CEP3 Encodes a Centromere Protein of Saccharomyces cerevisiae

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Abstract. We have designed a screen to identify mutants specifically affecting kinetochore function in the yeast *Saccharomyces cerevisiae.* The selection procedure was based on the generation of "synthetic acentric" minichromosomes. "Synthetic acentric" minichromosomes contain a centromere locus, but lack centromere activity due to combination of mutations in centromere DNA and in a chromosomal gene (*CEP*) encoding a putative centromere protein. Ten conditional lethal *cep* mutants were isolated, seven were found to be alleles of *NDC10* (*CEP2*) encoding the 110-kD protein of yeast kinetochore. Three mutants defined a novel essential gene *CEP3.* The *CEP3* product (Cep3p) is a 71-kD protein with a potential DNA-binding domain (binuclear Zn-cluster). At nonpermissive temperature the *cep3* cells arrest with an undivided nucleus and a short mitotic spindle. At permissive temperature the *cep3* cells are unable to support segregation of minichromosomes with mutations in the central part of element III of yeast centromere DNA. These minichromosomes, when isolated from *cep3* cultures, fail to bind bovine microtubules in vitro. The sum of genetic, cytological and biochemical data lead us to suggest that the Cep3 protein is a DNA-binding component of yeast centromere. Molecular mass and sequence comparison confirm that Cep3p is the p64 component of centromere DNA binding complex Cbf3 (Lechner, 1994).

CHROMOSOME segregation in mitosis requires the presence of a specialized chromosomal structure that facilitates binding of chromosomes to the microtubules (mt)[1] of the mitotic spindle. Functionally, this structure could be defined as a linker between DNA and microtubules, and is known as the centromere, or kinetochore (used interchangeably here). Structural organization of centromeres varies dramatically among evolutionary remote species and can be extremely complex (Brinkley et al., 1992; Grady et al., 1992). This complexity makes the structure-function analysis of a centromere a very difficult challenge. Fortunately, the centromere of the budding yeast, *Saccharomyces cerevisiae,* (Clarke and Carbon, 1980) provides an excellent model for studying centromere organization and activity. The centromere DNA (CenDNA) of *S. cerevisiae* is less than 150 bp in length (Bloom and Carbon, 1982), drastically smaller than CenDNA of other well studied eukaryotes (Pluta et al., 1990). The CenDNA in yeast thus provides a space for binding of only limited number of protein molecules. Also, only a single microtubule is attached to each kinetochore in the cells of budding yeast (Peterson and

Ris, 1976). These facts outline the unique status of *S. cerevisiae* centromere in the list of model segregation loci.

Upon the identification of the centromere locus of yeast chromosomes (Clarke and Carbon, 1980), the efforts of several groups were focused on the identification of proteins associated with centromere DNA in vivo, in conjunction with the dissection of structural properties of CenDNA itself. It has been suggested that yeast CenDNA can be subdivided into three distinct structural elements (recently reviewed in Hegemann and Fleig, 1993). The first, CDEI is associated with the CP1/Cbf1p/Cpf1p protein (Bram and Kornberg, 1987; Baker et al., 1989; Cai and Davis, 1990; Mellor et al., 1990). Both CDEI and CP1 are dispensable for centromere function under the standard laboratory conditions. The second element, CDEII, is an A- and T-rich region protected from nuclease digestion and/or modification in vivo (Bloom et al., 1984; Densmore et al., 1991). The CDEII element is required for proper centromere function, however there is no data, so far, indicating that it directly binds to a specific polypeptide. Finally, the CDEIII element is absolutely essential for centromere assembly and activity, both in vivo and in vitro (Hegemann et al., 1988; Lechner and Carbon, 1991; McGrew et al., 1986; Panzeri et al., 1985). The multisubunit CDEIII-binding complex Cbf3 has been purified and partially characterized (Lechner and Carbon, 1991). The Cbf3 complex has three major components: p58, p64, and p110. As these subunits have not been purified individually, the primary biochemical activity of each of them is un-

1. *Abbreviations used in this paper:* CenDNA, centromere DNA; ha, hemagglutinin; mt, microtubules; ORF, open reading frame; ts, temperature-sensitive.

known. The genes encoding p58 (*CTF13*) (Doheny et al., 1993) and p110 (*NDC10/CBF2/CTF14*) (Doheny et al., 1993; Goh and Kilmartin, 1993; Jiang et al., 1993a) have recently been characterized. The p58 and p110 proteins (henceforth, referred to as Ctf13p and Ndc10p, respectively) are indispensable for viability of yeast cell. The cells of the temperature-sensitive (ts) *ndc10-1* mutant do not exhibit cell cycle arrest at nonpermissive temperature, instead they undergo an abortive mitosis, leaving most of chromosomal DNA unsegregated but associated with an asymmetric spindle (Goh and Kilmartin, 1993). In contrast, the only known ts allele of *ctf13* (Doheny et al., 1993) causes cells to arrest at nonpermissive temperature in G2-M with an undivided nucleus and a short mitotic spindle.

Clearly, the identification of additional protein components of yeast kinetochore is necessary to understand its molecular structure and function. We expect such kinetochore proteins to have several characteristic features exemplified by CP1, Ctf13p and Ndc10p. First, they should be physically associated either directly or indirectly with CenDNA and/or the spindle microtubules. Second, cells deprived of a kinetochore protein should show some cytological defects consistent with the inability to segregate chromosomes properly. Third, the *trans*-mutations in the genes encoding centromere components should show genetic interaction with *cis*-CenDNA mutations (*cen*). These features provide at least three criteria to recognize a particular protein as a kinetochore component. Some other proteins satisfy at least one of these criteria and so, are the potential candidates for kinetochore components: p64 (Lechner and Carbon, 1991), Mck1 (Shero and Hieter, 1991), Cse1 and Cse2 (Xiao et al., 1993), Cbf5 (Jiang et al., 1993b), Top2 (Jiang et al., 1993b), Kar3 (Middleton and Carbon, 1994), and Mif2 (Brown et al., 1993). However, the DNA binding proteins essential for assembly of functional kinetochore remain unidentified. Particularly, proteins which make direct contacts with CDEIII are important, as CDEIII is absolutely required for centromere function. Based on our belief that the yeast centromere is a complex multisubunit structure, with many structural components yet to be found, we have designed a genetic screen aimed directly at identifying proteins involved in the formation of yeast centromeres.

Materials and Methods

Strains and Genetic Techniques

S. cerevisiae strains (S288C background) are listed in Table I. Strain YPH102 was used for mutagenesis, YPH499 was used for backcrosses of centromere proteins mutants (*cep*). Strain S42 (Doheny et al., 1993) was used as a tester for *ndc10* allelism. Media, incubation conditions, and strain manipulations were according to published protocols (Sherman et al., 1986). *Escherichia coli* strains DH5α (BRL), TOP10 (Invitrogen, San Diego, CA) and SCS110 (Stratagene, La Jolla, CA) were used for plasmid propagation. Standard methods of yeast genetics were done as described (Guthrie and Fink, 1991; Sherman et al., 1986). Mitotic stability of plasmids, defined as the fraction of cells in a culture that retain the plasmid when the cells are grown under selective conditions, was estimated as described previously (Strunnikov et al., 1993). As this parameter correlates well with the efficiency of plasmid transmission per cell division (Hieter et al., 1985a; Koshland et al., 1985), it was used as a routine estimate for the efficiency of minichromosome transmission. The disruption allele (*cep3-Δ1*) of *cep3* was constructed by transforming the AS260 diploid strain with pAS450 (see below) digested with EagI and XbaI, giving AS270. Disruption of one copy of *CEP3* gene with *HIS3* marker was confirmed by Southern

blot hybridization. After sporulation, segregation of two viable (His3⁻) to two inviable (His⁺) spores was observed in every tetrad. The His3⁺ spores were recovered only after transformation of AS270 with pAS452 prior to tetrad analysis. Haploid His3⁺ strains (*cep3-Δ1*) always carried the pAS452 (*CEP3*) plasmid.

Nomenclature

Standard nomenclature (Jones et al., 1992) for yeast genes and proteins was used. As most of yeast centromere factors and corresponding genes are known under redundant names (CP, Cep, Cpf, Cbf, Ctf, Ndc) we chose the existing "CEP" acronym (Baker and Masison, 1990) for the genes isolated in our screen. Centromere mutations where designated using the locus name, CDE number, and particular mutation name, e.g., *cen6-(III)15t*. The CDEIII mutations used are shown in Fig. 1 B.

