Ctf19p: A Novel Kinetochore Protein in Saccharomyces cerevisiae and a Potential Link between the Kinetochore and Mitotic Spindle

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Abstract. A genetic synthetic dosage lethality (SDL) screen using CTF13 encoding a known kinetochore protein as the overexpressed reference gene identified two chromosome transmission fidelity (ctf) mutants, YCTF58 and YCTF26. These mutant strains carry independent alleles of a novel gene, which we have designated CTF19. In light of its potential role in kinetochore function, we have cloned and characterized the CTF19 gene in detail. CTF19 encodes a nonessential 369–amino acid protein. ctf19 mutant strains display a severe chromosome missegregation phenotype, are hypersensitive to benomyl, and accumulate at G2/M in cycling cells. CTF19 genetically interacts with kinetochore structural mutants and mitotic checkpoint mutants. In addition, ctf19 mutants show a defect in the ability of centromeres on minichromosomes to bind microtubules in an in vitro assay. In vivo cross-linking and chromatin immunoprecipitation demonstrates that Ctf19p specifically interacts with CEN DNA. Furthermore, Ctf19-HA p localizes to the nuclear face of the spindle pole body and genetically interacts with a spindle-associated protein. We propose that Ctf19p is part of a macromolecular kinetochore complex, which may function as a link between the kinetochore and the mitotic spindle.

Key words: centromere • kinetochores • chromosome segregation • mitotic spindle apparatus • Saccharomyces cerevisiae

Mitotic cell division is a process which ensures that a cell’s chromosomes are faithfully replicated and equally segregated to two daughter cells. The kinetochore (centromere DNA and associated proteins) is essential to the high fidelity of chromosome transmission. The kinetochore mediates attachment of the chromosomes to the spindle microtubules (MTs) and directs chromosome movement during mitosis and meiosis (reviewed in Bloom et al., 1989; Koshland, 1994). The kinetochore–MT interaction is monitored by at least one centromere-based mitotic checkpoint system which delays onset of anaphase until stable bipolar attachment is achieved (Gorbsky, 1995; Rudner and Murray, 1996). Thus, kinetochores play both important structural and regulatory roles in ensuring faithful chromosome segregation during mitosis.

The kinetochores of Saccharomyces cerevisiae are relatively simple compared with the large trilaminar structures seen in multicellular eukaryotes (Rieder, 1982; Pluta et al., 1990). Major advances in defining components necessary for a functional kinetochore in yeast have implications for understanding the corresponding elements in more complex eukaryotes. The minimal functional centromere of S. cerevisiae (~125 bp) is comprised of three conserved centromere DNA elements (CDEs). The central CDEII element consists of 78–86 bp of AT-rich DNA, and is flanked by two highly conserved palindromic motifs, CDEI (8 bp) and CDEIII (25 bp). Of these, CDEIII is absolutely essential for centromere function. In vivo, a unique nucleosome-resistant chromatin structure encompassing 160–220 bp is associated with CEN DNA and presumably corresponds to the yeast kinetochore (Bloom and Carbon, 1982; Funk et al., 1989). Seven genes that encode centromere proteins have been identified. Four of these, NDC10/CTF14/CFB2...
(Goh and Kilmartin, 1993; Jiang et al., 1993), CEP3/CBF3 (Lechner, 1994; Strunnikov et al., 1995), CTF13 (Doheny et al., 1993), and SKP1 (Connelly and Hieter, 1996; Stemmann and Lechner, 1996) are essential for viability and encode the components of a multisubunit complex, CBF3, that binds to CDEIII DNA in vitro (Lechner and Carbon, 1991). CBF1/CEP1/CFP1 encodes a nonessential basic helix-loop-helix protein which binds to CDEI (Baker and Massion, 1990; Cai and Davis, 1990; Mellor et al., 1990). In addition, MIF2, a homologue of mammalian CENP-C, has been shown to interact with CEN DNA in a CDEIII- and CDEII-dependent manner (M eluh and Koshland, 1995, 1997), and CSE4, a homologue of mammalian CENP-A, is suggested to be involved in a specialized centromeric nucleosome (M eluh et al., 1998). Further analysis of the architecture of the CBF3 complex using DNA protein cross-linking (E spelink et al., 1997) suggests that three subunits of CBF3, Ndc10p, Cep3p, and Ctf13p, are in direct contact with CDEIII over a region spanning 80 bp of DNA.

The binding of the CBF3 complex to CDEIII is necessary, but not sufficient for attachment of chromosomes to MTs. Several studies indicate the existence of other factors which are required to link the CBF3-DNA complex to polymerized MTs (King sbury and Koshland, 1991; So rger et al., 1994). The CBF3 subcomplex is thought to act as the nucleation site onto which these as yet unidentified components assemble and form active MT-binding complexes. A rich resource for identification of these additional kinetochore components is the chromosome transmission fidelity (ctf) mutant collection, which was isolated by the sole criterion of an increased rate of missegregation of a nonessential chromosome (Spencer et al., 1990). Previously, two secondary in vivo genetic screens, the centromere transcriptional readthrough assay and the dicentric chromo-

some stabilization assay, were used to detect potential kinetochore mutants from this collection (Doheny et al., 1993). A subset of 12 ctf mutants screened positive for the transcriptional readthrough assay. Three of these also tested positive for the dicentric stabilization assay. Two mutants were characterized in further detail, ctf13-30 and ctf14-42, and were shown to correspond to CTF13 and CTF14 (NDC10/CBF2), genes which encode essential kinetochore proteins (Doheny et al., 1993).

To identify other proteins involved in kinetochore structure or function, a third in vivo genetic assay was developed as a tertiary screen. This novel screen, which we have called synthetic dosage lethality (SDL; K 9l et al., 1996), involves overexpressing a wild-type reference gene in a collection of target mutants. CTF13, encoding a known kinetochore protein (Doheny et al., 1993), was used as the inducibly overexpressed reference gene to screen for synthetic interactions within a subset of 12 potential kinetochore ctf mutants. Overexpression of CTF13 caused SDL in combination with four ctf mutants; one corresponded to a known kinetochore component, (CTF14/NDC10), and two were independent alleles of a previously uncharacterized gene, CTF19. We report here the cloning and detailed characterization of the CTF19 gene. The genetic and biochemical results presented here indicate that Ctf19p represents a new kinetochore protein which may play a role as a molecular link between kinetochore and the mitotic spindle.

