The Spindle Checkpoint of the Yeast Saccharomyces cerevisiae Requires Kinetochore Function and Maps to the CBF3 Domain

Richard D. Gardner,* Atasi Poddar,* Chris Yellman,* Penny A. Tavormina,*+ M. Cristina Monteagudo* and Daniel J. Burke*

*Department of Biochemistry and Molecular Genetics, University of Virginia Medical Center, University of Virginia, Charlottesville, Virginia 22908-0733 and Department of Cell Biology, University of Oklahoma Health Sciences Center, University of Oklahoma, Oklahoma City, Oklahoma 73190

Manuscript received June 30, 2000
Accepted for publication January 5, 2001

ABSTRACT

We have measured the activity of the spindle checkpoint in null mutants lacking kinetochore activity in the yeast Saccharomyces cerevisiae. We constructed deletion mutants for nonessential genes by one-step gene replacements. We constructed heterozygous deletions of one copy of essential genes in diploid cells and purified spores containing the deletion allele. In addition, we made gene fusions for three essential genes to target the encoded proteins for proteolysis (degron alleles). We determined that Ndc10p, Ctf13p, and Cep3p are required for checkpoint activity. In contrast, cells lacking Cbf1p, Ctf19p, Mcm21p, Skl19p, Cse4p, Mif2p, Mck1p, and Kar3p are checkpoint proficient. We conclude that the kinetochore plays a critical role in checkpoint signaling in S. cerevisiae. Spindle checkpoint activity maps to a discreet domain within the kinetochore and depends on the CBF3 protein complex.

Copyright © 2001 by the Genetics Society of America

THE spindle checkpoint is a regulatory system that controls the onset of anaphase in response to spindle malfunction (Amon 1999; Burke 2000; Gardner and Burke 2000). We identified seven mutants of the yeast Saccharomyces cerevisiae that could not arrest in the cell cycle in response to benzoimadazoles (such as nocodazole and benomyl) that inhibit microtubule assembly. Homologs of the genes have been identified in a number of different organisms, suggesting that the checkpoint is conserved from yeast to humans (Taylor and McKeon 1997; Bernard et al. 1998; Cahill et al. 1998; Chen et al. 1998; Basu et al. 1999; Chan et al. 1999; Yu et al. 1999). Mutants affecting microtubule assembly, microtubule-based motors, spindle pole body components, and kinetochore proteins induce checkpoint activity and inhibit anaphase (Wang and Burke 1995; Pangilinan and Spencer 1996; Skibbens and Hieter 1998; Hardwick et al. 1999). The kinetochore is a complex of proteins bound to centromere DNA (Ghoo 1997; Espelin et al. 1997; Meluh and Koshland 1997; Skibbens and Hieter 1998). Antibodies that recognize homologs of yeast checkpoint proteins stain kinetochores in a variety of cells (Gorbsky et al. 1998). This staining occurs at the time of checkpoint activity, suggesting that kinetochore localization of checkpoint proteins is an important part of checkpoint function (Taylor and McKeon 1997; Gorbsky et al. 1998; Kallio et al. 1998; Basu et al. 1999; Yu et al. 1999).

Formal proof that the kinetochore plays a role in checkpoint activity comes from analysis of kinetochore mutants in S. cerevisiae (Tavormina and Burke 1998; Sassoon et al. 1999). The core of the kinetochore in yeast is a protein complex, called CBF3, bound to a conserved centromere DNA element (CDEIII) that contains four essential proteins: Ndc10p, Ctf13p, Cep3p, and Ctf19p, and Sklp1p (Russell et al. 1999). A number of other proteins are also associated with CBF3, and at least three of them are essential (Brown et al. 1993; Stoler et al. 1995; Meluh and Koshland 1997; Meluh et al. 1998; Ortiz et al. 1999). Temperature sensitive (Ts-) mutants defining these genes have two different phenotypes. Most of the Ts- mutants arrest or show a pronounced delay in the cell cycle at the restrictive temperature (Brown et al. 1993; Doheny et al. 1993; Lechner 1994; Stoler et al. 1995; Strunnikov et al. 1995; Connelly and Hieter 1996; Ortiz et al. 1999). Ts- ndc10 mutants have a different phenotype. The cells, when grown at the restrictive temperature, are unable to attach chromosomes to the spindle microtubules and complete anaphase with massive nondisjunction of the chromosomes (Goh and Kilmartin 1993). This catastrophic missegregation of chromosomes does not cause cells to arrest in the cell cycle. Instead, they divide the DNA asymmetrically and replicate the unbalanced genomes in the subsequent cell cycle, resulting in genomic instability. ndc10-I mutants cannot maintain an arrest in the presence of nocodazole; therefore, ndc10-I mutants lack...
the spindle checkpoint (Tavormina and Burke 1998). The two different phenotypes associated with kineto-
chore mutants reflect the presence or absence of the spindle checkpoint. One explanation for the different
phenotypes associated with Ts mutants affecting kinet-
ochore activity is that there is reduced activity of the
gene products (hypomorphs) for some of the mutants.
Perhaps more kinetochore mutants would show a lack of spindle checkpoint activity if null alleles could be
used.

