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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. clf13-30 and clf13 mutants missegregate chromosomes at permissive temperature and transiently arrest at nonpermissive temperature as large-budded cells with a 62 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 establish checkpoints and regulate within the cell cycle. Biochemical purification of kinetochore proteins through sequence-specific affinity purification with CEN DNA (Ng and Carbon, 1967; Lechner and Carbon, 1991) has proven to be difficult, apparently owing to the low abundance of the kinetochore proteins.

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 Newton, 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; Piuta 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 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., 1962; Flavin et al., 1964; Funk et al., 1989). CDEI (8 bp) and CDEII (25 bp) exhibit dyad symmetry and are separated by CDEIII, a 76–86 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, 1997; 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, CFF1 (also known as CP1 or CBF1), has been extensively characterized (Baker and Maisin, 1990; Mellor et al., 1990; Cai and Davis, 1990). CFF1 is a member of the helix-loop-helix family of DNA-binding proteins and binds as a homodimer to CDEI. A null mutation in cff1 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-
The amino-terminal actin ORF is represented by the stippled boxes, coding sequence is shown as a hatched box. CEN DNA is indicated CDEII, and CDEIII, respectively. Transcription initiation from GAL70 transcription of a multicomponent protein-CEN DNA complex, been isolated with the primary criterion of chromosome ochore components. Several mutant collections have missegregation, including the ctf(chromosome transmission, some of which are expected to encode kinetochore components. 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 transmission 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 ctf 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. Phillipson, 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 CEN (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-
Secondary Screens

CTF13 Is an Essential Kinetochore Protein

Table 1. cff Mutants Identified in Kinetochore Integrity Secondary Screens

| Transcriptional Readthrough Screen | DICentric Chromosome Stabilization Screen |
|-----------------------------------|------------------------------------------|
| s10 (ctf7)                        | s26                                      |
| s9 (ctf6)                         | s26                                      |
| s30 (ctf13)                       | s58                                      |
| s42 (ctf14)                       |                                          |
| s61 (ctf17)                       |                                          |

The second assay we developed to screen for kinetochore mutants among the cff 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 cff 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 leu2-1t1 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 (ACDEI) or a highly defective (CDEIII-15C) secondary conditional CEN (Figures 3A and 3B). We predicted that upon activation of a relaxed transcriptional block by the colony color assay (Table 2).

Table 2: Quantitation of Transcriptional Readthrough in cff Mutants

| Strain | Transcription Block | β-Galactosidase (nmol/min per mg of protein) |
|--------|---------------------|---------------------------------------------|
| YPH278 (CTF+) | CEN6 (CDEIII-15C) | 22 ± 8                                      |
| YPH278 (CTF)  | CEN6 (CDEIII-15C) | 19 ± 6                                      |
| s16 (ctf9)  | CEN6 (CDEIII-15C) | 16 ± 9                                      |
| s10 (ctf7)  | CEN6 (CDEIII-15C) | 6 ± 12                                      |
| s9 (ctf6)   | CEN6 (CDEIII-15C) | 29 ± 12                                     |
| s30 (ctf13) | CEN6 (CDEIII-15C) | 24 ± 12                                     |
| s42 (ctf14) | CEN6 (CDEIII-15C) | 23 ± 12                                     |
| s61 (ctf17) | CEN6 (CDEIII-15C) | 24 ± 12                                     |
| 127 ± 42   | CEN6 (CDEIII-15C) | 72 ± 25                                     |

Assays were performed on strains grown at 30°C. CEN6*, wild-type CEN6.
* s16(ctf9) was not identified as a putative kinetochore mutant by the transcriptional readthrough assay and serves as a negative control.
* s42(ctf14) is inviable at 30°C.
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 monitoring phenotypes that resulted, shown in Figure 4, were mutants among the cff collection (Figure 3C).

