Point Mutations That Separate the Role of *Saccharomyces cerevisiae* Centromere Binding Factor 1 in Chromosome Segregation From Its Role in Transcriptional Activation

Pamela K. Foreman and Ronald W. Davis

Department of Biochemistry, Stanford University School of Medicine, Stanford, California 94305

Manuscript received March 12, 1993
Accepted for publication June 5, 1993

ABSTRACT

Centromere binding factor 1 (Cbf1p, also called CP1, CPFl) binds to the CDEI region of *Saccharomyces cerevisiae* centromeres and is a member of the basic helix-loop-helix (bHLH) class of proteins. Deletion of the gene encoding Cbf1p results in an increased frequency of chromosome loss, hypersensitivity to low levels of microtubule disrupting drugs (such as thiabendazole and benomyl) and methionine auxotrophy. By polymerase chain reaction-based random mutagenesis of the *CBFl* gene we have obtained a number of mutant alleles that make full-length protein with impaired function. The mutations in these alleles are clustered in or just downstream from the bHLH domain. Among the alleles obtained was a class that was more compromised for transcriptional activation and a class that was more compromised for chromosome loss and thiabendazole hypersensitivity. These results indicate that at least some aspects of the role of Cbf1p in chromosome segregation and transcriptional activation are distinct. In contrast, increased chromosome loss and thiabendazole hypersensitivity were not separated in any of the alleles, suggesting that these phenotypes reflect the same mechanistic defect. These observations are consistent with a model that suggests that one role of Cbf1p in chromosome segregation may be to improve the efficiency with which contact between the kinetochore and spindle microtubules is established or maintained.

Central binding factor 1 (Cbf1p, also called CP1, CPFl) binds to the CDEI region of *Saccharomyces cerevisiae* centromeres and is a member of the basic helix-loop-helix (bHLH) class of proteins. Deletion of the gene encoding Cbf1p results in an increased frequency of chromosome loss, hypersensitivity to low levels of microtubule disrupting drugs (such as thiabendazole and benomyl) and methionine auxotrophy. By polymerase chain reaction-based random mutagenesis of the *CBFl* gene we have obtained a number of mutant alleles that make full-length protein with impaired function. The mutations in these alleles are clustered in or just downstream from the bHLH domain. Among the alleles obtained was a class that was more compromised for transcriptional activation and a class that was more compromised for chromosome loss and thiabendazole hypersensitivity. These results indicate that at least some aspects of the role of Cbf1p in chromosome segregation and transcriptional activation are distinct. In contrast, increased chromosome loss and thiabendazole hypersensitivity were not separated in any of the alleles, suggesting that these phenotypes reflect the same mechanistic defect. These observations are consistent with a model that suggests that one role of Cbf1p in chromosome segregation may be to improve the efficiency with which contact between the kinetochore and spindle microtubules is established or maintained.

Unexpectedly, cells lacking Cbf1p are also unable to grow in the absence of exogenously provided methionine. This methionine auxotrophy is due, at least in part, to a requirement for Cbf1p in transcription of the *MET16* gene (Thomas, Jacquemin and Surdin-Kerjan 1992). The consensus Cbf1p binding sequence is located upstream of several genes involved in methionine biosynthesis including *MET16* (Che- rest, Thomas and Surdin-Kerjan 1990; Korch, Mountain and Bystrom 1991; Thomas, Barbey and Surdin-Kerjan 1990; Thomas, Chrest and Surdin-Kerjan 1989; Thomas, Jacquemin and Surdin-Kerjan 1992). Cbf1p may therefore function as a transcription factor as well as playing a role in chromosome segregation.
How does Cbf1p carry out such diverse functions in the cell? One model is that it plays a very generalized role such as in organizing chromatin. Consistent with this notion is the fact that the 8-bp CDEI consensus sequence is expected to occur ~1700 times in the haploid yeast genome (Baker, Fitzgerald-Hayes and O'Brien 1989). In this model the phenotypes observed in cbf1 mutants might reflect the cellular processes that are most sensitive to loss of this hypothetical generalized function.