We have used a variety of techniques to produce null
alleles of genes encoding kinetochore proteins. We used
targeted insertion to generate deletion mutations of non-
essential kinetochore genes. We developed a method to
recover null mutants after sporulation and germination
from diploids that were heterozygous for a targeted
deletion mutation. We also constructed “degron-
tagged” alleles of many of the essential genes encoding
kinetochore proteins. The degron targets the proteins
for proteolysis by the proteasome and results in tempera-
ture-sensitive null mutants. We show that null alleles of
NDC10, CEP3, and CTF13, which encode components
of CBF3, do not arrest the cell cycle in the absence of
the proteins. Null alleles of CSE4, MIF2, CBF1, CTF19,
MCM21, SLK19, MCK1, and KAR3 are checkpoint pro-
cient. We conclude that spindle checkpoint activity,
within the kinetochore, is dependent on CBF3.

MATERIALS AND METHODS

Strains and media: Strains are listed in Table 1. Cells were
grown in YM-1 medium (Hartwell 1967) and synthetic com-
plete medium lacking essential nutrients as required (Burke
et al. 2000). Meiosis was induced in diploid cells by incubation
in 1% potassium acetate (Burke et al. 2000). Benomyl (Sigma
St. Louis) and a gift from Dupont) was added to a final
concentration of 15 μg/ml in agar-containing plates. Nocoda-
zole (Sigma) was used in liquid medium at 15 μg/ml when
cells were grown at a temperature <36°C. A mixture of nocoda-
zole and benomyl (20 μg/ml of nocodazole and 30 μg/ml of
benomyl) was used when incubating cells at temperatures
>36°C (Tavormina et al. 1997). A stock solution of 1 mg/ml α-factor (Sigma) was diluted 1:20,000 for bar1 strains. Cyto-
heximide (Sigma) was used at a final concentration of 10 μg/
ml.

Plasmid and strain construction: Plasmids are listed in Table
1. Degron alleles were constructed and integrated based on
the method of Dohmen et al. (1994). The first 200–500 bp of
the gene were amplified by PCR (sequences of all primers are
available upon request). PCR products were subcloned into
the PCR2.1 plasmid (Invitrogen, Carlsbad, CA) according to
the instructions of the manufacturer. All plasmids were se-
quenced (University of Virginia DNA Core Facility) to confirm
the wild-type sequence. The PCR fragments were excised from
PCR2.1 and subcloned into the Yip degron vector pPW66R (Dohmen
et al. 1994). The correct sequence of the degron
fusion was confirmed by sequencing to assure that the pro-
ducts were cloned in-frame. The degron is a copper-regulated
promoter driving expression of an amino terminal fusion pro-
tein that targets the fusion protein for degradation by the 26S
proteasome in a temperature-dependent fashion (Dohmen
et al. 1994). The degron also contains an epitope derived from
haemagglutinin protein (HA) so that the fusion protein can be
detected by Western blots. The degron fusion was targeted to
the appropriate genomic locus by cutting at a unique restric-
tion site within the targeted gene.

Precise deletions of open reading frames were constructed
by integrating fragments derived by PCR (Wach et al. 1994).
The fragments contained 40 bp of homology to the 5’ and 3’
ends of the target genes flanking a fusion gene that expresses
aminoglycoside (kanamycin) resistance. Deletion alleles con-
structed in this manner were designated :KAN (see Table 1).
After transformation, cells were plated onto YPD agar
plates and grown overnight, and transformants were selected by
transferring cells using sterile velveteed pads, onto YPD agar
plates containing 500 μg/ml Geneticin (G418, Life Technolo-
gies). All integrations were confirmed by PCR. SLK19 was
deleted by integrating a PouIII-Sphi restriction fragment of plas-
mid pAdh24:URA3, selecting for integration on SC – ura
plates. Integrants were confirmed on Southern blots.

A Yip plasmid (pDB110) containing LEU2 and CYH2 was
constructed by subcloning the LEU2 and CYH2 genes, con-
tained in a BoXI fragment of pRS318, into the BoXI sites of
pRS305. Approximately 500 base pairs from the 3’ ends of
MIF2 and CTF13 were amplified by PCR and subcloned into
PCR2.1 as described above. The fragments were excised from
PCR2.1 and subcloned into pDB110 to produce plasmids
pDB135 and pDB112, respectively. The plasmids pDB135 and
pDB112 were targeted to their respective genomic loci, MIF2
and CTF13, by cutting with BglII (pDB133) and Sphi
(pDB112). Transformants were selected on SC – leu plates.

Flow cytometry: Cells were prepared for flow cytometry using
propidium iodide (Sigma) as described previously (Smith

