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# A zinc finger protein, essential for chromosome segregation, constitutes a putative DNA binding subunit of the *Saccharomyces cerevisiae* kinetochore complex, Cbf3

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**A multisubunit protein complex, Cbf3, is a component of the *Saccharomyces cerevisiae* kinetochore. Cbf3 was recently shown to be essential for chromosome segregation *in vivo* and for movement of centromere DNA (*CEN*) along microtubules *in vitro*. Cbf3 contains three proteins, Cbf3a, Cbf3b and Cbf3c. Here the characterization of Cbf3b is described. Cbf3b contains an N-terminal $Zn_2Cys_6$ type zinc finger domain, a C-terminal acidic domain and a putative coiled coil dimerization domain. Cbf3b is essential for growth. Mutations within the zinc finger domain result in cells that exhibit a $G_2$–M cell cycle delay and increased chromosome loss in each mitotic cell division. Therefore, Cbf3b has an essential function in chromosome segregation and the zinc finger domain executes part of this function presumably by providing the specific interaction between Cbf3 and *CEN*. Finally, data are provided to show that Cbf3c is encoded by *CTF13*, a gene that had been cloned recently by complementing a temperature sensitive mutant that exhibits chromosome loss as a result of a defective centromere.**

*Key words*: centromere/kinetochore/zinc finger

## Introduction

The components constituting kinetochores/centromeres in yeast and higher eukaryotes have received major scientific attention in recent years (Brinkley, 1990; Clarke, 1990; Pluta *et al.*, 1990; Schulman and Bloom, 1991; Bloom, 1993; Hegemann and Fleig, 1993). Centromeres/kinetochores play an essential role in chromosome segregation during mitosis and meiosis. Centromeres facilitate sister chromatid association. Kinetochores contain spindle attachment sites and molecular motors that move chromosomes on the microtubules during chromosome segregation (Pfarr *et al.*, 1990; Steuer *et al.*, 1990; Hyman and Mitchison, 1991; Yen *et al.*, 1992). Since centromeres/kinetochores execute this role at a particular time point in the cell cycle, it is feasible that the activity of centromeres/kinetochores is cell cycle controlled and that the successful assembly of centromeres is supervised. Evidence for both of these assumptions has been reported (Kingsbury and Koshland, 1991; Spencer and Hieter, 1992).

To understand centromere/kinetochore structure and function at the molecular level it is important to isolate the centromere components. The use of autoimmune sera of patients with the CREST syndrome and of monoclonal antibodies against chromosomal scaffold proteins led to the identification of several proteins, CENPA, B, C, D and E and INCENPA and B, that localize to the centromere region of higher eukaryotes, and to the cloning and sequencing of the corresponding genes (see Bloom, 1993 for review).

Investigating centromere structure and function in *Saccharomyces cerevisiae* has multiple advantages. First, in contrast to the centromere DNA of higher eukaryotes *S.cerevisiae* centromere DNA (*CEN*) is very small and extensively characterized. A functional *S.cerevisiae CEN* consists of only 125 bp (Hegemann *et al.*, 1988) and is organized into three domains, CDE I, CDE II and CDE III (Clarke and Carbon, 1985). CDE II, which contains 78–86 bp of AT-rich DNA, is flanked to the left by CDE I, consisting of an 8 bp consensus sequence [(A/G)TCAC(A/G)TG], and to the right by CDE III, consisting of a 25 bp consensus sequence [TGTTT(T/A)TGNTTT-CCGAAANNNAAAAA]. Extensive mutational analysis (McGrew *et al.*, 1986; Cumberledge and Carbon, 1987; Gaudet and Fitzgerald-Hayes, 1987; Ng and Carbon, 1987; Jehn *et al.*, 1991; Niedenthal *et al.*, 1991) revealed the central CCG triplet of CDE III to be absolutely essential for centromere function. Single basepair mutations in this sequence destroy the *S.cerevisiae* centromere. No other single basepair exchange within *CEN* nor partial deletion of CDE II or complete deletion of CDE I has such drastic effects on centromere function. Second, functional centromere assays (Clarke and Carbon, 1983; Hegemann *et al.*, 1988) can be applied to investigate centromere components.

Putative kinetochore proteins may be encoded by *CSE1* and *CSE2* (Xiao *et al.*, 1993). Mutations within these genes lead to increased chromosomal missegregation in strains carrying CDE II mutations. Also, CBF5p, a protein obtained from yeast extracts by low stringency *CEN* affinity chromatography (Jiang *et al.*, 1993b), could be a component of the yeast kinetochore. However, for neither of these proteins has a direct interaction with *CEN* been demonstrated. Presently, only two components of the *S.cerevisiae* kinetochore that can bind to centromere DNA *in vitro* have been isolated: Cbf1 (Cp1, Cpf1), a protein that binds to the CDE I domain of *CEN* as a dimer (Bram and Kornberg, 1987; Baker *et al.*, 1989; Cai and Davis, 1989, Jiang and Philippsen, 1989) and Cbf3, a multisubunit protein complex that binds to the CDE III domain (Lechner and Carbon, 1991). The gene encoding Cbf1 has been cloned and characterized (Baker and Marison, 1990; Cai and Davis, 1990; Mellor *et al.*, 1990). This work revealed that Cbf1 is important for high fidelity chromosome segregation. However, *cbf1* null mutants are viable and exhibit diminished, but not abolished, centromere function in addition to methionine auxotrophy.

The protein complex Cbf3 was originally defined as

**Table I.** Comparison of CBF3C peptide sequences and CTF13 protein sequence

| CBF3C peptides sequenced | Location of matching residues in the CTF13 protein sequence[a] |
|---|---|
| PSFNPVR | 2–8 |
| LELPIDIR | 10–17 |
| VAYIDLNS | 342–249 |

[a]Taken from Doheny *et al.* (1993)

the activity that binds to the CDE III element of *CEN* resulting in Cbf3–*CEN* complexes that were characterized by an electrophoretic gel mobility shift assay (Ng and Carbon, 1987; Lechner and Carbon, 1991). Cbf3 consists of the subunits Cbf3a, b and c. Minor protein components found in Cbf3 preparations are unlikely to contribute to Cbf3 for reasons discussed elsewhere (Lechner and Carbon, 1991). However, it cannot be absolutely excluded that other proteins beside Cbf3a, b and c are part of the Cbf3 complex. Cbf3 is absolutely essential for centromere function. This stems from various lines of evidence. First, Cbf3 binds *in vitro* specifically to wild type *CEN* but not to *CEN* with a point mutation in the CCG triplet of CDE III that inactivates the centromere *in vivo*. Second, Cbf3 preparations are able to move microtubules on *CEN* *in vitro*, and Cbf3 was shown to be a necessary component for this movement to occur (Hyman *et al.*, 1992). Third, analyzing the gene of Cbf3a (*CBF2*) (Jiang *et al.*, 1993a) showed that Cbf3a is essential for growth and that mutations in the Cbf3a-encoding gene are responsible for the phenotypes of *ndc10*-1 and *ctf14*-42, temperature senstitive (ts) mutants that exhibit severe chromosome non-disjunction (Goh and Kilmartin, 1993) and centromere defects (Doheny *et al.*, 1993).

