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Evidence that the *MIF2* Gene of *Saccharomyces cerevisiae* Encodes a Centromere Protein with Homology to the Mammalian Centromere Protein CENP-C

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The *MIF2* gene of *Saccharomyces cerevisiae* has been implicated in mitosis. Here we provide genetic evidence that *MIF2* encodes a centromere protein. Specifically, we found that mutations in *MIF2* stabilize dicentric minichromosomes and confer high instability (i.e., a synthetic acentric phenotype) to chromosomes that bear a *cis*-acting mutation in element I of the yeast centromeric DNA (CDEI). Similarly, we observed synthetic phenotypes between mutations in *MIF2* and *trans*-acting mutations in three known yeast centromere protein genes–*CEP1/CBF1/CPF1*, *NDC10/CBF2*, and *CEP3/CBF3B*. In addition, the *mif2* temperature-sensitive phenotype can be partially rescued by increased dosage of *CEP1*. Synthetic lethal interactions between a *cep1* null mutation and mutations in either *NDC10* or *CEP3* were also detected. Taken together, these data suggest that the Mif2 protein interacts with Cep1p at the centromere and that the yeast centromere indeed exists as a higher order protein-DNA complex. The Mif2 and Cep1 proteins contain motifs of known transcription factors, suggesting that assembly of the yeast centromere is analogous to that of eukaryotic enhancers and origins of replication. We also show that the predicted Mif2 protein shares two short regions of homology with the mammalian centromere Ag CENP-C and that two temperature-sensitive mutations in *MIF2* lie within these regions. These results provide evidence for structural conservation between yeast and mammalian centromeres.

INTRODUCTION

The proper segregation of eukaryotic chromosomes is mediated by a specialized chromosomal structure, termed the centromere or kinetochore. Studies of the centromere in vitro and in vivo suggest it is a multifunctional complex that can capture and stabilize microtubules, promote bidirectional chromosome movement along microtubules, facilitate polymerization and depolymerization of microtubules, and mediate sister chromatid association until the onset of anaphase (reviewed in Mitchison, 1988; Schulman and Bloom, 1991). To understand the molecular basis of these activities, it is necessary to identify the *cis*- and *trans*-acting components of the centromere and to elucidate how they assemble into a higher order structure with the appropriate biochemical properties.

Although some progress has been made in the molecular analysis of larger centromeres from mammals and fission yeast, the less complex centromeres of the yeast *Saccharomyces cerevisiae* have proved more amenable to detailed molecular genetic analysis of structure and function. Functional yeast centromeric DNA sequences (~125 bp) were identified by their ability to confer mitotic and meiotic stability to small recombinant DNA plasmids (minichromosomes) in yeast cells (Clarke and Carbon, 1980; Fitzgerald-Hayes *et al.*, 1982; Hieter *et al.*, 1985). These sequences are comprised of three conserved centromere DNA elements (Figure 1). The central element, CDEII (~85 bp), consists of alternating stretches of A and T residues, and is flanked by two highly conserved palindromic mo-

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tifs termed CDEI (8 bp) and CDEIII (26 bp). The relative contribution of each of these elements to mitotic fidelity has been assessed by extensive mutational analysis (reviewed in Hegemann and Fleig, 1993). In addition, in vivo, a unique nuclease-resistant chromatin structure (encompassing ~200 bp) is associated with the centromere DNA throughout the cell cycle (Bloom and Carbon, 1982; Saunders *et al.*, 1988; Funk *et al.*, 1989; Schulman and Bloom, 1991). In recent years, a combination of genetic and biochemical approaches has yielded a satisfying congruence in terms of identifying putative protein constituents of this chromatin structure, which presumably corresponds to the yeast centromere-kinetochore complex.

The integrity of CDEIII is essential for centromere function in vivo. Single point mutations in CDEIII can abolish measurable centromere function and disrupt centromere chromatin structure (McGrew *et al.*, 1986; Ng and Carbon, 1987; Hegemann *et al.*, 1988; Saunders *et al.*, 1988). A multisubunit complex (CBF3) that specifically binds to wild-type CDEIII DNA in vitro has been identified (Lechner and Carbon, 1991). The three major CBF3 components, CBF3A (110 kDa), CBF3B (64 kDa), and CBF3C (58 kDa) are encoded by *NDC10/CBF2*, *CEP3/CBF3B*, and *CTF13*, respectively (Doheny *et al.*, 1993; Goh and Kilmartin, 1993; Jiang *et al.*, 1993; Lechner, 1994; Strunnikov *et al.*, 1995). Several observations support the notion that the *NDC10*, *CEP3*, and *CTF13* gene products act as centromere proteins in vivo. First, these genes were identified in genetic screens for mutants defective in chromosome segregation and/or centromere function. Second, all three genes are essential, in keeping with the essential role of CDEIII DNA in centromere function. Third, mutations in these genes confer cytological defects indicative of aberrant DNA segregation and a mitotic delay. Fourth, the intranuclear localization pattern of Ndc10 protein (Goh and Kilmartin, 1993) is consistent with the distribution of centromeric DNA as visualized by fluorescent in situ hybridization (Guacci and Koshland, unpublished observations). Finally, some CBF3 preparations contain a microtubule binding activity (Hyman *et al.*, 1992; Middleton and Carbon, 1994), as is expected for an organelle that engages the microtubule-based mitotic spindle. Moreover, experiments with isolated minichromosomes or partially reconstituted centromeres suggest that CDEIII and the CBF3 proteins are essential for the centromere's ability to bind microtubules in vitro (Kingsbury and Koshland, 1991; Sorger *et al.*, 1994; Strunnikov *et al.*, 1995). It has been suggested, therefore, that CDEIII-CBF3 complexes represent partially reconstituted yeast centromeres (Hyman *et al.*, 1992; Sorger *et al.*, 1994). In this case, CDEIII can be viewed as the binding site for the machinery that mediates chromosome-microtubule interaction.

CDEI and its associated protein also have been well characterized. A deletion of CDEI has only a minor effect on chromosome segregation, indicating that this element is dispensible for centromere function (Panzeri *et al.*, 1985; Cumberledge and Carbon, 1987; Gaudet and Fitzgerald-Hayes, 1989). CDEI is bound in vitro by a protein encoded by the *CEP1* gene (also known as *CBF1* or *CPF1*; Baker *et al.*, 1989; Cai and Davis, 1989, 1990; Jiang and Philippsen, 1989; Baker and Masison, 1990; Mellor *et al.*, 1990). Cep1p is a member of the basic helix-loop-helix class of DNA binding proteins. Upon binding to CDEI-containing DNA fragments, Cep1p induces a strong bend centered in CDEI (Niedenthal *et al.*, 1993). It is unlikely that the possible role of Cep1p in modulating DNA structure is limited to centromeres, because the protein is quite abundant and CDEI sites exist both at the centromere and elsewhere in the genome (Bram and Kornberg, 1987; Baker *et al.*, 1989). Indeed, deletion of the *CEP1* gene confers pleiotropic defects, including slow growth and methionine auxotrophy, in addition to mitotic defects such as a 10-fold increase in the rate of chromosome loss and sensitivity to microtubule-destabilizing drugs (Baker and Masison, 1990; Cai and Davis, 1990; Mellor *et al.*, 1990). Thus, it has been proposed that Cep1p is a general DNA binding protein that acts in vivo to modulate chromatin structure at multiple loci including centromeres (Baker and Masison, 1990; Mellor *et al.*, 1990; Thomas *et al.*, 1992; Masison *et al.*, 1993).

Although CDEI and CDEIII and their associated proteins have been extensively characterized, less is known about CDEII. Although a complete deletion of CDEII eliminates centromere function, smaller deletions or insertions in this element retain partial activity (Gaudet and Fitzgerald-Hayes, 1987; Cumberledge and Carbon, 1987). These results suggest that CDEII is somewhat flexible and might comprise iterations of a protein binding site. To date, no CDEII-specific DNA binding proteins have been identified, although both nuclease protection experiments (Bloom and Carbon, 1982; Saunders *et al.*, 1988; Funk *et al.*, 1989) and in vivo DMS footprinting experiments (Densmore *et al.*, 1991) suggest that protein(s) are bound to CDEII in vivo. However, it remains possible that the intrinsic structural properties of homopolymeric A + T-rich DNA (e.g. a propensity to bend or a low melting temperature) contribute to CDEII function in the absence of an associated protein.

Clearly, understanding the contribution of CDEII and other aspects of centromere function will require the identification of additional centromere proteins. The *MIF2* gene was originally identified as a gene fragment that in high copy causes aberrant transmission of authentic yeast chromosomes (Meeks-Wagner *et al.*, 1986). Subsequently, *MIF2* was shown to encode an essential protein, and loss of *MIF2* function results

in chromosome missegregation, mitotic delay, and aberrant microtubule morphologies (Brown *et al.*, 1993). These phenotypes are reminiscent of those associated with mutations in components of the CBF3 complex. An additional hint that Mif2p might act at the centromere came from the analysis of its predicted gene product. The Mif2 protein has two features that suggest it interacts with DNA: an acidic domain and a proline-rich, "A-T hook" motif common to several chromatin proteins that bind A + T-rich DNA (e.g. mammalian HMGI(Y) proteins and *Drosophila* D1; reviewed in Churchill and Travers, 1991). Thus, it was reasonable to speculate that Mif2p might act at the centromere by binding to the A + T-rich CDEII (Brown *et al.*, 1993). In this paper we provide genetic evidence that *MIF2* indeed encodes a centromere protein. We suggest that the Mif2 protein plays a key role in mediating yeast centromere assembly and that this assembly is directly analogous to the stereospecific assembly of multiprotein complexes at enhancer loci and origins of replication.