The Screening Procedure for Isolation of cep Mutants

A schematic view of the selection procedure is given in Fig. 1. The first stage of screening included monitoring of plasmids with different reporter CenDNA to identify those suitable for large scale mutagenesis. Strain YPH102 was transformed with corresponding plasmid containing *leu2-d* gene and a CenDNA of interest. The cultures were mutagenized with EMS (50–90% viability). Cells where plated to form single colonies in the absence of selection for leucine prototrophs. The plates were then replica-printed onto media lacking leucine and incubated for 48 h. at 23°C. The frequency of Leu⁺ colonies was determined for each reporter CenDNA tested, and the corresponding colonies were picked from master plates. The *trans*-mutations were distinguished from other mutant classes via retransformation with the same minichromosome. The plasmid pAS97 (see below), which exhibited the highest rate of induced *trans*-mutations and, at the same time, the lowest rate of spontaneous Leu⁺, was chosen for large-scale mutagenesis. Only mutants reproducibly showing Leu⁺ phenotype and temperature sensitivity where chosen for further analysis. All mutant alleles were back crossed at least twice, to demonstrate that the generated mutations are single mutations of nuclear origin, and that temperature sensitivity cosegregates in meiosis with the accumulation of pAS97.

Plasmid Construction

Yeast-*E. coli* shuttle vectors, used for cloning purposes were pRS vectors (Christianson et al., 1992; Sikorski and Hieter, 1989). pT7blue (Novagen, Inc., Madison, WI) was used as a cloning vector for PCR-generated fragments.

Plasmid pAS93, the backbone of most of the minichromosomes used in mutagenesis experiments, was constructed from FAT-RS303 (*leu2-d, HIS3, 2μm-ORI, Ampʳ*) (contribution of D. Gottschling, University of Chicago, Chicago, IL). The *HIS3* marker was removed by digestion with BamHI and religation; the *URA3* gene (SmaI-HindIII fragment) and *ARSH4* (HindIII-XhoI fragment) were inserted into PvuII-XhoI sites to make pAS93. pAS93 has unique BamHI, SalI, and SmaI sites, used for cloning of CenDNA fragments, giving the following minichromosomes: pAS94 (*cen3-(II)X35* BamHI-BamHI fragment); pAS95 (*GAL1:CEN3* BamHI-BamHI fragment); pAS96 (*cen6-(I)8c* SalI-BamHI fragment); pAS97 (*cen6-(III)15t* SalI-BamHI fragment); pAS98 (*cen3-(III)BCT1* SalI-SalI fragment); pAS112 (*cen3-(III)BCT2* SalI-SalI fragment); pAS113 (*cen6-(III)19t20* SalI-BamHI fragment); pAS114 (*cen6-(III)14a* SalI-BamHI fragment); pAS122 (*CEN6* SalI-BamHI fragment). pAS76 has the *CEN4* XhoI-ScaI fragment inserted into SalI-SmaI sites of YEpFAT7 (Runge and Zakian, 1989). The corresponding pDK minichromosomes (without *leu2-d* and *2μm-ORI*) have been described before (Kingsbury and Koshland, 1991): pDK381 contains *CEN6* SalI-BamHI fragment, pDK377 contains *cen6-(III)15t* SalI-BamHI fragment, pDK371 contains *cen3-(III)BCT1* SalI-SalI fragment, pDK378 contains *cen6-(III)-19t20* SalI-BamHI fragment, pDK374 contains *cen3-(II) X78* BamHI-BamHI fragment and pDK380 contains *cen6-(I)8c* SalI-BamHI fragment. Descriptions of all CenDNA fragments used were published previously (Hegemann et al., 1988; Hill and Bloom, 1987; McGrew et al., 1986).

Several plasmids, containing the *CEP3* gene from the original isolate pAS300, have been constructed. pAS409 (pRS414 backbone) contains the entire genomic insert of pAS300 as 9-kb EagI-BamHI fragment. pAS420 is pRS414 with 3.2-kb BssHII-EcoRI fragment of pAS300. HincII-HincII fragment of pAS420 (Fig. 2 A) was introduced into pR406, pRS425, and pRS416 cut with PvuII producing pAS451, pAS452 and pAS461, respectively. To construct a deletion of *CEP3*, two PCR fragments corresponding

Table I.

Strain	Genotype	Source
YPH102	*MATα ade2 his3 leu2 lys2 ura3*	Ph. Hieter
YPH499	*MATa ade2 his3 leu2 lys2 trp1 ura3*	Ph. Hieter
S42	*MATα ctf14(ndc10)-42 ade2 leu2 lys2 ura3 his3*	Ph. Hieter
AS260	*MATa/MATα ade2 his3 leu2 lys2 trp1/TRP1 ura3*	This study
AS270	*MATa/MATα cep3-Δ1/CEP3 ade2 his3 leu2 lys2 trp1/TRP1 ura3*	This study
1dAS255	*MATa cep3-1 ade2 his3 leu2 lys2 trp1 ura3*	This study
6bAS255	*MATα cep3-1 ade2 his3 leu2 lys2 trp1 ura3*	This study
1cAS281	*MATa cep3-1 ade2 his3 leu2 lys2 trp1 ura3*	This study
2bAS282	*MATa cep3-2 ade2 his3 leu2 lys2 trp1 ura3*	This study
3dAS282	*MATa cep3-2 ade2 his3 leu2 lys2 trp1 ura3*	This study
2aAS257	*MATα cep3-3 ade2 his3 leu2 lys2 ura3*	This study

to upstream noncoding and downstream noncoding regions of *CEP3* ORF were cloned into pT7blue, making pAS446/1. In this plasmid the *CEP3* open reading frame (ORF) is substituted by single BamHI recognition site, in which the *HIS3* gene (BamHI-BamHI fragment) was inserted, to give pAS450.

To construct the epitope-tagged versions of *CEP3* the pAS451 plasmid was cut with either SpeI or HindIII (Fig. 2 *A*). In-frame insertion of six tandem tag sequences encoding the c-*myc* epitope (Strunnikov et al., 1993) into SpeI site produced pAS454 (*cep3::myc* allele). In-frame insertion of a triple tag sequence (contribution of M. Rose, Princeton University, Princeton, NJ; HindIII-ends introduced by PCR) encoding a hemagglutinin epitope (ha) into HindIII site of pAS451 produced pAS464 (*CEP3::ha* allele). Correct boundaries between *CEP3* and the tag-encoding fragments were confirmed by DNA sequencing.

Cloning, Mapping, and Sequence Analysis of the CEP3 Gene

The genomic DNA library used to isolate the *CEP3* gene was the *LEU2/CEN* library (Spencer et al., 1988) in pSB32 vector. Two ts strains, 14-YPH102 (*cep3-1*), and 10-YPH102 (*cep3-3*) were transformed with library plasmid DNA. Cells were spread on plates without leucine and incubated at 23°C for 24 h. The plates were then shifted to 37°C, and temperature-resistant colonies were picked. Total DNA was extracted and used to transform *E. coli* cells. Two plasmids were found to enable 14-YPH102 and 10-YPH102 to grow at 37°C. These plasmids also complemented the ts-lethal phenotype of back-crossed *cep3* strains. Restriction mapping showed, that the clones contain largely overlapping inserts of genomic DNA. The plasmid chosen for subcloning was designated pAS300.

Mapping of the cloned fragment to a chromosome was accomplished using the EagI-BamHI fragment of pAS300 as a hybridization probe for filters containing yeast chromosomes separated by PFGE. A single hybridization signal, corresponding to chromosome XIII, was detected. Preliminary sequencing starting from the vector and also from unique internal restriction sites was used for more accurate mapping of the clone. The cloned DNA fragment contained *SMP2* (Irie et al., 1993) and *MLH1* (Prolla et al., 1994), two known genes on chromosome XIII. *CEP3* gene is an immediate neighbor of *MLH1* and the order of open reading frames on the fragment is as follows: *CEP3>,<MLH1, . . ., SMP2>*.

To determine the nucleotide sequence of *CEP3*, nested deletions of the pAS420 plasmid were constructed (Henikoff, 1984). The resulting plasmids were sequenced using the fluorescent ddNTP PCR sequencing reaction and automated DNA sequencer (model 370A; Applied Biosystems, Foster City, CA), as recommended by the manufacturer. Both strands of *CEP3* were sequenced. Conflicting regions were resequenced using the custom synthesized 18-24-mer primers.

