Identification of Essential Components of the S. cerevisiae Kinetochore

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Summary

We have designed and utilized two in vivo assays of kinetochore integrity in S. cerevisiae. One assay detects relaxation of a transcription block formed at centromeres; the other detects an increase in the mitotic stability of a dicentric test chromosome. ctf13-30 and ctf14-42 were identified as putative kinetochore mutants by both assays. CTF14 is identical to NDC10/CBF2, a recently identified essential gene that encodes a 110 kd kinetochore component. CTF13 is an essential gene that encodes a predicted 478 amino acid protein with no homology to known proteins. ctf13 mutants missegregate chromosomes at permissive temperature and transiently arrest at nonpermissive temperature as large-budded cells with a G2 DNA content and a short spindle. Antibodies recognizing epitope-tagged CTF13 protein decrease the electrophoretic mobility of a CEN DNA–protein complex formed in vitro. Together, the genetic and biochemical data indicate that CTF13 is an essential kinetochore protein.

Introduction

The term chromosome cycle describes a fundamental aspect of the cell division cycle in which each of the chromosomal DNA molecules first is replicated and then undergoes a series of morphological changes and complex movements to ensure its faithful distribution at mitosis. The gene products responsible for execution of the chromosome cycle include structural components, such as those that assemble into the kinetochore, and regulatory components, such as those that establish checkpoints monitoring the proper completion of ordered events within the cell cycle.

Saccharomyces cerevisiae offers two major advantages as an experimental organism in which to study the chromosome cycle. First, it is possible to combine classical

genetics (isolation and phenotypic analysis of mutants) with recombinant genetics (manipulation of cloned DNA segments by recombinant DNA methods and subsequent reintroduction into the yeast host). Second, all of the cis-acting DNA sequence elements required for chromosome maintenance are cloned and well characterized, including functional centromere DNA, chromosomal origins of DNA replication, and telomere DNA (reviewed by Newlon, 1988).

One structure clearly essential to chromosome distribution is the kinetochore (centromere DNA and associated proteins), providing the site of attachment of spindle microtubules. The kinetochore is a relatively simple structure in S. cerevisiae in comparison with the large and complex trilaminar structures seen in higher eukaryotes (Rieder, 1982; Pluta et al., 1990). In electron microscopic studies of S. cerevisiae chromosomes, one microtubule is seen to interact with each chromatin molecule, but a structurally differentiated kinetochore is not visible (Peterson and Ris, 1976). The kinetochore of S. cerevisiae is composed of an approximately 160–220 bp nuclease-resistant core that is centered around the centromere (CEN DNA) sequence and flanked by an ordered array of nucleosomes (Bloom et al., 1984; Funk et al., 1989).

The CEN DNA sequence requirements for S. cerevisiae have been rigorously and extensively characterized through mutational analysis (reviewed by Carbon and Clarke, 1990). Approximately 125 bp is sufficient for centromere function (Cottarel et al., 1989). Comparison of centromeres from different chromosomes reveals that they consist of three centromere DNA sequence elements (CDEI, CDEII, and CDEIII) (Fitzgerald-Hayes et al., 1982; Hieter et al., 1985). CDEI (8 bp) and CDEIII (25 bp) exhibit dyad symmetry and are separated by CDEII, a 78–86 bp sequence of over 90% AT content. Deletions of CDEI and CDEII reveal that they are important but not essential for chromosome segregation, while single-nucleotide point mutations in CDEIII can completely destroy centromere function.

Although a great deal is known about CEN DNA sequence determinants in S. cerevisiae, little is known about the proteins required for kinetochore activity or its regulation within the cell cycle. Biochemical purification of kinetochore proteins through sequence-specific affinity purification with CEN DNA (Ng and Carbon, 1987; Lechner and Carbon, 1991) has proven to be difficult, apparently owing to the low abundance of the kinetochore proteins and the requirement of accessory factors for binding in vitro. To date, only one CEN DNA–binding protein, CPF1 (also known as CP1 or CBF1), has been extensively characterized (Baker and Masison, 1990; Mellor et al., 1990; Cai and Davis, 1990). CPF1 is a member of the helix-loop-helix family of DNA-binding proteins and binds as a homodimer to CDEI. A null mutation in cpf1 results in only a 10-fold decrease in chromosome segregation, indicating that it is important but not essential for kinetochore function. Lechner and Carbon (1991) have described the isola-

A

% β-galactosidase activity

100

1 ±1

22 ±7

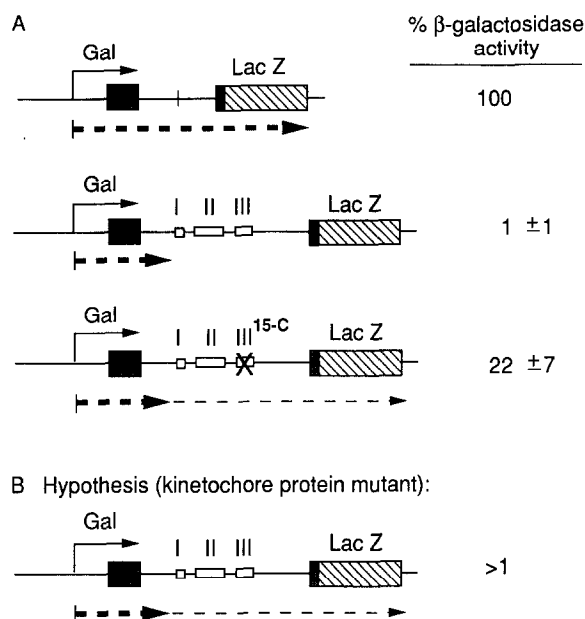B Hypothesis (kinetochore protein mutant):

>1

Figure 1. Transcriptional Readthrough Reporter Construct and Assay

The amino-terminal actin ORF is represented by the stippled boxes, separated by a line representing the actin intron. The in-frame LacZ coding sequence is shown as a hatched box. *CEN* DNA is indicated by open boxes, with roman numerals I, II, and III indicating CDEI, CDEII, and CDEIII, respectively. Transcription initiation from *GAL10* is indicated by the solid arrow, and the length and number of transcripts by the length and width of the dashed arrow.

(A) Control experiments (*CEN* DNA mutation in cis). *CEN* DNA transcriptional blocks (wild-type and CDEIII-15C mutant) were tested in a wild-type strain. β-galactosidase activity levels were normalized to 100% with a strain containing the reporter with no *CEN* transcriptional block (top).

(B) Proposed relaxation of the wild-type *CEN* transcriptional block due to mutation of a kinetochore protein component. Perier and Carbon (1992) recently described a reporter with *CEN* DNA within the *GAL1* promoter. This situation presumably sets up a competition for binding between kinetochore proteins and transcription initiation factors. The reporter used here is different in that it assays for the relief of a transcriptional block caused by a *CEN* placed downstream of the *GAL10* promoter.

tion of a multicomponent protein-*CEN* DNA complex, CBF3, which is defined as an in vitro activity that can bind *CEN* DNA sequences in a CDEIII sequence-specific manner. Three major protein species of 110 kd, 64 kd, and 58 kd apparent molecular weight are present in approximately equimolar amounts in the most highly purified preparations, although many substoichiometric species are also present (Lechner and Carbon, 1991). The CBF3 preparation has recently been shown to exhibit a minus end mechanochemical motor activity in vitro, observed as translocation of a latex bead covalently attached to *CEN* DNA along polymerized microtubules (Hyman et al., 1992).

Classical genetic approaches have also been undertaken to identify S. cerevisiae genes required for chromosome transmission, some of which are expected to encode kinetochore components. Several mutant collections have been isolated with the primary criterion of chromosome missegregation, including the *ctf* (chromosome transmis-

sion fidelity; Spencer et al., 1990), *chl* (chromosome loss; Kouprina et al., 1993), *cin* (chromosome instability; Hoyt et al., 1990), *mcm* (minichromosome maintenance; Maine et al., 1984), and *MIF* (mitotic fidelity; Meeks-Wagner et al., 1986) mutants. These mutants could have defects in any of the many components necessary for the chromosome cycle to proceed with high fidelity. Secondary criteria can be applied to identify those mutants defective in a particular structure or process. For example, sensitivity to Benomyl (a microtubule-destabilizing drug) was used as a secondary screen for the *cin* collection to identify mutants involved in microtubule function. This recently resulted in the identification of CIN8 and KIP1 (CIN9) (Hoyt et al., 1992; Saunders and Hoyt, 1992; Roof et al., 1992), two kinesin-related proteins that are involved in mitotic spindle function.

We have designed two secondary screens in order to identify kinetochore mutants. One assay monitors transcriptional readthrough of a centromere, and the other monitors the stability of a test dicentric chromosome. These in vivo assays of the integrity of a test kinetochore were used to screen the *ctf* mutant collection. The *ctf* collection consists of 136 independent mutants that exhibit increased loss of a nonessential chromosome. This collection represents approximately 50 genes whose products are required for high fidelity chromosome transmission in the mitotic cell cycle (Spencer et al., 1990). Two mutations, *ctf13-30* and *ctf14-42*, tested positive in both secondary screens. We found that CTF14 was identical to NDC10/CBF2, recently shown to encode a 110 kd kinetochore protein (Goh and Kilmartin, 1993; Jiang et al., 1993). Through a combination of genetic and biochemical approaches, we have shown that CTF13 is a previously unidentified essential protein that is a component of the S. cerevisiae kinetochore.

Results

Transcriptional Readthrough Assay and Secondary Screen of the *ctf* Collection

When transcription from a strong promoter is initiated toward a *CEN* DNA sequence, the mitotic segregational function of the centromere is destroyed (Hill and Bloom, 1987) without disruption of its 160–220 bp nuclease protected region (Bloom et al., 1984; Hill and Bloom, 1987), indicating that at least some of the kinetochore complex remains intact. Furthermore, it has been observed that the majority of transcripts terminate at the border of the *CEN* sequence (P. Phillipsen, personal communication). These observations suggest that the *CEN* DNA-protein complex is responsible for this transcriptional block.