An alternative model is that Cbf1p plays distinct, perhaps overlapping, roles in transcriptional activation and chromosome segregation. The specificity of its function could potentially be determined by the basic helix-loop-helix (bHLH) motif contained in the carboxyl region of the protein (Cai and Davis 1989; Mello et al. 1990). The bHLH motif consists of a basic DNA binding region upstream of two amphipathic α-helices. These α-helices mediate the formation of homo- and heterodimers with other bHLH-containing proteins (Murre et al. 1989; reviewed in Busch and Sassone 1990; Jones 1990; Li and Olson 1990; Olson 1990; Vinson and Garcia 1992; Weintraub et al. 1991). The DNA binding specificities of homo- and heterodimeric complexes may be quite different, allowing for functional diversity through various combinatorial interactions among different bHLH proteins. Thus, one mechanism by which Cbf1p could carry out different roles in the cell is through the formation of distinct classes of heterodimers. The nature of their dimerization partner might determine whether Cbf1p functions in chromosome segregation or in transcriptional activation.

A prediction of the former model, in which Cbf1p functions identically in each of its capacities, is that all mutations within the protein should affect chromosome loss, thiabendazole hypersensitivity, and methionine auxotrophy equivalently. However, if the latter model is correct, one might expect to find mutations that can separate its functions.

To determine whether the functions of Cbf1p could be separated as well as to define functional domains within Cbf1p, a random mutagenesis of the CBF1 gene was carried out. Nine alleles were obtained that made full-length protein which was defective in function. Among these were a class that was more compromised for transcriptional activation and a class that was more compromised for chromosome loss and thiabendazole hypersensitivity. Analysis of the mutations sustained in each of the alleles suggests that Cbf1p requires the bHLH domain for function and that this domain or a region just downstream, may be important in determining the specificity of its function. These results indicate that at least some aspects of the role of Cbf1p in chromosome segregation and transcriptional activation are distinct.

MATERIALS AND METHODS

Plasmids and sequencing: pCBBCF contains a 1.9-kb BamHI, HpaI restriction fragment including the CBF1 gene, subcloned into Bluescript KS (Stratagene). pCBFΔLEU was constructed from pCBBCF using an “inverse” polymerase chain reaction (PCR). Two oligonucleotide primers TTTGCTCGAGATTCATCGTTAATAAAA and TCGACCTCGAGGCTTGAGAGTTAAAA which anneal to the 5’- and 3’-ends of the CBF1 coding region, respectively, were used to direct outward synthesis of the entire plasmid less the coding region. A 2.5-kb Sall-Xhol fragment carrying the LEU2 gene was inserted at the junction between the primers, replacing the CBF1 coding sequence. YcpADE2 contains an 8.8-kb fragment bearing the ADE2 gene inserted into the BamHI site of pUN15 (Elledge and Davis 1988). Sequencing was carried out using Sequenase (U.S. Biochemical Corp.) and selected oligonucleotide primers from the CBF1 gene.

Strains and media: The strains used in this study are listed in Table 1. Strain YNN531 was derived from CRY2 (a W303 derivative) by single step gene disruption (Rothstein 1983). The CBF1 coding region (amino acids 3–351) was precisely deleted and replaced with the LEU2 gene carried on a BamHI, BamHI restriction fragment from pCBFΔLEU. All other strains were prepared by standard genetic crosses (Sherman 1991) and transformation (Ito, Fukuda and Kimura 1983). Standard yeast media were used (Sherman 1991) except the concentration of adenine was reduced to 6 μg/ml in the screen for mutants to aid in visualization of the color sectoring. Thiabendazole plates contained 75 μg/ml thiabendazole (Sigma) in YPD medium. Cycloheximide was used at a concentration of 10 μg/ml in YPD.