Spore enrichment: Spores were enriched using a modified
version of the protocol described by Rockmill et al. in Guthrie
and Fink (1991). Briefly, cells were pelleted, washed once in
a large volume of water (at least 40 ml), resuspended in 1 ml
of water, and transferred to a 15-ml conical polypropylene
tube containing 1 ml of water, and transferred to a 15-ml conical polypropylene
tube. Ascii were digested by adding 100 μl zymolase (10 mg/
ml; Seikagaku, Rockville, MD) and incubating at 30
°C for 30 min to 1 hr. Spores were concentrated by centrifugation, washed in
5 ml of water, and resuspended in 500 μl of water by stirring
with a sterile wooden dowel. The spores adhered to the surface
of the tube after suspending with the aid of a vortex mixer.
The remaining liquid was transferred to a second tube for
further dispersion onto the tube walls to recover additional
spores. Both tubes were gently washed twice with 500 μl of water
and sonicated. The two different phenotypes associated with kineto-
chore mutants reflect the presence or absence of the
gene were ampliﬁed by PCR (sequences of all primers are
available upon request). PCR products were subcloned into
plasmid pRS305. Approximately 500 base pairs from the 3’ ends of
MIF2 and CTF13 were amplified by PCR and subcloned into
PCR2.1 as described above. The fragments were excised from
PCR2.1 and subcloned into pDB110 to produce plasmids
pDB135 and pDB112, respectively. The plasmids pDB135 and
pDB112 were targeted to their respective genomic loci, MIF2
and CTF13, by cutting with BglII (pDB133) and Sphi
(pDB112). Transformants were selected on SC – leu plates.

Spore germination: Spores were inoculated into 25 ml of
YM-1 containing 2% glucose + 10 μg/ml cycloheximide and
incubated at 37°C. Budding of cycloheximide-resistant cells was
monitored by microscopy and usually began at 5–6 hr after
inoculation. Cycloheximide-resistant cells were purified from
the cycloheximide-sensitive spores after most had entered the
cell cycle. Cells were concentrated by centrifugation in a poly-
propylene tube. Many of the cells and spores adhered to the
walls. Spores that retained hydrophobicity could be removed
by gentle washing. The cells were resuspended in 500 μl of water
and applied to the tube walls by use of a vortex mixer
machine twice more and each time the centrifuge tube was
gently washed. Efficiency of initial spore enrichment was deter-
mined by testing viability after plating on YPD plates. If the
purification were completely successful, none of the cells
would be viable since they contain a deletion of an essential
gene. Viability was typically ~10%; therefore 90% of the cells