Recently a very likely candidate for the Cbf3c gene, *CTF13*, was described (Doheny *et al.*, 1993). *CTF13* was isolated by complementing a ts mutant, *ctf13*-30, that exhibits chromosome loss at the permissive temperature and a cell cycle delay at the $G_2$–M transition at the non-permissive temperature. Furthermore, *CTF13* encodes a 56 kDa protein that is a component of *in vitro* assembled Cbf3–*CEN* complexes.

Here the characterization of Cbf3b is presented. Data are also provided to show that Cbf3c is indeed encoded by *CTF13*.

## Results

### *Cbf3c is encoded by CTF13*

To obtain partial amino acid sequence data of Cbf3c, the Cbf3 complex was affinity purified as described (Lechner and Carbon, 1991) and subjected to SDS gel electrophoresis to fractionate the Cbf3 subunits (see Figure 2 in Lechner and Carbon, 1991). Purified Cbf3c was subjected to in-gel digestion with trypsin, and peptides were extracted from the gel and purified by reversed phase HPLC. Partial amino acid sequence from three peptides was obtained by gas phase sequencing (Table I). As mentioned above, the *CTF13* gene was cloned by complementation of a ts mutant exhibiting chromosome mis-segregation, which likely encodes Cbf3c. Therefore, the peptide sequences from Cbf3c and the deduced amino acid sequence of the *CTF13* gene product were compared. All three Cbf3c peptides matched amino acid sequences in CTF13p. Therefore, *CTF13* indeed encodes Cbf3c.

### *Cloning and characterization of the gene encoding Cbf3b*

To clone the gene encoding Cbf3b, three tryptic peptides were derived from Cbf3b as described above for Cbf3c. Polymerase chain reaction technology was used to amplify a Cbf3b-encoding gene fragment with degenerate primers made according to sequence data from two of the peptides (Figures 1 and 2). This DNA fragment was then used to screen an *S.cerevisiae* genomic library in λ Dash II. The DNA of a positive clone obtained revealed 1824 bp of open reading frame preceded by stop codons in all three reading frames (Figure 1). All of the partial amino acid data derived from peptide sequencing were found in this reading frame. The deduced amino acid sequence encodes a protein with a pI of 7.8 and a calculated mass of 71 kDa, which differs somewhat from the 64 kDa molecular mass estimated by SDS gel electrophoresis (Lechner and Carbon, 1991). The Cbf3b-encoding gene exhibits a codon usage indicative of a lowly expressed gene (Sharp *et al.*, 1986). This is in agreement with the finding that Cbf3 can be purified from yeast in only small amounts (Lechner and Carbon, 1991).

The predicted amino acid sequence of Cbf3b shows several noteworthy features all of which are reminiscent of fungal transcriptional activators like Gal4. The Cbf3b N-terminus contains a region (residues 14–42) highly homologous to the consensus sequence of the $Zn_2Cys_6$ type of zinc finger motif (Figure 1) that is an established DNA binding domain (Keegan and Ptashne, 1986).

The C-terminus of Cbf3b (residues 574–605) is highly acidic with a net charge of −11. Similar acidic regions have been shown to mediate protein–protein interaction in several cases (Mu and Ptashne, 1987; Ptashne and Gann, 1990; He *et al.*, 1993; Li and Botchan, 1993). A second possible protein–protein interaction domain includes residues 370–419. This region represents a heptad repeat that provides a hydrophobic protein–protein interface when folded into an α-helix. No significant homologies to other proteins besides the transcriptional activators mentioned above could be found for Cbf3b using the BLASTP program to search the SwissProt database. In particular, no similarities to known molecular motors like kinesin or dynein could be detected. Also, no homologies to those proteins, which localize to the centromere region of higher eukaryotes (CENPA–E and INCENPA and B), were found.

### *The cloned gene encodes Cbf3b, a component of the Cbf3–CEN complex*

To prove that the gene cloned encodes Cbf3b, a polyclonal antibody was raised against the *Escherichia coli* expression product of the cloned gene (see Materials and methods). This antibody specifically recognized the 64 kDa protein (Cbf3b) of Cbf3 preparations (Figure 3) and did not detect any proteins in a control preparation that did not contain Cbf3. The control preparation was obtained as described before (Lechner and Carbon, 1991) by affinity chromatography on *CEN* with a centromere-inactivating point mutation in CDE III.

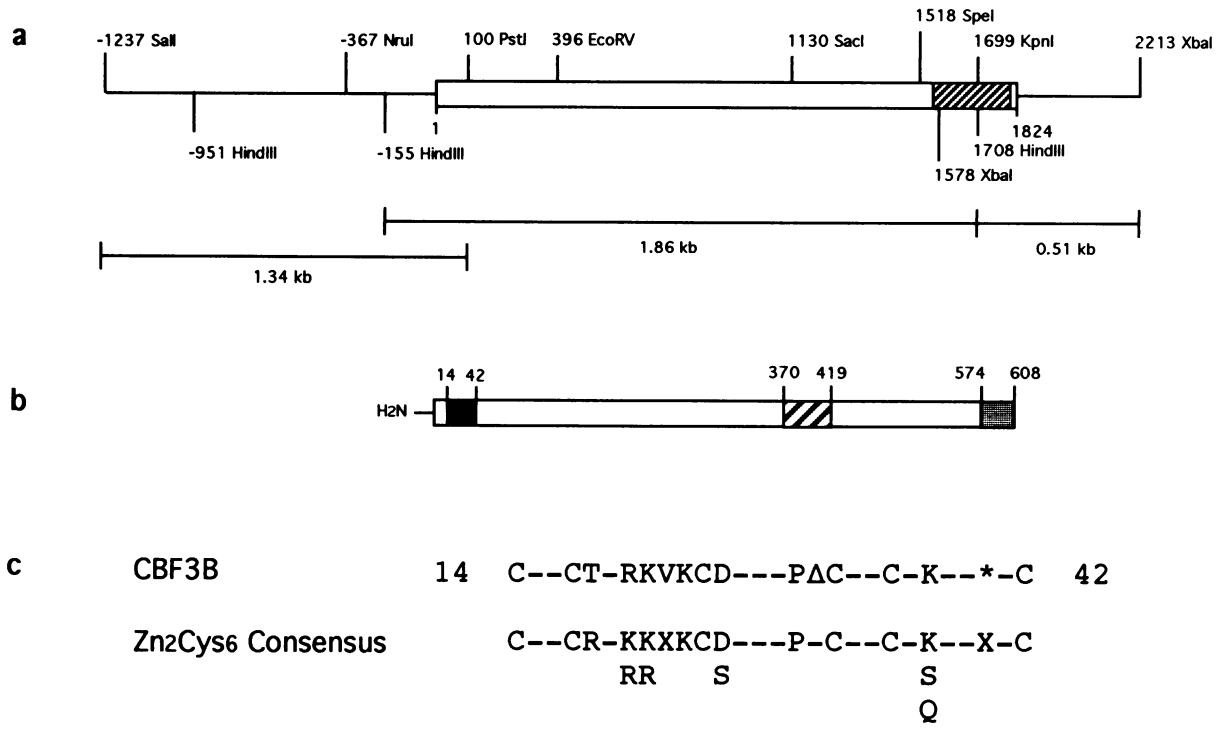


**Fig. 1.** (**a**) Restriction map of the *CBF3B* gene (open bar) and flanking regions. A striped box represents the *CBF3B* gene fragment produced by PCR that was used to identify the Cbf3b-encoding gene. (**b**) Organization of the Cbf3b polypeptide. The filled in region represents a putative zinc finger motif, the striped region contains a heptad repeat indicative of a coiled-coil protein interface and the stippled region represents an acidic domain with a negative net charge of −11. (**c**) Comparison of Cbf3b's primary structure with the $Zn_2Cys_6$ zinc finger consensus sequence (taken from Kraulis *et al.*, 1992). X is a hydrophobic amino acid, * represents a tripeptide and Δ indicates a missing amino acid.