MATERIALS AND METHODS

Genetic Manipulations and Molecular Techniques

Yeast strains used in this study are listed in Table 1. Temperature-sensitive alleles of *MIF2* were previously identified in a plasmid shuffle screen and characterized by Brown *et al.* (1993). Yeast transformations and genetic manipulations were as described by Rose *et al.* (1990). Yeast strains were routinely grown at 23°C. Yeast genomic DNA for Southern analysis or plasmid recovery was prepared by the method of Hoffman and Winston (1987). Double-stranded DNA sequencing reactions were performed according to a dye terminator cycle sequencing protocol and analyzed on an Applied Biosystems Model 373A DNA Sequencing System (Foster City, CA). Plasmid pDR11–6 was generously provided by Richard Baker and carries the *cep1* null allele *cep1::URA3* in which codons 53–351 and 92 bp of 3′-flanking DNA are replaced with the yeast *URA3* gene. A stable *cep1::URA3* strain was derived from wild-type strain 5371–10-2 by one-step gene replacement (Rothstein, 1983) using *Eco*RI-digested pDR11–6. Centromeric plasmids pPM3 and pPM4 each contain the 2.6-kb *Pst*I *MIF2* fragment from pMB024 (Brown *et al.*, 1993) on pRS314 (*CEN6 TRP1*) and pRS316 (*CEN6 URA3*), respectively (Sikorski and Hieter, 1989). To create plasmid pPM40, the 2.25-kb *Bam*HI fragment of plasmid pPM30 (see Figure 2A) containing the *CEP1* gene was cloned into the *Bam*HI site of pRS314. The coding and 5′-untranslated regions (~1.9 kb) of two *mif2* mutant alleles present on plasmids pMB041 (*mif2–2*) and pMB043 (*mif2–3*) were

Table 1. Strains used in experiments

Strain	Genotype	Source[a]
5371-10-2	*MATa ura3*	Brown *et al.* (1993)
6848-4-2	*MATa ura3 mif2-2*	Brown *et al.* (1993)
6849-10-1	*MATa ura3 mif2-3*	Brown *et al.* (1993)
6858-18-3	*MATa ura3 mif2-5*	Brown *et al.* (1993)
6801	*MATa/MATα ura3/ura3 leu2/leu2 his3/+ his7/+ can1/+ hom3/+ sap3/sap3*	Brown *et al.* (1993)
PM1101	*MATa/MATα ura3/ura3 leu2/leu2 his3/+ his7/+ can1/+ hom3/+ sap3/sap3 cen1::CEN3-URA3/CEN1*	
PM1102	*MATa/MATα ura3/ura3 leu2/leu2 his3/+ his7/+ can1/+ hom3/+ sap3/sap3 cen1::CEN3ΔcdeI-URA3/CEN1*	
6799	*MATa/MATα ura3/ura3 trp1/+ his3/+ his7/+ can1/+ hom3/+ sap3/sap3 mif2-3/mif2-3*	Brown *et al.* (1993)
PM1105	*MATa/MATα ura3/ura3 trp1/+ his3/+ his7/+ can1/+ hom3/+ sap3/sap3 mif2-3/mif2-3 cen1::CEN3-URA3/CEN1*	
PM1106	*MATa/MATα ura3/ura3 trp1/+ his3/+ his7/+ can1/+ hom3/+ sap3/sap3 mif2-3/mif2-3 cen1::CEN3ΔcdeI-URA3/CEN1*	
4513-216	*MATa ura3 leu2 ade2 ade3 his3 can1 sap3 gal1*	Koshland *et al.* (1987)
PM1002-4C	*MATa ura3 leu2 ade2 ade3 mif2-3*	
PM5371-101	*MATa ura3 cep1::URA3*	
PM1013-15D	*MATa ura3 trp1 cep1::URA3*	
6764-181	*MATα ura3 leu2 trp1 his3 can1 sap3 met2 mif2::HIS3* [pMB030]	Brown *et al.* (1993)
PM1002-28A	*MATa ura3 ade2 mif2-3*	
PM1002-28A-1	*MATa ura3 ade2 his3 mif2-3*	
PM1002-3A	*MATα ura3 leu2 trp1 mif2-3*	
YPH949	*MATα ura3 leu2 ade2 his3 lys2*	P. Hieter
s42[b]	*MATα ura3 leu2 ade2 his3 lys2 ndc10(ctf14)-42*	Doheny *et al.* (1993)
JK418[b]	*MATa ura3 leu2 trp1 ndc10-1 (Ade⁻His⁻Lys⁻)*	Goh and Kilmartin (1993)
CUY412	*MATα ura3 leu2 ade2 ndc10-2*	T. Huffaker
1cAS281[b]	*MATα ura3 leu2 ade2 trp1 his3 lys2 cep3-1*	Strunnikov *et al.* (1995)
s30	*MATα ura3 leu2 ade2 his3 lys2 ctf13-30*	Doheny *et al.* (1993)
MS524	*MATa ura3 leu2 ade2 kar3-101::LEU2*	Meluh and Rose (1992)
YPH698	*MATα ura3 leu2 ade2 trp1 his3 lys2 chl1Δ1::HIS3*	Gerring *et al.* (1990)

[a]Strains are from this study or as indicated.

[b]Strain corresponds to the original source of the mutant allele. Additional derivatives used in crosses are not shown.

sequenced on one strand using a set of 10 *MIF2* antisense primers. Plasmids pMB024, pMB041, and pMB043 were gifts from L. Hartwell and M. Brown.

Chromosome Stability Assays

All minichromosomes used in these studies were as previously described by Koshland *et al.* (1985) or Kingsbury and Koshland (1991). Plasmids pDK264 (*CEN3–111*) and pDK265 (*CEN3–1337*) were generated by linker insertion mutagenesis and previously characterized as bearing mutant alleles of *CEN3* (Koshland *et al.*, 1985). The sequences of both *CEN3* alleles were determined for this study and are described in Figure 1. Wild-type and mutant minichromosomes were introduced into strains 5371–10-2, 6848–4-2, and 6849–10-1 by a standard protocol (Ito *et al.*, 1983) and Ura^+ transformants were selected at 23°C. For each combination, single colonies corresponding to each of six independent transformants were inoculated separately into nonselective medium (YPD). For each culture, the frequency of Ura^+ cells was determined at the time of inoculation (F_0) and after approximately 24 h of nonselective growth at 23°C (F_{end}). Specifically, cultures were appropriately diluted and plated on YPD plates. After 2.5–3 days at 23°C, single colonies that arose were replica printed to synthetic complete medium lacking uracil. The rate of plasmid loss per generation was determined as described by Koshland *et al.* (1987), according to the relationship: $F_{end}=F_0(F_D)^G$, where F_D is the fraction of cells of each generation that retain the minichromosome (centromere efficiency) and G is the number of generations. The fraction of cells that lose the minichromosome per generation is $(1-F_D)$.

To measure the stability of an authentic chromosome, two centromere I replacement vectors were constructed based on plasmid pVG104. Plasmid pVG104 is based on pUC19 and contains an *Eco*RI insert (approximately 5 kb) corresponding to a 4.3-kb genomic *Eco*RI fragment from the centromeric region of chromosome I in which the central 0.55-kb *Nru*I–*Nar*I *CEN1*-containing fragment has been replaced with a 1.2-kb fragment containing the *URA3* gene and part of the pUC19 polylinker sequence. Plasmid pVG104 contains a unique *Bam*HI site adjacent to *URA3*. The details of plasmid pVG104 construction are available upon request. A 0.31-kb fragment containing wild-type *CEN3* was inserted into the unique *Bam*HI site of pVG104 to create the centromere replacement plasmid pPM101. A 0.25-kb fragment containing a CDEI-deletion derivative of *CEN3* (RB76; Gaudet and Fitzgerald-Hayes, 1989) was inserted into the *Bam*HI site of pVG104 to create pVG105. In both pPM101 and pVG105, the direction of *URA3* transcription is away from the CDEIII element of *CEN3*. Plasmids pVG104 and pVG105 were generously provided by Vincent Guacci.