Antibodies and Cytological Methods

Indirect immunofluorescence was performed as described (Kilmartin and Adams, 1984). Yeast nuclear DNA was stained by DAPI included into mounting media (Mowiol; Cal Biochem, La Jolla, CA). Microtubules were detected with the mouse monoclonal antibody YOL1/34 (1:200) (Kilmartin et al., 1982) and goat anti-mouse antibodies conjugated to rhodamine (Cappel Laboratories, Cochranville, PA). Mouse monoclonal anti-c-*myc* antibody 9E10 (Evan et al., 1985) and mouse monoclonal anti-hemagglutinin

antibody 12CA5 (BAbCO, Richmond CA) were used to monitor tagged Cep3p on Western blots. The pooled monoclonal antibodies against a 90-KD component of the spindle pole body (Rout and Kilmartin, 1990), were used undiluted for indirect immunofluorescent staining.
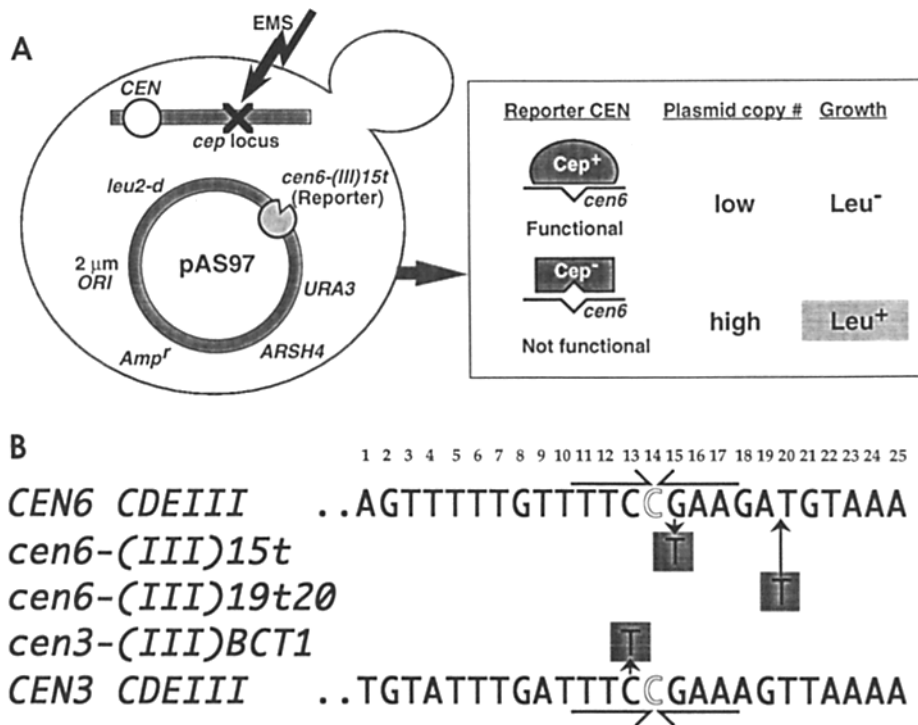
Minichromosome–Microtubule Binding Assays

The microtubule-minichromosome binding assays were performed essentially as described (Kingsbury and Koshland, 1991) with minor modifications (Kingsbury and Koshland, 1993). Binding experiments were done for two or four parallel cultures, always including YPH102/pDK381 strain as a control. For each minichromosome/strain combination (Fig. 6) the independent binding experiments have been performed at least twice.

Results

Isolation of cep Mutants

The first extrachromosomal replicon identified in yeast was the endogenous 2 μm plasmid (Beggs, 1978). It is a circular multicopy (20–30) double-stranded DNA molecule which uses its own specialized system to partition effectively during mitotic divisions (Broach and Volkert, 1991). In contrast, artificial minichromosomes with a centromere locus, segregate using the mitotic apparatus designed for chromosomes and are maintained as single copy per cell. The screen for genes encoding putative centromere proteins was based on the observation that the presence of both 2μm plasmid replication/segregation locus (*2μM-ORI*) and a yeast centromere on the same circular plasmid (2μm/CEN plasmid) creates an epistatic relationship between these two loci (Tschumper and Carbon, 1983). Such a plasmid behaves as a single-copy minichromosome and not as multicopy 2μm plasmid (Tschumper and Carbon, 1983). Several reports have been published however that compromising centromere function in such a minichromosome can convert it into a multicopy equivalent of the 2μm plasmid, at least in some cells in the population (Apostol and Greer, 1988; Chlebowicz-Sledziewska and Sledziewski, 1985; Schulman and Bloom, 1993). These results provided the primary basis for design of our screen (Fig. 1 *A*).

The second rationale for the *cep* screen was the use of a dose-dependent marker to follow changes in the copy number of the 2μm/CEN plasmid. Such markers have been used previously for other applications and genetic screens (Hieter et al., 1985*b*; Larionov et al., 1989; Runge et al., 1991; Smith et al., 1990; Toh-E, 1981). In our screen for *cep* mutants we used the *leu2-d* gene as that marker. The *leu2-d*

A

B

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                          1 2 3 4  5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25
CEN6 CDEIII      ..AGTTTTTGTTTTCCGAAGATGTAAA
cen6-(III)15t
cen6-(III)19t20
cen3-(III)BCT1
CEN3 CDEIII      ..TGTATTTGATTTCCGAAAGTTAAAA
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Figure 1. (*A*) Design of the screen for *cep* mutants. The schematic map of the minichromosome used to select *cep* mutants is shown with the *cen6-(III)15t* reporter. The "*cep* locus" designation stands for the putative target of mutagenesis in yeast chromosome. After the *cep* mutation is acquired by a cell, the defective product (*Cep⁻*) of the mutated gene interferes with already compromised reporter *cen6* DNA, allowing accumulation of minichromosomes and the appearance of Leu⁺ cells. (*B*) CDEIII mutations used to assess the function of *cep* genes. Nucleotides are numbered starting from base 1 of CDEIII. The CDEIII palindrome is marked with the half-arrows and its central base is outlined. The mutant centromere DNA fragments were resequenced to confirm their structure.

fragment has a truncation of the *LEU2* promoter, making the expression of the single *leu2-d* gene insufficient to complement the chromosomal *leu2* mutation. Approximately 100 copies of the *leu2-d* gene per Leu²⁻ cell are required to confer a Leu⁺ phenotype. That copy number can be easily achieved in yeast cells by the amplification of 2µm based *leu2-d* plasmid, when the cells are selected for Leu⁺ phenotype (Toh-E, 1981). This amplification is possible due to flexibility in 2µm plasmid copy number control, allowing transition from normal 20–30 copies per cell to 100–150 copies (under *leu2-d* selection) without any deleterious effect on cell physiology. Due to the epistatic effect mentioned above, the 2µm/CEN/*leu2-d* minichromosome can not undergo amplification from one copy per cell to 100 copies per cell without abolishing centromere function completely (Schulman and Bloom, 1993). This loss of function of the plasmid-born *CEN* locus (reporter centromere) as a result of unlinked (chromosomal) mutation in a *cep* locus (Fig. 1 *A*) is the essence of the screen described below.

Mutagenesis of YPH102 cells that contained the 2µm/CEN minichromosome with a wild-type CenDNA (*CEN4* or *CEN6*) failed to produce *trans*-mutations that could inactivate the reporter centromere. The most likely explanation for this failure is that these *cep* mutations not only inactivated the reporter centromere on the plasmid but also inactivated the chromosomal centromeres. Such *cep* mutants would not be recovered due to loss of viability. To overcome this problem, we decided to compromise the reporter centromere by using the CenDNA mutations that retain only partial centromere activity in vivo. We rationalized that this modification would allow the isolation of putative *cep* mutants displaying synergistic loss of the reporter centromere activity ("synthetic acentric" phenotype) due to the compound effect of *cis* and *trans* mutations. However, these *cep* mutants would be viable because the chromosomal centromeres with their

wild-type CenDNA would retain enough centromere activity to allow proper chromosome segregation.