Furthermore, to establish the identity of the cloned gene, it was shown that the protein encoded by the cloned gene is a component of the Cbf3−*CEN* complex as defined by the electrophoretic mobility shift assay (Lechner and Carbon, 1991). Affinity purified Cbf3 (1 pmol) was incubated with abundant *CEN* (4 pmol of low specific $^{32}P$ activity) (Figure 4a, lane 1) favoring the formation of Cbf3−*CEN* complexes over free Cbf3. After fractionation in a non-denaturing gel, Cbf3B−*CEN* complexes and bordering fractions were excised and subjected to SDS gel electrophoresis (Figure 4b). Western analysis with the polyclonal antibody described above detected a protein that was highly enriched in fractions containing Cbf3−*CEN* complexes in comparison with bordering fractions (Figure 4b, lanes 3–6) and that comigrated with the 64 kDa protein (Cbf3b) of Cbf3. To verify that the protein detected does not represent unbound protein that coincidentally comigrates with the CBF3−*CEN* complex, a control experiment was performed (Figure 4a, lane 2). In this case, affinity purified Cbf3 (1 pmol as above) was incubated with substoichiometric amounts of *CEN* (20 fmol with high specific $^{32}P$ activity). This limited the amount of Cbf3−*CEN* complexes that could form to a level that was barely detectable by the immunological method used while providing a visual way to identify the appropriate control fractions (Figure 4a, lane 2). When the control fractions were processed as above, only an insignificant amount of the 64 kDa protein was detected (Figure 4b, lanes 7–10). Thus, the 64 kDa protein detected does not comigrate with the Cbf3−*CEN* complexes. Consequently the protein detected in lane 1 (fractions II and III) of the non-denaturing gel is part of the Cbf3−*CEN* complex, demonstrating that the protein encoded by the cloned gene represents Cbf3b.

### *Anti-Cbf3b antibody inhibits Cbf3−CEN complex formation*

To examine further the role of Cbf3b in Cbf3−*CEN* complex formation, we investigated how the anti-Cbf3b antibody would influence the formation of Cbf3−*CEN* complexes. The anti-Cbf3b antibody inhibits Cbf3 binding to *CEN* when preincubated with affinity purified Cbf3 (Figure 5, lanes 1–4). Preincubation of the antibody with recombinant Cbf3b prevented the effect, showing that the inhibition is due to antibodies directed against Cbf3b epitopes (Figure 5, lane 5). This further proves that Cbf3b is part of Cbf3−*CEN*. In addition, this strongly indicates that Cbf3b is directly involved in the Cbf3−*CEN* interaction.

### *Phenotypical analysis of cbf3b mutants*

To address the *in vivo* function of Cbf3b, one copy of *CBF3B* in the diploid strain YP501 was disrupted by replacing the *Eco*RV−*Spe*I fragment of the coding region (Figure 1) with the TRP1 sequence. The resulting heterozygote, YJL1 (*cbf3b::TRP1/CBF3B*), was sporulated. Analysed tetrads revealed only two viable spores; all of them were $Trp^-$. The two nonviable spores germinated and produced a few undivided buds. Therefore, Cbf3b is essential for growth.

To examine further the Cbf3b function *in vivo*, a Pro to Leu mutation at residue 29 or a Lys to Glu mutation at residue 21 of the Cbf3b zinc finger region was engineered, resulting in *cbf3b*-L29 or *cbf3b*-E21 alleles (see Figure

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Met Phe Asn Arg Thr Thr Gln Leu Lys Ser Lys His Pro Cys Ser   15
Val Cys Thr Arg Arg Lys Val Lys Cys Asp Arg Met Ile Pro Cys   30
Gly Asn Cys Arg Lys Arg Gly Gln Asp Ser Glu Cys Met Lys Ser   45
Thr Lys Leu Ile Thr Ala Ser Ser Ser Lys Glu Tyr Leu Pro Asp   60
Leu Leu Leu Phe Trp Gln Asn Tyr Glu Tyr Trp Ile Thr Asn Ile   75
Gly Leu Tyr Lys Thr Lys Gln Arg Asp Leu Thr Arg Thr Pro Ala   90
Asn Leu Asp Thr Asp Thr Glu Glu Cys Met Phe Trp Met Asn Tyr  105
Leu Gln Lys Asp Gln Ser Phe Gln Leu Met Asn Phe Ala Met Glu  120
Asn Leu Gly Ala Leu Tyr Phe Gly Ser Ile Gly Asp Ile Ser Glu  135
Leu Tyr Leu Arg Val Glu Gln Tyr Trp Asp Arg Arg Ala Asp Lys  150
Asn His Ser Val Asp Gly Lys Tyr Trp Asp Ala Leu Ile Trp Ser  165
Val Phe Thr Met Cys Ile Tyr Tyr Met Pro Val Glu Lys Leu Ala  180
Glu Ile Phe Ser Val Tyr Pro Leu His Glu Tyr Leu Gly Ser Asn  195
Lys Arg Leu Asn Trp Glu Asp Gly Met Gln Leu Val Met Cys Gln  210
Asn Phe Ala Arg Cys Ser Leu Phe Gln Leu Lys Gln Cys Asp Phe  225
Met Ala His Pro Asp Ile Arg Leu Val Gln Ala Tyr Leu Ile Leu  240
Ala Thr Thr Thr Phe Pro Tyr Asp Glu Pro Leu Leu Ala Asn Ser  255
Leu Leu Thr Gln Cys Ile His Thr Phe Lys Asn Phe His Val Asp  270
Asp Phe Arg Pro Leu Leu Asn Asp Asp Pro Val Glu Ser Ile Ala  285
Lys Val Thr Leu Gly Arg Ile Phe Tyr Arg Leu Cys Gly Cys Asp  300
Tyr Leu Gln Ser Gly Pro Arg Lys Pro Ile Ala Leu His Thr Glu  315
Val Ser Ser Leu Leu Gln His Ala Ala Tyr Leu Gln Asp Leu Pro  330
Asn Val Asp Val Tyr Arg Glu Glu Asn Ser Thr Glu Val Leu Tyr  345
Trp Lys Ile Ile Ser Leu Asp Arg Asp Leu Asp Gln Tyr Leu Asn  360
Lys Ser Ser Lys Pro Pro Leu Lys Thr Leu Asp Ala Ile Arg Arg  375
Glu Leu Asp Ile Phe Gln Tyr Lys Val Asp Ser Leu Glu Glu Asp  390
Phe Arg Ser Asn Asn Ser Arg Phe Gln Lys Phe Ile Ala Leu Phe  405
Gln Ile Ser Thr Val Ser Trp Lys Leu Phe Lys Met Tyr Leu Ile  420
Tyr Tyr Asp Thr Ala Asp Ser Leu Leu Lys Val Ile His Tyr Ser  435
Lys Val Ile Ile Ser Leu Ile Val Asn Asn Phe His Ala Lys Ser  450
Glu Phe Phe Asn Arg His Pro Met Val Met Gln Thr Ile Thr Arg  465
Val Val Ser Phe Ile Ser Phe Tyr Gln Ile Phe Val Glu Ser Ala  480
Ala Val Lys Gln Leu Leu Val Asp Leu Thr Glu Leu Thr Ala Asn  495
Leu Pro Thr Ile Phe Gly Ser Lys Leu Asp Lys Leu Val Tyr Leu  510
Thr Glu Arg Leu Ser Lys Leu Lys Leu Leu Trp Asp Lys Val Gln  525
Leu Leu Asp Ser Gly Asp Ser Phe Tyr His Pro Val Phe Lys Ile  540
Leu Gln Asn Asp Ile Lys Ile Ile Glu Leu Lys Asn Asp Glu Met  555
Phe Ser Leu Ile Lys Gly Leu Gly Ser Leu Val Pro Leu Asn Lys  570
Leu Arg Gln Glu Ser Leu Leu Glu Glu Glu Asp Glu Asn Asn Thr  585
Glu Pro Ser Asp Phe Arg Thr Ile Val Glu Glu Phe Gln Ser Glu  600
Tyr Asn Ile Ser Asp Ile Leu Ser TER
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**Fig. 2.** Deduced amino acid sequence of Cbf3b. Regions that have been verified by peptide sequencing are underlined. Doubly underlined amino acid sequences were used to design oligonucleotides for PCR.