Ura^+ derivatives of diploid strains 6801 and 6799 were obtained by transformation with *Eco*RI-digested pPM101 or pVG105. Transformation resulted in replacement of the centromere on one homologue of chromosome I with a wild-type or a CDEI-deleted *CEN3* tightly linked to the *URA3* gene. Heterozygosity at the *CEN1* locus was confirmed by Southern blot analysis. Loss of the *URA3*-marked chromosome I produces Ura^-, 2N-1 aneuploids that can be selected on 5-fluoro-orotic acid (5-FOA; Boeke *et al.*, 1984). The rate of chromosome I loss in diploids was determined by fluctuation analysis of the frequencies at which 5-FOA–resistant cells arose in independent cultures (Lea and Coulson, 1949). For both wild-type strains (PM1101 and PM1102) and the *mif2–3* diploid with a wild-type *CEN3* (PM1105), the frequency of 5-FOA–resistant cells was measured for each of 10 or 11 single colonies after growth at 23°C on nonselective medium (YPD) for approximately 20 generations. For the *mif2–3* strain in which *CEN1* was replaced with the CDEI-deleted *CEN3* allele (PM1106), the rate of chromosome I loss was too great to be accurately measured in the same manner. For this strain, a single Ura^+ PM1106 colony was resuspended in YPD medium, diluted to a density of 2–4 cells per milliliter, and aliquotted into 30 independent 0.5 mL cultures. The frequency of 5-FOA-resistant cells in each culture was determined after approximately 9–11 generations of growth at 23°C.

To confirm that loss of the centromere-linked *URA3* marker generally reflected chromosome loss and not mitotic gene conversion, we sporulated and dissected 10 independent, 5-FOA–resistant derivatives from each of PM1105 and PM1106. Two or fewer viable spores were recovered per tetrad for 19/20 strains analyzed and viability showed centromere-linkage as judged by the segregation pattern of *TRP1* amongst the viable spores. The parental Ura^+ strains showed good viability. Thus, we estimate that at least 95% of Ura^- cells arise due to chromosome I loss.

The dicentric minichromosomes pDK306–10 and pDK310–1 were as described by Koshland *et al.* (1987) and contain the selectable *LEU2* gene and two centromeric DNA sequences. The centromeres flank the yeast *ADE3* gene, which in an *ade2 ade3* background provides a visual signal for the structural integrity of a given minichromosome. Briefly, *ade2 ade3* mutants carrying an intact *ADE3* gene will form red-pigmented colonies, whereas disruption of the *ADE3* gene by deletion or rearrangement results in formation of white colonies. Dicentric and control monocentric minichromosomes (pDK243, wild-type *CEN3*, and pDK318–1, *CEN3–1337*) were transformed into wild-type (4513–216) and a *mif2–3* mutant (PM1002–4C). For each combination, four independent Leu^+ transformant colonies were separately resuspended in water, diluted, and plated on YPD (complete medium) and synthetic medium lacking leucine (selective medium). Mitotic stability of a given minichromosome was measured as the percent of cells that retain the minichromosome under selection. In the case of dicentric minichromosomes, only cells bearing unrearranged plasmids at the time of plating were included in the calculation. The structural stability of dicentric minichromosomes was assessed by visual inspection of the color and morphology of colonies on selective medium as previously described (Koshland *et al.*, 1987).

Isolation of Suppressors of the mif2–3 Temperature-Sensitive Phenotype

The *mif2–3* mutant yeast strains 6849–10-1 and PM1002–28A were transformed with a yeast genomic library constructed by C. Connelly and P. Hieter in the high copy, 2 μ-based, *URA3*-containing vector pRS202 (a closely related vector, pRS425, is described by Christianson *et al.*, 1992). Approximately 50,000 total Ura^+ transformants selected at 23°C (~15 genomic equivalents) were replica printed onto synthetic medium lacking uracil at 37°C, a nonpermissive temperature for *mif2–3* mutants. Growth was scored after 1–3 days. From 105 original candidates, 19 transformants consistently retested. Plasmid DNA from each Ts^+ transformant was recovered in *Escherichia coli* by electroporation and retested for the ability to suppress the Ts^- phenotype of strain PM1002–28A. Eleven positive clones were analyzed by restriction enzyme digestion and corresponded to four classes, none of which were *MIF2*. One class of suppressor was defined by five independent clones representing three unique, but overlapping genomic DNA fragments. A restriction map of the smallest clone, pPM30, and its various subclones are shown in Figure 2A. Plasmid pPM30 and a derivative with an endpoint in the suppressor gene (pPM33) were sequenced using the T7 and T3 primers. Partial sequence analysis showed that the dosage suppressor on pPM30 corresponded to the previously characterized *CEP1* gene (Baker and Masison, 1990; Cai and Davis, 1990; Mellor *et al.*, 1990). Plasmid pPM45, which contains the 2.25-kb *Bam*HI fragment encompassing the *CEP1* gene on a 2 μ-*TRP1* vector, pRS424 (Christianson *et al.*, 1992), was unable to suppress the *mif2::HIS3* deletion allele (Brown *et al.*, 1993), as judged by a plasmid shuffle assay. High copy number plasmids bearing either *CTF13* (provided by P. Hieter), *NDC10* (pPM47), or *KAR3* (pMR794) failed to suppress *mif2–3*.

RESULTS

CDEI-Defective Centromeres Show Reduced Activity in mif2 Mutants

Centromere function can be partially inactivated by a mutation either in the centromeric DNA itself or in a centromere protein. Function may be further compromised by the combination of a *cis*- and a *trans*-acting defect. This synergistic effect could arise if the centromere protein normally binds to the site of the DNA mutation or if the centromere protein interacts with another protein normally bound to that site. Such a "synthetic acentric" phenotype would manifest as an increased rate of chromosome loss. This rationale is the basis of several genetic screens designed to identify genes that encode centromere proteins by virtue of mutations that inactivate a mildly defective centromere (Xiao *et al.*, 1993; Strunnikov *et al.*, 1995).

We employed the same rationale to examine the role of *MIF2* in centromere function. Specifically, we asked whether mutations in *MIF2* exacerbate the rate of loss of any one of several minichromosomes bearing *CEN* DNA mutations. Thus, minichromosomes bearing the yeast selectable marker *URA3* and either a wild-type centromere (*CEN3*) or one of several mutant centromeres (Figure 1) were introduced by transformation into wild-type and two *mif2* temperature-sensitive strains. All the centromere mutations chosen for this study have been shown to retain partial centromere function. Similarly, strains bearing either the *mif2–2* or *mif2–3* mutation grow well and exhibit only a modest chromosome loss phenotype at 23°C (Brown *et al.*, 1993; see also Table 3). The rate of plasmid loss per generation at 23°C was then determined for each combination as described in MATERIALS AND METHODS. The data are summarized in Table 2.

A significant effect on stability was observed for CDEI-defective minichromosomes (pDK265 and pDK380) in the *mif2* mutant background, corresponding to a two- to fourfold increase in loss rate over that seen in the wild-type control strain. It should be noted that the high basal loss rate for wild-type minichromosomes leads to a narrow range of measurable effect in this type of stability assay (i.e., 1–2% loss per generation for a wild-type minichromosome to 30% loss per generation for an acentric minichromosome or YRp plasmid; Koshland *et al.*, 1987; Kingsbury and Koshland, 1991). Therefore, the absolute loss rate per generation (12%) observed for the CDEI mutations in the *mif2* mutant background is significant. Mutations in *MIF2* had little or no apparent effect on the stability of either CDEII- or CDEIII-defective minichromosomes, although the modest increase in loss of the CDEII(X35) mutant minichromosome pDK375 was reproducible.