To identify CenDNA mutations with the appropriate level of partial centromere activity for our screen, we constructed the strains containing the 2µm/CEN minichromosomes with different CenDNA mutations. We then tested these strains for the generation of Leu⁺ clones before and after mutagenesis. Mutagenesis of strains harboring 2µm/CEN plasmids with *cen6-(I)8c* (dysfunctional CDEI) did not give significant numbers of Leu⁺ clones after the mutagenesis. The activity of this reporter centromere was apparently too similar to wild-type and hence, as discussed above, failed to give *cep* mutants. Strains that had 2µm/CEN plasmids with *GAL1::CEN3* (tanscriptionally inactivated centromere), *cen3-(III)-BCT1*, *cen3-(III)BCT2*, *cen3-(II)X35*, and *cen6-(III)14a* had a very high rate of spontaneous Leu⁺ clones. These reporter centromeres apparently were compromised so much that they already allowed the reporter plasmid to achieve high copy number in the absence of *cep* mutants. However strains that contained 2µm/CEN plasmids with either *cen6-(III)15t* or *cen6-(III)19t20* had undetectable levels of spontaneous Leu⁺ clones but numerous Leu⁺ clones after the mutagenesis. These induced Leu⁺ clones were good candidates for *cep* mutants, indicating that either of these CenDNA mutations would work as a reporter for our screen.

The minichromosome pAS97 (Fig. 1 *A*), having *cen6-(III)15t* as a reporter CEN, was primarily used for the screen described in this report (See Materials and Methods). When the YPH102/pAS97 strain was mutagenized, the estimated frequency of induced Leu⁺ clones carrying *cep* mutations was 9 × 10⁻⁴. The unwanted Leu⁺ clones, including the *cis*-mutants and clones with rearranged minichromosomes, originated with a similar frequency. More than 30 *cep* mutants were isolated. 10 of them were conditional lethal mutants recessive for temperature-sensitive growth. These 10

A

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BssHII
1  CGCGCAGCCATGTAAGCTAAGCGTAAGAGAATGAAATATTATAACCAACCTTCAGAGAGCAGGTGATGACAAAGTTAC  80

81 CAACACGAGTTTGCATCCTAATAATA ATG TTT AAC CGT ACC ACT CAA CTG AAA TCC AAG CAT CCC  145
1                            M   F   N   R   T   T   Q   L   K   S   K   H   P    13

146 TCT TCC CTG TGT ACG AGG CGA AAA GTC AAA TCT GAT CGT ATG ATA CCG TGT GCA AAC TGC  205
14  C   S   V   C   T   R   R   K   V   K   C   D   R   M   I   P   C   G   N   C     33

206 AGG AAG AGA GGA CAG GAC TCC GAA TGT ATG AAA TCA ACA AAA CTA ATA ACG GCT TCA TCT  265
34  R   K   R   G   Q   D   S   E   C   M   K   S   T   K   L   I   T   A   S   S     53
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(DNA sequence and translation continue; full sequence available from GenBank/EMBL/DDBJ under accession number U12339.)

B

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Cep3p   LKSKHP-SV-TRRKV-DRMI--GN-RKRGQDSE-MKSTKL
Mal2p   TLAKQA-DC-RVRRV-DRNK--NR-IQRNLN---TYLQPL
Pdr3p   SKVSTA-VN-RKRKI-TGKY--TN-ISYDCT---VFLKKH
Gal4p   SSIEQA-DI-RLKKL-SKEK-K-AK-LKNNWE--RYSPKT
Hap1p   NRIPLS-TI-RKRKV-DKLR-H-QQ-TKTGVAHL-HYMEQT
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Figure 2. Sequence analysis of the *CEP3* gene. (*A*) The primary DNA sequence of BssHII-HincII fragment of plasmid pAS420 and the putative polypeptide product are shown. Cysteine residues of the putative DNA-binding domain are italicized. Recognition sites for SpeI and HindIII endonucleases, used to construct tagged versions of Cep3p, are shown. Protein residues 367–402, able to form coil-coil structure, are underlined. These sequence data are available from GenBank/EMBL/DDBJ under accession number U12339. (*B*) Alignment of the metal-binding cluster of Cep3p to known fungal proteins: Mal2p (Kopetzki et al., 1989), Pdr3p (Genbank/EMBL/DDBJ accession number X79803), Gal4p (Marmorstein et al., 1992), Hap1p (Zhang and Guarente, 1994). The identical residues and conserved cysteins are highlighted.

mutations fell into two complementation groups designated *cep2* (seven alleles: *cep2-11* to *cep2-17*) and *cep3* (three alleles: *cep3-1* to *cep3-3*). All seven *cep2* alleles failed to complement *ndc10-42* for temperature sensitivity, indicating that *cep2* is allelic to the *ndc10* gene. Assuming, that the *NDC10* gene encodes a centromere protein, isolation of multiple *ndc10* alleles in our *cep*-screen indicates our approach was selective for centromere-specific genes.

CEP3 Is a Novel Essential Gene

Both *CEP2* and *CEP3* genes were cloned by complementation of the corresponding ts-mutations. Plasmids carrying inserts with the putative wild type genes conferred temperature resistant growth to the mutant strains. We isolated two independent DNA fragments which complemented the *cep3-1* and *cep3-3* mutations. Standard genetic procedures were performed to show that these plasmids indeed contained the *CEP3* gene. First, we showed that the integration of *URA3* marker into the chromosomal locus corresponding to the cloned fragment always segregated away from the *cep3* mutation in the meiotic progeny of diploid cells, heterozygous for both insertion and *cep3*. This result shows that the gene we cloned corresponds to the chromosomal locus of *cep3* ts mutation, and is not an ectopic suppressor gene. Second, using a stepwise subcloning procedure, we localized the complementing activity to a single ORF (see below). Minor truncations of the putative *CEP3* ORF from its 5' and 3' ends abolished complementing activity, indicating that we had identified the authentic *CEP3* ORF. The cloned gene also complemented the "synthetic acentric" phenotype of all three *cep3* mutants.

The DNA sequence of 2,074-bp fragment containing the *CEP3* gene was determined. The sequence of *CEP3* fragment revealed a 608-codon ORF, potentially encoding a novel protein with molecular mass of 71 kD (Fig. 2 *A*). The amino terminus of predicted polypeptide contained a binuclear zinc-cluster motif $Zn(II)_2Cys_6$ (Pan and Coleman, 1990) (Fig. 2 *B*) common to several DNA-binding proteins (Dhawale and Lane, 1993). The rest of Cep3p did not show any significant similarity to known protein sequences. According to secondary structure prediction (Rost and Sander, 1994) 56% of the polypeptide aminoacid residues are in alpha-helix, and 37% form loops. Only one short region of the protein was predicted (Lupas et al., 1991) to form coiled-coil structure with probability higher than 0.3 (Fig. 2 *A*).

The chromosomal copy of the *CEP3* gene was disrupted by substituting the *CEP3* ORF with the *HIS3* marker. We failed to recover the resulting allele, *cep3-Δ1*, as a haploid strain, thus showing that *CEP3* is an essential gene. The essential nature of *CEP3* and the presence of a DNA-binding motif suggest that Cep3p is a new DNA-binding component of yeast centromere.

To assess the expression level of *CEP3*, we introduced two alternative in-frame epitope tags (See Materials and Methods) into the COOH-terminal part of Cep3p (Fig. 2 *A*). The *cep3::myc* construct did not complement *cep3-1*, *cep3-2*, or *cep3-Δ1* defects. This result indicaties that the essential region of Cep3p is not limited to the putative Zn-binding cluster. However the alternative *CEP3::ha* construct did complement the *cep3-1* and *cep3-Δ1* mutations. The Cep3p is a rare protein as the Cep3p-ha protein can only be detected on Western blots when concentrated by immunoprecipitation (data not shown).

A

cep3-1   □ 25°C   cep3-2
           ■ 37°C (6 hr.)