6). Subsequently the cellular and nuclear morphology and the loss rate of a non-essential chromosomal fragment (Jehn *et al.*, 1991) was compared for *cbf3b::TRP1* haploid cells with plasmid-encoded *cbf3b*-L29, *cbf3b*-E21 or *CBF3B*(wt) alleles. A congenic *CBF3B* haploid strain (chromosomal wild type) served as a control. Both *cbf3b* mutants were affected in growth, *cbf3b*-E21 more severely than *cbf3b*-L29. Log phase cultures of the *cbf3b*-L29 mutant showed an accumulation of large budded cells with the nucleus at the neck of the presumable mother cell (Figure 6) indicating a cell cycle delay at $G_2-M$. Furthermore, the *cbf3b*-L29 mutant lost the chromosomal fragment six times more frequently per mitotic cell division than the control strains. For *cbf3b*-E21 cells, the accumulation of large budded cells with one nucleus was less pronounced than for the *cbf3b*-L29 mutant. Instead, *cbf3b*-E21 populations exhibited a high content (42%) of cells with abnormal morphology (giant, multi-budded and filamentous cells) and irregular nuclear staining. This included

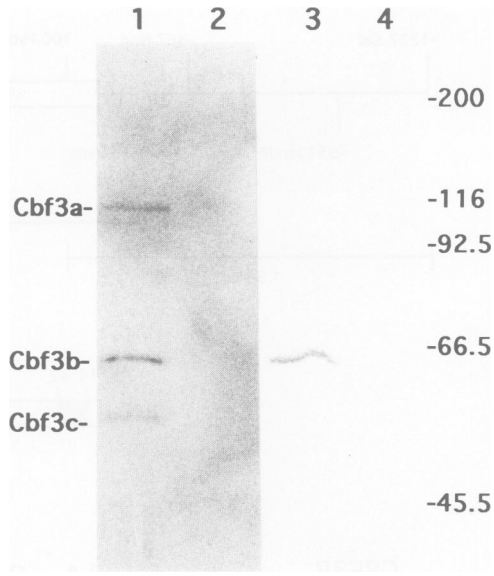



**Fig. 3.** Western analysis of Cbf3 preparations. A Cbf3 preparation (lanes 1 and 3) and a control preparation that contains no Cbf3 (lanes 2 and 3) produced as described elsewhere (Lechner and Carbon, 1991) were subjected to SDS−PAGE and silver staining (lanes 1 and 2) or Western analysis (lanes 3 and 4) using an antibody that was raised against the protein encoded by the cloned gene (see Materials and methods).

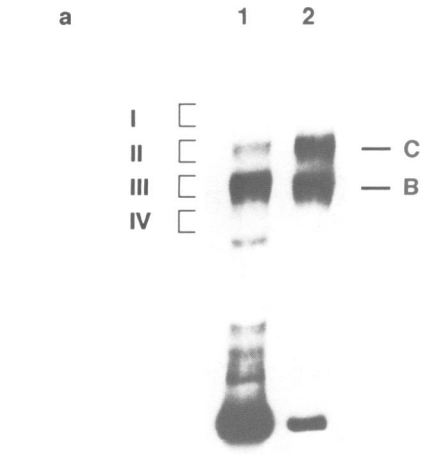
cells with no or a very diffuse nuclear staining, cells with many diamidinophenyl indole stainable structures of subnuclear size, presumably representing the accumulation of mitochondria in starving cells and cells that may contain multiple nuclei or nuclear fragments (Figure 6a). Consistent with these morphologies is the high percentage of nonviable cells (75%) observed in *cbf3b*-E21 populations. Also, *cbf3b*-E21 mutants lose the chromosomal fragment 18 times more frequently per mitotic cell division than the control strains. Therefore, the data presented are consistent with a proposed kinetochore function of Cbf3b that is essential for chromosome segregation. This also demonstrates that the zinc finger domain is important for Cbf3b to execute its role in chromosome segregation.

## Discussion

### *What is the role of Cbf3b in the Cbf3−CEN complex?*

A multisubunit protein complex, Cbf3, that constitutes part of the yeast kinetochore, contains three subunits: Cbf3a, b and c. Using peptide sequence data a putative *CBF3B* gene was cloned and it was demonstrated that the cloned gene encodes the 64 kDa subunit of Cbf3 (Cbf3b).