1494 R. D. Gardner et al.
### TABLE 1

Strains and plasmids

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>1293-R</td>
<td>MATa ade-2-101 his3Δ200 leu2Δ1 lys2-801 ura3-52 trp1Δ63 ctf13-30</td>
<td>P. Hieter</td>
</tr>
<tr>
<td>2114</td>
<td>MATa cep3-2 ade-2-101 his3Δ200 leu2Δ1 lys2-801 trp1Δ1 ura3-52 mad2::HIS3</td>
<td>This study</td>
</tr>
<tr>
<td>2124</td>
<td>MATa his3Δ1 ade-2-1 ade-6-1 cyh2 can1 hom3-H1 trp1-289 leu2-3,112 ura3-52 bar1::KAN</td>
<td>This study</td>
</tr>
<tr>
<td>2145</td>
<td>MATa his3Δ1 ade-2-1 ade-6-1 cyh2 can1 hom3-H1 trp1-289 leu2-3,112 ura3-52 bar1::KAN</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>(Yip URA3 cep3td)</td>
<td></td>
</tr>
<tr>
<td>2156</td>
<td>MATa his3Δ1 ade-2-1 ade-6-1 cyh2 can1 hom3-H1 trp1-289 leu2-3,112 ura3-52 bar1::KAN</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>(Yip URA3 skp1td)</td>
<td></td>
</tr>
<tr>
<td>2160</td>
<td>MATa his3Δ1 ade-2-1 ade-6-1 cyh2 can1 hom3-H1 trp1-289 leu2-3,112 ura3-52 bar1::KAN</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>(Yip URA3 ndc10td)</td>
<td></td>
</tr>
<tr>
<td>2162</td>
<td>MATa his3Δ1 ade-2-1 ade-6-1 cyh2 can1 hom3-H1 trp1-289 leu2-3,112 ura3-52 bar1::KAN</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>(Yip URA3 ctf13td)</td>
<td></td>
</tr>
<tr>
<td>2185</td>
<td>MATa/MATa MIF2::LEU2 CYH2 ste5-1/ste5-1 ade5-1/+ ura3-52/+ leu2-3,112 cyh2/cyh2</td>
<td>This study</td>
</tr>
<tr>
<td>2215</td>
<td>MATa/MATa MIF2::LEU2 CYH2/+ ste5-1/ste5-1 ade5-1/+ ura3-52/+ leu2-3,112 cyh2/cyh2</td>
<td>This study</td>
</tr>
<tr>
<td>2252</td>
<td>MATa/MATa cse4::KAN/CTF13::LEU2 CYH2 ste5-1/ste5-1 ade5-1/+ leu2-3,112/ leu2-3,112 cyh2/cyh2</td>
<td>This study</td>
</tr>
<tr>
<td>2419</td>
<td>MATa/MATa CTF13::LEU2 CYH2/+ ste5-1/ste5-1 ade5-1/+ ura3-52/+ leu2-3,112 cyh2/cyh2</td>
<td>This study</td>
</tr>
<tr>
<td>2268</td>
<td>MATa ade-2-1 can-1-100 his3-11,15 trp1-3,112 trp1-1 ura3-1 cj19::KAN</td>
<td>This study</td>
</tr>
<tr>
<td>2269</td>
<td>MATa ade-2-1 can-1-100 his3-11,15 trp1-3,112 trp1-1 ura3-1 cm21::KAN</td>
<td>This study</td>
</tr>
<tr>
<td>2272</td>
<td>MATa ade-2-1 can-1-100 his3-11,15 trp1-3,112 trp1-1 ura3-1 skh19::URA3</td>
<td>This study</td>
</tr>
<tr>
<td>2288</td>
<td>MATa cjy1 ura3-353 leu2-3 ade2-1 ade-3-1 cjy2 his4 mad2::URA3</td>
<td>This study</td>
</tr>
<tr>
<td>2289</td>
<td>MATa ade-2-101 his3Δ200 leu2Δ1 lys2-801 ura3-52 trp1Δ63 mck1::HIS3 mad2::URA3</td>
<td>This study</td>
</tr>
<tr>
<td>2290-5-1</td>
<td>MATa ade-2-101 his3Δ200 leu2Δ1 lys2-801 trp1-1 ura3-1😔5 skl9::URA3 mad2::KAN</td>
<td>This study</td>
</tr>
<tr>
<td>2291-22-2</td>
<td>MATa ade-2-1 can1 lys5 leu2 trp1 hom3-H1 his3 ura3 mad2::KAN (Yip URA3 cse4td)</td>
<td>This study</td>
</tr>
<tr>
<td>2407-5-3</td>
<td>MATa ade-2-1 hom3-H1 his3Δ1 leu2-2,113 trp1-289 ura3-52 lys2-1 ndc10-1::KAN</td>
<td>This study</td>
</tr>
<tr>
<td>2419</td>
<td>MATa ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 skp1-1::TRP1 skp1-4::LEU2 mad2::HIS3</td>
<td>This study</td>
</tr>
<tr>
<td>2bAS282</td>
<td>MATa ade-2-101 his3Δ200 leu2Δ1 lys2-801 trp1Δ1 ura3-32 cep3-2</td>
<td>A. Strunnikov</td>
</tr>
<tr>
<td>2AS282</td>
<td>MATa ade-2-1 hom3Δ1 leu2-3,112 trp1-289 ura3-52 cep13-30 mad2-1</td>
<td>This study</td>
</tr>
<tr>
<td>792-1-1</td>
<td>MATa ade-2-1 his3Δ1 leu2-3,112 trp1-289 ura3-52 cep13-30 mad2-1</td>
<td>This study</td>
</tr>
<tr>
<td>A364A</td>
<td>MATa ade-1-1 ade-2-1 tyr1-H1 lys2-1 hist7-H1 ura1-H1</td>
<td>L. Hartwell</td>
</tr>
<tr>
<td>MS1357</td>
<td>MATa trp1Δ1 ura3-52 leu2-3,112 kar3-1</td>
<td>M. Rose</td>
</tr>
<tr>
<td>R95-1-1-1</td>
<td>MATa cjy1 ura3-52 leu2-3,112 ade2-1 ade-3-1 his2-1 kar3-1</td>
<td>R. Baker</td>
</tr>
<tr>
<td>W305a</td>
<td>MATa ade-2-1 can-1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1</td>
<td>R. Rothstein</td>
</tr>
<tr>
<td>YPH1161</td>
<td>MATa ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 skp1-1::TRP1 skp1-4::LEU2 CEN5.LYPH983</td>
<td>P. Hieter</td>
</tr>
<tr>
<td>YPH636</td>
<td>MATa ade-2-101 his3Δ200 leu2Δ1 lys2-801 ura3-52 trp1Δ63 mck1::HIS3</td>
<td>P. Hieter</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Purpose</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pΔsb24:URA3</td>
<td>slk19::URA3</td>
<td>Joe Donovan</td>
</tr>
<tr>
<td>pDB110</td>
<td>Yip LEU2 CYH2</td>
<td>This study</td>
</tr>
<tr>
<td>pDB112</td>
<td>Yip CTF13 LEU2 CYH2</td>
<td>This study</td>
</tr>
<tr>
<td>pDB117</td>
<td>mif2 3Δ in pPW66R</td>
<td>This study</td>
</tr>
<tr>
<td>pDB118</td>
<td>cep3 3Δ in pPW66R</td>
<td>This study</td>
</tr>
<tr>
<td>pDB119</td>
<td>cse4 3Δ in pPW66R</td>
<td>This study</td>
</tr>
<tr>
<td>pDB120</td>
<td>skp1 3Δ in pPW66R</td>
<td>This study</td>
</tr>
<tr>
<td>pDB130</td>
<td>ndc10 3Δ in pPW66R</td>
<td>This study</td>
</tr>
<tr>
<td>pDB131</td>
<td>cj13 3Δ in pPW66R</td>
<td>This study</td>
</tr>
<tr>
<td>pDB133</td>
<td>Yip MFB2 LEU2 CYH2</td>
<td>This study</td>
</tr>
<tr>
<td>pPW66R</td>
<td>Yip URA3 degron vector plasmid</td>
<td>A. Varshavsky</td>
</tr>
<tr>
<td>pRC10.1</td>
<td>mad2Δ in Bluescript</td>
<td>R. Li</td>
</tr>
</tbody>
</table>
contained the deletion allele. Viable cells were contaminating wild-type spores or diploid cells, both of which were cycloheximide sensitive. The germinated cells were grown in YM-1 medium containing cycloheximide and any contaminating wild-type cells could not enter the cell cycle and did not add significantly to the population of cells in the analysis. Germinating the spores in rich medium resulted in reliable synchrony and the ste5-1 mutation prevented mating so that we could monitor changes in ploidy in haploid cells.