PCR-mediated mutagenesis: The CBF1 gene was mutagenized by performing a PCR in 50 mM KCl, 10 mM Tris, pH 8.3, 0.001% gelatin, 200 μM dNTPs, 2.5 mM MgCl₂, 5 ng linearized template plasmid containing the CBF1 gene, 1 μM each primer (AGTGGATCCTTCGGGACCACCAT, GAGTCTAAGAAAAATCATATTGACTATCTA), and 1.5 unit Taq polymerase (Cetus). The primer contains a recognition site for XbaI. The PCR products thus have an XbaI site at the C terminus of the coding region that was not present in the naturally occurring gene. Samples were heated to 94° for 7 min and amplified using 20 cycles of 94°/30 sec, 55°/30 sec, 72°/90 sec. To enrich for full-length products a final incubation at 72° for 7 min was performed. Mutagenesis was performed in aliquots and each mutant was chosen from a separate aliquot to ensure their independent derivation. The products were digested with BamHI and XbaI and ligated into the pU55-based vector (Elledge and Davis 1988) pCBFFL, which carries the 3’-noncoding region of CBF1, a yeast centromere, ARS and the URA3 gene. The ligation mixes were used to transform Escherichia coli by electroporation. At least 500 transformants were obtained from each ligation reaction. Plasmid preparations of pooled colonies were used to transform yeast.

Determination of growth phenotypes: Cells carrying each of the alleles cloned into pCBFFL were grown to logarithmic phase in SD medium lacking uracil to select for the presence of the plasmid. The cells were then cooled to 4° and sonicated for 1 min. The cell density was determined by counting using a hemacytometer. Serial fivefold dilutions were spotted onto various media and the cells were allowed to grow for 2 days.

Determination of combined rates of chromosome loss and recombination: Assays for rates of chromosome III loss
and recombination were performed as previously described (RUNGE, WELFLM.R AND ZAKIAN 1985). Each of the alleles, carried on pCBFFL, which has a URA3 marker, were introduced into strain YNN534 or YNN535. Approximately 3 x 10^9 logarithmically growing cells were picked from a colony grown on uracil-free medium and spread onto a cycloheximide plate. Since a significant fraction of the resulting colonies were found to lack the plasmid, the colonies were replica-plated to medium lacking uracil after 5 days of growth. The resulting colonies carried the CBF1-containing plasmid and arose from loss of, or recombination within, chromosome III. The frequency of these events was determined for 20-22 separate single colonies containing each allele and the rate was determined by the method of the median (LEA and COULSON 1949). Each allele was tested at least twice.

**Electrophoretic mobility shift assays and immunoblots:**

Protein extracts were prepared in 10 mM Tris pH 7.9, 50 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol (Miles Laboratories), 0.6 mM leupeptin, 2 mM pepstatin, 2 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 5% glycerol (buffer A). Cells were disrupted by vortexing for 5 min in the presence of 0.45-0.52-mm glass beads. Extracts were clarified by centrifugation at 12,000 g for 10 min. For gel retardation assays 30 μg extract were incubated with 6 μg poly(dI-dC) and ~1.25 ng 32P-end-labeled CEN4 probe in a volume of 25 μl for 30 min at room temperature. Gels were run as previously described (CAI and DAVIS 1989). For immunoblotting, 30 μg extract was separated on an 8% polyacrylamide gel (LAEMMLI 1970) and processed by standard techniques (HARLOW and LANE 1988). CBF1 was detected using rabbit polyclonal antiserum diluted 1:1000, horseradish peroxidase conjugated goat anti-rabbit secondary antibodies (Bio-Rad), and the ECL detection kit (Amersham Corp.).

**RESULTS**

**Random mutagenesis of CBF1:** A random mutagenesis of the CBF1 gene was performed to determine whether the functions of Cbf1p could be separated by point mutations and which parts of the protein are important in allowing it to carry out its diverse functions. Two oligonucleotides corresponding to sequences flanking the CBF1 gene were used as primers for PCR. Because Taq DNA polymerase incorporates nucleotides with a relatively low fidelity, the resulting products sustained mutations along their length. Under the conditions used, the misincorporation rate/nucleotide/cycle is estimated to be 2 x 10^-4 (SAIKI et al. 1988). Thus, if the number of mutations assumes a poisson distribution, in a 1200-bp fragment ~48% of the products are expected to have one or two base substitutions.