Cbf3b expressed in *E.coli* does not bind to *CEN in vitro* (data not shown) possibly due to improper folding, missing post-translational modifications, dependence on cooperative interactions with Cbf3a/c, or all of the above. However, there are two lines of evidence that strongly indicate that Cbf3b is the Cbf3 subunit that is responsible for specific *CEN* binding of Cbf3. First, anti-Cbf3b antibodies prevent the formation of the Cbf3−*CEN* complex. Interestingly, the anti-Cbf3b antibody does not produce a supershift of the Cbf3−*CEN* complexes even when added to the preassembled Cbf3−*CEN* complex. This indicates that Cbf3b might constitute the core of the Cbf3 complex



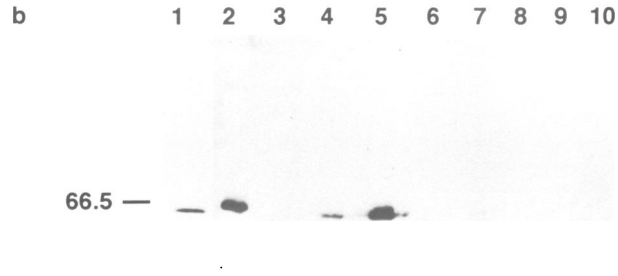




**Fig. 4.** Cbf3b is a component of the Cbf3–*CEN* complex. (**a**) Autoradiogram of Cbf3–*CEN* complexes (indicated by B and C) fractionated on a non-denaturing gel as described (Lechner and Carbon, 1991). Affinity purified Cbf3 (~1 pmol) was incubated with abundant (4 pmol) *CEN* DNA (lane 1) or with substoichiometric amounts (20 fmol) of *CEN* DNA (lane 2). Three times more $^{32}$P-labelled *CEN* DNA was included in lane 1 than in lane 2. Fractions of the gel that were excised are indicated by I–IV. (**b**) Fractions I–IV of the non denaturing gel were subjected to SDS–PAGE, blotted and probed with a polyclonal antibody that was raised against the protein encoded by the cloned gene. Lane 1, affinity purified Cbf3; lane 2, fusion protein used to produce the antibody ($His_{10}$-Cbf3b, see Materials and methods); lanes 3–6, fractions I–IV, respectively, from lane 1 of the non-denaturing gel; lanes 7–10, fractions I–IV respectively from lane 2 of the non-denaturing gel.

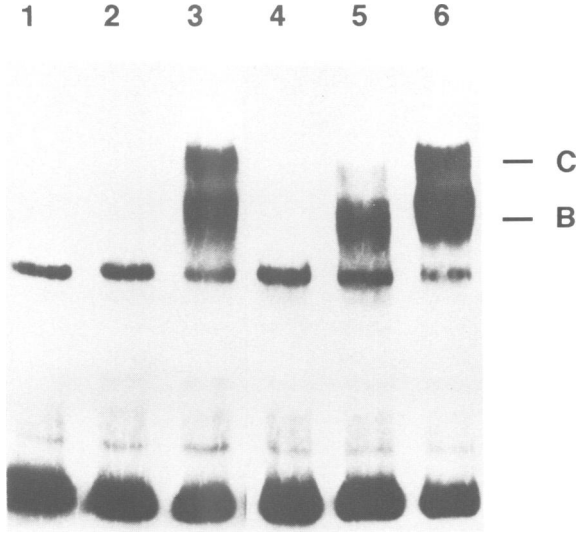
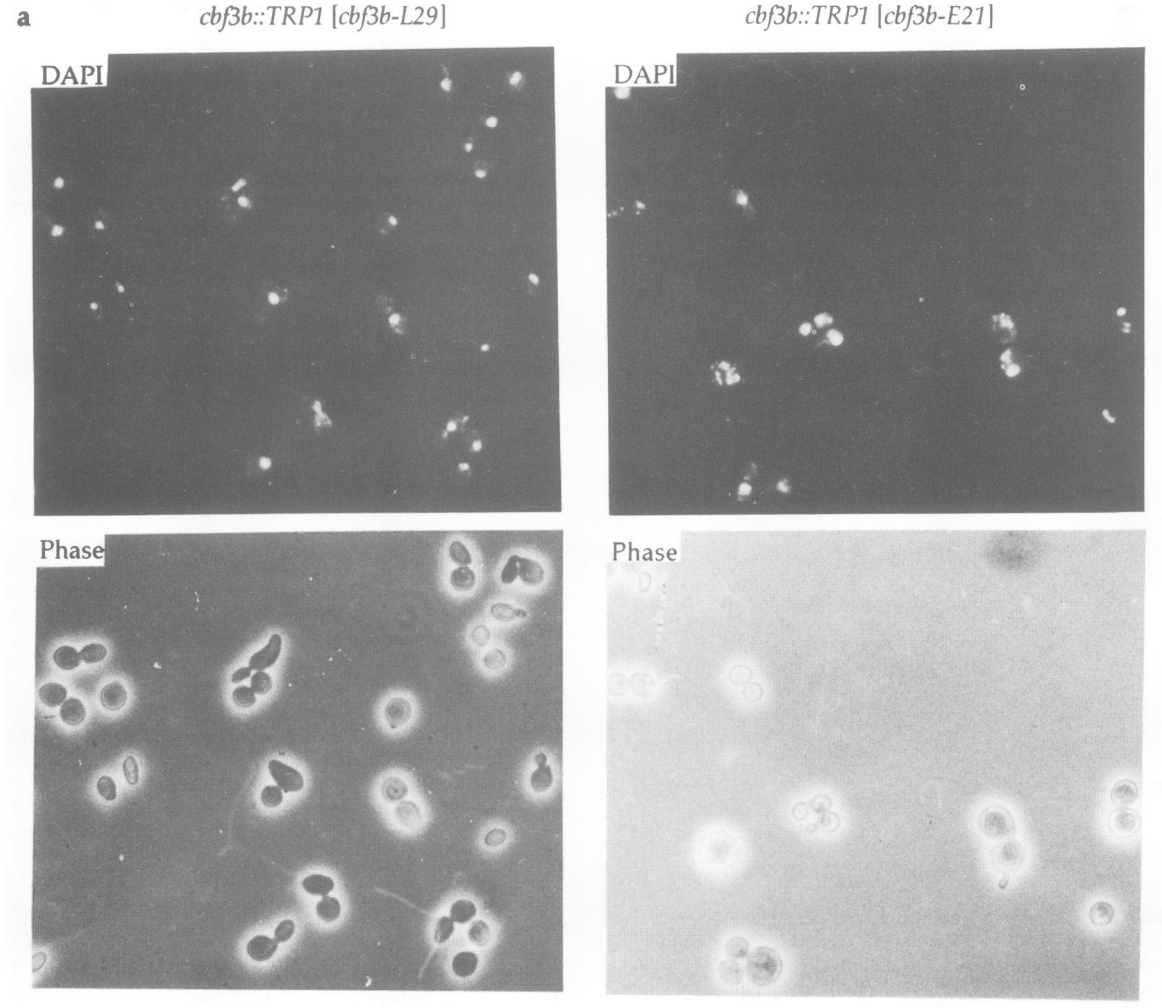



**Fig. 5.** Anti-Cbf3b antibody interferes with Cbf3b–*CEN* complex formation. Gel retardation assays with a $^{32}$P-labeled 350 bp *CEN3* DNA fragment and affinity purified Cbf3 were performed as described (Lechner and Carbon, 1991). Prior to the addition of *CEN* DNA, Cbf3 (~200 ng total protein) was incubated with 5 μg (lane 1), 0.5 μg (lane 2), 0.1 μg (lane 3), 1 μg (lane 4) or no (lane 6) affinity purified anti-Cbf3b antibody for 20 min at room temperature. Lane 5, 1 μg of anti-Cbf3b antibody was preincubated with 1 μg recombinant Cbf3b fusion protein (see Materials and methods) for 20 min at room temperature and subsequently with Cbf3 as above before addition of *CEN3* DNA. Formation of Cbf3–*CEN* complexes is indicated by the shifted bands B and C.