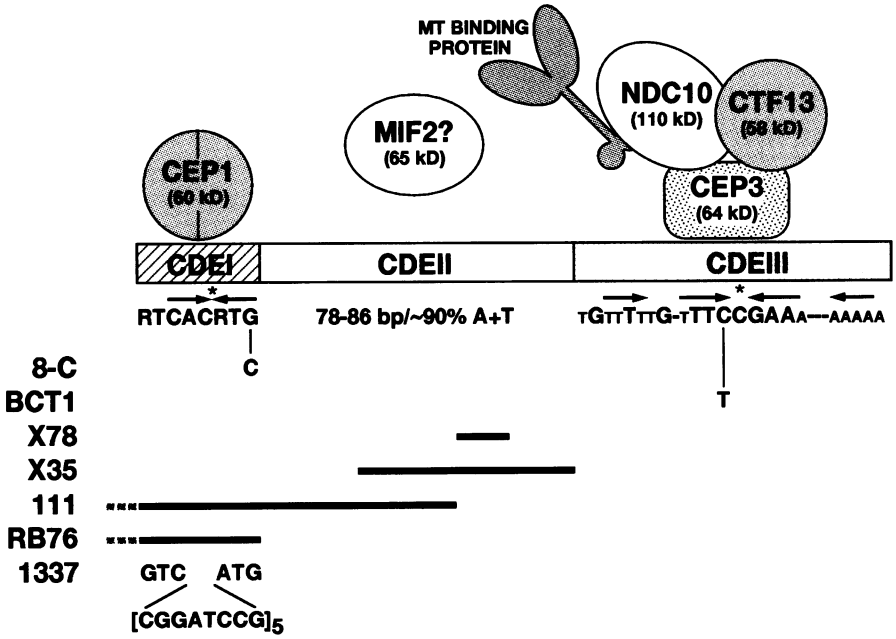


Figure 1. Structure of the yeast centromere. The consensus sequence of centromeric DNA from *S. cerevisiae* is indicated (adapted from Hegemann and Fleig, 1993), along with those proteins known to associate with CDEI and CDEIII in vitro. Cep1p (also known as Cbf1p or Cpf1p) binds as a dimer at CDEI (Mellor *et al.*, 1990; Dowell *et al.*, 1992). The multiprotein complex CBF3, which binds CDEIII, is comprised of at least three components: Ndc10p (also known as Cbf2p; Goh and Kilmartin, 1993; Jiang *et al.*, 1993), Cep3p (also known as Cbf3Bp; Strunnikov *et al.*, 1995; Lechner, 1994), and Ctf13p (Doheny *et al.*, 1993). This work provides genetic evidence that Mif2p is a centromere protein. In addition, the yeast centromere-kinetochore complex contains at least one, as yet unidentified, microtubule binding component (Hyman *et al.*, 1992; Sorger *et al.*, 1994). Apparent molecular mass (in kilodaltons) is also shown, except that the size of Mif2p was predicted from the gene sequence. Centromeric DNA mutations used in this study are indicated below. All except CDEI(8-C) are mutant alleles of *CEN3*. CDEI(8-C), a derivative of *CEN6*, is a point mutation that inactivates CDEI (Hegemann *et al.*, 1988). BCT1 is a point mutation in CDEIII (McGrew *et al.*, 1986). X78 and X35 are 14- and 57-bp deletions of CDEII, respectively, marked by an *Xho*I linker (Gaudet and Fitzgerald-Hayes, 1987). *CEN3–111* and *CEN3–1337* are deletions associated with *Bam*HI linkers (Koshland *et al.*, 1985). The 190-bp deletion in *CEN3–111* removes 130 bp of 5′-flanking DNA, CDEI, and the first 52 bp of CDEII, leaving part of CDEII (32 bp) and CDEIII intact. In *CEN3–1337*, the central 2 bp of CDEI have been replaced with five tandem copies of the *Bam*HI linker (CGGATCCG). RB76 is a precise deletion of CDEI also marked by a *Bam*HI linker (Gaudet and Fitzgerald-Hayes, 1989).

Table 2. Minichromosome stability in wild-type and *mif2* mutant backgrounds

Plasmid	Mutation	Element affected	Mean % loss per generation (± SEM)[a]		
			MIF2	*mif2-2*	*mif2-3*
pDK370	WT	–	1.0 ± 0.1	1.3 ± 0.3	0.9 ± 0.4
pDK373	WT	–	1.4 ± 0.1	1.7 ± 0.2	2.4 ± 0.4
pDK265	1337	CDEI	6.0 ± 0.6	11.9 ± 1.7	13.3 ± 2.4
pDK380	8-C	CDEI	2.8 ± 0.4	10.2 ± 2.1	11.8 ± 1.8
pDK374	X78	CDEII	2.2 ± 0.3	2.7 ± 0.4	2.5 ± 0.2
pDK375	X35	CDEII	12.4 ± 0.5	14.1 ± 1.1	14.7 ± 0.9
pDK264	111	CDEI+II	11.2 ± 1.0	12.4 ± 0.6	14.1 ± 0.6
pDK371	BCT1	CDEIII	11.2 ± 0.7	12.2 ± 0.4	12.2 ± 0.5

[a]Minichromosome stability reported as the mean percent of centromeric plasmid loss per generation at 23°C for at least six independent transformants ± SEM. All mutant centromeres are derivatives of *CEN3* except the CDEI(8-C) allele, which is a mutant allele of *CEN6*.

To more accurately assess the degree of synergism between mutations in *MIF2* and CDEI, the stabilities of authentic yeast chromosomes bearing either a wild-type centromere or a centromere lacking CDEI were measured in a *mif2–3* mutant. For this purpose, diploid strains were constructed in which the centromere of one chromosome I homologue was replaced with a cassette containing the *URA3* gene immediately adjacent to either the wild-type *CEN3* sequence or a CDEI-deleted *CEN3* derivative (RB76; Gaudet and Fitzgerald-Hayes, 1989). In the majority of cases, loss of the *URA3* marker should reflect chromosome I loss, because *URA3* is tightly linked (≤ 266 bp) to *CEN3* on the test chromosome I. Such Ura$^-$ derivatives, aneuploid for chromosome I, can be selected readily on 5-FOA (Boeke *et al.*, 1984) and show no obvious growth defect compared with their euploid counterparts (this study and J. Guacci, personal communication).

The rates of chromosome I loss per generation in the four diploid strains were estimated by fluctuation analysis of the frequencies at which 5-FOA–resistant cells arose in independent cultures (Lea and Coulson, 1949). These data are summarized in Table 3. Chromosome I was very stable in the wild-type diploid (PM1101), whereas the observed 50-fold increase in the *mif2–3* mutant (PM1105) is consistent with previous measurements of chromosome loss in this strain (Brown *et al.*, 1993). Likewise, deletion of CDEI caused a characteristic modest increase (~10-fold) in loss in the wild-type strain (PM1102). In contrast, the CDEI-deleted chromosome I was lost in approximately 5% of cell divisions in the *mif2–3* mutant (PM1106). This represents a >500-fold effect compared with the wild-type chromosome and indicates that in the *mif2–3* mutant background, a CDEI-defective centromere is severely compromised for function. Thus, in a *mif2* mutant background, CDEI, an otherwise nonessential DNA element, becomes critical for accurate chromosome segregation. This manifestation of a synthetic acentric phenotype is consistent with the notion that Mif2p is a component or effector of the yeast centromere.

Mutations in MIF2 Lead to Stabilization of Dicentric Minichromsomes

As an independent measurement of centromere function in the *mif2* mutant background, the behavior of a dicentric minichromosome pDK306–10 (Koshland *et al.*, 1987) was assessed in a *mif2–3* strain. Normally, chromosomes with two functional centromeres exhibit high rates of nondisjunction and undergo structural rearrangements that alter or eliminate one of the centromeric DNA sequences (Mann and Davis, 1983; Oertel and Mayer, 1984; Koshland *et al.*, 1987). However, dicentric chromosomes can be substantially stabilized either by *cis*-acting mutations in the centromeric DNA sequences (Koshland *et al.*, 1987; also see Table 4) or by *trans*-acting mutations in centromere factors (Doheny *et al.*, 1993). In a *mif2–3* mutant, both the mitotic and structural stabilities of pDK306–10 were enhanced relative to wild type, as evidenced by the increased frequency of cells bearing an unrearranged dicentric minichromosome during selective growth (Table 4). The degree of stabilization in the *mif2–3* mutant was even more pronounced for a dicentric minichromosome bearing a mutation in CDEI (pDK310–1), as might be expected given that CDEI-defective centromeres are dysfunctional in *mif2* mutants. Stabilization of dicentric chromosomes in the *mif2–3* mutant is consistent with data cited above, which implicates Mif2p in centromere function.

Loss of CEP1 Function Is Lethal in a mif2 Mutant Background

The preceding data provide clear evidence that CDEI-defective centromeres are much less active in a *mif2*

Table 3. Chromosome stability in wild-type and *mif2* mutant backgrounds

Strain	Genotype	*CEN3* allele replacing *CEN1*	Chromosome I loss per cell division[a]
PM1101	*MIF2/MIF2*	WT	0.8×10^{-5}
PM1105	*mif2-3/mif2-3*	WT	47×10^{-5}
PM1102	*MIF2/MIF2*	RB76 (ΔcdeI)	9.1×10^{-5}
PM1106	*mif2-3/mif2-3*	RB76 (ΔcdeI)	5600×10^{-5}

[a]Loss rate determined for strains grown at 23°C using fluctuation analysis (Lea and Coulson, 1949), as described in MATERIALS AND METHODS.

mutant and suggest that *MIF2* has at least one mitotic function that is dependent on or mediated through CDEI. Presumably, this dependence is indirect and the association or activity of Mif2p at the centromere is, in fact, facilitated by Cep1 protein bound at CDEI. If this is true, loss of *CEP1* function should affect Mif2 protein activity at all centromeres in a way similar to that described above for individual CDEI-defective centromeres. In the case of mutant Mif2p, this would lead to a dramatic and pleiotropic increase in the loss of all chromosomes of the magnitude observed for a CDEI-deleted chromosome. Therefore, *mif2 cep1* double mutants are predicted to be slow growing or inviable.

To test this prediction, we attempted to construct *mif2 cep1* haploids under conditions normally permissive for either single mutant. In crosses between *mif2–2* and *cep1::URA3* null strains, a pattern of reduced spore viability was observed (82% of spores were viable, compared with ≥95% for single mutant crosses) in which double mutants were under-represented amongst the viable progeny (Table 5). Only 15 of 38 expected double mutants (39%) were recovered. These rare *mif2–2 cep1::URA3* double mutants grew extremely slowly compared with either parent or wild type. Indeed, it is possible that growth of the *mif2–2 cep1::URA3* recombinants reflected the occurrence of secondary mutations (e.g. chromosome gain). In crosses with *mif2–3*, spore inviability was even more pronounced (only 72% of spores were viable) and only one putative *mif2–3 cep1::URA3* double mutant was recovered at 23°C in over 75 tetrads (of 87 inviable spores, at least 77 were predicted to be double mutants), suggesting that the *mif2–3* and *cep1::URA3* mutations are synthetically lethal when combined. We were able to recover *mif2–3 cep1::URA3* strains maintained by the presence of a *TRP1*-based centromeric plasmid bearing the wild-type *CEP1* gene (pPM40). Although readily lost from either single mutant during nonselective growth, the *CEP1* plasmid could not be lost by double mutants, confirming that *mif2–3 cep1::URA3* double mutants are inviable under vegetative growth conditions. The finding that mutations in *MIF2* and *CEP1* are synthetically lethal corroborates the idea that the synergistic effect seen with the CDEI-defective centromeres in *mif2* strains is a consequence of the loss of an interaction between Mif2p and Cep1p.