To screen the cff mutants for stabilization of a dicentric chromosome, the conditional nearly wild-type CEN, \( \Delta CEI \), was integrated into the chromosome fragment present in each strain. The strains were maintained on galactose to induce the \( \text{GAL}10 \) promoter and inactivate the conditional CEN (see Experimental Procedures and Figure 3A). Two transformants of each cff strain were streaked onto medium containing dextrose to activate the GAL10 promoter. The stability of the dicentric chromosome fragment in the cff strains was visually assessed and compared with the stability of the dicentric chromosome fragment in the \( CTF^* \) strain. If the dicentric chromosome fragment was as unstable as in the wild-type background (Figure 3B), the cff strain was scored negative in this assay. With 27 cff 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 those mutants are similar to that seen with a test dicentric chromosome carrying a weak secondary CEN.

Two cff 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 2. Diagram of the Two Chromatids of a Dicentric Chromosome](image)

(A) 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.

![Figure 3. Dicentric Stabilization Assay](image)

(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\(^{+\text{wt}}\)) and highly defective (CEN\(^{\text{wt}}\)) 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\(^{\text{wt}}\)).

(C) Proposed reduced sectoring phenotype, indicative of stabilization of the test dicentric chromosome fragment. Though the secondary CEN is nearly wild type (CEN\(^{\text{wt}}\)), mutation of a kinetochore protein component should result in a relatively mild red sectoring phenotype.
CTF13 is an essential kinetochore protein.

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 GenBank, GenPept, GUpdate, 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 method 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

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).
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 El 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 GAL7 promoter (Figure 5C). Both epitope-tagged CTF13 derivatives were able to rescue viability in a ctf13 Al::HIS3 null strain.

Extracts were prepared from cells carrying either wild-type or epitope-tagged CTF13, reacted with 32P-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 of ctf13 mutants 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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**Figure 6. Nucleotide and Predicted Protein Sequence of CTF13**

The amino acid translation, beginning with the first methionine of the ORF, is shown in frame in the amino terminus of CTF13 (Figure 5C). In the second construct, two tandem copies of the El 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 GAL7 promoter (Figure 5C). Both epitope-tagged CTF13 derivatives were able to rescue viability in a ctf13 Al::HIS3 null strain.

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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) and incubated with antibodies at various dilutions: lanes 7–9, extracts from ctf13::HIS3 null cells carrying an E1 epitope-CTF13 fusion (see Figure 5C) and incubated with antibodies at various dilutions: lanes 7–9, extracts from ctf13::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 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.

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 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.

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| Table 3. Rates of Missegregation Events in ctf13 Mutants at 25°C |
|-----------------|--------------|--------|
|                 | ctf13-30/   | CTF13/  |
|                 | ctf13-30    | CTF13  |
| Chromosome IIIa |              |        |
|                 |              | Ratio  |
| Missegregation  |              |        |
| (x 10^-5)      | 1.0 events  | 2.1    |
|                 | (x 10^-5)   | 0.04   |
|                 |              | 53     |
| Recombination   |              | 2.0    |
| (x 10^-5)      | 3.0          | 0.05   |
|                 |              | 60     |

a The rates of loss (1:0) and nondisjunction (2:0) events measured for the nonessential chromosome fragment present in YPH793 and YPH280. Methods were as described in Gerrits et al. (1990b). The numbers of colonies scored were 3,380 for YPH793 and 22,184 for YPH280.

b The rates of mitotic missegregation and recombination for chromosome III present in YPH786 and YPH899. Methods were as described in Gerrits et al. (1990b).
Figure 6. Phenotypic Analysis of cff13-30 (A) GZ/M accumulation at the permissive temperature and G2 arrest at nonpermissive temperature in cff13-30 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 YPH260. 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 cff73-30 in a cff13-30 background, while only 2% of wild-type cells had this morphology (Figure 8C).

cff13-30 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 cff13-30 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 cff13-30 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 cff13-30 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 kDa CBF3 Subunit

The cff strain s42 (ctf14) was also identified by both secondary screens as a putative kinetochore mutant. A clone that complemented the temperature sensitivity of clf73-42 was obtained and mapped to chromosome VII essentially as described for clf73-30 (data not shown). Nuclear division cycle 10 (NDC10), recently identified by Goh and Kil-

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26°C
CTF13/CTF13 2 5 88 ND
ctf13-30/ctf13-30 13 7 66 ND
38°C, 3 hrs.
CTF13/CTF13 20 17 44 ND
cff13-30/ctf13-30 9 9 3 ND
38°C, 7 hrs.
CFF13/CTF13 9 14 68 0/0
cff13-30/ctf13-30 45 32 15 2/2