with only the DNA binding domain of Cbf3b accessible for antibody interaction. Second, Cbf3b contains a $Zn_2Cys_6$ zinc finger domain. The DNA binding function of the $Zn_2Cys_6$ zinc finger domain has been clearly demonstrated for transcriptional activators (Keegan *et al.*, 1986; Marmorstein *et al.*, 1992). Thus Cbf3b is the only one of the Cbf3 components that contains a known DNA binding domain. X-ray crystallography data recently revealed that Gal4 makes direct base contacts only to a highly conserved CCG triplet at each end of the Gal4 binding site (Marmorstein *et al.*, 1992). CCG triplets are also present in binding sites of three other transcriptional activators (LEU3, LAC9 and PPR1) (Marmorstein *et al.*, 1992). Interestingly, the CDE III element of *CEN* DNA contains a highly conserved CCG triplet that is absolutely essential for centromere function and Cbf3 binding (Ng and Carbon, 1987; Jehn *et al.*, 1991). Therefore it could be speculated that Cbf3b might interact with this CCG triplet in the Cbf3–*CEN* complex. Since *CEN* DNA does not contain a pair of palindromic CCG triplets, Cbf3b may bind to *CEN* DNA not as homodimer, like known transcriptional activators, but as a heteromer with the other Cbf3 proteins. A proline to leucine substitution within the loop that connects the two α-helices of the Gal4 zinc finger domain results in a Gal$^-$ phenotype (Dover and Johnston, 1988), presumably because this mutation is incompatible with the formation of a *cis* peptide bond at this position. At the corresponding residue of Cbf3b a *cis* peptide bond is apparently less important since cbf3b-L29 mutants exhibit only diminished, but not abolished, Cbf3b function. The Cbf3b function is severely affected in the *cbf3b*-E21 mutant strain, suggesting that Lys21 of Cbf3b, like the corresponding Lys in Gal4 (Marmorstein *et al.*, 1992), might be involved in direct base interactions in the protein–DNA complex.

The amino acid sequence of the predicted Cbf3b zinc finger shows some variation from the $Zn_2Cys_6$ consensus sequence. The amino acid (residue 18) following the second cysteine is a threonine instead of an arginine. The loop between the third and fourth cysteines is one residue shorter than in most known $Zn_2Cys_6$ domains, although it is the same length as the equivalent loop in Mal63 (Kraulis *et al.*, 1992). The loop between the fifth and sixth cysteines is two residues longer than predicted by the $Zn_2Cys_6$ consensus sequence. The implications of these deviations from known $Zn_2Cys_6$ motifs for the binding properties of Cbf3b is unknown at present.

Cbf3b contains two regions that might enable protein–protein interactions with Cbf3a/c or other kineto-




b

| | Cell morphology | | | Nuclear morphology | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | ○ | | | | | | AM | DEAD | Mitotic CF loss rate |
| *CBF3B* | 49 | 45 | 6 | 1 | 5 | <1 | <1 | <5 | $9.1 \times 10^{-4}$ |
| *cbf3b::TRP1 [CBF3B]* | 49 | 39 | 12 | 6 | 5 | 1 | <1 | 16 | $9.9 \times 10^{-4}$ |
| *cbf3b::TRP1 [cbf3b-L29]* | 29 | 28 | 43 | 31 | 8 | 4 | 2 | 25 | $5.8 \times 10^{-3}$ |
| *cbf3b::TRP1 [cbf3b-E21]* | 21 | 12 | 25 | 20 | 4 | 1 | 42 | 75 | $1.6 \times 10^{-2}$ |

**Fig. 6.** Characterization of *cbf3b::TRP1* (*cbf3b*-L29) and *cbf3b::TRP1* (*cbf3b*-E21) mutants. (**a**) *cbf3b::TRP1* (*cbf3b*-L29) and *cbf3b::TRP1* (*cbf3b*-E21) morphologies. Exponentially growing cells were stained with diamidinophenyl indole (Sherman *et al.*, 1986) to visualize DNA and subsequently observed by fluorescence or phase contrast microscopy. (**b**) Quantification of nuclear and cellular morphologies; rate of mitotic loss of an artificial chromosome fragment. Strains YPH500 (*CBF3B*), YJL4/pJL52 [*cbf3b::TRP1* (*CBF3B*)], YJL4/pJL58 [*cbf3b::TRP1* (*cbf3b*-L29)] and YJL4/pJL59 [*cbf3b::TRP1* (*cbf3b*-E21)] were visualized as in panel a, and cellular morphology was scored as unbudded, small budded or large budded cells. Nuclear staining of large budded cells was scored as indicated by the drawings. 'AM' represents cells with abnormal cellular or nuclear morphology as explained in the text. At least 300 cells were examined for each strain and the numbers shown represent the percentage of the total cells scored. To determine the percentage of dead cells within an exponentially growing culture, the total amount of cells as determined using a haemocytometer was compared with the number of cells that were able to form single colonies on agar plates. The rate of loss of an artificial chromosome fragment (CF) was determined for the strains YJH 341(*CBF3B*), YJL6/pJL52 [*cbf3b::TRP1* (*CBF3B*)], YJL6/pJL58 [*cbf3b::TRP1* (*cbf3b*-L29)] and YJL6/pJL59 [*cbf3b::TRP1* (*cbf3b*-E21)] using the cycloheximide resistance/sensitivity system (Jehn *et al.*, 1991).

chore proteins. A C-terminal acidic domain and a central heptad repeat capable of providing a hydrophobic interface when forming an α-helix. Preliminary data revealed that a truncated version Cbf3b that has the acidic domain removed does not support cell viability. This indicates that the acidic domain is essential for Cbf3b function. The

Cbf3b heptad repeat could promote dimerization as has been seen for Gal4-like transcriptional activators. Notably, the distance between this putative protein−protein interaction domain and the DNA recognition domain is much larger for Cbf3b than for the related transcriptional activators. Since Cbf3b may not bind to one *CEN* as a dimer (see above), dimerization of Cbf3b could facilitate sister chromatid association. Alternatively, the hydrophobic interface could lead to heteromer formation.

Neither of the Cbf3 components resembles a known molecular motor. If the Cbf3 subunits do not represent a new type of motor, the motor activity associated with Cbf3 preparations (Hyman *et al.*, 1992) results from a minor component in the Cbf3 preparations that has yet to be identified.

### *The zinc finger domain of Cbf3b is essential for chromosome segregation*

Disrupting the Cbf3b-encoding gene downstream of the zinc finger domain revealed that Cbf3b is essential for growth and that the Cbf3b zinc finger domain is not sufficient to support viability. Nevertheless, the intactness of the Cbf3b zinc finger domain is essential for accurate chromosome segregation. Single amino acid substitutions within this domain result in two mutants, *cbf3b*-L29 and *cbf3b*-E21, that exhibit an increased chromosomal fragment loss per mitotic cell division. Furthermore, the *cbf3b*-L29 mutant strain is delayed at the $G_2$−M transition of the mitotic cell cycle. This is possibly due to a control mechanism surveying the intactness of kinetochores, or the successful attachment of the mitotic spindle onto the chromosome via the kinetochore, as has been proposed for *cbf3c* mutants (Doheny *et al.*, 1993). For the *cbf3b*-E21 mutant, the $G_2$−M delay may not be long enough to allow the assembly of functional kinetochores before the completion of cytokinesis. Alternatively, the *cbf3b*-E21 defect may not be detectable by the putative control system. Both scenarios would produce a subpopulation of inviable aneuploid or increasingly polyploid cells.