Table 4. Stability of dicentric minichromosomes in wild-type and *mif2-3* mutant background

	CEN3 allele at site			Mitotic stability (% cells with plasmid under selection)		*mif2-3*
Plasmid	1	2	Strain	Range	Average	WT
pDK243	WT	none	*mif2-3*	71.3-87.7	80.7	0.9
			MIF2	87.4-100.0	93.5	
pDK318-1	none	1337	*mif2-3*	46.5-58.8	55.3	0.7
			MIF2	76.3-88.3	81.0	
pDK306-10	WT	WT	*mif2-3*	22.2-33.9	30.0	3.6
			MIF2	6.4-10.8	8.4	
pDK310-1	WT	1337	*mif2-3*	59.1-72.7	66.3	3.1
			MIF2	16.4-25.0	21.5	

Indicated minichromosomes were transformed into a wild-type (4513-216) or a *mif2-3* (PM1002-4C) strain. Mitotic stability is given as the percent of total cells that harbors a given minichromosome during growth at 23°C on synthetic medium lacking leucine, which selects for the plasmid. For dicentric minichromosomes, the percentage includes only cells with unrearranged plasmids as judged by colony color and morphology (see MATERIALS AND METHODS). The range reported is for four independent transformants.

Increased Dosage of CEP1 Can Partially Suppress the Temperature-Sensitive Phenotype of mif2 Mutants

To further elucidate the role of Mif2p in mitosis, we have isolated multi-copy suppressors of the *mif2–3* temperature-sensitive phenotype (see MATERIALS AND METHODS), with the hope of identifying genes previously implicated in centromere function. Subcloning and partial sequence analysis revealed that one class of dosage suppressor, in fact, corresponds to the *CEP1* locus that encodes the CDEI-binding protein (Figure 2). 2 μ-*CEP1* also suppresses another temperature-sensitive allele, *mif2–5*; however, increased dosage of *CEP1* does not suppress a complete deletion of *MIF2*, indicating that *CEP1* is not functionally equivalent to *MIF2*. An in-frame deletion allele of *CEP1* (*CEP1–500*) lacking codons 77–154 also suppressed *mif2–3*. *CEP1–500* provides Cep1p function as judged by its ability to complement the slow growth phenotype and methionine auxotrophy of a *cep1* null mutant; therefore, amino acids 77–154 of Cep1p, which include an acidic cluster around residue 85 (Mellor *et al.*, 1990), are not required for suppression. This observation is consistent with previous reports that N-terminal deletion alleles of *CEP1* retain function (Mellor *et al.*, 1990; Kent *et al.*, 1994).

Thus, whereas loss of *CEP1* function is lethal in a *mif2* mutant background, the presence of extra copies of *CEP1* can partially alleviate the *mif2* mutant phenotype. Although it is formally possible that Cep1p enhances *MIF2* gene expression, it cannot be absolutely required because *CEP1* is a nonessential gene. Moreover, we note that the 5′ region of the *MIF2* gene lacks canonical CDEI consensus elements, and unlike *cep1::URA3* null mutants, *mif2* mutants grow normally at their permissive temperature and are not methionine auxotrophs. The chro-

Table 5. Genetic interactions between centromere protein genes

Parent 1	Parent 2	% Viable spores	4-Spore tetrads	3-Spore tetrads	2-Spore tetrads	# Tetrads	Predicted doubles	Observed doubles (% viable)	Growth of double mutants
mif2-2	WT	98	32	3	0	35	—	—	—
mif2-3	WT	95	80	6	6	92	—	—	—
mif2-2	***cep1::URA3***	**82**	**17**	**21**	**5**	**43**	**38**	**15 (39)**	**+/=**
mif2-3	***cep1::URA3***	**72**	**9**	**49**	**19**	**77**	**78**	**1 (1)**	**+/=**
mif2-3	*ndc10-1*	90	13	6	1	20	n.d.[a]	n.d.[a]	—
mif2-3	*ndc10-2*	98	21	2	0	23	n.d.[a]	n.d.[a]	—
mif2-3	***ndc10-42***	**76[b]**	**4**	**11**	**3**	**18**	**16**	**2 (12)**	**–**
mif2-3	***cep3-1***	**80**	**9**	**7**	**5**	**21**	**22**	**8 (36)**	**+/–**
mif2-3	*ctf13-30*	96	17	3	0	20	n.d.[a]	n.d.[a]	—
mif2-3	*chl1::HIS3*	100	10	0	0	10	11	11 (100)	++
mif2-3	*kar3::LEU2*	85[c]	12	7	3	22	19	12 (63)	+
cep1::URA3	WT	95	25	2	2	29	—	—	—
cep1::URA3	*ndc10-2*	97	19	3	0	22	18	17 (94)	+
cep1::URA3	***ndc10-42***	**72**	**4**	**18**	**7**	**29**	**29**	**0 (0)**	**–**
cep1::URA3	***cep3-1***	**82**	**8**	**11**	**2**	**21**	**15**	**4 (27)**	**+/=**
cep1::URA3	*ctf13-30*	99	20	1	0	21	24	24 (100)	+
ndc10-42	WT	93	91	14	8	113	—	—	—
cep3-1	WT	95	27	5	1	33	—	—	—

Indicated strains were mated, sporulated, and dissected at 23°C. In some cases, data shown are compiled from several independent crosses. The pattern of spore viability and segregation of relevant phenotypes were noted. Where necessary, complementation tests were performed to assign genotypes. The number of expected double mutants reflects the actual segregation of markers in the cross (theoretically, one would expect 1/4 of the progeny to be double mutants). Bold text indicates crosses with reduced spore viability indicative of a genetic interaction between the two genes. Wild-type and most single mutants grow well at 23°C ("++"); *cep1::URA3* and *kar3::LEU2* strains have a slow growth phenotype ("+").

[a]Expected double mutants were not determined (n.d.) because good spore viability and the distribution of wild-type versus temperature-sensitive spores in each tetrad (Meluh and Koshland, unpublished results) indicated that double mutants were certainly viable and grew well at 23°C.

[b]Data from only one cross is shown. Over 50 tetrads were dissected for analysis of *mif2-3 ndc10-42* recombinants, but genotypes could not be assigned to all spores for technical reasons. The distribution of phenotypically wild-type versus temperature-sensitive spores amongst all tetrads was consistent with double mutants being inviable. For example, in crosses where two mutations are synthetically lethal, the majority of tetrads, corresponding to tetratype asci, will contain three viable spores: two Ts^- single mutants and one wild type (2:1). The doubly mutant spore will be inviable and all tetrad classes indicative of a viable recombinant (i.e., 3 Ts^-:1 Ts^+; 2 Ts^-:2 Ts^+; and 1 Ts^-:2 Ts^+) will be absent. All *mif2-3 ndc10-42* tetrads conformed to this pattern. Two putative *mif2-3 ndc10-42* double mutants recovered presumably suffered additional mutations, because double mutants constructed with a *MIF2* plasmid were unable to lose the plasmid.

[c]Poor spore viability in the *kar3::LEU2* cross reflects nonrandom inviability of the *kar3* null spores and not a synthetic phenotype (Meluh and Rose, unpublished observations).

mosome loss data presented above implicate Mif2p rather directly in centromere function; therefore, we suggest that the genetic interactions between *MIF2* and *CEP1* reflect either a partial redundancy of function or protein-protein interaction at the centromere.

The three remaining multi-copy suppressors do not correspond to previously described centromere protein genes based upon their restriction maps and physical positions within the yeast genome. This is consistent with our observation that the *mif2–3* temperature-sensitive phenotype is not suppressed by increased dosage of either *CTF13* or *NDC10*. However, given the identification of *CEP1*, the suppressor genes might encode proteins that interact with Mif2p at the centromere and are currently being characterized.