In the reporter plasmid used to test this hypothesis (Figure 1), the *GAL10* promoter initiates transcription of an actin-*lacZ* fusion gene. A wild-type *CEN6* (165 bp) inserted in the actin intron allowed only 1% of the β-galactosidase levels seen when no *CEN* was present (Figure 1). The structurally dicentric reporter plasmid was maintained in a functionally monocentric state by keeping transformed strains on medium containing galactose. Transcription initiated from the *GAL10* promoter inactivates the segrega-

Table 1. *ctf* Mutants Identified in Kinetochore Integrity Secondary Screens

Transcriptional Readthrough Screen	Dicentric Chromosome Stabilization Screen
s10 (ctf7)	s30 (ctf13)
s9 (ctf8)	s42 (ctf14)
s30 (ctf13)	
s42 (ctf14)	
s61 (ctf17)	
s26	
s58	

tional function of the test *CEN* (Hill and Bloom, 1987). To test the hypothesis that a *CEN* DNA–protein complex was responsible for the transcriptional block, we replaced the wild-type *CEN6* sequence with a *CEN6* sequence containing a single-nucleotide point mutation (CDEIII-15C) in the central element of the palindrome of CDEIII (CCG). This transversion from G to C causes a 250-fold increase in the rate of mitotic missegregation of a chromosome fragment (Hegemann et al., 1988; Jehn et al., 1991). Similar central element mutations have been shown by in vivo footprinting to result in decreased methylation protection of *CEN* DNA (Densmore et al., 1991). The CDEIII-15C *CEN* mutant inserted in the actin intron allowed approximately 20% of the β-galactosidase levels seen when no *CEN* was present (Figure 1). Thus, a *CEN* DNA mutation affecting kinetochore integrity caused an increase in transcriptional readthrough that was detectable by increased levels of β-galactosidase activity. This increase in β-galactosidase activity could also be detected as blue colony color when strains were grown on solid medium containing X-gal (see Experimental Procedures), providing a sensitive visual assay for rapid screening of the *ctf* collection.

We proposed that the transcriptional block provided by the full-length wild-type *CEN6* might be relaxed in *ctf* strains with mutant kinetochore proteins. The reporter minichromosomes used in the control experiments would very likely be present in highly variable copy number in these *ctf* strains, owing to increased rates of nondisjunction and/or loss. This could result in the appearance of false positives and false negatives. To maintain the reporter in single copy, we integrated it into the *ctf* strains. Two independent transformants of each *ctf* strain, containing an integrated wild-type *CEN* reporter, were plated on medium containing X-gal and monitored for the appearance of blue colony color (see Experimental Procedures). Of 34 *ctf* mutants screened (see Experimental Procedures for list), 7 were identified as putative kinetochore mutants because they produced an intermediate level of blue colony color between the levels of the *CTF*⁺ strains carrying the wild-type and mutant *CEN* reporters. Five of these mutant strains (designated "s" followed by an isolate number) are members of complementation groups, s10 (ctf7), s9 (ctf8), s30 (ctf13), s42 (ctf14), and s61 (ctf17), and two contain independent mutations, s26 and s58 (Table 1). Quantitative measurement of β-galactosidase activity levels in protein extracts from these strains verify the identifi-

cation of a relaxed transcriptional block by the colony color assay (Table 2).

Dicentric Chromosome Stabilization Assay and Secondary Screen

The second assay we developed to screen for kinetochore mutants among the *ctf* collection is based upon the behavior of dicentric chromosomes as they undergo mitotic segregation. If a chromosome has two centromeres, kinetochores on the same chromatid may become attached to opposite poles of the mitotic spindle (Figure 2A). When this occurs, the DNA molecule usually breaks, and the dicentric chromosome is rapidly lost or is rearranged to a stable form (Mann and Davis, 1983; Haber and Thorburn, 1984). A kinetochore mutant might assemble kinetochores that have a weakened attachment of chromosomal DNA to microtubules. This could lead to microtubule detachment before chromatid breakage (Figure 2B), resulting in stabilization of the dicentric chromosome.

The artificial chromosome fragment present in the *ctf* strains was an appropriate substrate for the construction of a dicentric test chromosome. The chromosome fragment, a nonessential disome, possesses all the sequences required for proper chromosome segregation. Its stability can be visually monitored by the degree of colony color sectoring (see Figure 3B) (Spencer et al., 1990; Shero et al., 1991), and selective pressure for rearrangement to a stable form is absent because the chromosome fragment is not essential for viability. The *GAL–CEN* constructs developed in the transcriptional readthrough assay allow control of the mitotic activity of a centromere by the choice of carbon source in the medium. We constructed a vector that would direct integration of these test conditional centromeres to the *leu2-Δ1* locus present approximately 23 kb from the centromere on the chromosome fragment (see Experimental Procedures). In control experiments, we examined the stability of dicentric chromosome fragments containing either a nearly wild-type (ΔCDEI) or a highly defective (CDEIII-15C) secondary conditional *CEN* (Figures 3A and 3B). We predicted that upon activation

Table 2. Quantitation of Transcriptional Readthrough in *ctf* Mutants

Strain	Transcription Block	β-Galactosidase (nmol/min per mg of protein)
YPH278 (CTF⁺)	CEN6ʷᵗ	22 ± 8
YPH278 (CTF⁺)	CEN6 (CDEIII-15C)	135 ± 19
s16 (ctf9)ᵃ	CEN6ʷᵗ	19 ± 6
s10 (ctf7)	CEN6ʷᵗ	86 ± 13
s9 (ctf8)	CEN6ʷᵗ	50 ± 29
s30 (ctf13)	CEN6ʷᵗ	110 ± 24
s42 (ctf14)	CEN6ʷᵗ	NDᵇ
s61 (ctf17)	CEN6ʷᵗ	160 ± 23
s26	CEN6ʷᵗ	127 ± 42
s58	CEN6ʷᵗ	72 ± 25

Assays were performed on strains grown at 30°C. *CEN6*ʷᵗ, wild-type *CEN6*.
ᵃ s16(ctf9) was not identified as a putative kinetochore mutant by the transcriptional readthrough assay and serves as a negative control.
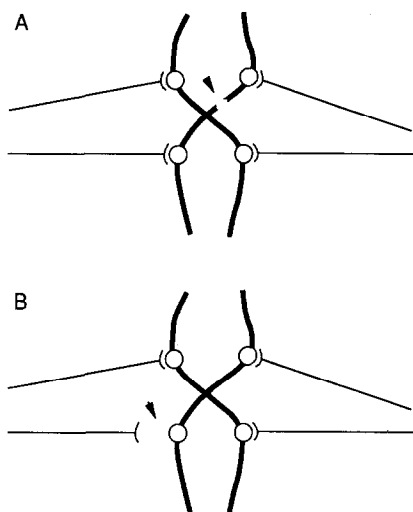ᵇ s42(ctf14) is inviable at 30°C.

A

B

Figure 2. Diagram of the Two Chromatids of a Dicentric Chromosome

In (A), the arrowhead indicates a break generated in the chromatid by the forces of the spindle pulling in opposite directions. In (B), the arrowhead indicates release of the microtubule attachment to the chromosome, allowing the dicentric chromatid to proceed intact to the spindle pole.

The hypothesis that a kinetochore mutant might result in the stabilization of a linear dicentric chromosome is based on a previous study of the behavior of dicentric minichromosomes (Koshland et al., 1987). A circular minichromosome carrying a single wild-type centromere is quite stable in S. cerevisiae (maintained in 98%–99% of the population under selection), whereas a minichromosome with two wild-type centromeres is highly unstable (maintained in only 6% of the population). However, when two identical partially defective centromeres (which by themselves allowed maintenance of minichromosomes in 91% of the population) were placed on the same minichromosome, the plasmid was not destabilized to the same degree (maintained in 49% of the population). In this case, defective kinetochore function was due to mutation of the CEN DNA sequence. By analogy, it is possible that kinetochore dysfunction due to a defective or aberrant protein will also result in stabilization of a test dicentric chromosome.

of the secondary CEN, the dicentric chromosome fragment containing a strong secondary CEN would be highly unstable, resulting in a frequent sectoring phenotype, and the dicentric chromosome fragment containing one wild-type and one defective CEN would be relatively stable, resulting in fewer sectors per colony (Figure 3B). The actual sectoring phenotypes that resulted, shown in Figure 4, were consistent with our hypothesis, indicating that the dicentric stability assay was a feasible screen for kinetochore mutants among the ctf collection (Figure 3C).

To screen the ctf mutants for stabilization of a dicentric chromosome, the conditional nearly wild-type CEN, ΔCDEI, was integrated into the chromosome fragment present in each strain. The strains were maintained on galactose to induce the GAL10 promoter and inactivate the conditional CEN (see Experimental Procedures and Figure 3A). Two transformants of each ctf strain were streaked onto medium containing dextrose to activate the dicentric state by repressing transcription from the GAL10 promoter. The stability of the dicentric chromosome fragment in the ctf strains was visually assessed and compared with the stability of the dicentric chromosome frag-

ment in the CTF⁺ strain. If the dicentric chromosome fragment was as unstable as in the wild-type background (Figure 3B), the ctf strain was scored negative in this assay. With 27 ctf mutants tested (see Experimental Procedures for list), 2 exhibited a reduction in sectoring frequency relative to the wild-type control and were thus identified as putative kinetochore mutants: s30 (ctf13), and s42 (ctf14) (see Table 2). The sectoring phenotypes exhibited by s30 (ctf13) and a representative mutant that was scored negative, s16 (ctf9), are shown in Figure 4. The sectoring phenotype of s42 (ctf14) carrying the dicentric chromosome fragment is similar to that seen for s30 (ctf13). The sectoring phenotypes of these mutants are similar to that seen with a test dicentric chromosome carrying a weak secondary CEN.

Two ctf strains, s30 (ctf13) and s42 (ctf14), were scored as putative kinetochore mutants in both secondary screens. The ctf14-42 mutation identifies a recently characterized kinetochore component, NDC10/CBF2 (see below). We therefore explored further whether the ctf13-30
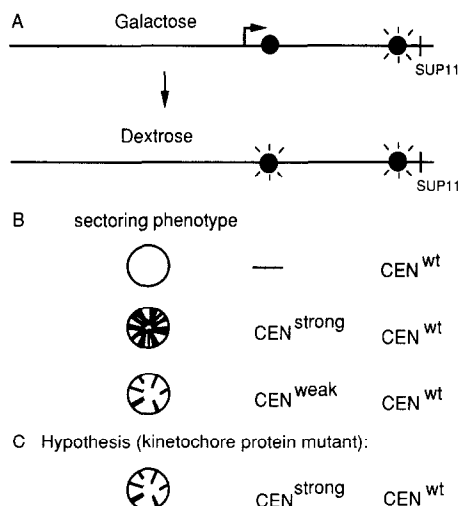

Figure 3. Dicentric Stabilization Assay

(A) Schematic of test dicentric chromosome fragment. The secondary conditional CEN is inactive on galactose medium (the test chromosome behaves as a monocentric) and active on dextrose medium (the test chromosome behaves as a dicentric).