The data presented describe *cbf3b* mutant strains that carry the mutant alleles on *CEN* plasmids. Since the segregation of these plasmids is likely to be affected by the Cbf3b mutations, an increased mitotic instability of the *cbf3b*-E21-expressing plasmid had to be considered, particularly for the *cbf3b*-E21 mutant strain. Thus, the inviable cells observed in the *cbf3b*-E21 populations may partly represent cbf3b-null phenotypes. Indeed, when the *cbf3b*-E21 allele was recently expressed from a 2μ vector (which segregates independently from the general mitotic apparatus), the mutant cell population contained fewer dead cells (40%) than the number given in Figure 6. Notably, the rate of chromosomal fragment loss, as determined by the method of Jehn *et al.* (1991), was even higher (28 times higher than the wild type control) than for cells expressing the *cbf3b*-E21 allele from a *CEN* plasmid. This indicates that the occurrence of dead cells within the *cbf3b*-E21 population may result in an underestimation of chromosomal loss events. However, it does not contradict the conclusion that the lysine to proline mutation at position 21 of the Cbf3b zinc finger severely interferes with chromosome segregation in yeast. This is also supported by the recent finding that moderate overexpression of the *cbf3b*-E21 allele (from a 2μ vector) in a *CBF3B/cbf3b::TRP1* heterozygote also results in cells with increased chromosomal fragment loss per mitotic cell division (my unpublished data).

## Materials and methods

### *Yeast strains and media*

The yeast strains used were: YPH501 (**a**/α *ade2*-101$^{ochre}$/*ade2*-101$^{ochre}$ *trp1*-Δ63/*trp1*-Δ63 *leu2*-Δ1/*leu2*-Δ1 *ura3*-52/*ura3*-52 *his3*-Δ200/*his3*-Δ200 *lys2*-801$^{amber}$/*lys2*-801$^{amber}$) and YPH500 (α *ade2*-101$^{ochre}$ *trp1*-Δ63 *leu2*-Δ1 *ura3*-52 *his3*-Δ200 *lys2*-801$^{amber}$) from Stratagene, YJH341(α *ade2*-101$^{ochre}$ *trp1*-Δ63 *leu2*-Δ1 *ura3*-52 *his3*-Δ200 *lys2*-801$^{amber}$ *cyh2*$^{R}$ [CF (wt *CEN6*) *URA3 SUP11 CYH2*$^{S}$]) from Johannes Hegemann, YJL1 (**a**/α *ade2*-101$^{ochre}$/*ade2*-101$^{ochre}$ *trp1*-Δ63/*trp1*-Δ63 *leu2*-Δ1/*leu2*-Δ1 *ura3*-52/*ura3*-52 *his3*-Δ200/*his3*-Δ200 *lys2*-801$^{amber}$/*lys2*-801$^{amber}$ *CBF3B/cbf3b::Trp1*), YJL2/pJL52 [**a** *ade2*-101$^{ochre}$ *trp1*-Δ63 *leu2*-Δ1 *ura3*-52 *his3*-Δ200 *lys2*-801$^{amber}$ *cbf3b::TRP1* (pJL52, *Cbf3B LEU2*)], YJL4/pJL53 [α *ade2*-101$^{ochre}$ *trp1*-Δ63 *leu2*-Δ1 *ura3*-52 *his3*-Δ200 *lys2*-801$^{amber}$ *cbf3b::TRP1* (pJL53, *CBF3B URA3*)] YJL6/pJL52 {**a** *ade2*-101$^{ochre}$ *trp1*-Δ63 *leu2*-Δ1 *ura3*-52 *his3*-Δ200 *lys2*-801$^{amber}$ *cbf3b::TRP1 cyh2*$^{R}$ [CF (wt *CEN6*) *URA3 SUP11 CYH2*$^{S}$] (pJL52, *Cbf3B LEU2*)} this study. YJL4/pJL52, YJL4/pJL58 and YJL4/pJL59 are identical to YJL4/pJL53 except that they contain the plasmids pJL52, pJL58 or pJL59, respectively, instead of pJL53. YJL6/pJL58 and YJL6/pJL59 are identical to YJL6/pJL52 except that they contain the plasmids pJL58 or pJL59 instead of pJL52.

Standard culture conditions and media were used (Sherman, 1991).

### *Peptide analysis of Cbf3b and Cbf3c*

Cbf3 was isolated as described (Lechner and Carbon, 1991), fractionated on an 8% polyacrylamide gel and Coomassie stained to reveal the three Cbf3 subunits (Cbf3a−c). A gel slice containing Cbf3b or Cbf3c was crushed and subsequently washed twice with 1 ml of Coomassie destainer, methanol/acetic acid/water (5:1:4) and 90% ethanol for 1 h each to remove Coomassie and SDS. After lyophilization the sample was incubated overnight with 0.01 mg/ml trypsin in 0.2 M ammonium bicarbonate at 37°C. Peptides were extracted three times with 1 ml of 0.2 M ammonium bicarbonate and 1 ml of 50% acetonitrile in 0.2 M ammonium bicarbonate, lyophilized, dissolved in 6 M guanidine hydrochloride and fractionated by reversed phase HPLC. Three peptides isolated from the Cbf3b and Cbf3c digests were each subjected to gas phase sequencing.

### *Cloning and sequencing of the Cbf3b-encoding gene*

Two degenerate oligonucleotides, 5′-TGGGA(C/T)AA(A/G)GTNCA-3′ and 5′-ATGTTGTATTC(G/T)GATTG(A/G)AA(C/T)TC-3′, derived from Cbf3b peptide sequence data (doubly underlined in Figure 2) were able to amplify a 257 bp DNA fragment from *S.cerevisiae* genomic DNA by polymerase chain reaction (PCR). Subcloning and sequencing of this DNA fragment revealed that the amino acid sequence encoded matches Cbf3b peptide sequence data. Therefore this DNA fragment is part of the Cbf3b-encoding gene. An *S.cerevisiae* genomic library, constructed essentially as described (Lechner and Sumper, 1987) using yeast DNA isolated according to Philippsen *et al.* (1991) and *Bam*HI cut λ Dash II arms from Stratagene, was screened with the Cbf3b-encoding gene fragment according to Sambrook *et al.* (1989). The DNA of a positive plaque was further analyzed by Southern hybridization using the *CBF3B* gene fragment as a probe. The positively identified 1.9 kb *Hin*dIII and 0.5 kb *Hin*dIII−*Xba*I fragments were subcloned into pUC18, resulting in pJL10 and pJL11 respectively. A nested set of deletions was produced according to a protocol by Stratagene to sequence one strand of the pJL10 insert. The second strand of the pJL10 insert and both strands of pJL11 insert were sequenced using specific oligonucleotides as primers. DNA sequencing was performed by the dideoxy chain termination method (Sanger *et al.*, 1977). A 1.3 kb *Sal*I−*Pst*I fragment that overlaps the 1.9 kb *Hin*dIII fragment and contains additional sequences upstream of the *CBF3B* coding region was identified subsequently from the above λ clone.