**Materials and Methods**

**Yeast Strains and Media**

Table I lists the genotypes of all yeast strains used in this study. Media for yeast growth and sporulation were as described (Rose et al., 1990), except as

| Strains     | Genotype                                      | Source                      |
|-------------|-----------------------------------------------|-----------------------------|
| YPH877      | MATa ura3-52 lys2-801 ade2-101 trp1Δ1 leu2Δ1   | F. Spencer and P. Hieter    |
| YPH892      | MATa ura3-52 lys2-801 ade2-101 his3Δ200 trp1Δ3 leu2Δ1 | P. Hieter                  |
| YPH898      | MATa/MATa ura3-52 lys2-801 his3Δ200 trp1Δ3 leu2Δ1 | P. Hieter                  |
| YPH912      | YCTF58 MATa ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 | This study                 |
| YPH926      | YCTF58 MATa ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 | Kroll et al., 1996         |
| YPH987      | MATa ura3-52 lys2-801 ade2-101 his3Δ200 trp1Δ1 ctf19Δ1 | F. Spencer and P. Hieter    |
| YPH989      | MATa/MATa ura3-52 lys2-801 ade2-101 his3Δ200 trp1Δ3 leu2Δ1 | P. Hieter                  |
| YPH991      | YPH991 MATa ura3-52 lys2-801 ade2-101 his3Δ200 trp1Δ3 leu2Δ1 | This study                 |
| YPH992      | YPH992 MATa ura3-52 lys2-801 ade2-101 his3Δ200 trp1Δ3 leu2Δ1 | This study                 |
| YPH993      | YPH993 MATa ura3-52 lys2-801 ade2-101 his3Δ200 trp1Δ3 leu2Δ1 | This study                 |
| YPH994      | YPH994 MATa ura3-52 lys2-801 ade2-101 his3Δ200 trp1Δ3 leu2Δ1 | This study                 |
| YPH995      | YPH995 MATa ura3-52 lys2-801 ade2-101 his3Δ200 trp1Δ3 leu2Δ1 | This study                 |
| YPH996      | YPH996 MATa ura3-52 lys2-801 ade2-101 his3Δ200 trp1Δ3 leu2Δ1 | This study                 |
| YPH997      | YPH997 MATa ura3-52 lys2-801 ade2-101 his3Δ200 trp1Δ3 leu2Δ1 | This study                 |
| YPH998      | YPH998 MATa ura3-52 lys2-801 ade2-101 his3Δ200 trp1Δ3 leu2Δ1 | This study                 |
| YPH999      | YPH999 MATa ura3-52 lys2-801 ade2-101 his3Δ200 trp1Δ3 leu2Δ1 | This study                 |
| YPH1000     | YPH1000 MATa ura3-52 lys2-801 ade2-101 his3Δ200 trp1Δ3 leu2Δ1 | This study                 |
| YPH1001     | YPH1001 MATa ura3-52 lys2-801 ade2-101 his3Δ200 trp1Δ3 leu2Δ1 | This study                 |
| YPH1002     | YPH1002 MATa ura3-52 lys2-801 ade2-101 his3Δ200 trp1Δ3 leu2Δ1 | This study                 |
| YPH1003     | YPH1003 MATa ura3-52 lys2-801 ade2-101 his3Δ200 trp1Δ3 leu2Δ1 | This study                 |
| YPH1004     | YPH1004 MATa ura3-52 lys2-801 ade2-101 his3Δ200 trp1Δ3 leu2Δ1 | This study                 |
otherwise indicated. For experiments monitoring the loss of a nonessential chromosome fragment, adenine was added to 8 μg/ml minimal media to enhance the development of the red pigment in ade2-101 strains. For galactose inductions, strains were grown on solid medium containing 2% galactose, either with or without additional 2% raffinose. For experiments using liquid cultures, strains were grown in liquid medium containing 2% raffinose overnight, and galactose was added to a final concentration of 2% for the induction times indicated. To inhibit MT function in liquid cultures, nocodazole (N2 Sigma Chemical Co.) was added to 20 μg/ml and cultures incubated at 25°C for 2 h, or to 15 μg/ml at 30°C for 90 min. To inhibit MT function on solid medium, benomyl (DuPont) was added at 5, 10, and 20 μg/ml as indicated. DMSO alone was added to the media as a control. All yeast transformations were done by the method of Ito et al. (1983).

### Table I. (continued)

| Strains | Genotype | Source |
|---------|----------|--------|
| YPH132  | MATaMATα ura3-52 ura3-52 lys2-801/lys2-801 ade2-101/ade2-101 HIS3/his3Δ200 trp1Δ1/CTF19-3HA leu2Δ1/leu2Δ1 ctf19Δ1::CTF19-3HA/CTF19-3HA-HIS3 | This study |
| YPH1329 | MATaMATα ura3-52 ura3-52 lys2-801/lys2-801 ade2-101/ade2-101 his3Δ200/his3Δ200 trp1Δ1/ctf19Δ1::CTF19-3HA-HIS3 | This study |
| YPH1330 | MATaMATα ura3-52 ura3-52 lys2-801/lys2-801 ade2-101/ade2-101 his3Δ200/his3Δ200 trp1Δ1/CTF19-3HA-HIS3 | This study |
| YPH1331 | MATaMATα ura3-52 ura3-52 lys2-801/lys2-801 ade2-101/ade2-101 his3Δ200/his3Δ200 trp1Δ1/CTF19-3HA-HIS3 | This study |
| YPH1332 | MATaMATα ura3-52 ura3-52 lys2-801/lys2-801 ade2-101/ade2-101 his3Δ200/his3Δ200 trp1Δ1/CTF19-3HA-HIS3 | This study |
| YPH1337 | MATaMATα ura3-52 ura3-52 lys2-801/lys2-801 ade2-101/ade2-101 his3Δ200/his3Δ200 trp1Δ1/CTF19-3HA-HIS3 | This study |
| YPH1338 | MATaMATα ura3-52 ura3-52 lys2-801/lys2-801 ade2-101/ade2-101 his3Δ200/his3Δ200 trp1Δ1/CTF19-3HA-HIS3 | This study |
| YPH1339 | MATaMATα ura3-52 ura3-52 lys2-801/lys2-801 ade2-101/ade2-101 his3Δ200/his3Δ200 trp1Δ1/CTF19-3HA-HIS3 | This study |
| YCTF30  | MATaMATα ura3-52 ura3-52 his3Δ200/his3Δ200 leu2Δ1/leu2Δ1 CIII (CEN3L URA3 SUP11 [YKH 65]) + pKH7 (CF19-3HA-LEU2; pRS315) | Spencer et al., 1990; Kroll et al., 1996 |
| YCTF42  | MATaMATα ura3-52 ura3-52 his3Δ200/his3Δ200 leu2Δ1/leu2Δ1 CIII (CEN3L YPH278) URA3 SUP11 nrd10-42 | Spencer et al., 1990; Kroll et al., 1996 |
| YPH971  | MATα ura3 leu2 trp1 lys2 nrd10-1 | J. Kilmartin |
| YPH1027 | MATα ura3-52 leu2-3,112 nrd10-2 | T. Huffaker |
| YPH1234 | MATα ura3-52 lys2-801 ade2-101 his3Δ200 trp1Δ1/CTF19-3HA-HIS3 | C. Connelly and P. Hieter |
| YPH1235 | MATα ura3-52 lys2-801 ade2-101 his3Δ200 trp1Δ1/CTF19-3HA-HIS3 | C. Connelly and P. Hieter |
| YAS281  | MATα ura3 lys2 ade2 his3 trp1 leu2 cep3-1 | Strunnikov et al., 1995 |
| YAS282  | MATα ura3 lys2 ade2 his3 trp1 leu2 cep3-2 | Strunnikov et al., 1995 |
| YFP2    | MATα ura3-52 ura3-52 ade2-101 his3Δ200 leu2Δ1/leu2Δ1 CIII (CEN3L YPH278) URA3 SUP11 nrd10-42 | F. Pangilinan and F. Spencer |
| YFS822  | MATα ura3-52 ura3-52 ade2-101 his3Δ200 leu2Δ1/leu2Δ1 CIII (CEN3L YPH278) URA3 SUP11 nrd10-42 | F. Pangilinan and F. Spencer |
| YPF74   | MATα ura3-52 ura3-52 ade2-101 his3Δ200 leu2Δ1/leu2Δ1 CIII (CEN3L YPH278) URA3 SUP11 nrd10-42 | F. Pangilinan and F. Spencer |
| YPH1238 | MATα ura3-52 lys2-801 ade2-101 his3Δ200 trp1Δ1/CTF19-3HA-HIS3 | M. Mayer and P. Hieter |
| YFS722  | MATα ura3-52 lys2-801 ade2-101 his3Δ200 trp1Δ1/CTF19-3HA-HIS3 | F. Spencer |
| YPH311  | MATα ura3-52 ade2(ochre) his3Δ200 leu2Δ1/leu2Δ1 cep3-1 | A. Hoyt and D. Botstein |
| YPH312  | MATα ura3-52 ade2(ochre) leu2Δ1/leu2Δ1 cep3-1 | A. Hoyt and D. Botstein |
| YN421   | MATα ura3-52 ade2 lys2 trp1::CTF19-3HA-HIS3 | Cai and Davis, 1990 |
| SBD220-8c| MATα ura3-52 ade2-101 his3Δ200 trp1Δ1/CTF19-3HA-HIS3 | Stoler et al., 1995 |
| PMY1002-3A| MATα ura3 ade2 trp1 myf3-3 | P. Meluh |
| SMD6-4b | MATα ura3-52 his3Δ200 ade2-101 his3Δ200 leu2Δ1/leu2Δ1 cep3-1 | Winet et al., 1991 |
| HC10-42D| MATα ura3-52 ade2 nrd10-1 | Winet et al., 1993 |
| ELW65-9d| MATα ura3-52 ade2 trp1 cep3-1/ade2 trp1 cep3-1 | M. Winey |
| TSY948  | MATα ura3-52 ade2-101 his3Δ200 ade2-101 cep3-1 | Marschall et al., 1996 |
| TSY502  | MATα ura3-52 ade2-101 his3Δ200 ade2-101 cep3-1 | Marschall et al., 1996 |
| Yspc42-10| MATα ade2-101 his3Δ200 ade2-101 cep3-1 | Donaldson and Kilmartin, 1996 |
| Yspc110-1| MATα ade2-101 his3Δ200 ade2-101 cep3-1 | J.V. Kilmartin |
| Ynde80-1| MATα ade2-101 his3Δ200 ade2-101 cep3-1 | Wigg et al., 1998 |
| Ynde80-2| MATα ade2-101 his3Δ200 ade2-101 cep3-1 | Wigg et al., 1998 |