(B) The stability of the test dicentric chromosome fragment can be monitored visually. A tRNA suppressor gene (SUP11) present on the chromosome fragment partially suppresses the accumulation of red pigment caused by the ade2-101 ochre mutation in our strain backgrounds. If the chromosome fragment is present, the strain is white; if it is lost, the strain is red. Thus, the number of red sectors that develop during the growth of a colony founded by a haploid cell containing the chromosome fragment (white) is indicative of the rate of loss of the chromosome fragment in the strain. The lines within the circles represent the presence of such red sectors in a white colony. The sectoring phenotypes pictured are those predicted and observed (see Figure 4) when nearly wild-type (CEN^strong) and highly defective (CEN^weak) CEN DNA are tested at the second site (left) in a wild-type strain background. The centromere originally present on the chromosome fragment is fully wild type (CEN^wt).

(C) Proposed reduced sectoring phenotype, indicative of stabilization of the test dicentric chromosome fragment. Though the secondary CEN is nearly wild type (CEN^strong), mutation of a kinetochore protein component should result in a relatively mild red sectoring phenotype.

YPH278 (CTF+)   s16 (ctf9)   s30 (ctf13)

— :CEN^{wt}

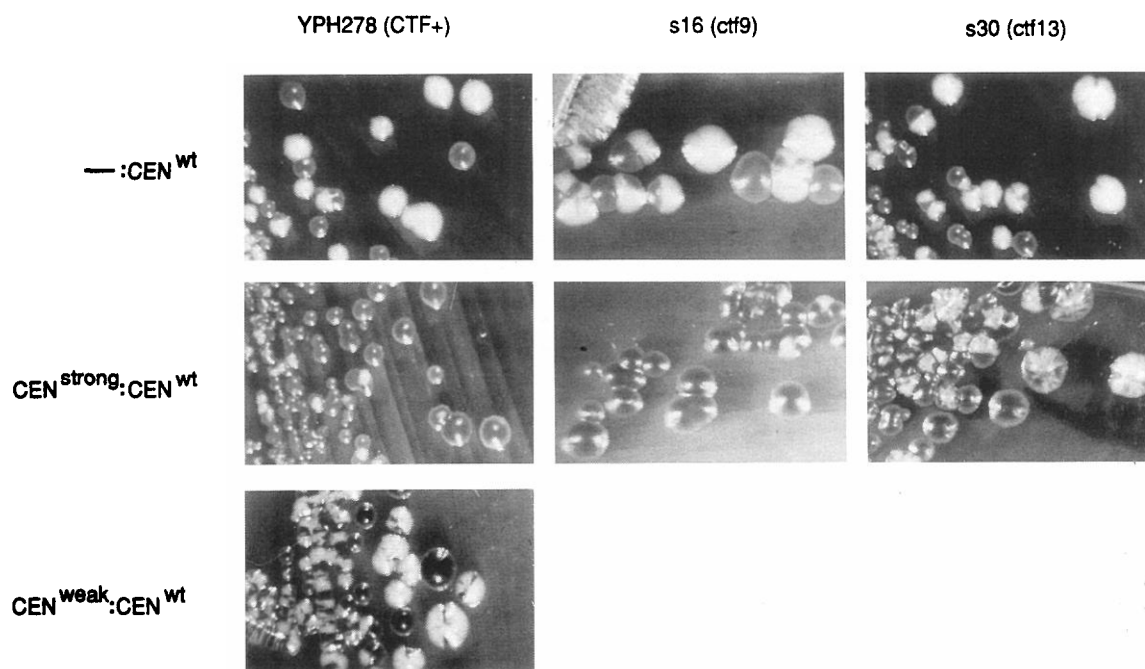CEN^{strong}:CEN^{wt}

CEN^{weak}:CEN^{wt}

Figure 4. Sectoring Phenotypes of Strains Carrying the Test Dicentric Chromosome Fragment

Labels at the left indicate the type and number of CEN DNAs present on the test chromosome fragment. Labels across the top indicate the relevant genotype of the pictured strain. s16 (ctf9) was scored negative; its sectoring phenotype with the test dicentric chromosome fragment (column 2, row 2) was the same as that seen in the CTF+ background (column 1, row 2). s30 (ctf13) was scored positive; its sectoring phenotype with the test dicentric chromosome fragment (column 3, row 2) was not as severe as that seen in the CTF+ background (column 1, row 2) and looked similar to that seen with the test dicentric chromosome carrying the weak secondary CEN (column 1, row 3).

mutation had identified a gene encoding a kinetochore component.

Molecular Cloning

ctf13-30 is completely deficient for growth at 37°C, and this temperature sensitivity was shown to cosegregate with its moderate sectoring phenotype at 25°C. CTF13 was cloned by complementation of lethality at 37°C (Spencer et al., 1988). A 2.2 kb Sau3A fragment that complemented the temperature sensitivity of ctf13-30 was shown to correspond to CTF13 by the directing of an integration event in a heterozygous diploid. This event introduced a prototrophic marker at the genomic site of the cloned DNA segment and deleted approximately half of the 2.2 kb genomic sequence (almost the entire CTF13 open reading frame [ORF]; see Experimental Procedures and Figure 5B). When the diploid transformants were dissected, it was found that viability segregated 2:2 (see Experimental Procedures). We concluded that the cloned DNA encodes wild-type CTF13 and that CTF13 is an essential gene in S. cerevisiae. CTF13 was localized to the right arm of chromosome XIII using both physical and genetic mapping methods (Figure 5A). From the mapping data, we concluded that CTF13 is a previously unidentified gene in S. cerevisiae.
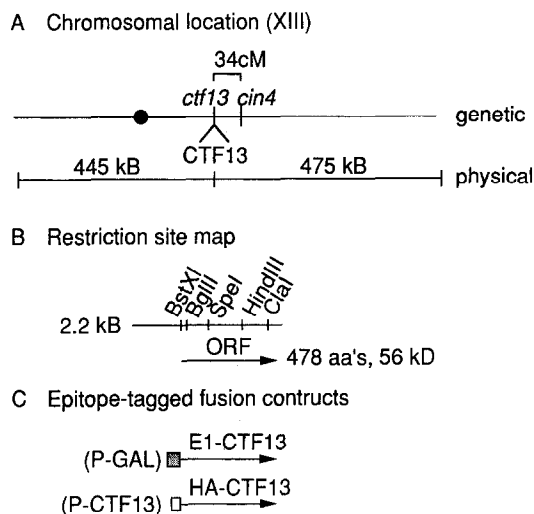
The nucleotide sequence of the 2.2 kb CTF13 clone contains a 1.4 kb ORF that encodes for a protein of 478 amino acids with a predicted molecular weight of 56 kd (Figures 5B and 6). The CTF13 protein shows no significant

overall homology at the amino acid level to entries in Gen-Bank, GenPept, GPUpdate, SwissProt, PIR, EMBL, and EMBLUpdate data bases as of January 1993. The homology searches were performed on the National Center for Biotechnology Information BLAST network (Altschul et al., 1990). The CTF13 protein contains a short acidic serine-rich region (amino acids 200–230) that is approximately 40% identical to the first acidic block found in a mammalian centromere-associated protein, CENP-B (Pluta et al., 1992). The significance of this small region of similarity is unclear, and there are no other significant homologies found outside this area. Interestingly, there is a possible CDC28 phosphorylation site in the CTF13 protein (SSPSS, amino acids 224–228) (Figure 6).

Biochemical Analysis

Lechner and Carbon (1991) have described a multiprotein complex (CBF3) present in nuclear extracts of S. cerevisiae cells that binds in vitro to a 350 bp fragment of CEN DNA. DNA footprinting reveals that CBF3 interacts with the CDEIII sequence element. Using a modification of the methods of Lechner and Carbon (1991), we were able to detect the binding of CBF3 complexes present in whole-cell extracts to an 88 bp DNA probe that spans CDEIII but lacks CDEI and CDEII.

To determine whether CTF13 is a component of the CDEIII-binding complex, the CTF13 ORF was fused to peptide epitopes against which antibodies had been previously raised. We constructed two epitope-tagged fusions

A Chromosomal location (XIII)

34cM

ctf13 cin4

——————●—╫╫—┤—————— genetic

|——— 445 kB ——|— CTF13 —|— 475 kB ———| physical

B Restriction site map

2.2 kB ——╫—┼—┼—┼—┼—

ORF → 478 aa's, 56 kD

C Epitope-tagged fusion contructs

(P-GAL)■ E1-CTF13 →

(P-CTF13) □ HA-CTF13 →

Figure 5. Molecular Cloning and Mapping of *CTF13*

(A) Physical and meiotic maps of *CTF13*. *CTF13* was localized to chromosome XIII of S. cerevisiae by Southern hybridization to an electrophoretic karyotype (Spencer et al., 1988; Gerring et al., 1990a). *CTF13* was positionally mapped by the method of chromosome fragmentation (Gerring et al., 1990a) and was localized to the right arm of chromosome XIII, 475 kb from the right arm telomere and 445 kb from the left arm telomere (see Experimental Procedures). This physical location was verified by using the 2.2 kb Sau3A fragment to probe a set of filters containing contiguous overlapping λ clones that cover 86% of the yeast genome (L. Riles and M. Olson, unpublished data). *CTF13* was localized to overlapping clones 4199 and 6543, placing it on an ~15 kb segment of DNA located on the right arm of chromosome XIII between *adh3* and *ilv2*. The temperature-sensitive *ctf13-30* mutation was meiotically mapped and found to be located 34 cM proximal to the *cin4* locus, which agrees well with the physical mapping data (see Experimental Procedures).

(B) Restriction site map of the 2.2 kb *ctf13-30*-complementing clone. The location of the ORF is indicated by the arrow.

(C) Schematic structures of the E1 and HA amino-terminal epitope–CTF13 fusion constructs. The E1–CTF13 fusion is under transcriptional control of the *GAL1* promoter; the HA–CTF13 fusion is under the transcriptional control of the endogenous CTF13 promoter.

of CTF13 (see Experimental Procedures). In the first construct, an 11 amino acid epitope derived from the HA1 protein of influenza virus (Field et al., 1988) was inserted in frame into the amino terminus of CTF13 (Figure 5C). In the second construct, two tandem copies of the E1 epitope (Pluta et al., 1992), derived from the carboxy-terminal 25 amino acids of an avian coronavirus glycoprotein (Machamer and Rose, 1987), were placed in frame at the amino terminus of the CTF13 ORF under the transcriptional control of the *GAL1* promoter (Figure 5C). Both epitope-tagged CTF13 derivatives were able to rescue viability in a *ctf13 Δ1::HIS3* null strain.