**Protein transfer:** Protein transfer and detection with antibodies was performed as described previously (Tavormina and Burke 1998). A mouse monoclonal anti-HA antibody 12CA5 (Babco) was used for detection in combination with enhanced chemiluminescence (Amersham, Buckinghamshire, UK).

**Immunofluorescence:** Antitubulin immunofluorescence was performed essentially as described previously (Adams and Pringle 1984). The antitubulin antibody YOL3/4 (Serotec, Oxford, UK) was cultured supernatant and was used undiluted. The monoclonal antibody was detected using a CY3 conjugated rabbit anti-rat antibody (Molecular Probes, Eugene, OR). Cells were mounted with Vecta-Shield (Vector Technologies) to prevent photobleaching during examination.

**Degron experiments:** Cells were grown to $1 \times 10^7$ cells/ml at 23°C in YM-1 medium containing 2% glucose and 100 μM CuSO₄. The mating pheromone α-factor was added and cells were incubated for 3 hr, washed three times in 50 ml of water to remove any residual copper, and resuspended in SC medium containing 2% glucose plus α-factor. Cells were incubated at 37°C for 90 min to destroy the degron-tagged protein and were released into the cell cycle at 37°C by adding pronase to 50 μg/ml. Samples were taken at 30-min intervals and processed for flow cytometry.

**RESULTS**

Temperature-sensitive ctf13-30 mutants delay in the cell cycle because of the spindle checkpoint (Wang and Burke 1995; Pangilinan and Spencer 1996). The activity of the spindle checkpoint in kinetochore mutants is easily assayed by constructing double mutants that lack one of the checkpoint genes, such as MAD2, and by growing the double mutants under conditions where they are limited for kinetochore protein function. The double mutants die because the spindle checkpoint is required for viability under these conditions. We confirmed that the spindle checkpoint is active in temperature-sensitive kinetochore mutants by assaying double mutants constructed by targeted deletion of MAD2. We grew cells at a semipermissive temperature and determined the viability by spotting serial dilutions of cells onto plates. Figure 1 shows the growth of the CBF3 mutants cep3-2, skp1-4, and ctf13-30. In each case the double mutants were compromised for growth compared to the single mutant. By examining budding morphology, we confirmed that the accumulation of large budded cells that is evident in all of the single mutants is eliminated in the double mutants (not shown). These data show that the kinetochore mutants activate the spindle checkpoint and that checkpoint activity is required for maximal viability.

The checkpoint is unaffected when nonessential kinetochore genes are deleted: We assayed the presence of the checkpoint in deletion mutants for all of the nonessential genes that encode kinetochore proteins. Deleting most of the nonessential genes that encode kinetochore proteins results in sensitivity to the drug benomyl that causes microtubule depolymerization (Foreman and Davis 1993; Hyland et al. 1999; Poddar et al. 1999). If the benomyl sensitivity is due to the loss of the spindle checkpoint, then mutants should be unable to arrest in the cell cycle in response to nocodazole, a similar microtubule inhibitor. We grew cells from the cbf1, mcm21, and ctf19 deletion mutants, treated them with nocodazole, and analyzed them by flow cytometry. In these and other experiments we also compared, where possible, the percentage of arrested cells (large buds and undivided nuclei) from each mutant to the percentage of arrested cells of each mutant in a mad2 background, to demonstrate that any arrest observed was checkpoint dependent. The majority of cells accumulated with a 2C content of DNA, suggesting that the mutant arrested in the cell cycle and that the spindle checkpoint was intact (Figure 2, A–C; 68% of cbf1 cells were arrested vs. 20% of cbf1 mad2 cells (data not shown). Double mutants were not possible for mcm21 and ctf19 because the double mutants are inviable (data not shown). In addition, we tested deletion mutants for nonessential genes that have been implicated in kinetochore function. SLK19 encodes a protein that associates with the kinetochore and is required for spindle integrity (Zeng et al. 1999). MCK1 encodes a protein kinase that was identified as a multicopy suppressor of the ndc10-42 mutant (Jiang et al. 1995). KAR3 encodes a kinesin-like protein that has been implicated in kinetochore function (Middleton and Carbone 1994). The spindle checkpoint was unaffected by deleting SLK19, MCK1, or KAR3 (Figure 2, D–F. slk19 cells showed a 91% arrest vs. 22% in slk19 mad2 cells (data not shown). mck1 cells showed a 65% arrest vs. an 18% arrest in mck1 mad2 cells (data not shown). Double mutants were not possible for kar3 because they are inviable. None of the six nonessential genes that encode either kinetochore
proteins or proteins that interact with kinetochores is required for the activity of the spindle checkpoint.

The checkpoint is eliminated when Cep3p is missing: We constructed degron-tagged alleles of three of the essential kinetochore genes. The efficiency of protein degradation in the three different degron mutants is shown in Figure 3. The degron-tagged protein fusion is detected by anti-HA Western blots when grown at the permissive temperature in the presence of copper. When grown in the absence of copper at the restrictive temperature, the fusion proteins are degraded within 90 min.