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**Genetic Analysis**

**Synthetic Lethality (SL) Studies.** Strains containing mutations in the gene of interest were mated to a ctf19 null strain to create the heterozygous diploids. These strains were sporulated, dissected, and cultured at 25°C. At least two independent diploid clones were analyzed for each mating. To test for conditional interactions, all double mutant spores recovered at 25°C were assayed for growth at higher temperatures, initially at 30°C and 37°C. Other temperatures were tested if there was an indication of a conditional effect at a permissive temperature to determine the lowest temperature at which lethality was observed. All synthetic lethal interactions indicated by the tetrad data were confirmed by plasmid shuffle. CBF3 mutant strains which demonstrated potential SL with a ctf19 null were mated to a ctf19Δ::TRP1 or ctf19Δ::HIS3 strain containing a wild-type copy of CTF19 on a URA3-CEN plasmid (pKH7), sporulated and dissected. The double mutant spores recovered containing a URA3-marked plasmid were streaked on plates containing 5-FOA, which does not allow growth of cells expressing URA3 (Boeke et al., 1987). Therefore, only viable double mutants can grow on 5-FOA, and true synthetic lethals can not.

**SDL Studies.** Methods are as described previously (Kroll et al., 1996). In brief, mutant strains were transformed under noninducing conditions, with overexpressing constructs containing either CTF13 (pKF88) or CTF19 (pKH21) reference genes under transcriptional control of a GAL1 promoter, and the corresponding vector, p415GEU2. Expression of the reference gene was induced on solid medium containing 2% galactose. The chromosome missegregation phenotype of CTF19 was confirmed to rescue the chromosome missegregation phenotype. The triple HA tag was lagged into the Stul site engineered at the NH2 terminus of CTF19, and this epitope-tagged fusion construct was subcloned into pRS313 and pRS315, generating pKH31 and pKH32, respectively (Fig. 1c). These constructs were transformed into ctf19Δ::TRP1 (YPH3129) and shown to rescue the chromosome missegregation phenotype. The CTF19-3H A fusion was integrated into the genome by gamma integration (Sikorski and Hieter, 1989). A 1.4-kb Xhol-Smal fragment containing CTF19-3H A from pKH32 was subcloned into a H1S3 marked gamma integration vector, p679 (Doheny et al., 1993), which is designed to direct an integration event at the leu2Δ1 locus on chromosome III. The resulting integrating construct, pKH35, was linearized at the NotI site, between the targeting sequences to the left (5′) and right (3′) of the leu2Δ1 locus, and transformed into a ctf19Δ::TRP1 deletion strain (YPH1317), creating leu2Δ1::CTF19-HA 3 (YPH1327). Integration at the leu2Δ1 locus was confirmed using PCR-mediated gene disruption (Loren et al., 1995). Oligonucleotides for PCR were synthesized as follows. OKH1 (5′-ATG TGG- GAT GTT GG TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT TGA TCTG TGT T
firmed by colony PCR analysis, using primers OM B-leu2Δ (5'-GTGTA-GAATTGAGATTCCC-3', provided by M. Basrai) and T7. An integration event targeted correctly to the genomic leu2Δ locus results in a 550-bp product. Both the plasmid based and genome integrated versions of the CTF19-3HA epitope-tagged fusion produce the same 48-kD band by Western blotting and probing with anti-HA antibody, which is not seen in control strains containing a wild-type untagged copy of CTF19.