B
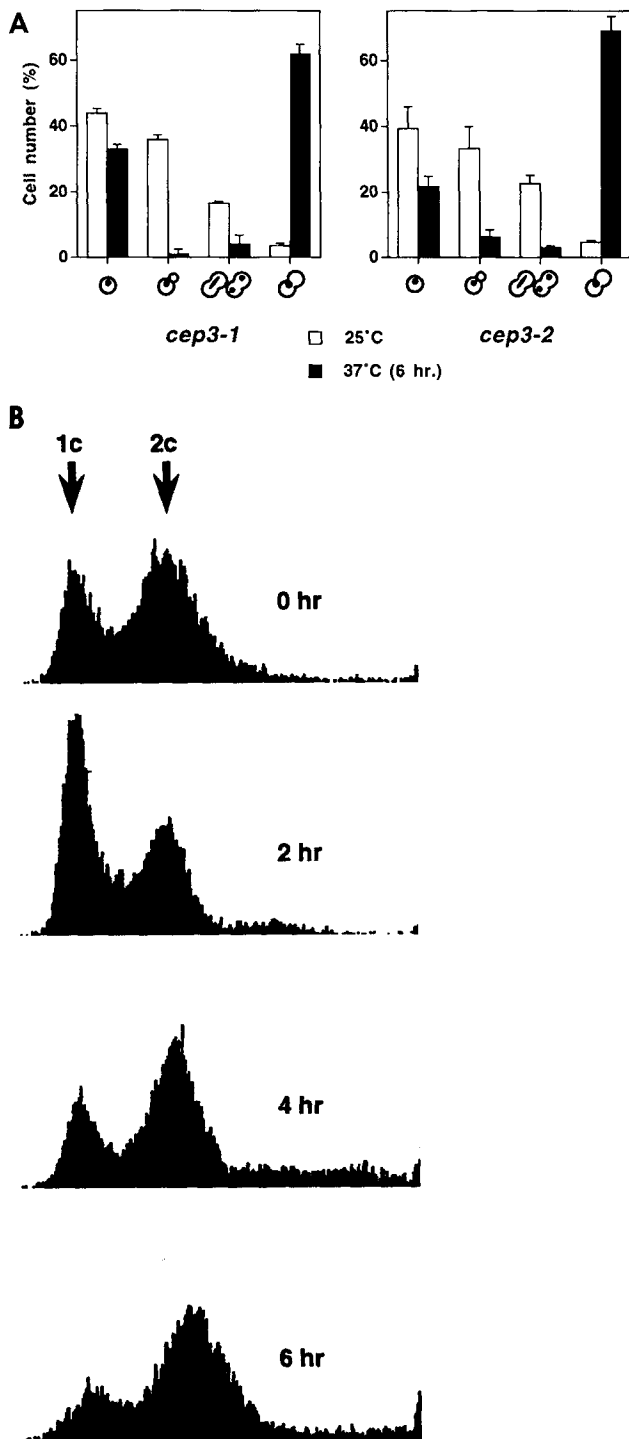
Figure 3. Characterization of *cep3* cultures arrested at the nonpermissive temperature. (*A*) The cell types of *cep3-1* (6bAS255) and *cep3-2* (2bAS282, 3dAS282) cultures were grouped into four classes according to the morphology of DAPI-stained cells. The first three classes are also present in wild type cells. The fourth class (large-budded cells with undivided nucleus) is generated in *cep3* culture upon its exposure to high temperature. Distribution of cell types in *cep3* cultures at permissive temperature is not different from a population of *CEP3* cells, except for the presence of large budded cells as a minor fraction. (*B*) Flow cytometry analysis (FACS) of a *cep3-1* culture after 0, 2, 4, and 6 h after shift to 37°C. Cells of a haploid *cep3-1* strain (6bAS255) were fixed with ethanol at the time points shown, and stained with propidium iodide after

Cytological Analysis of cep3 Mutants

Detailed cytological analysis of the *cep3* mutants was limited to *cep3-1* and *cep3-2*, as *cep3-3* cells showed practically identical characteristics in all preliminary tests. When the wild-type and *cep3* cells were shifted to 37°C (the nonpermissive temperature for *cep3*), their viability remained the same for 4 h. However, by 8 h the viability of *cep3* cells dropped below 20%. Time-course observations showed, that after the 6-h shift to nonpermissive temperature the *cep3* cells reach full arrest of cell division (Fig. 3 *A*). In the course of incubation at 37°C, the late anaphase cells and small-budded cells, present at 25°C (Fig. 4 *A*), disappear. The large-budded (dumbbell) cell with an undivided nucleus at the bud neck and a short spindle (Fig. 4 *B*) become the dominating class in the *cep3* population (Fig. 3 *A*). Most of the *cep3* cells arrested at 37°C have diploid DNA content (Fig 3 *B*). This complex of features is highly suggestive of G2-M phase arrest. The virtual absence of elongated mitotic spindles, was verified by the spindle pole body staining (Fig. 5 *C*). Therefore the phenotype of *cep3* is more similar to *ctf13-30* cells (Doheny et al., 1993) than to *ndc10-1* mutant (Goh and Kilmartin, 1993).

The staining of spindle microtubules in the *cep3* cells lacking their mitochondrial DNA also revealed that the dominant class of cells in the arrested *cep3* population is not homogeneous. We found some indications of the abnormal distribution of nuclear DNA, including the separation of chromosomal DNA from the mitotic spindle, nuclear DNA cut by the cytokinesis, and nuclear DNA unevenly spread between the mother and daughter cell (Fig. 5, *A* and *B*). The latter phenotype is probably due to the spindle movements known as the phenomenon of nuclear transits (Palmer et al., 1989), accompanied by the failure of the majority of chromosomes to attach to the mitotic spindle. The presence of cells with their spindles and nuclear DNA positioned in the different cell bodies (Fig. 5, *A* and *B*) suggests that in the arrested *cep3* cells chromosomes fail to maintain attachment to the microtubules of mitotic spindle.

Interestingly, at the restrictive temperature some of the examined *cep2* (*ndc10*) mutants arrest like the *cep3* cells (data not shown). This finding brings up the possibility that the *cep3*, *ctf13* (Doheny et al., 1993) and *ndc10* (Goh and Kilmartin, 1993) cells may display the same kind of mitotic arrest as a consequence of centromere inactivation.

Interaction between cep3 Mutations and Centromere Mutations In Vivo

Given the synthetic destabilization of the reporter centromere, *cen6-(III)15t*, in the *cep3* mutants and the presence of a DNA binding motif in the Cep3p, it is reasonable to postulate that Cep3p is a CDEIII-specific DNA-binding protein. To investigate this possibility we determined the specificity of interaction between the *cep3* mutations and different CenDNA mutations. *cep3* mutants were transformed with pDK minichromosomes (*URA3*, *ARS1*, CEN minichromo-

