Product Is the 110-kD Protein Involved in the Centromeric CBF3–CDEIII Binding Complex*

A polyclonal antibody directed against a 446-amino acid polypeptide (amino acid 453 to 898) from the COOH terminus of the CBF2 protein was purified from rabbit antiserum directed against *E. coli* overproduced protein (see Materials and Methods). This antibody specifically recognizes the 110-kD protein subunit of CBF3 purified as previously described (Lechner and Carbon, 1991, and Fig. 4 *A*). Further evidence that the *CBF2* gene specifies a protein involved in the CBF3-*CEN* DNA complex was derived from a gel mobility shift experiment in which affinity-purified CBF3 was bound to a 350-bp *CEN3* DNA fragment. Addition of this antibody gave supershifted bands for the CDEIII DNA–CBF3 protein complex (Fig. 4 *B*, lanes *3* and *4*), while preimmunserum did not (Fig. 4 *B*, lanes *5* and *6*). As expected, this antibody against CBF3A has no effect on the gel shift of the CDEI DNA-CBF1 protein complex (Fig. 4 *B*, lanes *8* and *9*). This clearly indicates that the *CBF2* gene product, i.e., the CBF3A protein, is a component of the centromere DNA–protein complex, specifically the CDEIII DNA–protein complex.

Discussion

The CBF2 *Gene Specifies the 110-kD Subunit of the Yeast Kinetochore Protein Complex CBF3*

Identification and purification of CBF3, a 240-kD multisubunit protein complex that binds specifically to the CDEIII region of the yeast *CEN* locus (Lechner and Carbon, 1991), has proven to be an important breakthrough in our efforts to understand the molecular mechanism of centromere function. The biological relevance of CBF3 previously was implied from in vitro analyses of differential binding of CBF3 to wild-type versus mutationally inactivated *CEN* DNA fragments. In addition, our most highly purified preparations of CBF3, when assayed in vitro by a microbead assay, dis-

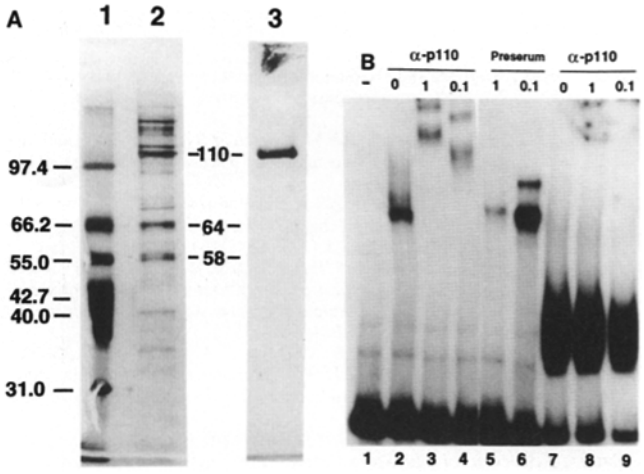

Figure 4. CBF2 encodes the large subunit (110 kD, CBF3A) of the multisubunit yeast centromere binding protein complex CBF3. (*A*) Immunoblot of the partially purified CBF3 protein with affinity-purified anti-CBF2p polyclonal antibody. 10 μl of CBF3 protein eluted from a *CEN* DNA affinity column (Lechner and Carbon, 1991) was loaded onto an 8% SDS-PAGE (lanes *2* and *3*). Lanes *1* and *2* are silver stained with the method described by Jiang and Philippsen (1989); lane *3* is an immunoblot of the same proteins as in lane *2*. Molecular weight standards (Promega Corp., lane *1*) are indicated on the left in kD. The three CBF3 subunits (110, 64, 58 kD) are indicated. (*B*) Antibody against CBF2p interacts with the CBF3-*CEN* DNA complex. 2 μl of the affinity-purified CBF3 proteins (lanes *2–6*) or 1 μl of the partially purified CBF1 protein (Jiang and Philippsen, 1989) (lanes *7–9*) were mixed with 0 (lanes *2* and *7*), 1 (lanes *3* and *8*), or 0.1 (lanes *4* and *9*) μl of the purified anti-CBF2p antibody, or with 1 (lane *5*) or 0.1 (lane *6*) μl of the preimmune serum, and then assayed for *CEN3* DNA binding activity on a native 0.5% agarose/3% polyacrylamide gel as described in Materials and Methods. A control containing *CEN* DNA but without centromere DNA-binding proteins is shown in lane *1*. The major shifted band in lanes *2*, *5*, and *6* and the weak shifted band in lane *6* are *CEN* DNA-CBF3 protein complexes *B* and *C*, respectively (Lechner and Carbon, 1991). Dilution of serum may also dilute inhibitory factors in the serum for the *CEN* DNA-CBF3 protein binding activities (lanes *5* and *6*).

play an ATP-dependent molecular motor activity that moves *CEN* DNA-coated beads along microtubules in a minus end-oriented direction (Hyman et al., 1992), the same direction that the chromatids move during anaphase A. The isolation of the *CBF2* gene specifying the 110-kD subunit of CBF3 has now enabled us to demonstrate that CBF3 functions in vivo as a nuclear component essential for cell viability.