Quantitation of Chromosome Missegregation

Colonial colony half sector analysis was performed as previously described (Koshland and Hieter, 1987; Gerring et al., 1990). In brief, homogenous diploid strains (YPH982, CTF19/CTF19; YPH1320, ctf19Δ::TRP1/ctf19Δ::TRP1; YPH1321, ctf19-58::ctf19-58; and YPH1322, ctf19-26::ctf19-26) containing a single SUP11-marked chromosome fragment were plated to single colonies on solid media containing a limiting amount of adenine (6 μg/ml), and grown at 30°C. The red pigment was allowed to develop at 4°C before scoring the sectors. Colonies scored as half-sectored were ≥50% red. A 1:0 missegregation event (chromosome loss) in the first division results in pink/red half-sectored colonies, whereas a 2:0 missegregation event (nondisjunction; chromosome gain) results in white/red half-sectored colonies.

Flow Cytometry and Cytological Analysis

Cells were processed for flow cytometry as previously described (Gerring et al., 1990), with a few modifications. In brief, logarithmically growing cells were pelleted, resuspended in 0.2 M Tris, pH 7.5, containing 70% ethanol, and fixed overnight at 4°C. Cells were treated with 1 mg/ml RNAse A at 37°C for 1 h. Proteinase K was added, and the cell suspension incubated at 55°C for 1 h. Protoplasts were then fixed overnight at 4°C in 0.2 M Tris containing 3 μg/ml propidium iodide (Sigma Chemical Co.). Before being subjected to flow cytometry, cells were sonicated, using a Branson sonifier 450, on a setting of two for 2-5 s. Aliquots of cells fixed with 3.7% formaldehyde, as described for immunofluorescence, were stained with 300 ng/ml 4,6-diamidino-2-phenylindole (DAPI) to analyze nuclear morphology, and anti–α-tubulin antibodies (Jackson ImmunoResearch Laboratories, Inc.). Anti–γ-tubulin was detected with fluorescein conjugated goat anti–mouse antibodies, anti–α-tubulin with rhodamine conjugated goat anti–rat antibodies, anti-HA and epitope-tagged fusion produce the same 48-kD band by

Isolation and Characterization of the CTF19 Gene

The methods used for chromatin immunoprecipitation and the PCR primers used to amplify both centromeric and noncentromeric test loci were as described in M.eluh and Koshland (1997). Purified HA, MA b12CA 5 (a gift of U. A. ntitbodies Co.) was added at either a 1:250 dilution (resulting in a 4 μg/ml final concentration), or 50 μl of HA antibody pre-cross-linked to CNBr-Sepharose beads was added to the chromatin solution. Crude anti–MIp2p rabbit antisera, provided by Pam M.eluh (Carnegie Institute of Washington, Baltimore, MD), was used at a 1:250 dilution and served as a positive control for this assay. For NZ treatment experiments, NZ was added to log phase growing cells at 15 or 20 μg/ml final concentration, and incubated for 90 min at 30°C to completely depolymerize M Ts before fixation.

Immunofluorescence

Ctf19p was localized by indirect immunofluorescence microscopy as described by Pringle et al. (1989), with a few modifications. To visualize

Results

Isolation and Characterization of the CTF19 Gene

A n SDS screen (Kroll et al., 1996) was performed in which CTF19 was inudably overexpressed in a subset of 12 potential kinetochore ctf mutants, which tested positive for the centromere transcription readthrough assay (Doheny et al., 1993). Overexpression of CTF19 caused SDL in combination with four mutants within this set: ndc10-42, ctf17-61, ctf19-26 (YCTF26), and ctf19-58 (YCTF58). The chromosome missegregation ctf phenotype (visualized by the formation of red sectors in a white colony) of ctf19-58 was confirmed by genetic analysis to be due to a single nuclear mutation, and the corresponding gene was cloned by complementation of this phenotype (see Materiais and Methods; Fig. 1 a). CTF19 was localized to the right arm of chromosome XVI using physical mapping methods. Nucleotide sequencing of the 2-kb CTF19 clone revealed a previously unidentified 1.2-kb ORF that encodes a protein of 369 amino acids with a predicted molecular mass of 40 kD. A database search revealed no significant overall homology at the amino acid level. Protein motif searching (ProSite) revealed a putative leucine zipper beginning at amino acid 131 (Fig. 1 d). The sequence and mapping data of CTF19 (YPL018W) was corroborated upon release of the complete sequence of the S. cerevisiae genome (Goffeau et al., 1996).

ctf19-26 was confirmed to be an independent allele of CTF19 using four lines of evidence: first, the cloned CTF19 DNA complements the ctf19-26 sectoring phenotype; second, the ctf19-58/ctf19-26 diploid exhibits a chromosome missegregation phenotype; third, when this diploid is sporulated, all spores which bear a chromosome fragment display the sectoring phenotype (Basrai, M., personal communication); and fourth, when ctf19-26 is crossed to a wild-type strain with the LEU2-marked genomic CTF19 locus (YPH1313), the ctf phenotype always segregated away from the LEU2 marker.

A complete deletion of the CTF19 ORF was generated using PCR-mediated gene disruption (Lorenz et al., 1995). The CTF19 ORF was replaced with two different marker genes, creating ctf19Δ::HIS3 and ctf19Δ::TRP1. The deletion strains are viable, display no temperature condi-
tional phenotypes, and exhibit a chromosome missegregation phenotype similar to that seen in strains containing either of the original mutant alleles, ctf19-58 or ctf19-26. Upon overexpression of CTF13 under the control of a GAL1 promoter, SDL was observed in the deletion strain, comparable to that seen with ctf19-58 and ctf19-26, albeit somewhat more pronounced.

**ctf19 Mutants Display Phenotypes Consistent with a Role in Kinetochore Function**

Strains carrying the ctf19Δ1, ctf19-58, or ctf19-26 mutations were tested for sensitivity to benzimidazole compounds. These agents cause depolymerization of MTs, and it has been observed that kinetochore mutants, as well as mitotic checkpoint mutants, are sensitive to compounds such as benomyl (Spencer et al., 1990; Hoyt et al., 1991; Li and Murray, 1991). All three strains are highly sensitive to 10 μg/ml of benomyl at 25°C, and slightly sensitive at the level of 5 μg/ml (Fig. 2 a). Isogenic wild-type strains grow well with 10 μg/ml of benomyl at 25°C, and are sensitive to 20 μg/ml. As an interpretive note, the bub (Hoyt et al., 1991) and mad (Li and Murray, 1991; Hardwick and Murray, 1995) mutants are also sensitive to 10 μg/ml of benomyl.

Examination of possible cell cycle defects revealed that a ctf19Δ1/ctf19Δ1 mutant shows a G2/M accumulation with 2C DNA content in a logarithmically growing culture (Fig. 2 b). Cytological analysis of these cells reveals an accumulation of large budded cells with the nucleus at or near the neck (29% in ctf19Δ1 versus 6% in wild-type). A similar G2 delay is seen in ctf13-30, ndc10-42 (Doheny et al., 1993), cep3-1/-2 (Strunnikov et al., 1995), and skp1-4 (Connelly and Hieter, 1996) kinetochore structural mutants at their nonpermissive temperatures. While there is an accumulation of large budded uninucleate cells, the number of telophase cells in the ctf19 null mutant remains similar to that seen for wild-type, 17% versus 16%, respectively. In contrast, the percentage of telophase cells in a ctf13-30 mutant at the nonpermissive temperature drops to 3%. This is consistent with the notion that ctf13-30 cells experience a strong arrest at G2/M at the nonpermissive temperature, whereas ctf19 null cells experience a shorter delay, most likely because the lesion is less severe.