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the RNAse treatment. The accumulation of G2-M cells with double (2c) content of DNA is evident. The "1c" peak corresponds to G1 cells. FACS profiles for the *cep3-2* strains look identical (not shown) to the profile of the *cep3-1* strain.
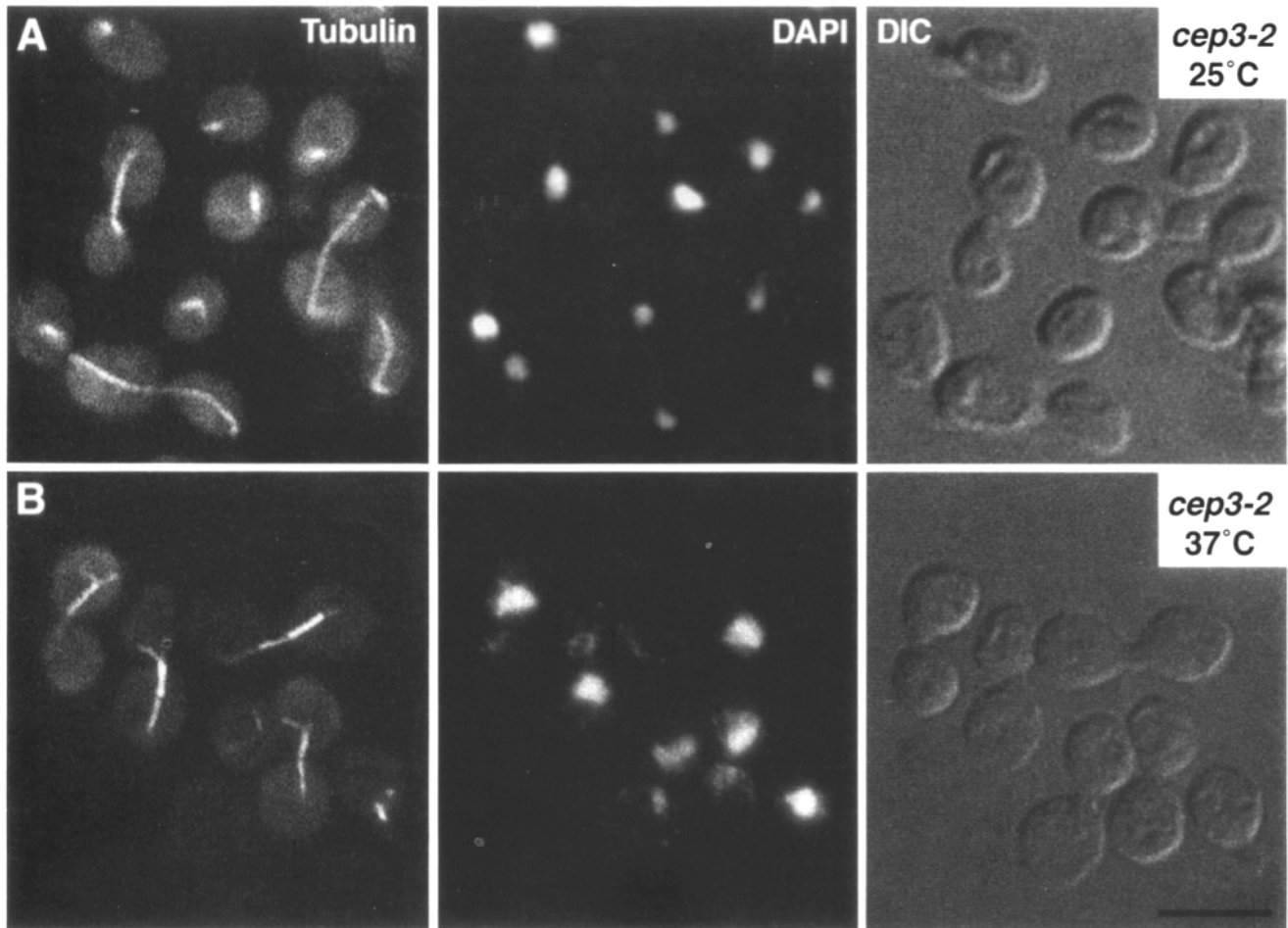
Figure 4. Micrograph of the *cep3* cells under permissive and restrictive temperatures. (*A*) The logarithmic population of *cep3-2* cells (3dAS282) at 25°C. DNA is stained with DAPI and the microtubules are stained with anti-tubulin antibodies. Differential interference contrast (DIC) images show general cell shape. The late anaphase cells with elongated mitotic spindles, as well as small-budded cells are present in the population. (*B*) The *cep3-2* cells (2bAS282 strain) after 6 h at 37°C. The dominating class of large budded cells with an undivided nucleus and a short spindle is evident. Bar, 10 μm.

somes without 2μm DNA), that differed only in their CenDNA sequences (Table II). We then tested the mitotic stability of these plasmids as a means to assay the function of their centromeres (Table II). It was evident that the *cep3* mutations inactivated centromeres in *trans* when the minichromosome CenDNA contained two CDEIII mutations (*cen6-(III)15t* and *cen3-(III)BCT1*). However, the *cep3* mutations did not inactivate significantly centromeres with another CDEIII mutation (*cen6-(III)19t20*), a CDEI mutation (*cen3-(I)8c*) or a CDEII mutation (*cen3-(II)X78*). Therefore, the *cep3* mutations appear to interact synergistically only with those centromeres that have two very specific mutations symmetrically flanking the central base in the CDEIII palindrome (Fig. 1 *B*). This specific interaction supports the hypothesis that the *CEP3* product is a CDEIII binding protein.

As *cep2* (*ndc10*) mutants were identified in the same screen as *cep3* mutants, it was of interest to analyze the mitotic stability of the minichromosomes with mutant CenDNA in *cep2* mutants as well. Similar inactivation of centromeres with specific CenDNA mutations was observed in *cep2* mutants as was observed in *cep3* mutants (Table II).

Therefore, the Ndc10p may also directly or indirectly participate in CDEIII binding (see Discussion). On the other hand, the *smc1-1* mutation, which also affects minichromosome transmission (Strunnikov et al., 1993), did not exhibit the synthetic acentric phenotype with any of centromeres harboring these CenDNA mutations (data not shown). Therefore, the synthetic acentic phenotype is not a general property of any mutation that affects minichromosome transmission. The similar pattern of interaction with centromere DNA mutations at the permissive temperature displayed by the *cep2* and *cep3* mutants suggests that the corresponding proteins act in close proximity in vivo.

The cep3 Mutation Interferes with Centromere Function In Vitro

The kinetochore of *S. cerevisiae* acts as a tripartite system (CenDNA-kinetochore proteins-microtubules) which acquires motility at the time of mitosis. Based on this concept, three types of biochemical assays have been applied previously to testing the yeast centromere function in vitro: protein–DNA interaction (Doheny et al., 1993; Ng and Carbon,
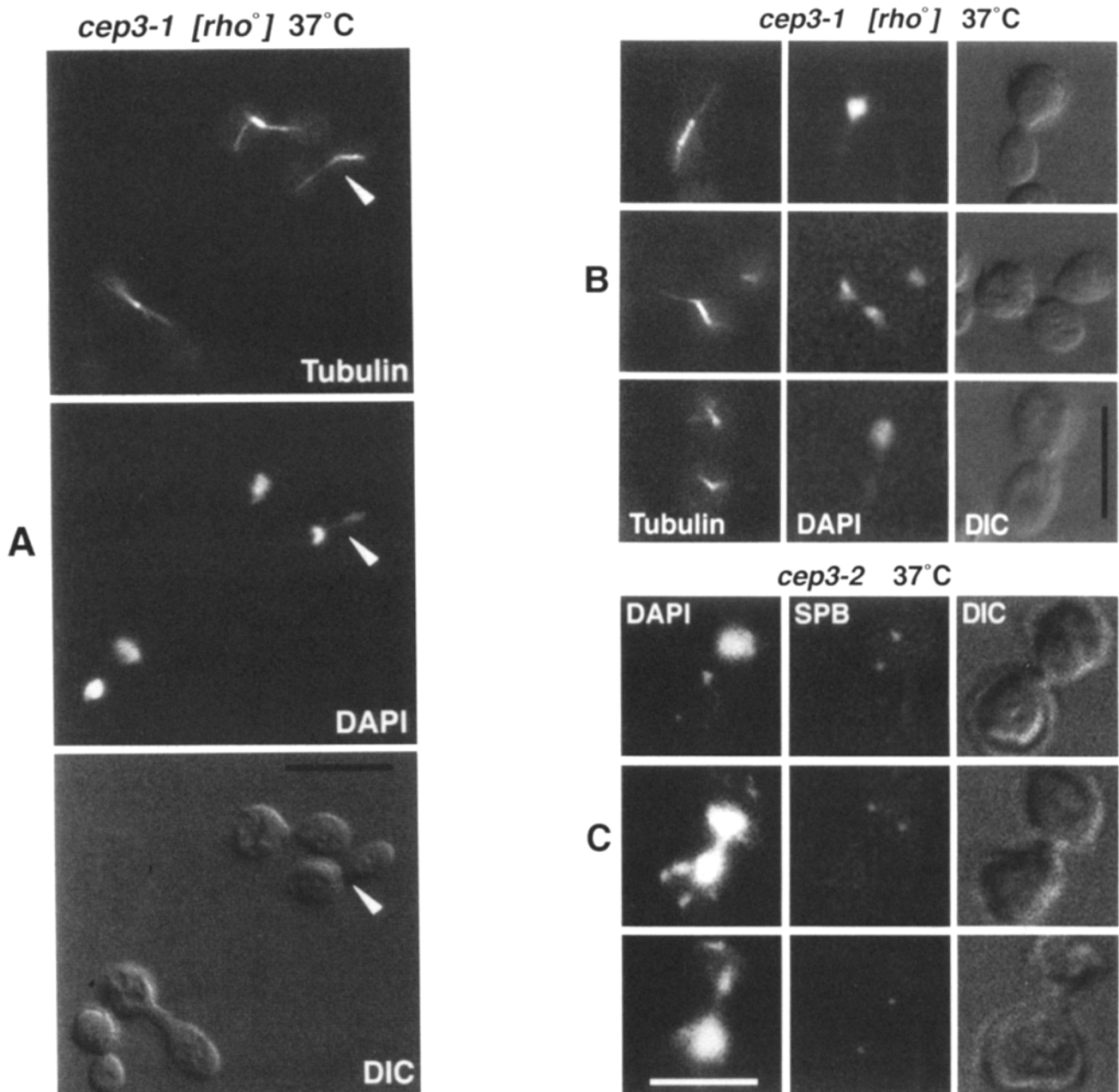
Figure 5. Arrested population of *cep3-1* cells lacking mitochondria. (*A*) The *cep3-1* (lcAS281) culture lacking mitochondrial DNA (*rho°*) after 6 h at 37°C. The cells where stained with DAPI and anti–tubulin antibodies. The cell marked with an arrow has underwent partial separation of chromosomal DNA; the bulk of chromosomal DNA and the spindle are separated from each other and positioned in different cell bodies, the phenotype never observed in wild type cells. (*B*) The *rho° cep3-1* cells (lcAS281) representing the abnormal subtypes of the large budded cells with an undivided nucleus. From top to the bottom: separation of chromosomes from the mitotic spindle; cut-like phenotype; formation of a potential aploid cell. Bar, 10 μm. (*C*) Spindle pole body staining of the *cep3-2* cells. 2bAS282 cells were stained with antibodies against the 90-kD SPB component after 6 h. at 37°C. The position of SPBs shows that corresponding mitotic spindles remain bipolar (no spindle collapse) but do not elongate, even in the cells with chromosomal DNA spread out between the mother and daughter cells. Bars, 10 μm.