We adopted a standard assay to determine if the degron mutants lacked checkpoint activity. We synchronized cells by arresting them with the mating pheromone and then induced protein degradation for 90 min. We released the cells into the cell cycle in the absence of the kinetochore protein and assayed cell cycle progression by flow cytometry. An example for the degron-tagged ndc10 mutant is shown in Figure 4B. The DNA is asymmetrically distributed to the daughter cells when the kinetochores do not attach and the spindle checkpoint does not restrain mitosis. The cells divide and enter the subsequent cell cycle to re-replicate the DNA to produce some cells with greater than 2C content of DNA and most of the remaining cells have a less than 1C content of DNA. We interpret the changes in ploidy to mean that the kinetochores are unable to attach to the mitotic spindle and the chromosomes are asymmetrically distributed to the daughter cells. In addition, the spindle checkpoint is inactive and fails to respond to the unattached chromosomes, resulting in re-replication of the genome producing some cells with a greater than 2C content of DNA. Furthermore, the phenotype of the degron-tagged ndc10 allele is indistinguishable from the ndc10-1 mutant, showing that the missense mutant has the null phenotype.

We tested other kinetochore components with similar expectations. The missense temperature-sensitive mutations (for example, cep3-2) result in cell cycle arrest due to checkpoint activation (Strunnikov et al. 1995; Wang and Burke 1995; Pangilinan and Spencer 1996; this study). If this were the null phenotype, we would expect that the degron mutants should similarly arrest in the cell cycle. The data for the degron-tagged cep3 mutant are shown in Figure 4C. The mutant has a phenotype

![Figure 2. Response of deletion mutants to checkpoint activation. Strains with deletion mutations (A) cbf1 (2264), (B) mcm21 (2269), (C) ctf19 (2268), (D) slk19 (2272), (E) mck1 (YPH636), and (F) kar3 (MS1353) were grown to midlog (time 0) at 30°C and nocodazole was added to 15 μg/ml. Cells were collected at the indicated times (hours), stained with propidium iodide, and analyzed by flow cytometry.](image)

![Figure 3. Stability of Cep3p, Ndc10p, and Cse4p fusion proteins in their respective degron mutants. Cells from degron strains 2145 (cep3), 2174 (ndc10), and 2162 (cse4) were grown in YM-1 medium containing copper at 23°C to 10^7 cells per milliliter. A protein sample was prepared from cells expressing the fusion protein at 23°C (0 min). The cells were washed three times and resuspended in SC-ura lacking copper and cells were incubated at 37°C to initiate proteolysis of the fusion protein. Protein samples were prepared from cells taken at 30, 60, and 90 min. The fusion protein was identified using a monoclonal anti-HA antibody after PAGE and transferring the proteins to a membrane. Asterisks indicate nonspecific bands of similar size that interact with the anti-HA antibody.](image)
similar to the ndc10 degron allele. Cells are not restrained in the cell cycle but divide and some cells re- replicate the DNA in the subsequent cell cycle. The cep3 degron mutant does not produce a phenotype as severe as the ndc10-1 or the ndc10 degron allele. There is a broader distribution of cells with increased ploidy and fewer aploid cells. The low degree of aploidy was confirmed by examining the DNA distribution in dividing cells by microscopy (data not shown). We interpret this to mean that the cep3 degron fails to destroy all of the Cep3 fusion protein. The result is that the degree of nondisjunction is less severe than in the ndc10 mutants. Regardless, kinetochores lacking Cep3p do not induce a spindle checkpoint arrest. Checkpoint activity within the kinetochore depends on both Ndc10p and Cep3p.

The checkpoint is intact when Cse4p is eliminated: A histone H3 variant, Cse4p, is present at the kinetochores of yeast and is thought to form a specialized nucleosome required for kinetochore function (Meluh et al. 1998). Missense Ts \(^{-}\) mutants show a prominent cell cycle delay at the restrictive temperature and the delay is dependent on the spindle checkpoint. We constructed a degron-tagged allele of CSE4 and determined whether the missense mutants had the null phenotype and whether the checkpoint was intact in the absence of Cse4p. The data in Figure 4D show that the majority of cells from the cse4 degron mutant accumulate with a 2C content of DNA. More than 80% of the cells in each sample, beginning at 60 min (\(n = 500\) for each sample), were large budded with a single undivided nucleus. A mad2 deletion in the degron-tagged cse4 strain resulted in only 6% of the cells accumulating with a large bud and an undivided nucleus. We conclude that the missense cse4 mutants have the null phenotype and that the spindle checkpoint is intact in the absence of Cse4p.