Chromosome missegregation in the ctf19 mutants was quantitated in homozygous diploid strains using colony color half sector analysis (Koshland and Hieter, 1987). Chromosome loss (1:0 segregation) results in a pink/red half-sectored colony, whereas nondisjunction (2:0 segregation) results in a white/red half-sectored colony. The rate of chromosome loss in a ctf19Δ1/ctf19Δ1 mutant is ~100 times greater than that of wild-type, and the rate of nondisjunction is 60-fold higher than wild-type (Table II). For ctf19 mutant alleles, the rates of chromosome loss and nondisjunction are 77- and 17-fold higher than wild-type for ctf19-58/ctf19-58, and 37- and 13-fold higher for ctf19-26/ctf19-26, respectively. Thus, both chromosome loss and gain are occurring in ctf19 mutant diploids, and the deletion strain is affected the most severely. Similar rates of chromosome loss and nondisjunction have been reported for essential kinetochore mutants ctf13-30 (Doheny et al., 1993) and skp1-4 (Connelly and Hieter, 1996).

**Ctf19p Interacts Genetically with Components of the Kinetochore and the Mitotic Spindle Checkpoint**

To explore a potential role of Ctf19p in kinetochore function, genetic analysis was used to look for synthetic pheno-
types between ctf19 mutants and known kinetochore mutants. As our first genetic test, we used the SDL screen (Kroll et al., 1996) to assay the effect of overexpression of CTF19 in a wild-type background, and in strains containing mutations in each of the four subunits of the CBF3 kinetochore complex. CTF19, the reference gene, was placed under a GAL1 promoter (pKH21, Fig. 1b) and this plasmid was transformed into a set of target mutants (ndc10, cep3, ctf13, and skp1). Growth was assessed upon galactose induction, as previously described (Kroll et al., 1996). Results of this dosage study are summarized in Table III. SDL was seen when CTF19 was overexpressed in the background of two independent mutant alleles of ND C10, ndc10-42, and ndc10-1, thus providing another genetic link between CTF19 and the kinetochore. Moreover, overexpression of ND C10 results in lethality in ctf19-26 and ctf19-58 mutants. The plasmid expressing CTF13 under a GAL1 promoter (pKF88) was also transformed into these mutant strains and viability assessed. Overexpression of CTF13 results in SDL in ndc10-42 (as previously described), and also in ndc10-1 at elevated temperature (30°C), as well as cep3-1 and cep3-2 (Table III).

To look for additional genetic interactions between

| Genotype* | 1:0 Events† | 2:0 Events§ | Total # colonies |
|-----------|------------|------------|-----------------|
| ctf19Δ::TRP1 | 2.8 | 1.8 | 2,737 |
| ctf19::TRP1 | 2.3 | 0.5 | 2,533 |
| ctf19-26 | 1.1 | 0.4 | 3,180 |
| CTF19 | 0.03 | 0.03 | 29,046 |

Mutants of the above genotypes were plated to single colonies and visual sectoring performed. Colonies scored as half-red, half pink. Rates are shown as events per 100 cells.

Table II. Rates of Chromosome Missegregation Events in ctf19 Mutants

| Mutant strain | Gene overexpressed* |
|---------------|---------------------|
| ctf13-30 | + | + |
| ndc10-42 | SDL | SDL |
| ndc10-1 | SDL† | SDL† |
| ndc10-2 | + | + |
| skp1-3 | + | + |
| skp1-4 | DS | + |
| cep3-1 | SDL | + |
| cep3-2 | SDL | + |
| ctf19Δ::TRP1 | SDL | + |
| ctf19-58 | SDL | + |
| ctf19-26 | SDL | + |

* Phenotypes are at 25°C, unless otherwise indicated.
† Temperature ±30°C.
missive temperature suggests that there is a more severe structural aberration than in the ctf13-30 mutant alone.

In light of the observations that the mitotic spindle checkpoint responds to impaired kinetochore function (Wang and Burke, 1995; Pangilinan and Spencer, 1996; Wells and Murray, 1996), we asked directly whether CTF19 genetically interacts with BUB1, BUB2, BUB3, or MAD2 mitotic checkpoint genes. To do this, we again used the SDL assay. The CTF19 overexpression plasmid (pKH21) was transformed into mitotic checkpoint target mutants, bub1Δ, bub2Δ, bub3Δ, and mad2Δ. Upon induction of CTF19 on galactose-containing medium, growth was assessed as previously described. SDL was observed in the bub1Δ and bub3Δ strains, but no defect of growth was seen with mad2Δ or bub2Δ. To interpret these results, we also determined the effect of overexpressing CTF13 in the same target mutants, especially since the ctf13-30 mutation triggers the mitotic checkpoint resulting in a G2/M delay, as described above. When CTF13 was overexpressed in combination with bub2Δ, bub3Δ, and mad2Δ, SDL was observed with bub3Δ, but not with mad2Δ or bub2Δ.

To test for synthetic lethal interactions, crosses were made between strains carrying a ctf19 null mutation and strains carrying either bub1Δ, bub2Δ, bub3Δ, or mad2Δ mutations (Table V). A rad9Δ mutation, previously characterized as defective in monitoring incomplete replication and DNA damage (Weinert and Hartwell, 1988; Lydall and Weinert, 1995), was also tested. SL was seen when ctf19Δ was combined with bub1Δ, bub3Δ, or mad2Δ, but not with bub2Δ or rad9Δ (Table V). Bub1p, Bub3p, and Mad2p are all necessary for the initiation of the mitotic checkpoint pathway and are sufficient for shorter delays, as seen with low levels of NZ, whereas Bub2p is required for maintaining longer delays, as seen with high levels of NZ (Wang and Burke, 1995; Pangilinan and Spencer, 1996). These synthetic dosage lethal and synthetic lethal genetic interaction results are consistent with the notion that ctf19 mutations invoke a mild premitotic delay, which does not require Bub2p. Taken together, these genetic studies strongly suggest that Ctf19p plays a structural role at the kinetochore.

**Ctf19p Interacts with Centromere DNA In Vivo**

Sucrose gradient sedimentation analysis revealed that Ctf19-HA p sediments with an S value of 20 (data not shown). This suggests that Ctf19p may be part of a large protein complex in the cell. There is no evidence that Ctf19p is part of the CBF3 CDEIII-binding complex, as Ctf19p is not required for the normal CEN DNA bandshift in vitro, and antibodies to an epitope-tagged Ctf19p do not result in a supershift of the CBF3-CEN DNA complex (data not shown; Stemmman et al., 1999). In addition, communoprecipitation experiments using crude yeast cell extracts were unable to detect a direct interaction between Ctf19p and CBF3 proteins.

