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

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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 mitotic 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.

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]TGNTCTGGAAANNNAAAA) is absolutely essential for centromere function (McGrew et al., 1986; Ng and Carbon, 1987; Hegemann et al., 1989; Jehn et al., 1991), while mutations in or deletion of CDEI (PuT-CACpTG) impair but do not abolish function (Cumberledge and Carbon, 1987; Gaudet and Fitzgerald-Hayes, 1987; Niedenthal et al., 1991). A protein (CPI or CBFI) 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 (CBFI/CPFI/CEFI) 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 CENV 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 kD (CBF3A), 64 kD (CBF3B), and 58 kD (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: YPH774 (a/a ade2-101 leu2-112 trpl-1 Δ/Δ try2Δ/Δ trp1::URA3 trp2::URA3, (c) DLLVQIFPEI; (d) (? × 8)ENSFK. Strains, Media, and Microbial Techniques

Gene and Sequence Analysis

Materials and Methods

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Disruption of the CBF2 Gene

A 2.1-kb Ndel fragment containing more than two thirds of the CBF2 gene (including the NH2-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 YPH774, selecting for tryptophan phototrophs. The transplacement of one copy of the CBF2 gene in these transformants was verified by Southern hybridization of HindIII-Xbal-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+.

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 SUC4 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

Probes were end labeled with T4 polynucleotide kinase and [γ-32P]ATP and used to screen a yeast genomic Agt11 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 Bluescript SK; plasmid 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 dyeoxy 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/VAAX computer. Searches for sequence homology were performed through GenBank, EMBL, and NBRF/PIR data bases via the FASTA-MAIL program.

Peptide Analysis of CBF3 Protein

The CBF3 protein complex was purified as described by Lechner and Carbon (1991) from a cbf3-deficient diploid yeast strain YW90. ~10% 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). "lYyp-transfer onto Immobilon polyvinylidene difluoride (PVDF) membranes (Millipore Corp., Bedford, MA) as described by Mellor et al., 1990); mating type tester strains H272a (a/c~ ade2-1Ol°Ch'e/ade2-1Ol°Chre/trpl-Al/trpl-Al leu2-Al/leu2-Al ura3-52/ura3-52 his3/HlS3 gal2/GAL2 pral/PRAl prbl/prcl cpsl/cpsl URA3 URA3, (c) DLLVQIFPEI; (d) (? × 8)ENSFK. Strains, Media, and Microbial Techniques

the construction, resulting in an in-frame fusion, but with one less strand sequencing as described above, except that a specific oligonucleotide was used as a primer. Three nucleotides at the BamHI site were deleted during subcloning. A 1.3-kb EcoRI fragment containing the TRP1 gene was derived from YW90, selecting for tryptophan phototrophs. The transplacement of one copy of the CBF2 gene in these transformants was verified by Southern hybridization of HindIII-Xbal-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+.

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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) after end-ligation of the fragment 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 subcloning, resulting in an in-frame fusion, with one less amino acid. A 3.3-kb Clal-Aval 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 YEp3, resulting in pWJ110-316B and pWJ110-13B, respectively. A 4.2-kb Psvil DNA fragment from pWJ110-6.5B was subcloned into the Smal sites of plasmid Bluescript SK, pRS313, pRS315, and pRS316 (Silkowsi and Hieter, 1989), resulting in pWJ110-4.2P, pWJ110-313P, pWJ110-315P, and pWJ110-316P, respectively.

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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 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 containing 1% of NP-40. Analysis by SDS-PAGE revealed the expressed proteins. Both the pellet and the gel were used to produce antibodies in rabbits (Babco, Richmond, CA). The antibody either kept for injection or subjected to SDS-PAGE for excision of gel bands of the expressed proteins. Both the pellet and 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 1015 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 (CBF1) protein was partially purified by heat treatment and Sephacryl S-200 chromatography as described by Jiang and Philippens (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 Xgt11 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) 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

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

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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.

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 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 DI00, MGMI, SPO15/VPS, Mx) and from Bourne et al. (1991) (for ERA and IF2).
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 cryptic 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 resulting peptides fractionated by HPLC, and partial inhibitory factors in the serum for the CEN DNA-CBF3 protein binding activities (lanes 5 and 6).

rected 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 mutationaly 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 nonpermissive 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 mechaonomy 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.

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