We could not produce degron-fusion proteins, useful for these studies, using two of the kinetochore genes. The amino terminal fusion of the degron to Mif2p results in lethality. The degron fusion to Ctf13p did not result in temperature sensitivity. Excess expression of Ubr1p, a ubiquitin ligase, can improve the efficiency of proteolysis of degron-tagged proteins (Labib et al. 1999). We found that excess expression of Ubr1p in the strain containing the Ctf13p degron caused the cells to be Ts\(^{-}\). However, the cells could form small microcolonies of \(~50\) cells at the restrictive temperature. Therefore, we could not use the Ctf13 degron-tagged allele for cell synchrony experiments.
An alternative method for constructing null mutants:

We developed a new method to recover null mutants (Figure 5). In this method, we modify “your favorite gene” (YFG1) to create and sporulate a heterozygous diploid strain Δyg1/YFG1-CYH2 (see MATERIALS AND METHODS). The diploid is also homozygous for cyh2 and the temperature-sensitive ste5-1. The strain is sporulated and two types of spores are produced. The spores containing the wild-type allele of the gene of interest are CYH2 and therefore cycloheximide sensitive. The spores containing the deletion allele are cycloheximide resistant. Germination requires protein synthesis (HERMAN and RINE 1997). Therefore only the spores containing the deletion allele can germinate in the presence of cycloheximide. Spores are hydrophobic and can be purified away from cells on the basis of their hydrophobicity. The purified germinated spores were grown at 36°C to inactivate Ste5p and prevent mating. The resulting haploid cells were analyzed by flow cytometry to determine the effect of the null allele on cell cycling and to determine if there is a change of ploidy in cells that lack the protein.

We purified spores with a null allele of MIF2 and analyzed the mutant cells by flow cytometry. The data in Figure 6B show that the mij2::KAN mutant arrests in the cell cycle with a 2C content of DNA. The cells had an undivided nucleus, suggesting that the cells were arrested in mitosis. We stained cells with antitubulin antibodies and found that the arrested cells had typical-looking mitotic spindles with a mean length of 1.9 ± 0.4 μm (n = 50). Therefore cells arrest prior to anaphase in a mij2 null mutant.

We also used the strategy to construct a null allele of CTF13 and recovered mutant cells for flow cytometry (Figure 6D). The cells were clearly cycling as approximately half of the cells passed through the G2/M stage of the cell cycle (9 hr). We spread unpurified spores from the diploid onto a YPD plate containing cycloheximide and determined the fate of the cells after 12 hr of incubation (Figure 6E). Half of the spores (wild type) did not germinate as expected and produced cells with one cell body. The ctf13::KAN cells divided 2–3 times after germinating before finally ceasing cell division. The cells did not uniformly accumulate at a stage in the cell cycle as determined by bud morphology. Therefore, the cells were inviable due to a loss of Ctf13p and therefore ceased dividing, but the cells did not arrest homogeneously in the cell cycle. We conclude that Ctf13p, like Ndc10p and Cep3p, two other proteins in the CBF3 complex, is required for spindle checkpoint activity.

DISCUSSION

CBF3 is required for spindle checkpoint activity: We have analyzed null mutants to determine which kinetochore proteins are required for spindle checkpoint activity. NDC10, CEP3, and CTF13 are required for the spindle checkpoint. CSE4, MIF2, CBF1, MCM21, CTF19, SLK19, MCK1, and KAR3 are not required for checkpoint function. We conclude that the kinetochore plays an active role in the spindle checkpoint in S. cerevisiae and that the activity is dependent on the CBF3 complex within the kinetochore.

Our data show that the ndc10 degron-tagged allele has the same phenotype as ndc10-1, a temperature-sensitive missense mutant. The spindle checkpoint is eliminated in cells lacking Ndc10p. Both the ndc10 and cep3 degron-tagged mutants lacked the spindle checkpoint and showed dramatic genetic instability as assayed by flow cytometry. Therefore eliminating Cep3p has the same effect as eliminating Ndc10p: the spindle checkpoint is nonfunctional. We constructed a degron-tagged allele of the fourth CBF3 gene, SKP1, but the experiments were complicated to execute and interpret because of the dual requirement of Skp1p in the cell cycle (BAI et al. 1996; CONNELLY and HETTER 1996). We conclude that the CBF3 components Ndc10p and Cep3p are required for spindle checkpoint activity in S. cerevisiae.

We obtained a different phenotype with the ctf13 null mutant compared to ndc10 and cep3 null alleles. Cells lacking Ctf13p were isolated after sporulating a hetero-
zygous diploid. The spores containing the deletion allele divided two to three times and did not arrest at mitosis. We interpret the multiple divisions to mean that there is some carryover of the Ctf13p from the heterozygous diploid parent. However, the absence of a homogeneous mitotic arrest indicates that the spindle checkpoint is unaffected by the loss of Ctf13p.

Previous data have also suggested that Ctf13p is required for spindle checkpoint activity. Cells that progress through the cell cycle in the absence of DNA replication have an intact spindle checkpoint that is dependent on Ctf13p (Tavormina et al. 1997). The data are also consistent with kinetochore assembly experiments. The loss of Ndc10p has a drastic effect on kinetochore structure in vivo. Kinetochore proteins, including those in the CBF3 complex, do not localize with centromeric DNA in a ndc10-1 mutant (Ortiz et al. 1999). This agrees with the results from experiments showing that all of the CBF3 proteins must be present to assemble a stable kinetochore complex in vitro (Russell et al. 1999). The in vivo and the in vitro data predict that eliminating one of the CBF3 proteins should eliminate the entire complex. Our data give the expected result; mutations that eliminate CBF3 proteins result in similar phenotypes with respect to checkpoint activity.