### Table IV. Synthetic Lethal Interactions between ctf19Δ and Kinetochore Mutants

| Double mutant genotype | Phenotype | 4-Spoke tetrads | 3-Spoke tetrads | 2-Spoke tetrads | 1-Spoke tetrads | Total tetrads | Viable spores* |
|------------------------|-----------|-----------------|-----------------|-----------------|----------------|---------------|---------------|
| ctf19Δ::HIS3 ctf13-30  | CSL       | 19              | 3               | 0               | 0              | 22            | 97            |
| ctf19Δ::HIS3 ndc10-42  | SL         | 4               | 15              | 9               | 2              | 30            | 68            |
| ctf19Δ::TRP1 ndc10-1   | Viable     | 21              | 4               | 0               | 0              | 25            | 96            |
| ctf19Δ::TRP1 cep3-1    | SL         | 7               | 15              | 5               | 2              | 29            | 72            |
| ctf19Δ::TRP1 cep3-2    | SL         | 5               | 11              | 4               | 0              | 20            | 76            |
| ctf19Δ::TRP1 skp1-3    | Viable     | 14              | 2               | 0               | 0              | 16            | 97            |
| ctf19Δ::TRP1 skp1-4    | CSL        | 15              | 2               | 1               | 0              | 18            | 94            |
| ctf19Δ::TRP1 sgt1-3    | SL         | 18              | 2               | 8               | 2              | 30            | 80            |
| ctf19Δ::TRP1 sgt1-5    | Viable     | 24              | 0               | 2               | 1              | 27            | 94            |
| ctf19Δ::TRP1 mig3-2    | SL         | 5               | 20              | 4               | 1              | 30            | 74            |

*Numbers presented are for 25°C. Percent viable spores = number of viable spores from a given mating/total number of possible spores.

### Table V. Synthetic Lethal Interactions between ctf19Δ and Mitotic Checkpoint Mutants

| Double mutant genotype | Phenotype | 4-Spoke tetrads | 3-Spoke tetrads | 2-Spoke tetrads | 1-Spoke tetrads | Total tetrads | Viable spores* |
|------------------------|-----------|-----------------|-----------------|-----------------|----------------|---------------|---------------|
| ctf19Δ::HIS3 bub1Δ::LEU2 | SL         | 2               | 6               | 8               | 4              | 20            | 58            |
| ctf19Δ::HIS3 bub2Δ::LEU2 | Viable     | 12              | 3               | 3               | 1              | 19            | 84            |
| ctf19Δ::HIS3 bub3Δ::LEU2 + pBUB3-URA3 | SL        | 16              | 8               | 6               | 1              | 31            | 81            |
| ctf19Δ::TRP1 mad2::HIS3 | SL         | 6               | 10              | 12              | 0              | 28            | 70            |
| ctf19Δ::TRP1 rad9::HIS3 | Viable     | 26              | 1               | 3               | 0              | 30            | 94            |

*Numbers presented are for 25°C. 

Analysis of spores from this diploid required the inclusion of a plasmid (pBUB3-URA3) before sporulation to achieve sufficient sporulation efficiency and acceptable viability of spore products. Double mutant spores containing pBUB3-URA3 were not viable on FOA plates, thus confirming synthetic lethality between bub3 and ctf19.
Knowing that Ctf19p is not a part of the CDEIII-binding complex detected by CEN DNA bandshift in vitro, we investigated other ways in which it could be involved in kinetochore function. One possibility is that Ctf19p plays a role in binding of MTs to kinetochores. To test this hypothesis, an in vitro assay was used which measures the ability of yeast centromeres to bind to MTs (Kingsbury and Koshland, 1991). In this assay, minichromosomes containing a centromere are introduced into wild-type or mutant strains, and purified chromatin is prepared. Taxol-stabilized bovine MTs are added to the lysate and sedimented. The supernatant and pellet are separated, DNA from each fraction is extracted, and the relative amount of the minichromosome in each fraction is determined. In wild-type cultures with wild-type centromeres on the minichromosomes, the minichromosomes sediment with the MTs. CEN DNA mutations in CDEII and CDEIII abolish the centromere’s ability to bind MTs (Kingsbury and Koshland, 1991). Similarly, trans-acting mutations in CEN-binding proteins also inhibit the ability of the minichromosome to bind to MTs, exemplified by the cep3-1 mutant (Strunnikov et al., 1995). Two independent alleles of CTF19 were tested in this assay, ctf19-58 and ctf19-26, and both were shown to exhibit a dramatic defect in centromere-dependent binding of minichromosomes to MTs (Fig. 3). Therefore, knowing that Ctf19p interacts genetically with the CBF3 complex, is important for binding MTs to centromeres of minichromosomes, and sediments with an S value consistent with being part of a protein complex, we next investigated whether Ctf19p is involved in a higher order centromere complex.

To examine whether Ctf19p physically interacts with a kinetochore macromolecular complex, we used an in vivo cross-linking and immunoprecipitation strategy (Meluh and Koshland, 1997). Formaldehyde cross-linked chromatin (2 h fixation) prepared from an epitope-tagged CTF19-3HA strain and a wild-type untagged control strain were sonicated to shear the DNA and immunoprecipitated with anti-HA antibody. After reversing the cross-links, the presence of specific DNA sequences in the immunoprecipitates was assessed by PCR analysis. Mif2p, which was previously shown to associate with CEN DNA in vivo (Meluh and Koshland, 1997), served as a positive control for this assay. Two CEN DNA sequences were tested, CEN3 and CEN16, and both were found to be present in the Ctf19-HA p immunoprecipitate, but not in the untagged control strain or in the mock-treated control (Fig. 4 a). The anti-Mif2p immunoprecipitate also contained these CEN DNA sequences. To test if the interaction detected is specific for CEN DNA, two noncentromeric loci, PGK1 and HMRa, which are AT-rich intergenic regions located on yeast
Ctf19p Localizes Near the Spindle Pole Body (SPB)

The localization of Ctf19p in yeast cells was determined by indirect immunofluorescence using an HA epitope fusion construct (pKH32). To avoid potential copy number effects, the CTF19-3HA fusion was integrated into the genome at the LEU2Δ1 locus (LEU2Δ1::CTF19-3HA, YPH1327). For immunofluorescence studies, an asynchronous population of cells from strains containing CTF19-3HA (either plasmid-based or integrated, YPH1324 or YPH1327) or an untagged control (YPH1323 or YPH500) were formaldehyde-fixed, and stained with anti-HA antibody and anti-α-tubulin, which stains spindle MTs. The HA antibody detected a bright dot at the vertex of the MTs in interphase cells, and at the ends of the spindle in mitotic cells (Fig. 5). This staining pattern, reminiscent of SPB staining, is similar to those seen with other centromere proteins, including Ndc10p (Goh and Kilmartin, 1993), Mif2p (Meluh and Koshland, 1997), and Cse4p (Meluh et al., 1998), and is consistent with the notion that centromeres display a non-random localization throughout the cell cycle, clustering near the SPB during interphase (G1) and late mitosis (anaphase and telophase; Guacci et al., 1997; Straight et al., 1997). A key difference between the staining pattern observed for Ctf19-HAp and those reported for other centrromere proteins occurs during early mitosis. Both Ndc10p and Cse4p are reported to stain the spindle MTs, evident as a short bar, in cells with short spindles. Ctf19-HAp staining, however, resolves into two distinct foci even in cells with short spindles.