Figure 8. Phenotypic Analysis of cff13-30

(A) G2/M accumulation at the permissive temperature and G2 arrest at nonpermissive temperature in cff13-30 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 YPH793 and YPH260. 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 cff13 30

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CTF14 Encodes the 110 kDa CBF3 Subunit

The cff strain s42 (ctf14) was also identified by both secondary screens as a putative kinetochore mutant. A clone that complemented the temperature sensitivity of cff14-42 was obtained and mapped to chromosome VII essentially as described for cff13-30 (data not shown). Nuclear division cycle 10 (NDC10), recently identified by Goh and Kil-

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26°C
CTF13/CTF13 2 5 88 ND
ctf13-30/ctf13-30 13 7 66 ND
38°C, 3 hrs.
CTF13/CTF13 20 17 44 ND
cff13-30/ctf13-30 9 9 3 ND
38°C, 7 hrs.
CFF13/CTF13 9 14 68 0/0
cff13-30/ctf13-30 45 32 15 2/2

Figure 8. Phenotypic Analysis of cff13-30

(A) G2/M accumulation at the permissive temperature and G2 arrest at nonpermissive temperature in cff13-30 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 YPH793 and YPH260. 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 cff13 30

in a cff13-30 background, while only 2% of wild-type cells had this morphology (Figure 8C).

cff13-30 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 cff13-30 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 cff13-30 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 cff13-30 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 kDa CBF3 Subunit

The cff strain s42 (ctf14) was also identified by both secondary screens as a putative kinetochore mutant. A clone that complemented the temperature sensitivity of clf73-42 was obtained and mapped to chromosome VII essentially as described for clf73-30 (data not shown). Nuclear division cycle 10 (NDC10), recently identified by Goh and Kil-
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 cff collection, Spencer et al., 1990) for altered kinetochore integrity. In combination, those 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 kinetochore 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 (Loohnor 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 (ctf 13.1::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 segregation 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
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, an aberrant kinetochore on a single chromosome can cause a mitotic delay (Spencer and Hieter, 1992).

\textbf{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, an aberrant kinetochore on a single chromosome can cause a mitotic delay (Spencer and Hieter, 1992).

\textbf{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 between 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.

\textbf{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 \textit{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.\footnotemark
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 cff 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 cff collection of 136 originally isolated mutants can be represented in 18 complementation groups and 41 single isolates. All cff mutant isolates that are members of a complementation group retain the original isolate number as an allele number (e.g., s30 contains cff13-30). One member of each complementation group (the isolate with the most severe segregating phenotype) and 19 single isolates (those that were his3-) were tested in the two kinetochore screens. All cff mutant strains tested have the genotype of the original parent, YPH278 (ura3-52 lys2-801 ade2-101 his3-1200 leu2-111 CFI1 [CEN3.L. YPH278]) URA3 SUP11, unless otherwise indicated.

s30 derivatives used in further phenotypic characterization of the cff13-30 mutation were YPH793 MATa/MATc ura3-52 lys2-801 ade2-101 his3-1200 trpl-11111 CFI1 [CEN3.L. YPH278] URA3 SUP11, YPH794 MATa/MATc ura3-52 lys2-801 ade2-101 his3-1200 trpl-111111 df11-11 CFI1 [CEN3.L. YPH278] URA3 SUP11, YPH795 MATa/MATc ura3-52 lys2-801 ade2-101 his3-1200 trpl-111111 df11-11 CFI1 [CEN3.L. YPH278] URA3 SUP11, and YPH796 MATa/MATc ura3-52 lys2-801 ade2-101 his3-1200 trpl-111111 df11-11 CFI1 [CEN3.L. YPH278] URA3 SUP11, respectively.

CTF13 is an Essential Kinetochore Protein

In this context, the S. cerevisiae kinetochore contains the simplest ancestral unit of the eukaryotic kinetochore (Fitzgerald-Hayes et al., 1982; Koshland et al., 1987).

The reporter construct schematically pictured in Figure 1 was modified to incorporate two new HindIII sites (Mullis and Faloona, 1987). The wild-type strain YPH276 selecting for replacement of the his3-200 locus on chromosome XV. All yeast strains did not contain a reporter plasmid.