1987), centromere-microtubule interaction (Kingsbury and Koshland, 1991; Kingsbury and Koshland, 1993), and motility assays (Hyman et al., 1992; Middleton and Carbon, 1994). To reveal the molecular basis of the "synthetic acentric" phenotype, we carried out experiments testing the fidelity of centromere binding to bovine microtubules in a cell-free system. This minichromosome–microtubule binding

assay has been described previously (Kingsbury and Koshland, 1991, 1993). It involves quantitative precipitation of minichromosomes isolated from yeast cells (in the form of chromatin) by their association with taxol-stabilized bovine microtubules. All experimental data presented in this report were obtained for Cep3+ or Cep3− cells arrested with nocodazole prior to the preparation of the extracts contain-

Table II.

Strain	Mitotic stability of minichromosomes with reporter centromere:					
	CEN6	cen6-(III)15t	cen3-(III)BCT1	cen6-(III)19t20	cen3-(II)X78	cen6-(I)8c
Cep$^+$	88.9 ± 4.3	85.4 ± 6.2	77.0 ± 5.0	84.8 ± 3.8	86.3 ± 4.5	87.9 ± 0.9
cep3-1	86.3 ± 4.2	6.0 ± 3.8	3.6 ± 3.5	78.7 ± 7.0	80.3 ± 1.8	80.0 ± 4.0
cep3-2	87.4 ± 0.8	9.3 ± 2.1	11.8 ± 5.1	81.3 ± 2.7	83.3 ± 4.5	65.4 ± 13.2
cep2-12	81.8 ± 3.9	10.3 ± 2.6	15.8 ± 2.2	63.9 ± 17.8	83.7 ± 4.3	65.8 ± 5.0

ing minichromosomes. Arrest with nocodazole (G2-M transition), as was previously shown (Kingsbury and Koshland, 1991), confers the highest mt-binding potential for wild type cultures. This provides a wide range for the comparison of experimental data obtained for mutant and wild type extracts and CenDNA. The G2-M transition is also the phase when the function of a centromere is actually required for progression of the normal cell division cycle through mitosis, i.e., when the kinetochore is in its most active state. In fact, comparison of mt-binding activity of *cis*-mutant centromeres from the asynchronous culture (Kingsbury and Koshland, 1991) to activity from nocodazole arrested cells (Fig. 6) suggests that centromeres assembled with some *cis*-mutant CenDNA (e.g., *cen6-(III)15t*) achieve almost full wild-type activity in the cells arrested in G2-M.

Four types of cultures (grown at 23°C) were used for microtubule-binding experiments: Cep3$^+$ cells with wild type *CEN 6* minichromosome, Cep3$^+$ cells with mutant CenDNA (*cen6-(III)15t*) minichromosome, Cep3$^-$ cells with wild type *CEN6* minichromosome and Cep3$^-$ cells with mutant CenDNA (*cen6-(III)15t*) minichromosome (Fig. 6). These combinations are analogous to ones used for assaying minichromosome stability in vivo (Table II). For both *cep3/CEN6* or *CEP3/cen6* combinations, only a slight difference in the capacity of minichromosome–microtubule binding was observed at 23°C (Fig. 6). However, combining the *cep3* (*trans*) and *cen* (*cis*) mutation in the same cell produced a striking effect: the microtubule–minichromosome binding became indistinguishable from the background level (i.e., of acentric plasmid precipitation; Kingsbury and Koshland, 1991). This result indicates that there is an intimate link between the structure of CDEIII and the Cep3p function. These cell-free system experiments are in agreement with data obtained in vivo for these minichromosomes. Results of these experiments provide a basis for a hypothesis addressing the mechanism of the "synthetic acentric" phenotype: the synthetic acentrics are probably generated in vivo, due to the inability of *cen6-(III)15t* minichromosomes to bind yeast microtubules.

The structural properties of Cep3p, as well as the cytological, genetic, and biochemical data obtained for *cep3* mutants, provide a basis for assuming that Cep3 protein is the CDEIII-binding subunit of yeast centromere, indispensable for both kinetochore assembly and function. To summarize, several properties of Cep3p suggest that it is a centromere protein: (a) mutant forms of the protein show allele specific interactions with particular CenDNA mutations; (b) minichromosomes isolated from *cep3* cells exhibit a defect in the centromere-dependent binding to microtubules in vitro; (c) the Cep3 protein is essential for cell viability, and the *cep3* mutants exhibit complex mitotic defects consistent with a

failure of centromere function; and (d) the amino acid sequence of the Cep3 protein is identical to the sequence of p64 found in the Cbf3 complex (Lechner, 1994). The fact, that both Cep3p and Cep2p, are the subunits of Cbf3 complex, shows that our screen was very selective for genes encoding the components of yeast centromere.

Discussion

Developing a Selective Primary Screen for Kinetochore Components

Centromere proteins have been previously identified in cells of vertebrates as centromere (or kinetochore) antigens (Compton et al., 1991; Earnshaw et al., 1987a, b; Yen et al., 1992). The yeast centromere proteins have been identified by their biochemical properties (CenDNA association) (Jiang et al., 1993a; Lechner and Carbon, 1991) or by secondary screening of existing collections of temperature-sensitive mutants (Goh and Kilmartin, 1993) or mutants with frequent chromosome loss (Doheny et al., 1993). However, the identification of additional centromere components by these approaches has proved limited, mainly because they are labor intensive. In this report we described a new approach for identifying centromere proteins by selecting mutants displaying the "synthetic acentric" phenotype. Using this approach we have isolated multiple mutations in two genes en-
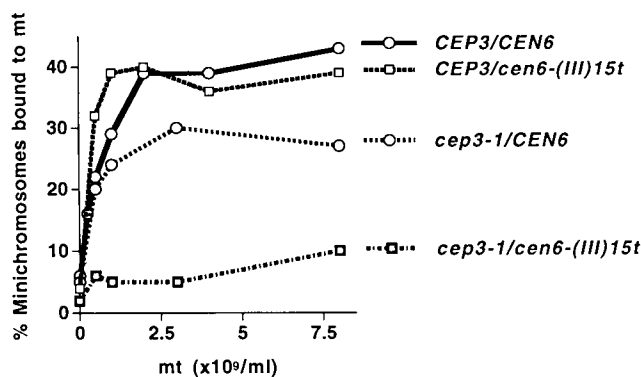
Figure 6. Minichromosome-microtubule binding experiments. Four types of extracts used for microtubule binding experiments: *CEP3/CEN6*, strain YPH102 transformed with pDK381; *CEP3/cen6-(III)15t*, YPH102 with pDK377 minichromosome; *cep3-1/CEN6*, 1dAS255 with pDK381 minichromosome; *cep3-1/cen6-(III)15t*, 1dAS255 with pDK377 minichromosome. Binding at mt concentrations, higher than required for saturation of binding in wild type strain (Kingsbury and Koshland, 1991), was done to exclude the possible rescue of binding activity in *cep3* mutant extracts by the excess of mt.

coding probable centromere components; one of these genes, *NDC10* (*CEP2*), had been identified previously (Goh and Kilmartin, 1993; Jiang et al., 1993a) while the other, *CEP3*, is a novel gene.