MIF2 and CEP1 Genetically Interact with Genes that Encode Components of the CBF3 Complex

The observed synthetic phenotypes between mutations in *MIF2* and either a *cis*-acting mutation in the centromeric DNA or a mutation in the centromere protein gene *CEP1* imply that the Mif2p acts at the centromere. To strengthen this conclusion, we asked whether *MIF2* genetically interacts with other genes that encode centromere proteins. Thus, *mif2* mutants were crossed to several putative centromere protein mutants and the diploid strains were sporulated and dissected at 23°C (Table 5). In this way, an allele-specific synthetic lethal interaction with *NDC10* was detected. Although *mif2–3 ndc10–1* and *mif2–3 ndc10–2* double mutants could be constructed, only two putative *mif2–3 ndc10–42* strains were recovered

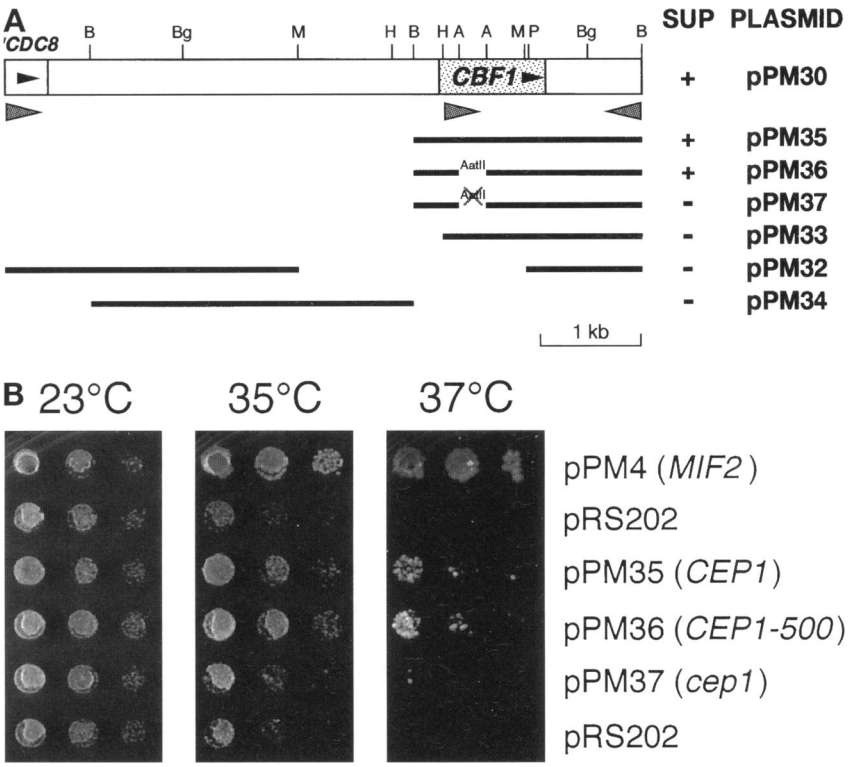
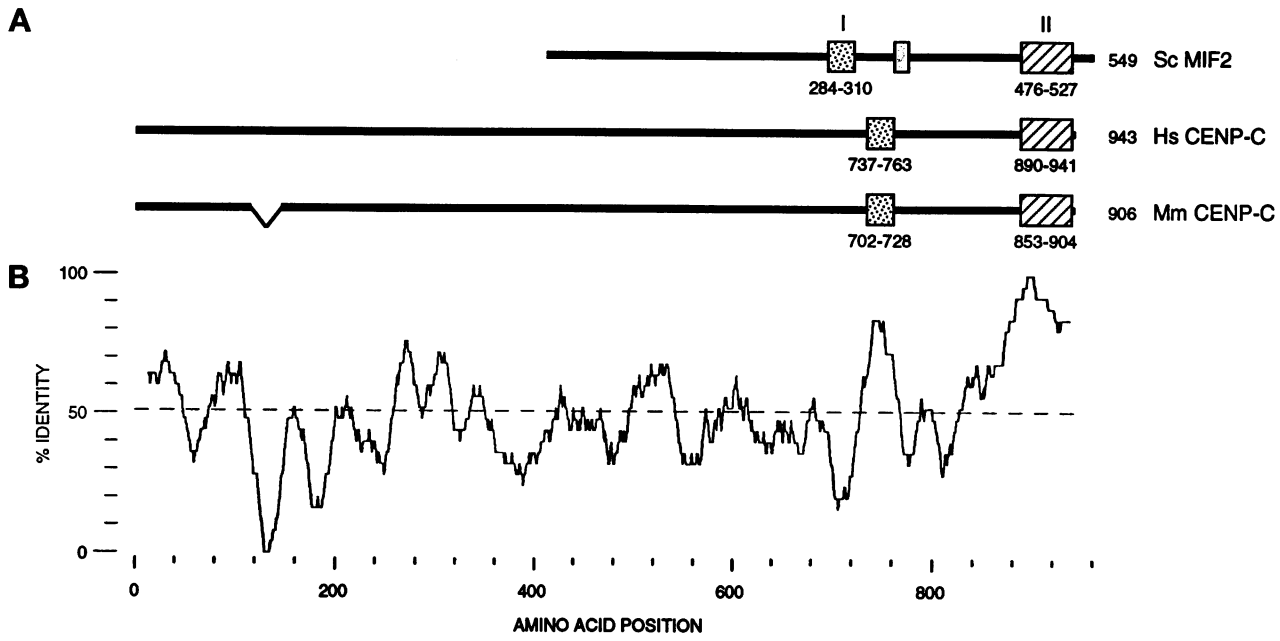


Figure 2. *CEP1* is a dosage suppressor of the *mif2–3* temperature-sensitive phenotype. (A) Schematic of the *CEP1* genomic locus. The original genomic clone (pPM30) isolated as a dosage suppressor of the *mif2–3* mutation is indicated, as well as various pRS202-based (2 μ vector) subclones of pPM30. Dark lines indicate sequences present. The ability, "+", or inability, "−", to suppress the temperature-sensitive phenotype is indicated. Arrows beneath pPM30 indicate sites of partial sequence analysis. Plasmid pPM36 carries a functional, in-frame deletion allele of *CEP1*, *CEP1–500*, that lacks codons 77–154, inclusive. Symbols correspond to restriction endonuclease sites: B = *Bam*HI, Bg = *Bgl*II, M = *Mlu*I, H = *Hin*dIII, P = *Pst*I. Two *Aat*II sites (A) within the *CEP1* coding region used to construct *CEP1–500* are also shown. (B) Suppression of the *mif2–3* temperature-sensitive phenotype by a high copy *CEP1* plasmid. Derivatives of the *mif2–3* strain PM1002–28A bearing each of the indicated plasmids were resuspended in sterile H_2O and spotted onto synthetic medium lacking uracil using a multi-prong inoculating device. Shown are the concentrated suspensions, as well as 10-fold (middle) and 100-fold (right) dilutions, thereof. Plates were incubated at 23°C, 35°C, and 37°C for approximately 2 days. Plasmid pPM4, a positive control, is a centromeric plasmid containing *MIF2*. The other plasmids are derivatives of the 2 μ-based vector pRS202 as described in Figure 2A.

as viable ascospore colonies at 23°C. Similar results were obtained with the *mif2–2* allele. A plasmid-borne copy of the wild-type *MIF2* gene facilitated the construction of *mif2–3 ndc10–42* double mutant strains; however, these strains were unable to subsequently lose the *MIF2* plasmid. Therefore, we presume the two putative *mif2–3 ndc10–42* ascospores were viable due to secondary mutations. In addition, *mif2–3 cep3–1* double mutant ascospore colonies were recovered at a lower than expected frequency (i.e., 14 of 22 predicted double mutants were inviable). At 23°C, those *mif2–3 cep3–1* double mutants that were recovered grew slowly compared with either parent. In contrast, recombinants bearing the *mif2–3* mutation and the temperature-sensitive *ctf13–30* allele (Doheny *et al.*, 1993) were readily recovered and showed no apparent growth defect. Obviously, because only one *CTF13* allele was available for testing, we cannot rule out a possible interaction with *CTF13*. Nonetheless, the ability to recover *mif2 ctf13* and certain *mif2 ndc10* double mutants argues that the observed genetic interactions are not simply the additive consequence of defects in chromosome transmission. Specificity is further supported by the viability of recombinants between *mif2–3* and null mutations in either of two other genes required for accurate chromosome transmission, namely, *CHL1/CTF1* (Gerring *et al.*, 1990) and *KAR3* (Meluh and Rose, 1990). Thus, *MIF2* genetically interacts with at least two components of the CDEIII-binding complex, *NDC10* and *CEP3*, as well as with *CEP1*. These observations support the idea that Mif2p acts at the centromere, and further, suggest that all these proteins exist as part of a larger complex. In support of the latter idea, we found a similar pattern of genetic interactions between the *cep1* null mutation and the various centromere protein genes. Thus, loss of *CEP1* function is synthetically lethal with either the *ndc10–42* or the *cep3–1* mutation, but not with *ndc10–2* or *ctf13–30*. It is tempting to speculate that the genetic interaction (or cross-talk) between Cep1p at CDEI and components of the complex present at CDEIII (Ndc10p and Cep3p) is mediated through Mif2p.