### *Expression of Cbf3b in E.coli and anti-Cbf3b antibody preparation*

Using PCR technology, an *Nde*I site was introduced at the start codon and a *Bam*HI site was introduced immediately downstream of the *CBF3B* coding sequence. Sequences that were produced by PCR were confirmed by DNA sequencing. The *Nde*I−*Bam*HI fragment containing the *CBF3B*

gene was ligated into the *Nde*I and *Bam*HI sites of the *E.coli* expression vector pET16b (Novagene) and the recombinant plasmid, pJL33, was transformed into the *E.coli* strain BL21(DE3). After induction with isopropyl thiogalactoside, a Cbf3b fusion protein was obtained in the *E.coli* cytosol. This protein ($His_{10}$-Cbf3b) has the peptide $MGH_{10}$SSGHIEGRH linked to the Cbf3b N-terminus. The Cbf3b fusion protein was purified to near homogeneity by chromatography on chelating Sepharose according to a protocol by Novagene, precipitated by trichloroacetic acid, dissolved in 0.2 M SDS and injected into a New Zealand white rabbit. The polyclonal antiserum obtained was purified by affinity chromatography on $His_{10}$-Cbf3b linked to NHS-activated Sepharose (High trap, Pharmacia) according to the manufacturer's recommendations. One millilitre of serum was diluted 10-fold in 10 mM Tris−HCl (pH 7.5) and applied to the affinity column. After washing the column with 10 ml of the above buffer, anti-Cbf3b antibody was eluted with 0.1 M glycin and immediately neutralized to pH 7–8 by addition of 1 M Tris−HCl (pH 8.0). Most of the antibody was obtained in the first 1 ml fraction at a protein concentration of 1 mg/ml. This fraction was concentrated 10-fold using Centricon 30 (Amicon) and stored at 4°C.

### Gel mobility shift assay

Gel mobility shift assays were performed as described (Lechner and Carbon, 1991) with the exception that 0.5 μl of yeast nuclear extract (Lechner and Carbon, 1991) was added to 30 μl of incubation mix.

### 2D analysis of Cbf3−CEN complexes

Gel mobility shift assays with a 350 bp *CEN3* DNA fragment and 5 μl affinity purified Cbf3 were performed as described above. One microgram of $^{32}$P-labelled *CEN3* fragment of low specific activity (60 000 c.p.m./μg) was included in one sample, favouring the formation of Cbf3−*CEN* complex over free Cbf3. Five nanograms of $^{32}$P-labelled *CEN3* fragment of high specific activity ($4\times10^6$ c.p.m./μg) was included in the control sample limiting the amount of Cbf3−*CEN* complex that could be formed to 20 fmol. An autoradiogram obtained from the wet gel was used to locate and excise gel fractions with Cbf3−*CEN* complexes and neighbouring fractions. The gel slices were incubated in SDS probe buffer for 45 min at 37°C prior to loading into the wells of an 8% SDS−polyacrylamide gel. Protein blots were probed with the affinity purified anti-Cbf3b antibody and primary antibody binding was detected by chemiluminescence using a commercial kit from Tropix.

### Disruption of CBF3B

To construct an integration vector, the 1.9 kb *Hin*dIII fragment and the 0.5 kb *Hin*dIII−*Xba*I fragment described in Figure 1a were ligated, reconstituting the *CBF3B* reading frame, and cloned into pBS (Stratagene) resulting in plasmid pJL29. The 1.1 kb *Eco*RV−*Spe*I fragment of the *CBF3B* coding sequence was deleted from pJL29 and replaced with the 1.3 kb *Drd*I fragment of pRS414 (Stratagene) that contains the *TRP1* gene of yeast resulting in pJL73. Prior to ligation, the *Spe*I site of digested pJL29 was filled in with Klenow fragment and deoxynucleotide triphosphates; the *Drd*I ends of the *TRP1* containing fragment were blunt ended by T4 DNA polymerase and deoxynucleotide triphosphates. pJL73 was digested with *Sac*I and *Xho*I and transformed into the diploid *S.cerevisiae* strain YPH501 according to Ito *et al.* (1983) selecting for $Trp^+$ transformants. This directs an integration event that leaves the first 396 bp of the Cbf3b coding sequence intact. Successful disruption of one *CBF3B* copy was verified by Southern analysis of *Hin*dIII-digested yeast genomic DNA using the 1.9 kb *Hin*dIII fragment of pJL10 as a probe. Transformants were sporulated and tetrads were dissected. Spore viability segregated 2:2 in tetrads dissected. All viable spores were $Trp^-$.

### Construction of cbf3b mutants

The 1.34 kb *Sal*I−*Pst*I fragment, the 1.61 kb *Pst*I−*Hin*dIII fragment and the 0.51 kb *Hin*dIII−*Xba*I fragment described in Figure 1a were ligated in-frame between the *Sal*I and *Xba*I sites of pRS415 and pRS416 (Sikorski and Hieter, 1989) resulting in plasmids pJL52 and pJL53 which express wild type Cbf3b in yeast. The 0.47 kb *Nru*I−*Pst*I fragment of pJL52 was replaced by corresponding NruI−*Pst*I fragments containing single point mutations introduced by 'recombinant PCR' (Innis *et al.*, 1990). This resulted in plasmids pJL58 (*cbf3b*-L29) and pJL59 (*cbf3b*-E21) which express Cbf3b with a Pro to Leu mutation at residue 29 or a Lys to Glu mutation at residue 21.

YJL1 (*CBF3B/cbf3b::TRP1*) was transformed with pJL52 and pJL53. Sporulation of YJL1/pJL52 or YJL1/pJL53 and selection of $Trp^+$ haploids resulted in strains YJL2/pJL52 [*cbf3b::TRP1* (pJL52, *Cbf3B LEU2*)] and YJL4/pJL53 [*cbf3b::TRP1* (pJL53, *CBF3B URA3*)] respectively. A plasmid shuffling strategy (Sikorski and Boeke, 1991) was used to exchange plasmid pJL53 for pJL52, pJL58 or pJL59 in YJL4, resulting in *cbf3b::TRP1* (pJL52, *CBF3B LEU2*), *cbf3b::TRP1* (pJL58, *cbf3b*-L29 *LEU2*) or *cbf3b::TRP1* (pJL59, *cbf3b*-E21 *LEU2*) cells.

### Chromosome fragment loss assay

Chromosome fragment loss was assayed as described by Jehn *et al.* (1991). To obtain *cbf3b* mutant strains carrying artificial chromosome fragments, YJL2/pJL52 was crossed with YJH341. The resulting diploid strain was either directly sporulated or cured of pJL52, transformed with pJL58 or pJL59 and then sporulated. $Trp^+$ haploids were selected and haploids that tested cycloheximide resistant after chromosome fragment loss were identified. This resulted in strain YJL6 carrying plasmids pJL52, pJL58 or pJL59.

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## Note added in proof

The nucleotide sequence data has been deposited in the EMBL/GenBank/DDBJ databases under the accession number X81396.