The mutagenized PCR products were subcloned into the yeast plasmid, pCBFFL, which carries the URA3 gene, and transformed into strain YNN532. The CBF1 gene has been precisely deleted in this strain, from three amino acids downstream of the start codon to the stop codon, to preclude artifacts resulting from gene conversion in the coding region. The transformants were screened for an increased frequency of plasmid minichromosome loss by plating them at low density on medium containing a limiting amount of adenine. Under these conditions colonies that lost the centromere-bearing plasmid YCpADE2 at an elevated frequency (e.g., those with compromised Cbf1p function) displayed an increased frequency of red sectors. This phenotype arises from loss of the wild-type ADE2 gene carried on YCpADE2. After scoring the sectoring phenotype, the transformants were replica-plated onto medium lacking methionine and incubated at 30° or at 37°. Mutants were picked that displayed either an increased frequency of sectoring or methionine auxotrophy or both. The products of 50 separate PCR reactions were subcloned and screened separately to ensure that each mutant chosen was of independent origin.

The most informative class of mutants was likely to be those that encoded full-length stable Cbf1p. To identify mutant genes that met these criteria, one or two mutants were chosen randomly from each PCR pool and crude protein extracts from these cells were analyzed by immunoblotting. Of 80 mutants screened, seven synthesized full-length Cbf1p that was of comparable abundance to that encoded by the wild-type gene. These seven mutants were chosen for further study. An additional 14 mutants had partial function that depended on the presence of the mutagenized cbf1 gene. However, the level of Cbf1p was below the

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRY2</td>
<td>MATa ade2 his3 leu2 trp1 ura3 CYH2</td>
<td>R. FULLER (Stanford)</td>
</tr>
<tr>
<td>YNN531</td>
<td>MATa ade2 his3 leu2 trp1 ura3 CYH2 cbf1::LEU2</td>
<td>This study</td>
</tr>
<tr>
<td>YNN532</td>
<td>MATa ade2 his3 leu2 trp1 ura3 CYH2 cbf1::LEU2 [YCpADE2]</td>
<td>This study</td>
</tr>
<tr>
<td>YNN533</td>
<td>MATa ade2 his3 leu2 trp1 ura3 CYH2 cbf1::LEU2 [YCpADE2]</td>
<td>This study</td>
</tr>
<tr>
<td>YNN534</td>
<td>MATa ade2 his3 leu2 trp1 ura3 CYH2 cbf1::LEU2 [YCpADE2]</td>
<td>This study</td>
</tr>
<tr>
<td>YNN535</td>
<td>MATa ade2 his3 leu2 trp1 ura3 CYH2 cbf1::LEU2 [YCpADE2]</td>
<td>This study</td>
</tr>
</tbody>
</table>

**TABLE 1**

<table>
<thead>
<tr>
<th>Strains used</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRY2</td>
<td>MATa ade2 his3 leu2 trp1 ura3 CYH2</td>
</tr>
<tr>
<td>YNN531</td>
<td>MATa ade2 his3 leu2 trp1 ura3 CYH2 cbf1::LEU2</td>
</tr>
<tr>
<td>YNN532</td>
<td>MATa ade2 his3 leu2 trp1 ura3 CYH2 cbf1::LEU2 [YCpADE2]</td>
</tr>
<tr>
<td>YNN533</td>
<td>MATa ade2 his3 leu2 trp1 ura3 CYH2 cbf1::LEU2 [YCpADE2]</td>
</tr>
<tr>
<td>YNN534</td>
<td>MATa ade2 his3 leu2 trp1 ura3 CYH2 cbf1::LEU2 [YCpADE2]</td>
</tr>
<tr>
<td>YNN535</td>
<td>MATa ade2 his3 leu2 trp1 ura3 CYH2 cbf1::LEU2 [YCpADE2]</td>
</tr>
</tbody>
</table>

Separation of Functions of CBF1 289
limit of detection in these mutants suggesting that the protein was unstable. Among the remaining 59 mutants, which had a null phenotype, we did not distinguish among those that synthesized unstable protein, those that had sustained nonsense mutations, and those that contained plasmids with no CBF1 insert.

**Sequences of the mutant genes:** Each of the seven cbfl alleles that synthesized full-length stable Cbf1p were sequenced. Figure 1 shows the amino acid alterations sustained in these alleles. Two alleles, cbfl-10 and cbfl-15, suffered two amino acid alterations. To assess the individual effects of each of these alterations, an SphI-XbaI restriction fragment carrying the downstream mutation in each allele was exchanged with the corresponding wild-type gene fragment. Four new alleles, cbfl-11, cbfl-12, cbfl-16 and cbfl-17 were derived from the original set of mutant alleles in this manner.