Mif2p Shows Limited Similarity to Highly Conserved Regions of CENP-C, a Mammalian Centromere Antigen

Comparison of the predicted Mif2 protein with current databases using the sensitive TBLASTN algorithm (Altschul *et al.*, 1990) revealed that in addition to the previously noted HMGI(Y) motif (Brown *et al.*, 1993), the C-terminal one-half of Mif2p also possesses two short regions of similarity to the mammalian centromere antigen, CENP-C (Figure 3A). CENP-C was first identified as a human centromere-associated antigen recognized by several autoimmune sera (Earnshaw and Rothfield, 1985) and has been shown to be a component of the inner kinetochore plate (Saitoh *et al.*,



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HsCENP-C  HHKLVLPSNTPNVRRTKRTRLKPLEYWRGERIDYQGRPSGGFVISGVL..  772
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MmCENP-C  HHKLVLPSNSPNVRRSNRIRLKPLEYWRGERVDYQESSSGQLVL.EII..  736
                :. .:|:|.|::: ||:|||.|:| |. :|. .::  : |
  ScMIF2  ...TDSIIDRNGLRKSTRVKVAPLQYWRNEKIVYKRKSNKPVLDIDKIVT  321
                                          *
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                    .         .         .         .         .
HsCENP-C  .SPDTISSKRKAKENIGKVNKKSNKKRIC.....................  800
           ||..:..| ||. |:|||||| .||..
MmCENP-C  .SPSSVPTKIKAQRNLGKVNKKVTKKPTH.....................  764
           ..|. ...| |.:.  | .||.|..|.:
  ScMIF2  YDESEDEEEILAAQRRKKQKKKPTPTRPYNYVPTGRPRGRPKKDPNAKEN  371


                    .         .         .         .         .
HsCENP-C  .......LDNDERKTNLMVNLGIPLGDPLQPTRVKDPETREIILMDLV..  841
                  |:..| |..: : |::.||||:|:| .||||| |:: |||:
MmCENP-C  .......LNSHE.KAKMELPLDMRLGDPFQATLAKDPETAELVPMDLI..  804
                  |  || .. ::: | :| ..: ...|| .:|:| .. ::|
  ScMIF2  LIPEDPNEDIIERIESGGIENGEWLKHGILEANVKISDTKEETKDEIIAF  421


                    .         .         .         .         .
HsCENP-C  .....RPQDTYQFFVKHGELKVYKTLDTPFFSTGKLILGPQEEKGKQHVG  886
               ||.|||.|||.: :|||:|||| :||||||:||| |||||||||
MmCENP-C  .....RPRDTYRFFVEQHGLKVFKTLDTIYFSTGKLVLGPYEEKGKQHVG  849
               ..::. :   |: :|.:: . ...||..| | |.:.:. |   :
  ScMIF2  APNLSQTEQVKDTKDENFALEIMFDKHKEYFASGILKLPAISGQKKLSNS  471

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                    .         .         .         .         .
HsCENP-C  .QDILVFYVNFGDLLCTLHETPYILSTGDSFYVPSGNYYNIKNLRNEESV  935
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MmCENP-C  .QDILVFYVNFGDLLCTLHETPYKLTTGDSFYVPSGNHYNIKNLLNVESS  898
           ...:.|.|  | : .|: ...:: ..|..| :|. |.|.| |  |:|..
  ScMIF2  FRTYITFHVIQGIVEVTVCKNKFLSVKGSTFQIPAFNEYAIANRGNDEAK  521
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HsCENP-C  LLFTQIKR*.....................  943
          ||||||||
MmCENP-C  LLFTQIKR*.....................  906
          ::|.|:.
  ScMIF2  MFFVQVTVSEDANDDNDKELDSTFDTFG*  549
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Figure 3. Mif2p show limited homology to highly conserved regions of the mammalian centromere protein CENP-C. (A) Schematic alignment of Mif2p with human and mouse CENP-C. The C-terminus of the predicted Mif2 protein contains two regions (Region I, stipled box; Region II, striped box) of homology with the mammalian centromere antigen, CENP-C. The position of the putative A-T hook motif is indicated by the gray box. Scale is as in Figure 3B. (B) Sequence identity between human and mouse CENP-C sequences. An alignment of the full-length human CENP-C (GenBank accession M95724; Saitoh *et al.*, 1992) and mouse CENP-C (GenBank accession U03113; submitted by S.J. McKay *et al.*, 1993) protein sequences were generated using the PileUp program of the GCG Sequence Analysis Software Package (Genetics Computer), using the default parameters. The level of identity between the two proteins was then plotted as a function of position using the PlotSimilarity program (comparison window size = 25). The dashed line indicates overall average identity (53%) between the sequences. Note that the two regions of highest identity between human and mouse CENP-C lie in the C-terminal 200 amino acids and coincide with the regions of homology between both CENP-C proteins and Mif2p. (C) Amino acid sequence alignment of Mif2p and mammalian CENP-C proteins. This alignment was also generated by PileUp, using the default parameters except that the GapWeight was 5.0. Subsequences used were: human CENP-C, amino acids 725–943; mouse CENP-C, amino acids 690–906; and yeast Mif2p, amino acids 275–549 (GenBank accession Z18294; Brown *et al.*, 1993). Vertical lines indicate identities; colons and periods indicate similarities according to the Dayhoff PAM-250 matrix. Gaps introduced to facilitate alignment are also indicated with periods. Identities and similarities between the predicted human and mouse CENP-C sequences or between either of these and Mif2p are shown. Accordingly, Mif2p is 22% identical (41% similar) to human CENP-C and 24% identical (41% similar) to mouse

1992). The sequences for both the human and the mouse CENP-C genes have been determined (Saitoh *et al.*, 1992; submission to GenBank by S.J. McKay *et al.*, 1993; Lanini and McKeon, unpublished observations). The predicted CENP-C protein sequences are very similar throughout, showing 53% identity overall; however, the C-termini are most highly conserved (70% identity over the last 220 residues; Figure 3B). Figure 3C shows a partial sequence alignment of Mif2p with the highly conserved C-terminal portions of the human and mouse CENP-C proteins. With this arrangement, Mif2p is 22% identical to human CENP-C and 24% identical to mouse CENP-C, (41% similar). Within the two regions detected by TBLASTN analysis (Regions I and II) the homology between Mif2p and CENP-C is greater, corresponding to ~40% identity over 27 amino acids in Region I and 28% identity over 52 amino acids in Region II. Importantly, the regions of Mif2p/CENP-C homology coincide with the two longest regions of sequence identity between the human and mouse CENP-C sequences (Figure 3B). Thus, human CENP-C (amino acids 725–759) is 86% identical to mouse CENP-C (amino acids 690–724) over 35 residues that include Region I and 88% identical over their last 88 residues, which encompass Region II (human CENP-C, amino acids 856–943 compared with mouse CENP-C, amino acids 819–906). It is also noteworthy that the C-terminal location and the approximate spacing between Regions I and II are also conserved. Moreover, these regions are apparently important to Mif2p function because molecular characterization of the temperature-sensitive alleles *mif2–2* and *mif2–3* revealed bp changes that correspond to substitutions in these regions of the encoded mutant proteins (Figure 3C). Although it is unlikely that CENP-C can functionally substitute for Mif2p, this remains to be tested. Nonetheless, the limited similarity between Mif2p and CENP-C is intriguing given the implication of both proteins in centromere function and raises the possibility that these regions specify protein surfaces that interact with centromere components conserved from yeast to humans.

Figure 3 cont. CENP-C. Regions of high sequence similarity originally identified using TBLASTN (Altschul *et al.*, 1990) are overscored. In region I (27 amino acids), Mif2p is 37% identical (70% similar) to human CENP-C and 40% identical (77% similar) to mouse CENP-C. In region II (52 amino acids), Mif2p is 28% identical (55% similar) to human CENP-C and 28% identical (51% similar) to mouse CENP-C. Two mutations were found for the *mif2–2* allele (indicated by * below the alignment); a single mutation was found for *mif2–3* (indicated by ◇). The putative A-T hook motif in Mif2p (residues 357–364; Brown *et al.*, 1993) is indicated by bold text.

DISCUSSION

MIF2 Encodes a Centromere Protein

In this paper we present several observations that implicate the product of the *MIF2* gene as a centromere protein. First, mutations in *MIF2* stabilize dicentric minichromosomes and dramatically reduce the activity of centromeres bearing *cis*-acting mutations in element I of the yeast centromere (CDEI). Second, *MIF2* genetically interacts with three genes that encode centromere proteins: *CEP1*, *NDC10*, and *CEP3*. In particular, temperature-sensitive mutations in *MIF2* show synthetical lethality with either a *cep1* null mutation or with a specific temperature-sensitive allele of *NDC10*, whereas viable *mif2–3 cep3–1* strains exhibit a slow growth phenotype. Also, increased dosage of *CEP1* can partially suppress the *mif2* temperature-sensitive phenotype. Finally, the predicted Mif2 protein has similarity to a mammalian centromere protein, CENP-C. The functional importance of this homology is underscored by the fact that bp changes associated with two temperature-sensitive mutations in *MIF2* correspond to amino acid substitutions within the two regions of highest similarity with CENP-C (see Figure 3). All these observations, coupled with the phenotypic similarities of *mif2*, *ctf13*, *ndc10*, and *cep3* mutants, suggest that Mif2p is a centromere component.