Extracts were prepared from cells carrying either wild-type or epitope-tagged CTF13, reacted with ^{32}P-labeled CDEIII DNA, and complexes were resolved on a nondenaturing gel (Figure 7). A single band corresponding to a CDEIII–protein complex was observed (Figure 7, lanes 1, 7, and 10); no complex was observed with a nonfunctional CDEIII variant (data not shown). The addition of anti-epitope antibodies to binding reactions containing extracts

from *ctf13* mutant strains rescued by the respective CTF13–epitope fusion protein resulted in the appearance of a complex with significantly decreased electrophoretic mobility (Figure 7, lanes 2–4 and 11). This supershift is

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   1 AATCAGTTTAAATGGAGCGAAGCACTCGACAATGCCTTTGTACCAGGTATGGCCAAAGAG
  61 TTGACACTTACTGTTCTTCAAGAACTACGCAAACGTGGCAAAGTTCGTGTAGCGCTATAT
 121 GGTAGAGATGAATCTACATTAGAGCCGCTACTGAATTGGTGCTTAAAAGGTATTGAAGAT
 181 GTGAGATCAGCATCTATTGTAGCGGATTGGGTCGCCGTGGTTTTAGAATTATATGGGAAT
 241 ACGTTAGAAAGCTCTCCAGTTCTTCAAGAATTGATGATAGATTTGAAAACTAAAGTTAGA
 301 CATGAAATTCATAAGTCTAAAGAAGCTCAAAGAATTGAAGGTATGCTTCAACTTTTAACG
 361 AGAAGAAATATTAGCATATAGATTTTAATTTGTATCTATTTTATAGATATTCAGGCATAC
 421 ATATATATCTCTTTCTCATTGTAATCGAAAAAAATAATATCTTCTCTTTTTATTGAAAAA
 481 AGGAACCTGTATAAATTTCATTTTAGAGTGCTTTGCATTTTGATACGTTTTCGCGTTTAA
 541 CTACCTTCGAGGGCTGAACAAACATTGTGAGATGAGATGCTATCAACAGAAAGCTGACTG

 601 TGAGTCCCCAGAAGTCCATGTCGCTTGATGCCTTCTTTCAATCCTGTTAGATTCTTAGAG
   1                       M  P  S  F  N  P  V  R  F  L  E

 661 TTACCAATTGACATTAGAAAAGAAGTTTACTTCCATTTAGATGGTAATTTTTGCGGAGCA
  12 L  P  I  D  I  R  K  E  V  Y  F  H  L  D  G  N  F  C  G  A

 721 CATCCCTACCCAATAGATATATTATACAAATCTAATGATGTGGAGCTTCCAGGAAAACCA
  32 H  P  Y  P  I  D  I  L  Y  K  S  N  D  V  E  L  P  G  K  P

 781 AGTTATAAAAGGTCTAAGAGATCTAAGAAATTATTGAGATACATGTATCCTGTTTTTGCC
  52 S  Y  K  R  S  K  R  S  K  K  L  L  R  Y  M  Y  P  V  F  A

 841 ACGTATTTGAACATTTTCGAATACTCTCCGCAACTCATTGAAAAATGGCTTGAGTATGCA
  72 T  Y  L  N  I  F  E  Y  S  P  Q  L  I  E  K  W  L  E  Y  A

 901 TTTTGGTTGCGCTATGATTGCCTGGTTTTGGACTGTTTCAAAGTAAACCATCTTTATGAC
  92 F  W  L  R  Y  D  C  L  V  L  D  C  F  K  V  N  H  L  Y  D

 961 GGAACGCTTATAGATGCATTAGAATGGACGTACCTTGATAATGAACTTCGACTGGCCTAC
 112 G  T  L  I  D  A  L  E  W  T  Y  L  D  N  E  L  R  L  A  Y

1021 TTCAATAAGGCCAGTATGTTAGAAGTTTGGTATACTTTTAAAGAGTACAAGAAGTGGGTG
 132 F  N  K  A  S  M  L  E  V  W  Y  T  F  K  E  Y  K  K  W  V

1081 ATAGACAGTGTTGCTTTTGACGAGCTTGACTTACTTAATGTAAGTAATATTCAATTCAAC
 152 I  D  S  V  A  F  D  E  L  D  L  L  N  V  S  N  I  Q  F  N

1141 ATTGATAATCTAACTCCACAACTAGTAGACAAATGCTTATCAATTTTAGAACAGAAAGAT
 172 I  D  N  L  T  P  Q  L  V  D  K  C  L  S  I  L  E  Q  K  D

1201 TTATTCGCAACAATCGGGGAGGTACAATTTGGACAAGATGAAGAAGTAGGAGAAGAGAAA
 192 L  F  A  T  I  G  E  V  Q  F  G  Q  D  E  E  V  G  E  E  K

1261 GATGTTGATGTTAGTGGAGCTAATTCTGACGAAAACTCCTCACCTAGCTCCACAATTAAA
 212 D  V  D  V  S  G  A  N  S  D  E  N  S  S  P  S  S  T  I  K

1321 AACAAGAAAAGGTCAGCATCCAAAAGATCACATTCAGATAATGGCAATGTGGGAGCAACG
 232 N  K  K  R  S  A  S  K  R  S  H  S  D  N  G  N  V  G  A  T

1381 CACAACCAACTAACAAGTATTTCTGTTATAAGAACAATAAGAAGTATGGAATCCATGAAG
 252 H  N  Q  L  T  S  I  S  V  I  R  T  I  R  S  M  E  S  M  K

1441 AGCCTGCGGAAGATCACTGTGAGAGGAGAGAAATTATATGAGTTGCTAATAAATTTTCAT
 272 S  L  R  K  I  T  V  R  G  E  K  L  Y  E  L  L  I  N  F  H

1501 GGGTTCAGAGACAATCCTGGTAAAACGATTAGTTACATAGTGAAACGTAGGATAAATGAA
 292 G  F  R  D  N  P  G  K  T  I  S  Y  I  V  K  R  R  I  N  E

1561 ATACGCCTATCACGCATGAATCAAATTTCCAGAACCGGACTAGCTGACTTTACAAGATGG
 312 I  R  L  S  R  M  N  Q  I  S  R  T  G  L  A  D  F  T  R  W

1621 GACAACTTGCAAAAGCTTGTATTAAGTAGAGTAGCTTACATCGACCTGAATAGCATTGTC
 332 D  N  L  Q  K  L  V  L  S  R  V  A  Y  I  D  L  N  S  I  V

1681 TTTCCCAAAAATTTTAAATCTCTGACAATGAAACGTGTAAGTAAAATCAAGTGGTGGAAC
 352 F  P  K  N  F  K  S  L  T  M  K  R  V  S  K  I  K  W  W  N

1741 ATCGAGGAAAATATACTAAAGGAACTAAAAGTCGATAAGAGAACTTTTAAATCGTTGTAC
 372 I  E  E  N  I  L  K  E  L  K  V  D  K  R  T  F  K  S  L  Y

1801 ATAAAGGAAGATGATAGTAAATTCACAAAATTTTTTAATTTACGACACACAAGAATAAAA
 392 I  K  E  D  D  S  K  F  T  K  F  F  N  L  R  H  T  R  I  K

1861 GAGCTTGACAAAAGTGAAATTAATCAAATAACATACCTTCGATGTCAAGCAATAGTTTGG
 412 E  L  D  K  S  E  I  N  Q  I  T  Y  L  R  C  Q  A  I  V  W

1921 CTATCTTTTCGGACTTTGAACCATATCAAGCTACAAAATGTATCTGAAGTTTTTAACAAT
 432 L  S  F  R  T  L  N  H  I  K  L  Q  N  V  S  E  V  F  N  N

1981 ATCATCGTACCTAGAGCATTGTTCGATAGTAAACGAGTAGAGATTTATCGATGTCAAAAA
 452 I  I  V  P  R  A  L  F  D  S  K  R  V  E  I  Y  R  C  E  K