The nucleotide changes that gave rise to each of the amino acid alterations are listed in Table 2. In addition to the mutations giving rise to the amino acid changes shown in Figure 1, five of the alleles sustained one or more nucleotide misincorporations that did not alter the encoded amino acid. Both transitions and transversions occurred with thymidine to cytosine and adenine to guanosine changes predominating.

**Growth phenotypes of the mutants:** The growth phenotypes of cells carrying each of the mutant alleles were carefully examined by spotting serial dilutions of logarithmically growing cells on various solid media. In Figure 2A, the cells were plated onto medium lacking uracil to demonstrate that the number of cells spotted was approximately equivalent for each allele.

Figure 2B shows the ability of the mutant cbfl alleles to promote growth on medium containing the microtubule disrupting drug thiabendazole at 30°C. As previously reported (CAI and DAVIS 1990), cells lacking Cbf1p (cbflΔ) are unable to grow on this medium. Alleles cbfl-3, cbfl-4, cbfl-10 and cbfl-11 are also unable to support growth, whereas cbfl-21 supports minimal growth. Moderate growth is sustained in cells containing cbfl-9 and cbfl-16. Surprisingly, cbfl-15, which contains the same Glu-283 to proline alteration as cbfl-16, promotes growth almost as well as wild-type CBF1. Thus the second amino acid alteration in cbfl-15, Glu-86 to valine, partially ameliorates the defect caused by changing Glu-283 to proline. However, the Glu-86 to valine mutation alone (cbfl-17) does not affect growth. cbfl-12, in which Glu-86 is changed to lysine, also shows no growth defect. cbfl-38 confers wild-type growth levels.

Since deletion of Cbf1p causes methionine auxotro-
phy, the ability of each of the alleles to support growth on medium lacking methionine was also examined. At 30° (Figure 2C), the vigor of growth paralleled that observed for growth on thiabendazole with the following exceptions. *cbf1-9* did not support growth on methionine-free medium but did support moderate growth on thiabendazole. In contrast, *cbf1-16* conferred wild-type growth levels on methionine-free medium at 30°, but compromised growth on thiabendazole. These mutations therefore separate the functions of Cbf1p.

Whereas *cbf1-15*, *cbf1-16* and *cbf1-38* support growth on methionine-free medium at 30°, cells containing these alleles are unable to grow on the same medium at 37° (Figure 2D). We were unable to perform the equivalent experiment for growth on thiabendazole at 37° as even wild type cells do not grow under these conditions.

**Chromosome loss in cells containing the mutant alleles:** The combined rate of chromosome loss and recombination within chromosome *III* was determined for each of the alleles by the method of Runge and colleagues. (RUNGE, WELLINGER and ZAKIAN 1991). Previous studies have shown that the rate of recombination within chromosome *III* is not appreciably altered in *cbf1* mutants (CAI and DAVIS 1990) and that recombination accounts for only ~12–17% of the events scored in this type of assay (CAI and DAVIS 1990; RUNGE, WELLINGER and ZAKIAN 1991). Variations in the values determined thus primarily reflect

---

**Figure 2.**—Growth phenotypes of *cbf1* mutants. YNN532 cells containing each of the listed alleles were serially diluted (fivfold) and were spotted onto various media. The most concentrated spot contains ~6250 cells. Cells labeled *cbf1Δ* carried the *URA3*-containing plasmid pUN55 alone. Each of the other alleles (including wild-type *CBFI*) was present on a pUN55-based vector pCBFFL. (A) SD medium lacking uracil; (B) YPD medium containing thiabendazole; (C) SD medium lacking methionine, growth at 30°; (D) SD medium lacking methionine, growth at 37°.
differences in the rate of chromosome loss among the cbfl alleles. Therefore for simplicity, the combined rate of chromosome III loss and recombination will hereafter be referred to as the rate of loss. Figure 3A shows the rate of loss sustained in YNN535 cells bearing each of the alleles and grown at 30°. In this strain background the wild-type rate of chromosome III loss is 0.71 ± 0.17 × 10⁻⁴. In the strain completely lacking Cbf1p (cbflΔ), the rate is elevated ~10-fold.