The *CBF2* gene was cloned by using *CEN* DNA affinity-purified CBF3 as a starting point. The individual subunits separated by SDS-PAGE were subjected to tryptic digestion, the resulting peptides fractionated by HPLC, and partial amino acid sequences determined on a few purified peptides. Synthetic oligodeoxynucleotides with degenerate sequences based on these peptides were used as probes to identify hybridizing clones in yeast genomic libraries. One danger inherent in this experimental strategy is that the cloned gene might actually be derived from an irrelevant protein in the original CBF3 preparation. What lines of evidence support our view that the *CBF2* gene described here actually codes for synthesis of a yeast kinetochore protein? Antibodies directed against the protein obtained by over-expression of the *CBF2* gene in *E. coli* bind to the CBF3–*CEN* DNA complex and reduce the electrophoretic mobility of this complex in a standard fragment mobility shift assay (Fig. 4 *B*). Thus, this antibody recognizes a protein component of a complex which binds specifically to wild-type *CEN* DNA, but which cannot bind to mutationally inactivated *CEN* DNA.

In an accompanying paper in this issue of *The Journal of Cell Biology*, Goh and Kilmartin (1993) describe a yeast temperature-sensitive mutant strain (*ndc10*) that, at the non-permissive temperature, displays a massive chromosome missegregation phenotype. The wild-type *NDC10* function is necessary for chromosome segregation but not for assembly of the mitotic spindle. In mutant cells at restrictive temperatures, the chromosomes remain at one pole and do not move to the bud junction. Cloning and sequencing of the *NDC10* gene has now revealed it to be essentially identical to *CBF2* (a single amino acid difference at position 775 appears to be due to strain variation). This mutant offers further strong evidence that CBF2p is in some way involved in the action of the kinetochore.

Properties of the Kinetochore Protein CBF2p (CBF3A)

The *CBF2* gene encodes a protein of 956 amino acids with a calculated molecular mass of 112 kD, agreeing quite well with estimates of molecular weight from SDS-PAGE on purified CBF3 (110 kD; Lechner and Carbon, 1991). CBF2p is quite hydrophilic, especially toward the COOH-terminal one third of the molecule; no long hydrophobic regions are present.

CBF2p prepared by over expression in *E. coli* was incapable of forming a complex with a 350-bp *CEN* DNA fragment, when subjected to the typical fragment mobility shift assay (see Materials and Methods). *CEN* DNA recognition may depend upon the presence of one or more of the other CBF3 subunits, or a posttranslational modification might be required. In that regard, phosphatase treatment of CBF3 inactivates the complex in terms of *CEN* DNA binding (Lechner and Carbon, 1991). In addition, over expression of a protein kinase gene (*MCK1*) has been found to suppress a partially inactivating mutation in CDEIII (Shero and Hieter, 1991; Dailey et al., 1990).

Purified preparations of CBF3 contain a minus end-directed molecular motor activity on bovine microtubules (Hyman et al., 1992). What is the relationship, if any, between CBF2p and this motor activity? Kinesin from bovine brain is also a multisubunit protein complex, containing two 120-kD heavy chains and two 62-kD light chains (Bloom et al., 1988; Kuznetsov et al., 1988). Because of the coincidence of molecular weights and the motor activity of CBF3, we extensively compared the sequence of CBF2p with known kinesin heavy chains (Yang et al., 1989; Kosik et al., 1990; Wright et al., 1991) and four kinesin-related yeast proteins: KAR3 (Meluh and Rose, 1990); SMY1 (Lillie and Brown, 1992); CIN8 (Hoyt et al., 1992); KIP1 (Roof et al., 1992), and with the bovine kinesin light chain (Cyr et al., 1991). No homology with the kinesin consensus motor domain or light chain was detected, although CBF2p does contain sequences partially homologous to the consensus GTP binding site in various G-proteins (Bourne et al., 1991) (Fig. 3). Interestingly, dynamin, a 100-kD protein that in vitro induces microtubules to form hexagonally packed bundles and has

microtubule-stimulated ATPase and GTPase activities (Shpetner and Vallee, 1989, 1992), also contains this consensus GTP binding sequence (Obar et al., 1990). It is also possible that the sequence homologies pointed out in Fig. 3 are indicative of an ATP-binding site in CBF2p. The motor activity associated with CBF3 is functional in the presence of GTP, although velocities are greater when ATP is used (Hyman et al., 1992). No significant homologies were detected when the CBF2p sequence was compared with the other known microtubule-based motor protein, dynein (Gibbons et al., 1991; Ogawa, 1991).

Thus, the role of CBF2p in the mechanochemical activity associated with purified CBF3 is still unclear. An antiserum directed against CBF2p prepared by over-expression in *E. coli* inhibits the CBF3-associated motor activity as determined by a microtubule gliding assay (Middleton, K., W. Jiang, and J. Carbon, unpublished results), suggesting that CBF2p is associated in some way with the motor subunit. However, preliminary results indicate that *E. coli*-overexpressed CBF2p assayed alone has no motor activity in the microtubule gliding assay. At this point, we cannot exclude the possibility that one of the other subunits of CBF3 is the actual motor subunit. Because of the presence of a consensus GTP binding domain in CBF2p, it is possible that this subunit, as is the case with most G-proteins, plays a regulatory role in kinetochore function.

We thank Kim Middleton for help in purifying CBF3 proteins and for providing samples of pure CBF3; Louise Clarke for many helpful discussions; Claire Fouquet for proof-reading sequencing gels; Michael Centola and Mohamed Khan for help with the OFAGE gels; Mary Baum and Arlicia Salter for technical assistance; Jeff Miller and Andrew Billin for advice on construction of *E. coli* expression vectors; Linda Riles and Maynard Olson for providing dot blots of overlapping yeast DNA clones; Ann Sperry and Phil Hieter for the *CEN4/LEU2* genomic library. Peptide sequences were determined by Ralph Reid at the University of California at San Francisco Biomolecular Resource Facility.

This research was supported by a grant from the National Cancer Institute, National Institutes of Health (CA-11034). J. Carbon is an American Cancer Society Research Professor.

Received for publication 18 November 1992 and in revised form 26 January 1993.

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