2041 ATCTCTCAGGTTTTGGTAATATAAAAATGTGGAAAACCGGCGGTATTTTAATTAGAAACA
 472 I  S  Q  V  L  V  I  *

2101 AACTGTCTGATAAACCAATCATGAAATCTTGTATCACTGTCAGCAAGCTCTGGATGAAAA
2161 GAAGTCACAAGGATATTATGATTTTGCGTTGCAGCTACAACCACATCCTTTCCATTCACT
2221 GGCAATTCATATAAACTTTTAACCGCGATAGGATCGCGCGCTCTAGACTA
```
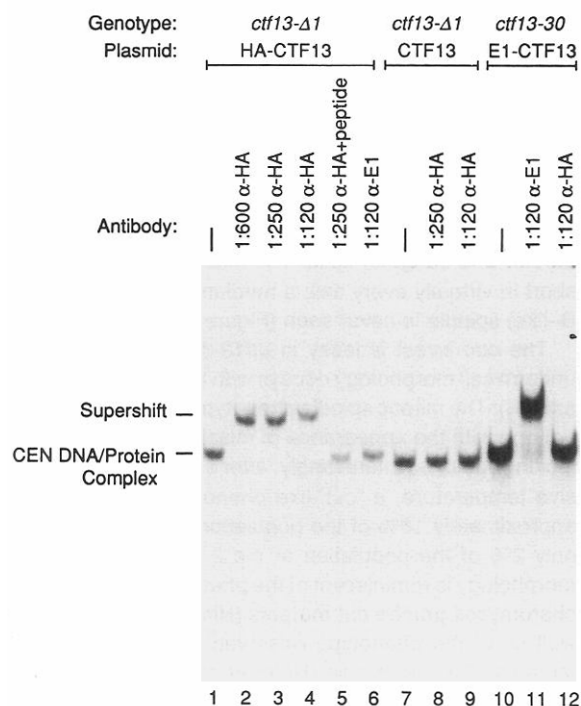
Figure 6. Nucleotide and Predicted Protein Sequence of CTF13

The amino acid translation, beginning with the first methionine of the ORF, is shown. The acidic serine-rich region is underlined (amino acids 200–230), and within that, the putative CDC28 phosphorylation site (amino acids 224–228) is italicized.

Genotype: ctf13-Δ1   ctf13-Δ1   ctf13-30
Plasmid: HA-CTF13   CTF13   E1-CTF13

Antibody:
1:600 α-HA
1:250 α-HA
1:120 α-HA
1:250 α-HA+peptide
1:120 α-E1
1:250 α-HA
1:120 α-HA
1:120 α-E1
1:120 α-HA

Supershift —

CEN DNA/Protein —
Complex

1 2 3 4 5 6 7 8 9 10 11 12

Figure 7. Immunodetection of CTF13 in DNA–Protein Complexes

DNA–protein complexes formed with ³²P-labeled CDEIII probe and whole-cell extracts were analyzed on a nondenaturing acrylamide gel. Antibodies were added to preformed complexes, and samples were incubated for 20 min at room temperature before gel analysis. Unbound probe was run off the bottom of the gel. Lanes 1–6, extracts from ctf13Δ1::HIS3 null cells carrying an HA epitope–CTF13 fusion (see Figure 5C) and incubated with antibodies at various dilutions; lanes 7–9, extracts from ctf13Δ1::HIS3 null cells carrying a CTF13 plasmid reacted with the indicated antibody (controls for lanes 1–6); lanes 10–12, extracts from ctf13-30 cells carrying an E1 epitope–CTF13 fusion (see Figure 5C) and incubated with indicated antibodies (control is lane 6). HA indicates the addition of 12CA5 monoclonal antibody, which is directed against the HA epitope; E1 indicates the addition of a polyclonal serum directed against the E1 epitope; peptide indicates the addition of HA peptide to 1 mM prior to the addition of 12CA5 antibody.

clearly antibody specific, because antibodies directed against the E1 epitope did not recognize the HA–CTF13 fusion protein (Figure 7, compare lanes 6 and 4), and antibodies directed against the HA epitope did not recognize the E1–CTF13 fusion protein (Figure 7, compare lanes 12 and 11). As expected, the supershift was also shown to require the presence of E1–CTF13 (Figure 7, compare lanes 6 and 11) or HA–CTF13 (Figure 7, compare lanes 8 and 9 with lanes 3 and 4) and to be competed with HA peptide (Figure 7, compare lanes 5 and 3). These results show that the supershifted band shown in lanes 2–4 and 11 of Figure 7 is composed of a complex containing proteins, DNA, and antibody.

These results demonstrate that CTF13 is present in the protein complex that binds to the essential CDEIII region of S. cerevisiae CEN DNA in vitro. Because all of the CDEIII–protein complex formed in our reactions were able to be supershifted by antibodies directed against epitope-

tagged CTF13, the stoichiometry of CTF13 and DNA in the complexes must be at least 1 to 1. We conclude from these data that CTF13 is a major component of the yeast kinetochore, which, probably in combination with other proteins, interacts with CDEIII.

Phenotypic Analysis

The ctf13-30 mutant allowed transcriptional readthrough of a test CEN and stabilized a test dicentric chromosome fragment. Further phenotypic analysis of this mutant revealed defects consistent with defective kinetochore function.

The colony color assay for chromosome fragment stability can be used to monitor the rates of chromosome fragment loss and nondisjunction events in diploids. These rates were measured for a ctf13-30 homozygous diploid and its wild-type parent at permissive temperature (25°C). The ctf13-30 homozygous diploid exhibited an approximately 50-fold elevation both in the rates of nondisjunction and in loss of the chromosome fragment (Table 3A). The rates of mitotic missegregation and recombination of a suitably marked endogenous chromosome III were also measured. The mitotic missegregation rate of chromosome III was elevated 19-fold in the ctf13-30 homozygous diploid, while the mitotic recombination rate was only elevated 4-fold (Table 3B). We conclude that the ctf13-30 mutation confers mitotic segregation and recombination phenotypes consistent with a role in the segregational machinery.

ctf13-30 causes cells to arrest at the G2/M phase of the cell cycle when shifted to the nonpermissive temperature. Flow cytometric analysis of DNA content per cell revealed an accumulation of cells with a G2 DNA content during log phase growth at the permissive temperature and a single peak of G2 content DNA after arrest at the nonpermissive temperature (Figure 8A). Quantitation of cell and nuclear morphology at the permissive temperature also indicated an accumulation of cells with a G2 content; 13% of cells were large budded with the nucleus at the neck

Table 3. Rates of Missegregation Events in ctf13 Mutants at 25°C

	ctf13-30/ ctf13-30	CTF13/ CTF13	Ratio
Chromosome fragment[a]			
1:0 events (× 10⁻²)	2.1	0.04	53
2:0 events (× 10⁻²)	3.0	0.05	60
Chromosome III[b]			
Missegregation (×10⁻⁵)	14	0.74	19
Recombination (× 10⁻⁵)	4.9	1.3	3.8

[a] The rates of loss (1:0) and nondisjunction (2:0) events measured for the nonessential chromosome fragment present in YPH973 and YPH280. Methods were as described in Gerring et al. (1990b). The numbers of colonies scored were 3,380 for YPH973 and 22,184 for YPH280.
[b] The rates of mitotic missegregation and recombination for chromosome III present in YPH976 and YPH699. Methods were as described in Gerring et al. (1990b).

A

B

C
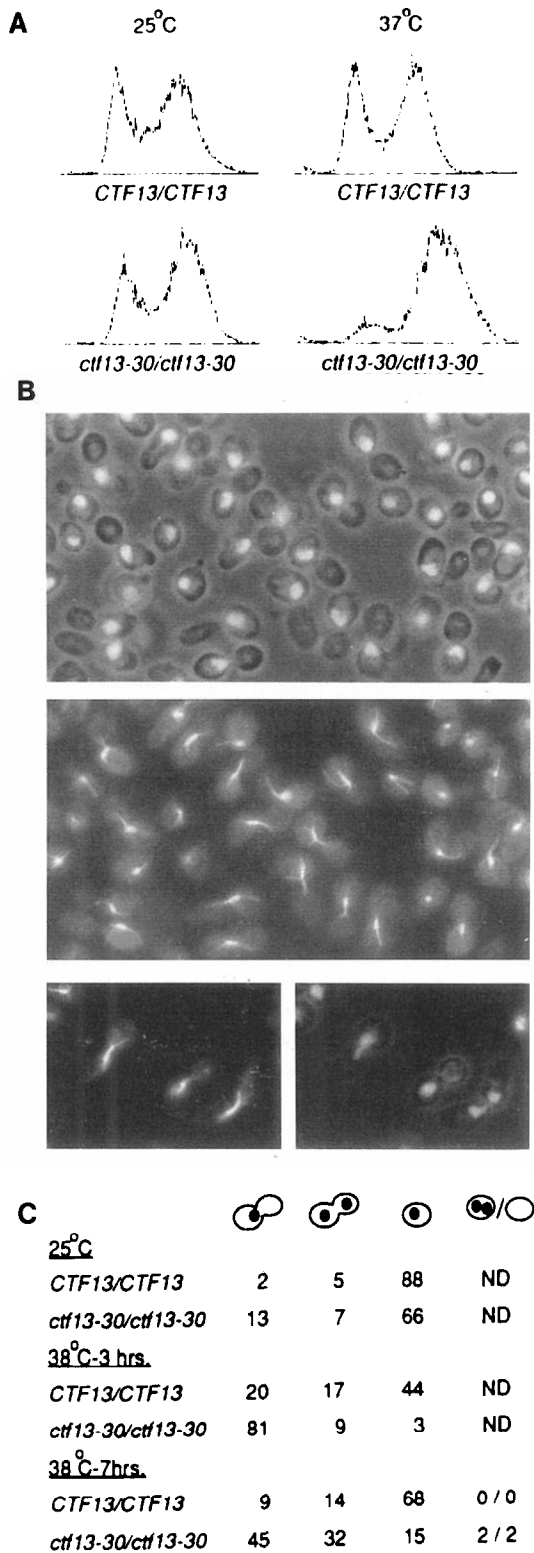
Figure 8. Phenotypic Analysis of *ctf13-30*

(A) G2/M accumulation at the permissive temperature and G2 arrest at nonpermissive temperature in *ctf13-30* cells, as analyzed by flow cytometry. The number of cells is depicted on the vertical axis, with fluorescent intensity of emitted light (proportional to DNA content) on the horizontal axis. Strains used were YPH973 and YPH280. Logarithmically growing cells were processed after 6 hr at 25°C and 3 hr at 37°C as described by Gerring et al. (1990b). The G2 arrest of *ctf13-30*

in a *ctf13-30* background, while only 2% of wild-type cells had this morphology (Figure 8C).

ctf13-30 is a *cdc*-like mutation that arrests with a cell morphology indicative of the G2/M preanaphase portion of the cell cycle. After 3 hr at nonpermissive temperature (38°C), approximately 80% of *ctf13-30* homozygous diploid cells had arrested as large-budded cells with an undivided nucleus positioned at or near the neck between the mother and daughter cells. The mitotic spindle was very short in virtually every cell; a medium or long (anaphase B–like) spindle is never seen (Figure 8B, upper panels).

The *cdc* arrest is leaky in *ctf13-30* at 38°C, and the uniform cell morphology decays with time (see Figures 8B and 8C). The mitotic spindle phenotype also becomes less uniform with the appearance of misaligned and aberrant-looking spindles. Interestingly, after 5 hr at the nonpermissive temperature, a "cut"-like phenotype is observed in approximately 10% of the population (though present in only 2% of the population at the 2 hr time point). This morphology is reminiscent of the phenotype of Schizosaccharomyces pombe cut mutants (Hirano et al., 1986), as well as of the phenotype observed in topoisomerase II mutants of S. cerevisiae (Holm et al., 1985). We define this cut-like cell morphology as a very narrow-necked, large-budded cell in which the nucleus straddling the neck has a pinched appearance (see Figure 8B, lower panel).

These data demonstrate that the *ctf13-30* mutation results in a defect revealed in the G2/M phase of the cell cycle, consistent with a defect in kinetochore function.

CTF14 Encodes the 110 kd CBF3 Subunit

The *ctf* strain s42 (ctf14) was also identified by both secondary screens as a putative kinetochore mutant. A clone that complemented the temperature sensitivity of *ctf14-42* was obtained and mapped to chromosome VII essentially as described for *ctf13-30* (data not shown). *Nuclear division cycle 10 (NDC10)*, recently identified by Goh and Kil-

cells is slightly tighter (a single G2 peak) when incubated at 38°C for 3 hr (data not shown).

(B) *ctf13-30* terminal arrest morphology. Upper panel: 4′,6-diamidino-2-phenylindole staining and phase contrast photograph of *ctf13-30/ctf13-30* cells after a 2 hr incubation at the nonpermissive temperature (38°C). Middle panel: fluorescein isothiocyanate staining of α- and β-tubulin of cells shown in upper panel. Notice the uniform phenotype: large budded with the nucleus at the neck and a short spindle. Lower right panel: 4′,6-diamidino-2-phenylindole staining and phase contrast photograph of *ctf13-30/ctf13-30* cells after 5 hr incubation at the nonpermissive temperature (38°C). Lower left panel: fluorescein isothiocyanate staining of α- and β-tubulin of cells shown in lower right panel. Notice the morphology of the two flanking cells, large budded with the appearance of a pinched nucleus spanning the neck (cut-like). Logarithmically growing cells were processed for immunofluorescence as described by Gerring et al. (1990b). Samples shown are aliquots of cells quantitated in (C).

(C) Quantitation of nuclear and cell morphology. Strains used were YPH973 and YPH280. Nuclear morphology was scored by staining with propidium iodide. The criteria used for each morphological class scored are schematically shown above the columns. The numbers shown represent the percentage of total cells scored; small-budded cells with a single nucleus were quantitated (percentage is 100 minus the sum indicated) but are not shown. At 25°C, 1500 cells were scored; 200 cells were scored for each time point at 38°C.

martin (1993) as an essential gene involved in chromosome segregation in S. cerevisiae, is identical to CBF2, a gene recently identified by Jiang et al. (1993) that encodes the 110 kd subunit of the CBF3 complex (Lechner and Carbon, 1991). Multiple internal restriction fragments from the NDC10 clone were found to comigrate with fragments from the ctf14-42 complementing clone. Moreover, the temperature-sensitive mutation ctf14-42 failed to complement the temperature sensitivity of ndc10-1. We conclude that the ctf14-42 mutation is present at the NDC10/CBF2 locus. Thus, the only two ctf mutants identified by both secondary screens as putative kinetochore mutants have now been shown to be defective in essential kinetochore components.