For screening of the cff 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, pSB2–XhoI (McLeod et al., 1986), kindly provided by J. Broach. The resulting S. cerevisiae strain containing HIS3 and the lacZ reporter fragment was transformed into YPH278 selecting for replacement of the his3-1200 locus on chromosome XV. Individual His- transformants were picked and analyzed by Southern blotting to verify insertion of the reporter construct into chromosome XV. The resulting S. cerevisiae strain containing HIS3 and the lacZ reporter inserted into the HIS3 BamHI fragment YPH797, and the resulting S. cerevisiae strain containing HIS3 and the lacZ reporter inserted into the HIS3 BamHI fragment YPH797 contains the pck72-1 and pck72-2-derived DAPI 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 beta-galactosidase, X-gal. YPH797 colonies appear white (this progresses to a very faint blue color after several days), while YPH797 colonies develop a deep blue color.

The reporter containing the wild-type CEN6 (pck71) was inserted into chromosome XV in each of the cff mutants as follows. Each cff strain was made competent for transformation in synthetic complete (SC) medium by transformation with the wild-type CEN6 reporter, and p-galactosidase assays were done on independent transformants and the lacZ reporter inserted into the HIS3 BamHI fragment YPH4477, and the resulting S. cerevisiae strain containing HIS3 and the lacZ reporter inserted into the HIS3 BamHI fragment YPH797 contains the pck71-1 and pck72-1-derived DAPI 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 beta-galactosidase, X-gal. X-gal YPH797 colonies appear white (this progresses to a very faint blue color after several days), while YPH797 colonies develop a deep blue color.

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 cff strains. A HIS3-containing vector was used in the construction of the test dicentric chromosome.
The plasmid containing the HA epitope fused to CTF13, pSF197a, 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 Apal and Xhol 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 EcoR1 site of p414GEU1 in the appropriate translational orientation. The 5' - 600 bp of the CTF13-containing fragment (up to the BgIII 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 YPH792 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). pSF197a was transformed into YPH972 and shown to rescue the temperature sensitivity (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, +/chr:URA3). The meiotic distance was calculated from the following data by using the formulas of Perkins: ctf13-cin4 34 cm (parental diploids: nonparental diploids/tetratype = 42/256). CTF13 was placed proximal to cin4 by probing the CTF13 chromosome fragment OFAGE blots with a 2 kb Sacl–Kpnl 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 Apal and Xhol 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 EcoR1 site of p414GEU1 in the appropriate translational orientation. The 5' - 600 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 YPH792 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 the CTF13 locating fragment was also cloned into pRS315 (Sikorski and Hieter, 1989), downstream of 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 YPH792 and shown to rescue the temperature sensitivity caused by the ctf13-30 mutation on both galactose- and dextrose-containing media.

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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 fructose-1,6-bisphosphate, 1 mM KCl, 6 mM EGTA, 6 mM EDTA, 6 mM NaF, 10% glycerol, 1 mM phenylmethanesulfonyl 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 35S-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 CDEII, from 5 bp to the left of CDEII to 9 bp to the right of CDEII. Binding reactions were ectoprophosphorylated on 4% polyacrylamide gels as described (Nig and Carbon, 1987) and visualized by autoradiography.

Acknowledgments

We thank C. Connelly for significant contributions to this work. We would like to thank J. Kilmartin, J. Carbon, and J. Lechner for communicating results prior to publication. We thank D. Koshland, W. Earnshaw, and T. Kelly for critical reading of the manuscript. K. F. D. is a student in the predoctoral training program in human genetics at Johns Hopkins (National Institute of General Medical Sciences grant P32GM07814). G. T. is supported by the National Institutes of Health Departmental Training Grant 5T32CA09139. P. K. S. and I. A. H. are biomedical scholars of the Lucille P. Markey Charitable Trust. This work was supported in part by a National Institutes of Health grant (CA18639) to P. H. and an American Cancer Society grant (CD-509) to F. S.

Received January 25, 1993; revised March 10, 1993.

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GenBank Accession Number

The accession number for the CTF73 sequence reported in this paper is L10993.