Possible insight into the role of *MIF2* in centromere function is provided by the homology between the C-terminus of the predicted Mif2p protein and the C-terminal 200 residues of the mammalian centromere protein CENP-C. Based on antibody microinjection experiments and the apparent localization of CENP-C to an interior region of the kinetochore (i.e., the inner plate), it has been proposed that CENP-C is a structural component of the mammalian kinetochore upon which other factors assemble (Tomkiel *et al.*, 1994). In this capacity, CENP-C should contain at least two distinct functional domains–one that mediates its own localization to the centromere and another that recruits other proteins to the centromere. As the C-terminal 250 residues of human CENP-C are not absolutely required for proper localization (Lanini and McKeon, unpublished observations), we propose that the regions of homology between Mif2p and CENP-C specify a conserved surface(s) that is used to tether other evolutionarily conserved centromere components such as mechanochemical proteins or tubulin. Outside of the C-terminal domain, the Mif2 and CENP-C proteins differ dramatically both in size and amino acid composition, and presumably, the precise mechanisms whereby Mif2p and CENP-C are directed to the centromere are different. Such a bifunctional model is similar to that in which otherwise unrelated transcription factors use a conserved motif (e.g. "acid blobs") to tether the conserved transcription machin-

ery to distinct promoters. Interestingly, truncated Mif2p or CENP-C proteins lacking the region of similarity have dominant negative effects as might be expected if they could localize to the centromere but were unable to bind additional essential factors (Meeks-Wagner *et al.*, 1986; Lanini and McKeon, unpublished observations). In principle, candidate proteins that interact with the Mif2p/CENP-C region of similarity might be encoded by the remaining high copy suppressors of *mif2–3*. Thus, it is possible that certain aspects of centromere function are broadly conserved and that, in yeast, Mif2p serves a structural role in centromere function analogous to that of CENP-C in mammalian cells. In this regard, the observed similarity between Mif2p and CENP-C provides one of the first examples of possible structural conservation between yeast and mammalian centromeres. The homology between Mif2p and CENP-C has also recently been noted (Brown, 1995).

The surprising results of these studies are that the activity of CDEI-defective centromeres is dramatically reduced in a *mif2* mutant background and that *mif2* mutants require the CDEI-binding protein Cep1p for viability. Normally, disruption of either CDEI or the *CEP1* gene has only modest effects on chromosome stability (Baker and Masison, 1990; Cai and Davis, 1990; Mellor *et al.*, 1990; see also Tables 2 and 3). In contrast, Mif2p performs an essential function in the cell. How can we explain the dependency of an essential activity on nonessential factors? A tenable hypothesis is that Cep1p bound to CDEI helps to position or stabilize Mif2p in the centromere-kinetochore complex, where in turn, Mif2p executes its essential function (e.g. by recruiting other conserved components). In the absence of Cep1p or when CDEI is mutated, the association of wild-type Mif2p with the centromere is weakened, but not abolished, leading to the observed mild increase in chromosome loss. If stable association of mutant forms of Mif2p with the centromere were to require Cep1p, mutant Mif2p protein would fail to associate with centromeres lacking bound Cep1p. Hence centromere activity would be diminished synergistically and chromosome loss would ensue. In the case of *mif2 cep1*Δ mutants, increased chromosome loss due to a pleiotropic diminution in centromere activity would lead to reduced viability or death, as is observed.

This model necessarily supposes that centromere association of Mif2p is mediated by at least two mechanisms, one of which is normally independent of Cep1p. A possible second interaction between Mif2p and components of the CBF3 complex is suggested by the observed genetic interactions between *MIF2*, *NDC10*, and *CEP3*. Alternatively, as was the original premise, Mif2p protein might bind to the A + T-rich DNA of CDEII by virtue of its potential A-T hook motif (Brown *et al.*, 1993). The absence of a strong effect of *mif2* mutations on the stability of two CDEII-defective minichromosomes might be taken as evidence against this idea. However, we note that the minimal sequence recognized by A + T-rich DNA binding proteins such as HMGI(Y) is often as little as 6 bp (reviewed in Churchill and Travers, 1991) and that the mutations tested, *CEN3-X78* and *CEN3-X35*, correspond to partial deletions within CDEII (Gaudet and Fitzgerald-Hayes, 1987). Thus, it remains possible that multiple binding sites for Mif2p (or another protein) exist within CDEII and that occupation of only one or a few of these sites is essential to centromere function. Moreover, *MIF2* alleles that specifically lack the region encoding the HMGI(Y) motif do not fully complement *mif2* temperature-sensitive mutations (our unpublished observation), suggesting that the A-T hook is important to Mif2p function.

The idea that Cep1p facilitates assembly of Mif2p at the centromere is essentially a refinement of the view that Cep1p mediates its diverse chromosomal functions by modulating chromatin structure, thereby restricting or "facilitating" the association of other DNA binding proteins (Baker and Masison, 1990; Mellor *et al.*, 1990; Thomas *et al.*, 1992; Masison *et al.*, 1993). The premise that an erstwhile transcription factor like Cep1p could also mediate the assembly of a protein-DNA complex unrelated to transcription is not without precedent. For example, several transcription factors have been shown to enhance the assembly of the DNA replication machinery at origins of replication in vitro and in vivo (reviewed in Heintz, 1992; DePamphilis, 1993). According to these paradigms, the role of Cep1p as an auxiliary factor in centromere assembly could entail a direct protein-protein interaction with Mif2p or an indirect effect on the accessibility or conformation of potential Mif2p binding sites in the centromeric DNA. Although there is evidence that Cep1p does indeed influence nucleosome positioning around CDEI motifs in some promoters (Kent *et al.*, 1994), its effect at the centromere is unclear. Obviously, definitive proof that Mif2p is a centromere protein will require appropriate biochemical tests, such as those that have been applied to components of CBF3 (Doheny, *et al.*, 1993; Sorger *et al.*, 1994; Strunnikov *et al.*, 1995), as well as experiments that establish whether Mif2p physically interacts with centromeric DNA and/or Cep1p.

A Complex View of the Yeast Centromere

To date, analysis of the yeast centromere has focused largely on its individual *cis*- and *trans*-acting components. Nonetheless, it is presumed that these components assemble into a higher order protein-DNA complex. It is likely that the limited set of binary genetic interactions described here reflect the existence of such a higher order complex in vivo. Genetic interac-

tions between components of the CBF3 complex also have been detected (Hyland and Hieter, personal communication). These important observations support the existence of the CBF3 complex in vivo, but do not address the existence of a higher order complex. Our data do provide some insight into aspects of centromere assembly. For example, the genetic interactions between Cep1p, which binds to CDEI, and components of the CBF3 complex (Ndc10p and Cep3p), which bind CDEIII, might be indicative of direct protein-protein interactions. However, juxtaposition of CDEI and CDEIII in space would require looping out, and possibly bending, of the intervening DNA because these elements are separated by at most 85 bp and the minimum length for linear DNA looping is 130 bp. (Shore *et al.*, 1981; reviewed in Schlief, 1992). Several factors could potentially contribute to bending of the centromeric DNA. First, the centromeric DNA itself apparently contains an intrinsic bend in vitro (Murphy *et al.*, 1991). Also, as noted earlier, Cep1p induces DNA bending at CDEI elements (Niedenthal *et al.*, 1993). Finally, it remains possible that Mif2p, like mammalian HMGI(Y) protein, interacts with A + T-rich DNA through its A-T hook motif (see above). Because the binding of HMGI(Y) to short A + T-rich elements in the human IFN β gene enhancer is known to induce DNA bending (Thanos and Maniatis, 1992) and facilitate a cooperative interaction between two transcription factors (Du *et al.*, 1993), it is conceivable that association of Mif2p with CDEII could cause centromeric DNA bending and facilitate an interaction between Cep1p and the CBF3 complex.

A model for the yeast centromere-kinetochore complex that invokes multiple cooperative interactions, and possibly DNA looping, not only provides for high specificity, but is intriguing because a similar structure has been proposed for a so-called prokaryotic centromere, namely, the P1 bacteriophage DNA segregation locus (Hayes and Austin, 1994). Our model is further supported by several reported observations. For example, it previously has been suggested that the protein-DNA complexes at CDEI and CDEIII interact in vivo based on the observation that doubly mutant centromeres containing point mutations in CDEI and in CDEIII are less active than centromeres with either single mutation (Niedenthal *et al.*, 1991). Also, although no systematic face-of-helix phasing studies have been conducted, it is clear that the relative position and orientation of individual centromeric DNA elements are important to centromere function (Gaudet and Fitzgerald-Hayes, 1987; Murphy *et al.*, 1991), a feature reminiscent of stereospecific enhancers (Tjian and Maniatis, 1994). In particular, the relative orientation of CDEIII with respect to CDEII is critical both for centromere function (Murphy *et al.*, 1991) in vivo and, under certain conditions, for the binding of partially reassembled yeast kinetochores to microtubules in vitro (Sorger *et al.*, 1994). These data have been taken as evidence that proteins bound to CDEII (possibly Mif2p) and CDEIII might interact directly.

Thus, by analogy to eukaryotic enhancers, such as that of the IFN-β gene (Du *et al.*, 1993; Tjian and Maniatis, 1994), or origins of replication (DePamphilis, 1993), the yeast centromere might best be viewed as a highly specific three-dimensional nucleoprotein complex that assembles onto a multipartite regulatory sequence within the context of chromatin. As with these better characterized elements, some proteins within the centromere-kinetochore complex presumably serve as architectural or "tethering" components (e.g. Cep1p and Mif2p) that in turn place or position an enzymatic machinery that, in this case, interacts with microtubules (e.g. the CBF3 complex). Thus, prudent application of paradigms for the assembly of eukaryotic transcriptional enhancers and origins of replication should enlighten future biochemical analyses of the yeast centromere. In this regard, we anticipate that further study of *MIF2* and elaboration of the genetic interactions put forth here will elucidate how yeast centromeres are assembled.

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