The relationship between CBF3 and the spindle checkpoint: What role does CBF3 play in the spindle checkpoint? There is abundant evidence, from a variety of organisms, showing that checkpoint proteins localize to the kinetochore when the spindle checkpoint is active (Skibbens and Hieter 1998). The simple explanation is that at least one kinetochore protein interacts with one of the spindle checkpoint proteins and the interaction is required to signal that the chromosome is misaligned. The consequence is that the cell arrests in the cell cycle. Preliminary data from our lab have shown that the checkpoint protein Mad2p associates with the kinetochore and the association is CDEIII dependent (data not shown). This suggests that a CBF3-dependent Mad2p kinetochore interaction is required for spindle checkpoint activity.

We propose three possible roles for CBF3 in the pro-
cess on the basis of the hypothesis that kinetochore association of checkpoint proteins is required for checkpoint activity. The simplest is that one of the CBF3 proteins directly interacts with Mad2p (or other checkpoint proteins) and when CBF3 is missing the interaction cannot occur. The second possibility is that the loss of CBF3 from the kinetochore indirectly affects the interaction of the kinetochore with checkpoint proteins. Mif2p, Cse4p, Ctf19p, Mcm21p, and Okp1p do not localize to the kinetochores in a nde1-1 mutant, suggesting that kinetochore structure is severely impaired (Meluh and Koshland 1997; Ortiz et al. 1999). A loss of any CBF3 component probably eliminates many proteins from the kinetochore. If there is a kinetochore protein that interacts with the spindle checkpoint and is dissociated by a loss of CBF3, then it is not encoded by any of the known kinetochore genes. Null mutations that eliminate each of the other known kinetochore proteins do not eliminate the spindle checkpoint. Therefore, if checkpoint proteins interact with some kinetochore protein, other than a CBF3 component, it has not been identified yet. This is an appealing possibility because the spindle checkpoint is conserved from yeast to humans and the expectation is that a conserved protein that is at the kinetochore plays a role in the spindle checkpoint. Two kinetochore proteins that have obvious mammalian homologs (Mif2p and Cse4p) are not required for checkpoint signaling. The third possibility is that the spindle checkpoint signal depends on combinations of the kinetochore proteins that are already known. This model predicts that there must be some redundancy in function to allow checkpoint activity when one protein is absent.

The robust phenotype of checkpoint mutants overcomes limitations in the analysis of null alleles: Both methods that we have chosen to produce null alleles of essential genes have limitations. Deletion mutants isolated after sporulation inherit an unknown amount of wild-type protein from the heterozygous diploid into the mutant spores. The phenotype of cells having a mif2 deletion mutation is the same as the Ts− mutants; cells arrest uniformly in the cell cycle. Therefore the Ts− allele results in the null phenotype and this validates the data produced from analyzing null mutants produced by this method. The important difference in phenotypes between the spores from the ctf13 null mutant and the spores from the mif2 null mutant is that the lack of Ctf13p did not result in a uniform cell cycle arrest. A uniform arrest is expected if the mutant cells with defective kinetochores were checkpoint proficient. The different phenotypes associated with deletion mutations allow us to come to opposite conclusions with respect to functions of the two genes in the spindle checkpoint.

The degron-tagged alleles contain an epitope tag to provide an assay for the presence of the protein. However, there are also limitations in this approach. Following the degradation of the protein by Western blots does not assure that 100% of the protein is eliminated. Proteins may assemble only one molecule per kinetochore and it is possible that we could not detect one or two functional kinetochores. Therefore we cannot say with certainty that every kinetochore is affected in a degron-tagged mutant. It is possible that only a few kinetochores are affected in the cse4 mutant. The mutant arrests in the cell cycle but we cannot say with certainty how many kinetochores lack Cse4p. Cells adopt a uniform cell cycle arrest in a checkpoint-dependent manner when only a single kinetochore is affected (Skibbens and Hieter 1998; Gardner and Burke 2000). In the cases of cep3 and nde10 degron-tagged alleles, the phenotypes are quite different. The effects on chromosome stability are drastic so that we assume a large number of kinetochores are affected in each mutant. The important observation is that the cells fail to arrest uniformly in the cell cycle and therefore the dysfunctional kinetochores fail to induce the spindle checkpoint. The robust nature of the phenotypes has allowed us to assign a role for each kinetochore protein in the spindle checkpoint.

We thank Lee Hartwell, Mitch Smith, Doug Koshland, Alex Strunnikov, Mark Rose, Phil Hieter, Rodney Rothstein, Rick Baker, John Carbon, Alex Varshavsky, Angelika Amon, Joe Donovan, and John Diffley for strains and plasmids. We thank Gary Gorbsky, Mitch Smith, and members of the Burke lab for many helpful discussions. We are especially grateful to Mitch Smith for critical comments on the manuscript. We also thank the anonymous reviewers for insightful comments and suggestions. The work was supported by U.S. Public Health Services Grant GM-40334.

LITERATURE CITED


Bai, C., P. Sen, K. Hofmann, L. Ma, M. Goebi et al., 1996 SKP1 connects cell cycle regulators to the ubiquitin proteolysis machin- ery through a novel motif, the F-box. Cell 86: 263–274.


Chan, G. K., S. A. Jarlonski, V. Sudakin, J. C. Hittle and T. J. Yen, 1999 Human BUBR1 is a mitotic checkpoint kinase that...