Discussion

Although the CEN DNA sequence elements from budding yeast have been cloned for over 10 years (Clarke and Carbon, 1980), identification of the genes encoding proteins essential for centromere function has proven difficult. We describe a genetic approach using two independent in vivo genetic assays to screen an existing large reference set of mitotic segregation mutants (the ctf collection; Spencer et al., 1990) for altered kinetochore integrity. In combination, these assays identified two mutant strains, s30 (ctf 13) and s42 (ctf14), as putative kinetochore mutants. Biochemical and further phenotypic analysis indicated that the CTF13 gene product was indeed an essential structural component of the kinetochore, and the CTF14 gene product was shown to be identical to NDC10/CBF2, a recently characterized essential kinetochore component (Goh and Kilmartin, 1993; Jiang et al., 1993).

The Genetic Screens for Kinetochore Mutants
Theoretically, the transcriptional readthrough assay might result in both false negatives (e.g., kinetochore protein mutations that fail to relieve a transcription block) and false positives (e.g., mutations that affect transcriptional regulation or chromatin structure). Similarly, a dicentric chromosome could be stabilized by mutants affecting DNA metabolism or spindle integrity and assembly. In light of these caveats, we used these assays to screen a set of mutants previously shown to have defects in mitotic chromosome segregation. It is not known how efficient either of these secondary assays would be in a primary screen.

Our experience suggests that kinetochore mutants can be recognized by the combined phenotype of transcriptional readthrough and dicentric chromosome stabilization. In theory, the degree to which the integrity of the kinetochore must be compromised to result in either of these phenotypes could be quite different. In a simplified view, mutations affecting the interaction of the kinetochore complex with the CEN DNA should be detected by both assays, while mutations affecting kinetochore to microtubule interactions may only be detected by the dicentric stabilization assay. However, we note that kinetochores participate in several distinct processes in vivo, including microtubule capture, congression to the metaphase plate, and poleward migration. In addition, a viable kinetochore

mutation would most likely be a leaky mutation, which might exhibit complex consequences following the primary defect. Thus, in reality, it is quite possible that some of the mutations identified by only one of these secondary screens indeed disrupt kinetochore integrity but perhaps lead to more subtle alterations than ctf13-30 or ctf14-42. It is clear that these two screens, whether used alone or in combination, have the potential to aid in the identification of additional regulatory and structural components of the S. cerevisiae kinetochore. They may also be adaptable to other organisms.

CTF13 Is an Essential Kinetochore Component
We have presented a combination of in vivo and in vitro evidence demonstrating that the CTF13 protein is a component of the S. cerevisiae kinetochore. In vivo, the ctf13-30 mutation confers relaxation of a transcriptional block mediated by the kinetochore and stabilizes a test dicentric chromosome fragment. In vitro, we demonstrate that the CTF13 protein is a component of the CEN DNA–protein complex and, specifically, that it interacts with CDEIII. The predicted molecular mass of 56 kd for the CTF13 protein is the approximate size seen on a Western blot (data not shown). Therefore, the CTF13 protein seemed to be a very good candidate for the 58 kd subunit of the CBF3 complex (Lechner and Carbon, 1991), and in fact, the predicted amino-terminal amino acid sequence of the CTF13 protein was found to be identical to tryptic peptide sequence obtained from the purified 58 kd protein component of the CBF3 complex (J. Lechner, personal communication).

The CTF13 protein appears to be limiting for CDEIII–protein complex formation in vitro. When extracts derived from a strain overproducing CTF13 are used in the band shift assays (see Figure 7, lanes 10–12), the amount of CDEIII–protein complex formed is increased relative to the amount seen with extracts from nonoverproducing cells (see Figure 7, lanes 1–9). Also, a ctf13 heterozygous diploid strain (ctf13Δ1::HIS3/CTF13) exhibits a mild but detectable sectoring phenotype. This indicates that the amount of CTF13 protein produced by one copy of the CTF13 locus is not sufficient to keep the fidelity of chromosome segregation at a wild-type level. These observations suggest that CTF13 may be limiting for kinetochore function in vivo.

Phenotypic analysis of the temperature-sensitive ctf13-30 mutation is consistent with a kinetochore defect. ctf13-30 causes an increase in the mitotic rate of chromosome missegregation and results in a terminal phenotype indicative of a defect in the G2/M phase of the cell cycle. It has been previously proposed that missegregation mutants will fall into two broad groups: those affecting the pathways of DNA metabolism and those affecting the mitotic segregational machinery. A mutation affecting DNA metabolism was expected to cause increased rates of both chromosome loss and mitotic recombination, while a mitotic segregation mutant was expected to cause only an increase in chromosomal loss events. Phenotypic analysis of a known DNA metabolic mutant, DNA polymerase α (cdc17; Hartwell and Smith, 1985), and a known spindle mutant, β-tubulin (tub2; Huffaker et al., 1988), supported this

model. Examination of these phenotypes for *ctf13-30* strains revealed a significant increase in the rate of chromosomal missegregation with only a very slight elevation in the rate of mitotic recombination (Table 3B). In addition, we now have the ability to distinguish between loss (1:0) and nondisjunction (2:0) missegregation events, and we find that the rates of both of these events are significantly elevated in a *ctf13-30* background (Table 3A). Thus a known kinetochore mutation, *ctf13-30*, has been shown to result in phenotypes consistent with previous expectation, and we can perhaps extend this expectation to include a predicted increase in the rates of both chromosomal loss and nondisjunction in mitotic segregation mutants.

The *ctf13-30* Kinetochore Defect May Be Recognized by a Cell Cycle Checkpoint

Critical events in the cell cycle are temporally ordered and coordinated by a series of dependency pathways in which late events are dependent on the successful completion of earlier events. These dependencies can result from a substrate–product mechanism or from extrinsic control by a monitoring function termed cell cycle checkpoint control (Hartwell and Weinert, 1989). Checkpoints are responsible for a subset of observed cell cycle arrests or delays. Cell cycle arrests or delays associated with kinetochore defects have been reported in several systems. In animal cells, a delay in the initiation of anaphase is correlated with the failure of chromosomes to achieve bipolar attachment to the spindle (Rieder and Alexander, 1989; Zirkle, 1970), and a metaphase arrest is observed with kinetochore disruption by injection of anti-centromere antibodies (Bernat et al., 1990). In yeast, one aberrant kinetochore on a single chromosome can cause a mitotic delay (Spencer and Hieter, 1992).

ctf13-30 strains exhibit a G2/M phase accumulation in logarithmic cultures at permissive temperature and a pre-anaphase arrest morphology at nonpermissive temperature. Cell morphology and DNA content do not critically distinguish G2 and M phases in yeast. However, at permissive temperature, *ctf13-30* strains exhibit a detectable increase in H1 kinase activity relative to *CTF13* controls, and at nonpermissive temperature, H1 kinase activity levels in *ctf13-30* strains are equivalent to nocodazole-arrested strains (data not shown). Thus, H1 kinase activity measurements suggest that the *ctf13-30* mutation causes an accumulation in M phase. It is tempting to speculate that this cell cycle alteration is similar to those described above and that these are a result of checkpoint control exerted in the presence of defective kinetochores.

Checkpoints are defined by two experimental criteria: first, identification of mutations or conditions that allow bypass of an arrest or delay (resulting in the accumulation of errors) and second, an observed error correction when cell cycle delay is reintroduced experimentally. Alternatively, defective substrate–product conversion that becomes rate limiting for progress may also result in cell cycle delay. These alternatives have not been distinguished for the delays seen associated with kinetochore defects. Conditional mutations in kinetochore proteins will provide important tools for exploring the relationship be-

tween kinetochore structure and cell cycle progression.

Examination of the terminal phenotype of *ctf13-30* mutants raises several interesting questions. The fact that the *ctf13-30* defect does not lead to a permanent and uniform arrest morphology may simply be a result of the presence of a small amount of active CTF13 protein that eventually allows completion of mitosis, or mitosis may never be completed but cytokinesis may still eventually be attempted in some cells. Consistent with the latter possibility, we have observed the accumulation of cells with a cut-like phenotype: 10% of all cells exhibit this phenotype after 5 hr at the nonpermissive temperature. Bernat et al. (1990) describe a similar cut-like phenotype after injection of mammalian cells with anti-centromere antibodies and propose that it is a result of the cells' eventual attempt to undergo cytokinesis after prolonged mitotic arrest. Because it is not known whether this subset of the *ctf13-30* population is still capable of dividing, it is unclear whether cytokinesis has trapped nuclei in these cells or whether they are caught undergoing nuclear transits at the time of fixation (Palmer et al., 1989).