The improved efficiency of our approach relies on three major principles. First, by requiring the reporter minichromosome to achieve a very high copy number, we selected only those mutants that completely inactivate the reporter centromere, thus eliminating the possible background of mutants, able to accumulate only few extra copies of minichromosome (Larionov et al., 1989). Second, we used a reporter centromere that was already partially impaired by a *cis*-mutation. In this way mutant centromere proteins, that inactivated the reporter centromere, would still be active on chromosomal centromeres, allowing the mutant cells to survive. Third, since we assumed that many kinetochore proteins would be essential for viability, we imposed the requirement that the *cep* mutants be conditionally lethal.

The "synthetic acentric" approach has at least two obvious virtues for the future studies on centromere proteins. First, it provides a very rapid and efficient means to isolate mutant alleles of genes encoding the p110 and p64 components of the kinetochore. These mutations may serve as powerful tools for dissecting the molecular organization and the cellular role of these proteins. For example, some new alleles of *ndc10* (*cep2*) isolated by our synthetic acentric approach (see below) are phenotypically distinct from known *ndc10* alleles. Second, although this approach may be saturated for the *cen6-(III)15t* reporter, as evidenced by the isolation of multiple alleles of *ndc10* and *cep3*, other reporter CenDNA sequences with CDEII or CDEIII mutations may be used to identify genes encoding additional centromere proteins. For example, the *cen6-(III)19t20* mutation is not synthetically acentric when combined with the *ndc10* or *cep3* alleles, yet potential *cep* mutants were obtained with this centromere as a reporter (Strunnikov, A. V., unpublished results).

The CEP3 Gene Encodes a Putative CenDNA-binding Protein That Is Necessary for Microtubule-binding Activity of the Centromere

DNA sequence analysis revealed that *CEP3* encodes a protein with a predicted NH_2-terminal DNA-binding domain $Zn(II)_2Cys_6$ (Pan and Coleman, 1990). In other fungal proteins this domain mediates DNA binding in a sequence-specific manner and has been found only in transcription factors-like proteins, dispensable under standard laboratory conditions. The most characterized member of this family, Gal4p, binds a palindromic DNA sequence as a dimer (Marmorstein et al., 1992). Interestingly, CenDNA mutations that interact with *cep3* alleles lie in symmetrical positions of the CDEIII palindrome, suggesting that Cep3p could also bind DNA as a dimer. Previously, a limited functional symmetry of CDEIII was described (Jehn et al., 1991) based on data obtained for *cis* mutations.

Of three major proteins in the Cbf3 complex, Cep3p is the only subunit with an evident DNA-binding motif. Therefore, it is reasonable to assume that Cep3p mediates binding of the Cbf3 complex to CDEIII. Surprisingly, all tested *cep2* (*ndc10*) mutations have similar pattern of interaction with CDEIII mutations as do *cep3* alleles (this study). One possible explanation for this phenomenon is that Ndc10p also binds to the same nucleotides of CDEIII as does Cep3p. However, this seems unlikely, as Ndc10p and Cep3p do not share significant sequence similarity and Ndc10p lacks any known DNA-binding motif. More likely, Ndc10p is a cofactor of Cep3p binding to CDEIII. Alternatively, Ndc10p could be an unconventional DNA-binding protein affecting Cep3p due to the close proximity of Cep3p and Ndc10p binding sites. These models are yet to be verified by biochemical studies, however, our genetic data provide the first evidence that Cep3p and Ndc10p physically interact in vivo.

What would be a hypothetical way to construct a functional centromere, taking into account that Cep3p has the only known DNA-binding motif among the Cbf3 components? One possibility is, that Cep3p Zn-cluster is solely responsible for tethering all other centromere components to the CenDNA. This seems unlikely because the binding of kinetochore proteins to CenDNA should have special requirements to withstand the pulling forces that are exerted during mitosis. The interaction between CDEIII and the relatively simple DNA-binding motif of Cep3p, likely, would not be sufficient for this purpose. Also, since CDEII, CDEIII and flanking sequences form a large special chromatin structure (Bloom et al., 1984), it seems likely that other DNA-binding components exist. Based on the fact, that CDEIII and the proteins bound to it are the key elements for the assembly of this entire complex (mutations in CDEIII can both abolish centromere function [Hegemann et al., 1988; McGrew et al., 1986] and prevent formation of centromere chromatin [Saunders et al., 1988]), we can suggest that the DNA-binding motif of Cep3p first recognizes CDEIII during replication of centromere chromatin ("centromere priming" role) and then nucleates assembly of other centromere proteins. These proteins (including Cep3p) could then be locked on the CenDNA using a special mechanism, yet to be identified. For example, the relative richness of Cep3p in cysteine residues (beyond the Zn cluster) provides a possibility for disulfide bonds which could covalently lock Cep3p as well as other subunits wrapped around CenDNA. Partial DNA-wrapping has been documented for the Gal4 protein previously (Marmorstein et al., 1992).

In addition to the putative DNA-binding properties of Cep3p in vivo, we examined the role of Cep3p in organizing microtubule-binding activity of the kinetochore. We showed that the minichromosomes isolated from *cep3* cells exhibit a dramatic defect in centromere-dependent binding of minichromosomes to microtubules in vitro. This observation has three important implications. First, it validates the use of isolated minichromosome as the means to identify and characterize structural components of kinetochore, required for microtubule binding. Second, *cep3-1* is the first described mutation in a Cbf3 component that disrupts the microtubule binding activity of centromere, corroborating the importance of Cbf3 complex for centromere structure and function (Lechner and Carbon, 1991; Hyman et al., 1992). Third, the microtubule-binding data suggest a molecular model for the "synthetic acentric" phenomenon. While it is theoretically possible that Cep3p directly binds microtubules, it is more likely that the presence of Cep3p is needed to assemble a multi-protein complex which has mt-binding subunits, in ac-

cordance with our model published previously (Kingsbury and Koshland, 1993). This hypothetical "centromere priming" role for the Cep3 protein, if verified by additional biochemical experiments, could help to identify other centromere proteins physically associated with Cep3p.

Cellular Response to the Loss of Centromere Function

The cells of multicellular organisms developed a sensitive mechanism which delays anaphase if even one chromosome fails to achieve its metaphase position (Nicklas and Arana, 1992). It has been suggested that this delay reflects a "checkpoint" that monitors chromosome congression and kinetochore–microtubule attachment. As some aspects of cell cycle regulation in *S. cerevisiae* may be quite different from multicellular organisms (Nasmyth, 1993), it is possible that loss of kinetochore function would not generate a cell cycle delay. In fact no delay was observed in *ndc10-1* cells, the first mutants demonstrated to be defective in an essential yeast centromere protein (Goh and Kilmartin, 1993). At restrictive temperature these cells undergo asymmetric elongation of the mitotic spindle leaving most of the chromosomes unsegregated. However, experiments with CenDNA mutations (Spencer and Hieter, 1992) and analysis of *ctf13-30* mutant suggested that yeast cells could respond to the loss of centromere function in more classical fashion. The inactivation of Ctf13p results in a metaphase-like delay with no elongation of the mitotic spindle. Since *CTF13* encodes an essential Cbf3 component, the *ctf13-30* arrest could reflect an authentic "checkpoint" in response to the loss of centromere integrity (Doheny et al., 1993). Our data obtained for three independent alleles of *cep3* support the latter idea. The arrest in *cep3* mutant cells is phenomenologically close to the published description of *ctf13*-arrested cells. Moreover we found an indication that the arrest in *cep3* cells is probably due to the separation of chromosomes from mitotic spindle. If the arrest observed in *cep3* and *ctf13* cells is a checkpoint activated by the inability of chromosomes to establish stable contacts with kinetochore microtubules, the *ndc10-1* mutant could still retain partial kinetochore activity, resulting in a catastrophic anaphase. This interpretation makes the control of chromosome segregation in *S. cerevisiae* more relevant to the metaphase–anaphase control in the cells of *Metazoa*.

In conclusion, we described an effective and non-laborious screen for *cep* genes. This screen allowed isolation of the *CEP3* gene, a new essential gene encoding a putative centromere component in yeast, as well as new alleles of the *CEP2* (*NDC10*) gene, encoding a known centromere protein. These results bring us closer to understanding the centromere structure, however, more proteins need to be identified. Our system based on the generation of synthetic acentric mutants should be very useful in the identification of these unknown centromere proteins.

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