The rates of chromosome loss paralleled the degree of sensitivity to thiabendazole the mutant cbfl alleles exhibited. Cells containing cbfl-3, cbfl-4, cbfl-10 and cbfl-11 showed rates of chromosome III loss comparable to that observed in cells that lack Cbf1p. A slightly smaller increase in the loss rate was observed in cells containing cbfl-21, an allele that supports minimal growth on thiabendazole. cbfl-16-containing cells show an intermediate rate of loss and, as with growth on thiabendazole, this defect is partially ameliorated by the mutation in Glu-86 in cbfl-15. However, cbfl-12 and cbfl-17, which sustained only mutations of Glu-86, do not display elevated rates of chromosome loss. In addition cells containing cbfl-9 and cbfl-38 display only marginally increased rates.

The growth of cells containing cbfl-15, cbfl-16 and cbfl-38 on medium lacking methionine was markedly reduced at 37°. To determine whether these alleles also displayed temperature sensitivity with respect to chromosome loss, cells were grown at 37° and the rate of chromosome loss was determined (Figure 3B). In wild type cells, chromosome loss was elevated ~two-fold at this temperature. Similarly the rate of loss for cells containing cbfl-15 was elevated ~two-fold. However, cells containing cbfl-16 and cbfl-38 displayed a loss rate comparable to that observed in cells lacking CBF1. These alleles therefore appear to confer temperature sensitivity with respect to chromosome loss whereas the protein encoded by cbfl-15 behaves similarly to wild type.

DNA binding activity in cells containing the mutant alleles: To determine whether any of the mutations interfered with the ability of Cbf1p to bind to its recognition sequence at the centromere, gel retardation assays were performed. Crude extracts were prepared from cells containing each of the cbfl alleles and incubated with a CDEI-containing DNA fragment. As shown in Figure 4A, cells containing cbfl-3, cbfl-10 and cbfl-11 have no detectable specific CDEI DNA binding activity. Each of these alleles sustained a mutation of Glu-231, an amino acid that is highly conserved in the DNA binding regions of bHLH proteins. cbfl-4 also lacked detectable CDEI binding activity. The mutation in this allele occurs in the first amphipathic helix of the bHLH domain. In addition, cbfl-21, which also has a mutation in the helix 1, displays markedly reduced CDEI DNA binding. The mutation in this allele occurs in the first amphipathic helix of the bHLH domain. In addition, cbfl-21, which also has a mutation in the helix 1, displays markedly reduced CDEI DNA binding.

Among the remaining alleles (CBF1, cbfl-9, cbfl-12, cbfl-15, cbfl-16, cbfl-17, cbfl-38) the variation in apparent DNA binding arose primarily from variations in the abundance of the protein rather than from differences in binding affinity. The steady state levels of Cbf1p were determined by immunoblotting cell extracts and probing with antibodies against Cbf1p (Figure 4B). The intensities of the signals corresponding to Cbf1p in this experiment paralleled those arising from binding to CDEI DNA (in the cases where binding was observed). This experiment also demonstrates that the proteins encoded by cbfl-3, cbfl-4, cbfl-10, cbfl-11 and cbfl-21, which did not bind CDEI DNA, are reasonably stable and abundant.

Interestingly, the protein encoded by cbfl-15 is present at a higher level than wild type Cbf1p. This increased abundance is reflected in an increase in CDEI binding activity. However, an additional band (which is more distinct in shorter film exposures) of
FIGURE 4.—DNA binding activity and abundance of Cbf1p encoded by each of the mutant alleles. Crude extracts were prepared from cells bearing each of the indicated alleles. The extracts were either (A) incubated with a 32P-labeled DNA fragment containing the CDEI sequence and separated on a nondenaturing polyacrylamide gel, or (B) immunoblotted and probed with antibodies raised against a Cbf1p fusion protein. The same extracts were used for both experiments. In (A) the arrow indicates the retarded band representing CDEI bound specifically to Cbf1p. In (B) the arrow indicates the Cbf1p band.

Faster mobility is also apparent in the gel retardation assay (Figure 4A). This band is unlikely to arise from proteolysis of the protein since an aliquot from the same extract was applied in the immunoblot (Figure 4B) and no lower molecular weight species was detected. The band may therefore arise from an altered conformation of Cbf1p or from an altered interaction with other constituents of the DNA binding complex in these crude extracts.