The terminal phenotype of *ctf13-30* is quite different from the terminal phenotype of the other described temperature-sensitive kinetochore mutant, *ndc10-1* (Goh and Kilmartin, 1993). *ndc10-1* mutants exhibit detachment of the chromosomes from one spindle pole and progression through the cell cycle in the absence of chromosome segregation (most cells produce one aploid daughter and one daughter of increased ploidy). If there is checkpoint control exerted in response to events at the kinetochore, the *ndc10-1* defect is not recognized. Perhaps this is because the *NDC10* protein itself is involved in the recognition and/ or signaling of a dysfunctional complex, or alternatively, checkpoint control may be disabled by complete disruption of kinetochore structure. Future experiments addressing the relationships of kinetochore proteins to the control of progression through mitosis should help define important molecular determinants of the temporal order of events in chromosome segregation.

Will the Molecular Dissection of the S. cerevisiae Kinetochore Aid the Understanding of Kinetochore Function in More Complex Eukaryotes?

At this time, analysis of the DNA sequence and protein component requirements of the kinetochore is significantly more advanced in S. cerevisiae than in any other eukaryotic organism, although there is great speculation about the relevance of these studies to the understanding of the much larger and morphologically more elaborate kinetochores present in other eukaryotes. While there may be a need for additional components to ensure fidelity in more complex eukaryotes, we think it is probable that the basic mechanisms of the segregational process, including those involved in centromere function, will have been conserved through evolution. A repeat subunit model for the centromere–kinetochore complex has recently been proposed by Zinkowski et al. (1991). This model describes the kinetochore as organized in multiple small repeat units that fold together into a contiguous plate-like structure when condensed at metaphase. Zinkowski et al.

propose that each unit is capable of microtubule binding and segregational function. In this context, the S. cerevisiae kinetochore, which binds a single microtubule, could represent the simplest ancestral unit of the eukaryotic kinetochore (Fitzgerald-Hayes et al., 1982; Koshland et al., 1987).

Identification and characterization of the S. cerevisiae kinetochore components will facilitate the definition of the activities necessary for the completion of proper mitotic segregation in this organism and may well provide substrates for the identification of kinetochore components in other eukaryotic organisms.

Experimental Procedures

Yeast Strains and Media

The ctf and wild-type parental strains containing chromosome fragments that can be monitored by a visual assay have been previously described (Spencer et al., 1990; Shero et al., 1991). The ctf collection of 136 originally isolated mutants can be represented in 18 complementation groups and 41 single isolates. All ctf mutant isolates that are members of a complementation group retain the original isolate number as an allele number (e.g., s30 contains ctf13-30). One member of each complementation group (the isolate with the most severe sectoring phenotype) and 19 single isolates (those that were his3⁻) were tested in the two kinetochore screens. All ctf mutant strains tested have the genotype of the original parent, YPH278 MATa ura3-52 lys2-801 ade2-101 his3-Δ200 leu2-Δ1 CFIII (CEN3.L.YPH278) URA3 SUP11, unless otherwise indicated.

s30 derivatives used in further phenotypic characterization of the ctf13-30 mutation were: YPH973 MATa/MATα ura3-52/ura3-52 lys2-801/lys2-801 ade2-101/ade2-101 HIS3/his3-Δ200 trp1-Δ1/TRP1 leu2-Δ1/leu2-Δ1 ctf13-30/ctf13-30 CFIII (CEN3.L.YPH278) URA3 SUP11 (isogenic wild-type strain is YPH280), YPH972 MATα ura3-52 lys2-801 ade2-101 his3-Δ200 trp1-Δ1 leu2-Δ1 ctf13-30 CFIII (CEN3.L.YPH278) URA3 SUP11, YPH974 MATa/MATα ura3-52/ura3-52 lys2-801/lys2-801 ade2-101/ade2-101 his3-Δ200/his3-Δ200 trp1-Δ1/trp1-Δ1 leu2-Δ1/leu2-Δ1 CTF13/ctf13-30 CFVII (RAD2.d.YPH275) TRP1 SUP11, YPH975 MATa ura3-52 lys2-801 ade2-101 his3-Δ200 trp1-Δ1 leu2-Δ1 ctf13Δ1::HIS3 pRS316/CTF13 CFVII (RAD2.d.YPH275) TRP1 SUP11, and YPH976 MATa/MATα ura3-52/ura3-52 lys2-801/lys2-801 ade2-101/ade2-101 his3-Δ200/his3-Δ200 trp1-Δ1/TRP1 LEU2/leu2-Δ1 ctf13-30/ctf13-30 (isogenic wild-type strain is YPH699). Media for yeast growth and sporulation were as described (Rose et al., 1990), except that where sectoring was examined, adenine was added to 6 μg/ml to minimal (SD) medium to enhance the development of red pigment in ade2-101 strains. X-gal plates were made as described for synthetic complete (SC) medium (Rose et al., 1990) except for the addition of 0.1 M NaPO₄ (pH 6.8) and 40 μg/ml X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) (use a 20 mg/ml stock in dimethylformamide). All yeast transformations were done by the method of Ito et al. (1983).

Transcriptional Readthrough Assay

The reporter construct schematically pictured in Figure 1 was modified from pGAB (U. Vijayraghavan and J. Abelson, unpublished data) obtained from R. Parker. (pGAB is a modification of pYAHB2 [Vijayraghavan et al., 1986], a CEN–ARS plasmid containing an actin–lacZ fusion gene that has been used extensively to study splicing in S. cerevisiae [Vijayraghavan et al., 1986; Cellini et al., 1986]. EcoRI restriction sites were created in pYAHB2 just upstream of the actin AUG by J. Rossi and just after the lacZ sequence, through the conversion of BalI to EcoRI, by U. Vijayraghavan. The resulting actin–lacZ EcoRI fragment was inserted into the EcoRI site of pBM150 [Johnston and Davis, 1984], placing it under control of the GAL10 promoter.)

We introduced a unique HindIII site into the XhoI site of pGAB at position 62 of the actin intron by ligation to a 14 bp double-stranded oligonucleotide containing an internal HindIII site with XhoI half-sites at each end. The resulting plasmid is pKF18. Test centromeres were obtained by polymerase chain reaction amplification of CEN6 sequences (Mullis and Faloona, 1987) and inserted into the actin intron

at the unique HindIII site of pKF18. Polymerase chain reaction products were created with oligonucleotides containing CEN6 sequences (boldface) plus a HindIII site at the 5′ ends. Oligonucleotides OKF4, CCGGAAGCTTATAATACTAATTTCTAAC, and OKF5, CCGGAAG-CTTGAAGACTATATTTC, amplify a 186 bp CEN6 product that includes 19 bp to the left of CDEI, CDEI, CDEII, CDEIII and 29 bp to the right of CDEIII. The wild-type and mutant (CDEIII-15C) test CEN6 sequences were amplified from pJS2 and pJS18, respectively (Hegemann et al., 1988). The DNA sequence and orientation of the test CEN6 sequences in pKF18 were verified by sequencing (Sanger et al., 1977; Hattori and Sakaki, 1986). The resulting plasmids, pKF19 and pKF44, contain wild-type and mutant (CDEIII-15C) CEN6 sequences, respectively, in the orientation placing CDEI 5′ to CDEIII. In control experiments, pKF18, pKF19, and pKF44 were transformed into the wild-type strain YPH102 MATα ura3-52 lys2-801 ade2-101 his3-Δ200 leu2-Δ1, and β-galactosidase assays were done on independent URA⁺ transformants (see Figure 1). The structurally dicentric plasmids (all YCp50 derivatives) were maintained in a functionally monocentric state by keeping transformed strains on medium containing galactose as the carbon source causing transcriptional inactivation of the test centromeres (Hill and Bloom, 1987). β-galactosidase assays were performed essentially as described (Rose et al., 1990), following the protocol for assay of crude extracts, except that cells were grown in SCGal–His liquid medium or scraped off SCGal–His plates. The values for optical density at 420 nm were zeroed to an isogenic yeast strain that did not contain a reporter plasmid.

For screening of the ctf collection, the reporter was integrated into chromosome XV as follows: the GAL1-10–actin-test CEN6–lacZ fragment described above was inserted into a genomic XhoI site immediately 3′ to the HIS3 gene contained on a pBR322-based plasmid, pSZ62-XbaI (McCleod et al., 1986), kindly provided by J. Broach. The resulting BamHI fragment containing HIS3 and the test reporter fragment was transformed into YPH278 selecting for replacement of the his3Δ200 locus on chromosme XV. Independent His⁺ transformants were picked and analyzed by Southern blotting to verify insertion of the reporter construct into chromosome XV at the HIS3 locus. pKF71 contains the wild-type CEN6 reporter, and pKF72 contains the mutant CDEIII (15C) CEN6 reporter inserted into the HIS3 BamHI fragment. YPH977 and YPH978 contain the pKF71- and pKF72-derived BamHI fragments, respectively, and were maintained in medium containing galactose. Strains were tested for the production of blue color on medium containing the chromogenic substrate of β-galactosidase, X-gal. YPH977 colonies appear white (this progresses to a very faint blue color after several days), while YPH978 colonies develop a deep blue color.

The reporter containing the wild-type CEN6 (pKF71) was inserted into chromosome XV in each of the ctf mutants as follows. Each ctf strain was made competent for transformation in SC medium containing 2% galactose and transformed with the BamHI fragment of pKF71. Transformants were selected, and the colony was purified on SCGal–His plates at 25°C. Two independent transformants of each ctf strain tested were then plated at a low density (~ 200 colonies per plate) on SCGal–His plates containing 40 μg/ml X-gal and grown at 25°C. The development of blue colony color was monitored in comparison with that of the control strains YPH977 and YPH978. YPH978 colonies developed a deep blue color after 9 days, while YPH977 colonies remained white at this time point. ctf strains were scored as positive for the transcriptional readthrough assay if they displayed an intermediate blue colony color phenotype after 9 days.

The ctf strains tested with the transcriptional readthrough assay were: YPH754 MATα ura3-52 lys2-801 ade2-101 his3-Δ200 trp1-Δ1 ctf1Δ1::TRP1, s59 (ctf2), s2 (ctf3), s50 (ctf4), s31 (ctf5), s53 (ctf6), s10 (ctf7), s9 (ctf8), s16 (ctf9), s69 (ctf10), s67 (ctf11), s18 (ctf12), s30 (ctf13), s42 (ctf14), YPH980 MATa his3-Δ200 ade2-101 lys2-801 leu2-Δ1 ctf16-124 CFVII (RAD2.d.YPH275) URA3 SUP11, s61 (ctf17), YPH979 MATa lys2-801 ade2-101 his3-Δ200 trp1-Δ1 ctf18Δ1::URA3, s3, s4, s11, s17, s22, s26, s41, s47, s48, s49, s54, s55, s56, s58, s62, s63, and s64.

Stabilization of a Dicentric Chromosome Fragment Assay