To examine the specificity of this localization, we obtained antibodies against Tub4p (provided by L. Marshall, Stanford, CA), which served as an SPB marker (Sobel and Snyder, 1995; Marshall et al., 1996; Spang et al., 1996). Tub4p, the α-tubulin homologue in S. cerevisiae, is part of a complex that localizes to the inner and outer plaques of the SPB (Geissler et al., 1996; Knop et al., 1997), where nuclear and cytoplasmic MTs are organized, respectively. Costaining experiments revealed that Ctf19-HAp localizes to the same region as Tub4p (Fig. 6, a and b). Further analysis of cells stained for both Ctf19-HAp and Tub4p revealed that the Ctf19-HAp signal appears to be just interior to (on the nucleoplasmic side of) the

Tub4p signal in mid-mitotic cells with short spindles (Fig. 6 d). This observation was examined in more detail by measuring the distance between the Ctf19-HAp signals and Tub4p signals in individual cells. The average distance between Ctf19-HAp signals for 50 mitotic cells was 1.5 μm ± 1.1, whereas the comparable distance between Tub4p signals was 1.8 μm ± 1.1. These measurements correspond to an average distance of 0.2 μm between Ctf19Ap and Tub4p in mitotic cells. To interpret the significance of this difference, it should be considered that Tub4p resides on both nuclear and cytoplasmic surfaces of the SPB and that the fluorescent signal observed for Tub4p is an average of both signals. Therefore, the distance from the Ctf19-HAp signal to the nuclear side of the SPB may be less than indicated by these measurements. These data suggest that Ctf19p localizes near the SPB, but, as expected, is not likely to be an integral component of the SPB. Consistent with this hypothesis, we were unable to demonstrate a di-
rect interaction between Ctf19-HAp and Tub4p by coimmunoprecipitation (data not shown). It is possible that Ctf19p is part of a complex which resides at the nucleoplasmic surface of the SPB and transiently interacts with kinetochores. However, it is more likely that the staining pattern observed for Ctf19-HAp corresponds to a centromere localization, as noted above for other kinetochore proteins, and is thus compatible with data implicating a kinetochore function for Ctf19p.

To test if we could differentiate between these two hypotheses, we asked whether Ctf19p requires MTs for its localization. Assuming that kinetochores are tethered to the SPB in a MT-dependent manner (Guacci et al., 1997), cells were treated with 15 μg/ml NZ (a concentration that depolymerizes all MTs) and examined by immunofluorescence. For proteins that are known to be integral components of the SPB, localization is unchanged by the presence of NZ (Marschall et al., 1996). Fluorescent in situ hybridization analysis has revealed that centromere localization is, however, changed in the presence of NZ as they are no longer clustered (Guacci et al., 1997). It should be noted that, although NZ and other benzimidazole drugs depolymerize most of the MTs, it is possible that a small amount of the polymer which is undetectable by immunofluorescence remains (Marschall et al., 1996; Jacobs et al., 1988). Interestingly, Ctf19p localization in most cells remained near the SPB, visualized as one (z 66% of cells) or two (z 27% of cells) brightly staining dots (Fig. 6 c). This compares to 69% and 31%, respectively, in cells not treated with NZ. However, z 7% of the cells (out of 500 cells analyzed) contained three or four discrete signals for Ctf19-HAp. There were also cells observed in which two signals were seen for Ctf19-HAp, but only one signal was seen for Tub4p. These observations indicate that the majority of Ctf19p remains near the SPB in the absence of MTs, however, a portion of the protein in some cells has an altered localization in the presence of NZ. These results may indicate that Ctf19p is present both at the centromere...
and near the nucleoplasmic surface of the SPB. Whether or not these localization data are suggestive of a function for Ctf19p at the SPB, or merely a consequence of the limitations of the assay, has yet to be determined.

CTF19 Interacts Genetically with a Spindle- and Pole-associated Protein

As a preliminary investigation into a potential role for Ctf19p at the SPB, we tested for genetic interactions between CTF19 and several genes encoding either integral SPB proteins or proteins involved in SPB duplication. No synthetic lethal interactions were detected in any double mutants created between ctf19Δ1 and mps2-1, ndc1-1, cdc31-1, spo42-10, spo110-1, tbu4-32, or tbu4-34 (data not shown). In comparison to the unequivocal genetic interactions detected between CTF19 and the genes encoding the CBF3 kinetochore components, we conclude that the function of Ctf19p lies at the kinetochore. We also tested for genetic interactions between CTF19 and several genes encoding either integral SPB proteins or proteins involved in SPB duplication. No synthetic lethal interactions were detected in any double mutants created between ctf19Δ1 and mps2-1, ndc1-1, cdc31-1, spo42-10, spo110-1, tbu4-32, or tbu4-34 (data not shown). In comparison to the unequivocal genetic interactions detected between CTF19 and the genes encoding the CBF3 kinetochore components, we conclude that the function of Ctf19p lies at the kinetochore. We also tested for genetic interaction between CTF19 and CBF3, which encodes a spindle- and pole-associated protein. Interestingly, a conditional synthetic lethal effect was detected in ndc80-1 ctf19Δ1 double mutants at 28°C. This genetic interaction is allele specific, as no conditional SL was detected in ndc80-2 ctf19Δ1 double mutants. NDC80 mutants display phenotypes very similar to a temperature-sensitive mutant of NDC10, which encodes a component of the CBF3 kinetochore complex (Wigge et al., 1998). Although the phenotypes and immunofluorescent staining pattern of Ndc80p are consistent with localization to the kinetochore, no genetic interactions have been detected between NDC80 and CBF3 kinetochore components (Wigge et al., 1998). These authors have concluded that an indirect interaction may exist between Ndc80p and the kinetochore. It is quite possible that such an indirect interaction may occur through Ctf19p.

Discussion

We have identified and characterized the CTF19 gene of S. cerevisiae, and show that it encodes a novel protein which is required for faithful chromosome transmission and efficient binding of centromeres to MTs, associates with a centromere DNA complex in vivo, and localizes to the SPB region. Previously, two uncharacterized mutations, ctf19-58 and ctf19-26, were classified as putative kinetochore mutants by an in vivo centromere transcription readthrough assay (Doheny et al., 1993). Ctf19p mutants were further implicated in kinetochore function by our SDL screen (Kroll et al., 1996), in which overexpression of the CTF13 reference gene, encoding an essential CBF3 kinetochore protein, resulted in lethality in the context of the ctf19-58 and ctf19-26 mutations. The phenotypes described here for ctf19 mutants, including chromosome missegregation, increased sensitivity to benomyl, and a G2/M accumulation of logarithmically growing cells, are all consistent with a defect in kinetochore function.