*cbf1-10* is a dominant mutation: Several naturally occurring helix-loop-helix motif-containing proteins lack a functional DNA binding region (Benezra et al. 1990; Christy et al., 1991; Ellis, Spann and Posekony 1990; Garrell and Modoelli 1990; Sun et al. 1991). These proteins exert a dominant negative effect, presumably by sequestering their dimerization partners from forming productive DNA binding complexes. To determine whether a similar phenomenon might be occurring in alleles of *CBFI* that sustained mutations within the putative DNA binding region, *cbf1-3, cbf1-10, cbf1-11* and *cbf1-12* were tested for dominance. Each of these alleles was introduced into a diploid strain heterozygous for *CBFI* (YN533). These strains were then spotted onto various media to determine their phenotype (Figure 5). As in Figure 2, cells were plated on medium lacking uracil to control for the number of cells spotted (Figure 5A). When grown on medium containing thiabendazole, cells bearing *cbf1-10* showed markedly compromised growth (Figure 5B). Each of the other alleles (data not shown for *cbf1-4, cbf1-9, cbf1-15, cbf1-16, cbf1-17, cbf1-21, cbf1-38*) displayed wild-type growth indicating that they were fully recessive. The dominant behavior of *cbf1-10* was not manifest with respect to growth at 30°C on methionine-free medium (Figure 5C). However, at 37°C, a modest reduction in growth was apparent (Figure 5D).

We also carried out chromosome loss/recombination assays on strains heterozygous for these alleles. Figure 3C shows that *cbf1-10* is dominant with respect to this phenotype as well. The rate of chromosome loss is increased in cells bearing this allele relative to cells carrying *cbf1Δ, cbf1-3, cbf1-11* and *cbf1-12*.

DISCUSSION

This study describes nine alleles of the *CBFI* gene that encode protein with altered function. Since the mutagenesis used to obtain these alleles was not site directed and the screen required production of a stable protein, the mutations are likely to reveal, without bias, the regions of Cbf1p that are most important for performing its diverse functions.

Use of PCR for random mutagenesis: The polymerase chain reaction protocol used in this study was ideal for mutagenizing the ~1350-bp *CBFI* gene. This procedure generated two loss of function alleles that sustained one point mutation, three alleles that sustained two point mutations, and two alleles that sustained three point mutations. As shown in Table 2, some of these mutations did not change the encoded amino acid. As a result, five of the alleles suffered one amino acid alteration, and two suffered two amino acid alterations. Among the alleles that were sequenced, T to C and A to G transitions predominated. However, since only loss of function alleles that synthesized full-length Cbf1p were sequenced, it is not clear whether this preference reflects the distribution of base changes within the whole mutagenized population or whether it reflects a bias related to loss of function. Interestingly, other studies have shown that Taq polymerase preferentially substitutes dCTP for dTTP under mutagenic conditions (Eckert and Kunkel 1991; Tindall and Kunkel 1988).

Functional domains of Cbf1p: With two exceptions...
the mutations that alter the function of Cbf1p are clustered in or just downstream from the bHLH motif. This result confirms a role for the bHLH domain in Cbf1p function. This and other studies demonstrate the importance of Glu-231 (which is altered in cbf1-3, cbf1-10 and cbf1-11) and the corresponding residue in the basic region of the bHLH protein myoD for DNA binding (Davis et al. 1990; MELLOR et al. 1991). However, it is interesting that mutation of Leu-244 (cbf1-4) and Leu-248 (cbf1-21) also dramatically decrease DNA binding despite the fact that they lie outside the canonical DNA binding domain (within helix 1). The compromised ability of the proteins encoded by cbf1-4 and cbf1-21 to bind DNA is consistent with the notion that dimerization mediated by the amphipathic α-helices is important for DNA binding.

MELLOR et al. (1991) used site directed mutagenesis to create a mutation, YAG214, that has an alanine in place of Glu-231. Like cbf1-3, cbf1-10 and cbf1-11, the protein encoded by YAG214 fails to detectably bind CDE I. However, unlike the alleles used in this study, the mutation in YAG214 does not appear to cause methionine auxotrophy. Surprisingly, the alterations in cbf1-3, cbf1-10 and cbf1-11 therefore have a more severe effect, presumably unrelated to DNA binding, on Cbf1p than the change to alanine in YAG214.