The test dicentric chromosome used in this assay was constructed by inserting a conditional centromere into the nonessential chromosome fragment present in the ctf strains screened. A HIS3-containing vector

carrying the conditional centromere was constructed that would direct a γ integration event (Sikorski and Hieter, 1989) at the *leu2-Δ1* locus present on the left arm of chromosome III. Polymerase chain reaction products derived from sequences to the 5' (left) and the 3' (right) side of the *leu2Δ1* locus (Sikorski and Hieter, 1989) were blunted into the SacI and SpeI sites, respectively, of pRS303 (Sikorski and Hieter, 1989), creating vector p679. The conditional mutant *CEN* (CDEIII-15C) containing fragment derived from pKF44 was inserted into the BamHI and EcoRI sites of p679, resulting in pKF76. The conditional nearly wild-type *CEN*-containing fragment derived from pKF45 was blunt end ligated into the SmaI site of p679, resulting in pKF77. (pKF45 has the same basic structure as pKF19, except that the *CEN6* sequence inserted into the unique HindIII site of pKF18 is present in the opposite orientation and contains CDEII, CDEIII and 29 bp to the right of CDEIII. This *CEN6* sequence [ΔCDEI] was amplified from pJS2 [Hegemann et al., 1988] using oligonucleotides OKF7, CCGGAAGCTTCTATAA AAATAATTATAAT, and OKF4.) NotI-linearized p679, pKF76, and pKF77 direct integration at either the *leu2-Δ1* locus present on the chromosome fragment or the *leu2-Δ1* locus present on the endogenous chromosome III. The test centromeres were maintained in an inactive state by growth of strains in galactose-containing medium. His⁺ transformants demonstrating mitotic linkage between the HIS3 locus present on the integrating vector and the URA3 locus present on the chromosome fragment were identified and used in the dicentric stabilization assay. By the same procedure, *ctf* mutant strains were obtained containing p679 (no *CEN*) and pKF77 (ΔCDEI *CEN*) sequences integrated into the nonessential chromosome fragment at the *leu2-Δ1* locus.

Two p679 and two pKF77 transformants of each *ctf* strain tested were streaked onto synthetic complete dextrose plates containing a limiting amount of adenine. The switch to dextrose as a carbon source causes the *GAL10* promoter to be turned off, resulting in activation of the second conditional centromere and a functionally dicentric chromosome fragment. Sectoring phenotypes were directly compared with those of a YPH278 p679 or pKF77 transformant streaked onto the same plate.

The *ctf* strains tested with the stabilization of a dicentric assay were: s59 (ctf2), s50 (ctf4), s31 (ctf5), s53 (ctf6), s10 (ctf7), s9 (ctf8), s16 (ctf9), s67 (ctf11), s18 (ctf12), s30 (ctf13), s42 (ctf14), YPH980 *MATa his3-Δ200 ade2-101 lys2-801 leu2-Δ1 ctf16-124 CFVII (RAD2.d.YPH-275) URA3 SUP11*, s61 (ctf17), YPH981 *MATa ura3-52 lys2-801 ade2-101 his3-Δ200 trp1-Δ1 leu2-Δ1 ctf18-160 CFIII (CEN3.L.YPH278) URA3 SUP11*, s3, s4, s12, s17, s20, s22, s41, s47, s55, s56, s62, s63, and s64.

s31 (ctf5) and s20 were unscorable in this screen because the chromosome fragment present in the p679 derivative strains was extremely unstable. True positives were verified in multiple independent transformants. One source of false positives was transformants containing two chromosome fragments, only one of which carried the conditional secondary centromere. These false positives were easily identified by the demonstration that sectored colonies were His⁺. For s30 (ctf13), the phenotype of stabilization of the test dicentric chromosome was shown to be due to a mutation in the *CTF13* gene product by transformation of a pKF76 derivative of s30 with pKF11, a pRS314-based (Sikorski and Hieter, 1989) plasmid carrying the *CTF13* locus. The presence of the wild-type *CTF13* gene product resulted in destabilization of the dicentric chromosome fragment back to the level seen in the wild-type parent, YPH278.

Molecular Characterization of *CTF13*

The 2.2 kb Sau3A subclone that rescues the temperature sensitivity of s30 (ctf13) was inserted into the polylinker of pRS314 (Sikorski and Hieter, 1989), resulting in pKF11. Deletion derivatives of the pKF11 insert were made using existing restriction sites (see Figure 5). A BglII to polylinker deletion, as well as a ClaI to polylinker deletion, was unable to rescue the temperature sensitivity of s30 (ctf13). The DNA sequence encoding the entire ORF was determined using a set of unidirectional deletions (Henikoff, 1987) by standard methods (Sanger et al., 1977; Hattori and Sakaki, 1986). The sequence of the second strand of the ORF was obtained using synthetic oligonucleotides as primers.

The *CTF13* clone was shown to correspond to the *ctf13-30* locus, and *CTF13* was shown to be an essential gene in S. cerevisiae by

using the *CTF13* clone to direct an integration event (Sikorski and Hieter, 1989) that replaced a majority of the *CTF13* ORF with vector and *HIS3* sequences. The integration vector, pKF93, was constructed by inserting the ~800 bp BglII (polylinker)-BglII fragment and the ~200 bp ClaI-EcoRI (polylinker) fragment from pKF11 (see Figure 5B) into the BamHI site and ClaI-EcoRI sites of pRS303 (Sikorski and Hieter, 1989), respectively. pKF93 was linearized with EcoRI and transformed into a *ctf13-30*/CTF13 heterozygous diploid strain, YPH974, selecting for His⁺ transformants. Integration of pKF93 should delete the *CTF13* ORF from amino acid 57 to amino acid 467 (see Figure 5B). Approximately half of the His⁺ diploid transformants obtained exhibited the *ctf13-30* sectoring phenotype, indicating that the *ctf13-30* locus was being targeted by pKF93. The integration of pKF93 and deletion of *CTF13* sequences was confirmed by Southern analysis (data not shown). Two sectoring diploid isolates (*CTF13* locus deleted) and two nonsectoring diploid isolates (*ctf13-30* locus deleted) were sporulated, and tetrads were dissected. Viability segregated 2:2 in all 31 tetrads dissected, and all viable spores were his⁻. All viable spores resulting from the sectoring diploids were temperature sensitive, and all of the viable spores resulting from the nonsectoring diploids were not temperature sensitive.

CTF13 was physically mapped by the previously described method of chromosome fragmentation (Gerring et al., 1990a), using the 2.2 kb *CTF13* fragment. The sizes of the resulting stable chromosome fragments were determined by orthogonal field–alteration gel electrophoresis (OFAGE) analysis (Carle and Olson, 1984), and assignment of CTF13 to an arm of chromosome XIII was accomplished by hybridization of a left arm telomere–adjacent probe, *TUB3*, to a Southern blot of the OFAGE gel. *TUB3* was obtained from P. Schatz, and the probe used was a 1.2 kb HindIII fragment, radioactively labeled with ³²P (Feinberg and Vogelstein, 1984). *TUB3* hybridized to the 445 kb proximal fragment. To obtain a meiotic map position, a diploid strain was constructed that was heterozygous for *ctf13* and *cin4* (*ctf13-30/+*, *+/cin4::URA3*). The meiotic distance was calculated from the following data by using the formula of Perkins: *ctf13-cin4* 34 cM (parental ditype/ nonparental ditype/tetratype = 42/2/56). *CTF13* was placed proximal to *cin4* by probing the *CTF13* chromosome fragmentation OFAGE blots with a 2 kb SacI–KpnI *CIN4* fragment obtained from A. Hoyt. *CIN4* hybridized to the 475 kb distal *CTF13* chromosome fragment, placing *CTF13* proximal to *CIN4*.

Biochemical Analysis

The plasmid containing the E1 tag fused to CTF13, pKF80, was constructed from the base plasmid p414GEU1 (J. Kroll, unpublished data). p414GEU1 has a 460 bp *GAL1* promoter fragment cloned into the KpnI site and two tandem copies of the E1 tag sequence, described by Pluta et al. (1992), inserted in frame into the ApaI and XhoI sites of pRS414 (Sikorski and Hieter, 1989). The *GAL1* promoter directs transcription from its own ATG toward the polylinker. An EcoRI fragment containing the entire 2.2 kb insert of pKF11 was cloned into the EcoRI site of p414GEU1 in the appropriate transcriptional orientation. The 5' ~800 bp of the *CTF13*-containing fragment (up to the BglII site; see Figure 5B) were removed and replaced with an ~200 bp polymerase chain reaction product containing sequences from the ATG of *CTF13* to the BglII site. This allowed the in-frame fusion of the tandem E1 tags to *CTF13* under the transcriptional control of *GAL1* (see Figure 5C). pKF80 was transformed into YPH972 and shown to rescue the temperature sensitivity caused by the *ctf13-30* mutation on both galactose- and dextrose-containing media.

The plasmid containing the HA epitope fused to CTF13, pSF197a, was constructed by using a synthetic oligonucleotide to fuse the HA epitope and linker sequences to the amino terminus of CTF13 (see Figure 5C). The fusion protein and ~200 bp of 3' noncoding sequence from the CTF13 locus were cloned into pRS315 (Sikorski and Hieter, 1989), downstream of a 625 bp fragment of 5' flanking DNA that is presumed to include the *CTF13* promoter. pSF197a was transformed into YPH975. Transformants were streaked onto medium containing 5-fluoroorotic acid to select against the CTF13–URA3 plasmid (Boeke et al., 1987), and it was shown that pSF197a would rescue viability in the resulting *ctf13Δ1::HIS3* strain.

The preparation and analysis of CBF3–DNA complexes was performed using a modification of procedures previously described by Lechner and Carbon (1991). Cells in log phase were harvested by

centrifugation, frozen in liquid nitrogen, and mechanically disrupted by fragmentation with a liquid nitrogen–cooled mortar and pestle in 30 mM sodium phosphate (pH 7.0), 60 mM β-glycerophosphate, 1 M KCl, 6 mM EGTA, 6 mM EDTA, 6 mM NaF, 10% glycerol, 1 mM phenyl-methylsulfonyl fluoride, and 10 μg/ml (each) leupeptin, pepstatin, and chymostatin. Whole-cell extract (40 μg) was incubated for 30 min at room temperature with 20 fmol of ^{32}P-labeled DNA probe, 5 μg of salmon sperm DNA, 5 μg of poly(dI–dC), and 10 μg of bovine serum albumin in 30 μl of 10 mM HEPES (pH 8.0), 1 mM NaF, 6 mM MgCl₂, 10% glycerol, and KCl at a final concentration of 125 mM. The 88 bp DNA probe was derived from CEN3 and spans the core region of CDEIII, from 5 bp to the left of CDEIII to 59 bp to the right of CDEIII. Binding reactions were electrophoresed on 4% polyacrylamide gels as described (Ng and Carbon, 1987) and visualized by autoradiography.

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