Extensive genetic analysis revealed that CTF19 exhibits strong genetic interactions with both kinetochore structural mutants and mitotic checkpoint mutants. Genetic interactions (SDL, SL, or both) were detected between the ctf19 null mutation and mutant alleles of all four subunits of the CBF3 kinetochore complex, as well as with Mif2p, which has been shown to interact with CEN DNA in a CDEIII-specific manner (Meluh and Koshland, 1997). We also demonstrate that cells defective in, or overexpressing Ctf19p require a functional mitotic checkpoint pathway, as do cells defective in, or overexpressing Ctf13p. The extent and specificity of these interactions strongly implicates Ctf19p as playing a role in kinetochore structure or function.

Ctf19p Functions as Part of a Macromolecular Kinetochore Complex

Consistent with the numerous genetic interactions linking Ctf19p to the kinetochore, as well as the sedimentation data, we demonstrate that Ctf19p associates specifically with centromeric DNA in vivo through formaldehyde cross-linking followed by chromatin immunoprecipitation. Two known kinetochore proteins, Ndc10p and Cbf1p, as well as two implicated kinetochore proteins, Mif2p and Cse4p, have been shown to cross-link wild-type CEN DNA in vivo (Meluh and Koshland, 1997; Meluh et al., 1998). Ndc10p and Mif2p both demonstrate a specific dependence on the integrity of CDEIII (Meluh and Koshland, 1997). A similar specificity of interaction between Ctf19p and CDEIII is described by Lechner and colleagues, who have independently identified Ctf19p as part of a complex of proteins that interacts with CBF3 (Stemmann et al., 1999). These data are consistent with genetic interactions reported here, which are also specific for CBF3 components.

The fact that Ctf19p is able to specifically cross-link CEN DNA does not differentiate between a direct and indirect association. Ctf19p is not a subunit of the CBF3 complex (Stemmann and Lechner, 1996), and is not necessary for the gel mobility shift seen with the assembled kinetochore complex on a CEN DNA fragment (data not shown; Lechner, J., personal communication). Therefore we propose that Ctf19p interacts indirectly with CEN DNA, likely through interactions with the CBF3 complex, or perhaps through a larger macromolecular complex whose assembly is initiated by recruitment of CBF3 to CDEIII (Sorger et al., 1994; Meluh and Koshland, 1997). Given the ability of Ctf19p to cross-link CEN DNA, and the defect seen in the ability of ctf19 mutants to bind centromeres of minichromosomes to MTs, Ctf19p would be a good candidate for a factor which interacts with CBF3 and is necessary to form active spindle MT-binding complexes. The fact that Ctf19p is able to associate with centromeric DNA in the presence of NZ suggests that MTs are not required for this association, and places Ctf19p at the kinetochore instead of on the spindle. Similar results were observed for Mif2p and Ndc10p. We are currently testing whether Ctf19p is required for attachment of reassembled kinetochore complexes in vitro to polymerized MTs.

Implications of a Kinetochore Protein with SPB Localization

The immunolocalization pattern of Ctf19p is consistent with kinetochore proteins, which is strongly supported by
the genetic and biochemical data presented here. Other proteins which have been biochemically placed at the centromere, including Ndc10p, Mif2p, and Cse4p, show immunofluorescent staining patterns similar to Ctf19p. The SPB staining pattern observed in G1 cells and in late mitotic cells could be due to the effect of centromere clustering, a phenomenon seen in higher eukaryotes and demonstrated in yeast by fluorescent in situ hybridization (Guacci et al., 1997). Studies on cell cycle dependent centromere positioning in S. cerevisiae reveal that in G1 cells, centromeres are loosely clustered around the SPB, similar to that reported for fission yeast and mammalian cells (Ferguson and Ward, 1992; Funabiki et al., 1993; Vourch et al., 1993). In mitosis before anaphase (mid M), centromeres are centrally localized within the nucleus, away from the SPB, reminiscent of metaphase in larger eukaryotic cells. In anaphase and telophase cells, centromeres are clustered tightly and proximal to the SPBs. One caveat to interpreting the Ctf19p localization as consistent with it being a CEN binding protein is that, unlike Ndc10p (Goh and Kilmartin, 1993) and Cse4p (Meluh et al., 1998), Ctf19p does not display spindle staining, as may be expected for centromere associated proteins when kinetochores are forming bipolar attachments to the spindle MTs. The absence of spindle staining for Ctf19p may reflect limitations of the immunofluorescence assay, as other kinetochore proteins, including Ctf13p and Cep3p, have not been visualized in the cell by traditional immunofluorescent techniques, or it may reflect a unique role for Ctf19p in mitosis, perhaps functioning to tether kinetochores to the SPB. Given the prominent role of anaphase B in sister chromatid separation in budding yeast (Straight et al., 1997), Ctf19p may be important in stabilizing the link between kinetochores and each SPB during spindle pole separation.

The results of immunofluorescence with Ctf19-HAp in the presence of NZ suggest that at least some amount of Ctf19p localizes adjacent to the SPB, as opposed to Ctf19p residing solely at the kinetochores, because the positioning of kinetochores near the SPB is believed to be dependent on MTs. In the presence of NZ, it has been observed that centromeres are no longer clustered, but rather disperse throughout the nucleus (Guacci et al., 1997), presumably because MTs are no longer present for the kinetochores to remain attached to the SPB. However, the procedures used to fix and process nuclei for in situ hybridization in yeast may be more disruptive than that used for immunofluorescence, and theoretically could contribute to an aberrant delocalization of centromeres after treatment with NZ. Although Ctf19-HAp does maintain a discrete localization in the presence of NZ, we did observe a few examples (~7% of cells analyzed) in which more than two fluorescent signals were seen for Ctf19-HAp which did not correlate with a Tub4p signal. We reason that, because a small polymer of MTs may still be present at the face of the SPB even after NZ treatment (Morschall et al., 1996; Jacobs et al., 1998), Ctf19p may be part of a complex that is peripheral to the SPB. The localization of this complex may be less stable in the presence of MT depolymerizing agents, accounting for the small population of cells that exhibit more than two Ctf19-HAp signals. Wigge et al. (1998) have identified several new spindle pole and spindle-associated proteins through analysis of purified spindle preparations with MALDI mass spectrometry. Ndc80p is of particular interest because it stains spindles as well as poles, and ndc80-1 mutants display phenotypes similar to yeast kinetochore mutants, including chromosome missegregation and anaphase defects. In addition, Ndc80p is a potential homologue of human HEC protein, which localizes to the centromere. However, no genetic interactions were detected between NDC80 and three of the CBF3 components tested. Ndc80p partially copurifies with the factors which bind kinetochores to MTs (Sorger et al., 1994), but is absent from the final fraction (Wigge et al., 1998). Since Ctf19p interacts with the centromere, both genetically and biochemically, and it genetically interacts with Ndc80p, we propose that Ctf19p provides a link between the mitotic spindle and the kinetochore in budding yeast.

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