Two alleles, cbf1-10 and cbf1-15, obtained in this study sustained two amino acid alterations. In each case, one mutation occurred in Glu-86, and the second occurred in or near the bHLH domain. The mutations in Glu-86 alone, represented by cbf1-12 and cbf1-17, do not detectably alter Cbf1p function. Rather, these mutations modify the phenotype caused by the mutations lying downstream. In cbf1-15 changing Glu-86 to valine partially ameliorates the thiabendazole sensitivity and elevated rate of chromosome loss caused by changing Glu-283 to proline. In cbf1-10, changing Glu-86 to lysine causes the allele to become partially dominant. How can altering Glu-86 effect these modifications of phenotype? Glu-86 does not play an essential role in interfacing with other proteins since previous studies have shown that amino acids 1–209 are dispensable for Cbf1p function (MELLOR et al. 1990). Therefore one possibility is that the mutations sustained in cbf1-10 and cbf1-15 increase the stability of the encoded protein. However, Figure 3B shows that the abundance of Cbf1p encoded by cbf1-10 is actually decreased relative to wild type protein. Another possibility is that the mutations in Glu-86 alter nonessential interactions between Cbf1p and another protein, either directly or by altering the conformation of Cbf1p. Consistent with this notion is the fact that an additional band is observed in gel retardation assays carried out on crude extracts from cells bearing cbf1-15. It is also a formal possibility that the different alterations of Glu-86 in cbf1-10 and cbf1-15 affect the function of the protein by two different mechanisms.

Separation of Cbf1p functions: Several of the cbf1 alleles obtained in this study separated the growth phenotypes associated with mutations in CBF1. Cells containing cbf1-9 were more compromised for their ability to grow on medium lacking methionine than for their ability to grow on medium containing thiabendazole. In contrast, cbf1-16 supported vigorous growth on methionine-free medium (at 30°), but only moderate growth on thiabendazole. Similarly, the
dominant behavior of cbfl-10 was more pronounced with respect to growth on thiabendazole than with respect to growth on methionine-free medium. The separation of functions in these alleles does not reflect differential sensitivity of all cells with impaired Cbf1p function to one of the growth media since both an allele more compromised for its ability to grow on medium lacking methionine and alleles that are more compromised for their ability to grow on medium containing thiabendazole were observed. Therefore at least some aspects of Cbf1p function in each of these capacities must be mechanistically distinct.

The region immediately downstream from the bHLH domain may determine the specificity of Cbf1p function. Both cbfl-9 and cbfl-16, which separate the functions of Cbf1p, suffered mutations in this region. However, the mutation in each of these alleles resulted in a change to the helix-destabilizing amino acid proline. Potential destabilization of adjacent regions such as the bHLH domain might therefore also account for the separation of function in these alleles. Interestingly, the CDEI DNA binding affinities of the proteins encoded by the separation of function alleles appeared to be quite similar.

Whereas methionine auxotrophy and thiabendazole hypersensitivity could be separated by point mutations in Cbf1p, mutations that separated the thiabendazole hypersensitivity from the increased frequency of chromosome loss were not observed. The correlation between these two phenotypes was even evident in the partially dominant allele cbfl-10. Thiabendazole hypersensitivity and chromosome loss may therefore occur through the same functional mechanism. While other possibilities are not excluded, these observations are consistent with a model in which one role of Cbf1p in chromosome segregation may be to improve the efficiency with which contact between the kinetochore and spindle microtubules is established or maintained.

We thank M. WAHLBERG, S. CUMBERLIDGE, R. BENEZRA, R. LEHMAN and A. VILLENUEVE for critical reading of the manuscript and S. RAMER and C. ALFANO for many helpful discussions. We also thank K. RUNGE and C. BRENNER for useful suggestions. This investigation was supported by National Institutes of Health grant R37HG00198 to R.W.D. P.K.F. was a recipient of National Research Service Award GM12588.

LITERATURE CITED


Korch, C., H. A. Mountain and A. S. Bystrum, 1991 Cloning,


Communicating editor: F. WINSTON