A suppressor of a centromere DNA mutation encodes a putative protein kinase (MCK1)

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A new approach to identify genes involved in Saccharomyces cerevisiae kinetochore function is discussed. A genetic screen was designed to recover extragenic dosage suppressors of a CEN DNA mutation. This method identified two suppressors, designated MCK1 and CMS2. Increased dosage of MCK1 specifically suppressed two similar CEN DNA mutations in CDEIII, but not comparably defective CEN DNA mutations in CDEI or CDEII. A strain containing a null allele of MCK1 was viable under standard growth conditions, had a cold-sensitive phenotype (conditional lethality at 11°C), and grew slowly on Benomyl (a microtubule-destabilizing drug). Furthermore, when grown at 18°C or in the presence of Benomyl, the null mutant exhibited a dramatic increase in the rate of mitotic chromosome loss. The allele-specific suppression and chromosome instability phenotypes suggest that MCK1 plays a role in mitotic chromosome segregation specific to CDEIII function. The MCK1 gene encodes a putative protein–serine/threonine kinase, which suggests a possible role for the MCK1 protein in regulating the activity of centromere-binding proteins by phosphorylation. MCK1 was identified and cloned independently for its involvement in the induction of meiosis and is identical to a gene that encodes a phosphotyrosyl protein with protein kinase activity.

[Key Words: S. cerevisiae; kinetochore function; CEN DNA; allele-specific suppression]

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The eukaryotic kinetochore is a complex macromolecular structure that plays a critical role in the segregation of chromosomes during mitosis and meiosis, providing the site of chromosome attachment to the spindle apparatus. Although the kinetochore is morphologically well defined in higher eukaryotes, little is known about specific proteins that are required for proper function. An understanding of how the structure mediates proper chromosome segregation will require the identification of gene products that are physically or temporally important for kinetochore function.

In Saccharomyces cerevisiae, single microtubules are seen directly attached to chromatin without structurally differentiated kinetochores (Peterson and Ris 1976). Nuclease digestion experiments of intact chromatin, however, demonstrate that the centromeric DNA exists in a highly resistant 250-bp core that is flanked by nuclease hypersensitive sites (Bloom and Carbon 1982). It has been suggested that this core acts as the microtubule attachment site and may represent a primitive kinetochore structure. The centromere [CEN] DNA from S. cerevisiae has been cloned (Clarke and Carbon 1980) and characterized extensively (for review, see Newlon 1988). Completely functional CEN DNA is only ~120 bp in length and contains three conserved centromere DNA elements [CDEs]. An 8-bp conserved sequence [CDEI] is separated from a 25-bp conserved sequence [CDEIII] by a 78- to 86-bp AT-rich region [CDEII]. Mutation and deletion analysis of several yeast centromeres indicates that CDEI and CDEII are not absolutely required but are important for optimal chromosome stability and that CDEIII is essential for centromere function. These studies indicate that understanding the role of gene products involved in CDEIII function will be critical for understanding kinetochore function. On this basis, investigators have begun biochemical characterization of proteins that specifically bind to CDEIII (Hegemann et al. 1986; Ng and Carbon 1987; Lechner and Carbon 1991). Here we describe a genetic strategy involving suppressor analysis to isolate genes important for S. cerevisiae kinetochore function. Our experiments were designed to identify genes that play a role in CDEIII function.

Suppressor analysis has proved to be useful for identifying protein–protein interactions in a variety of biological investigations (for review, see Botstein and Maurer 1982). In principle, if two proteins interact, a deleterious amino acid substitution in one protein can be suppressed by a compensatory change in the other. By starting with a mutation in a gene encoding one component of a system, interacting gene products can be identified. This method has been extended to DNA–protein interactions as well. For example, mutations in the SV40 origin of replication are suppressed by mutations in T antigen, a

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known origin-binding protein [Shortle et al. 1979]. Another related strategy for identifying interacting proteins, termed dosage suppression, relies on overexpression of an extragenic wild-type gene to rescue a mutant phenotype (for examples, see Hayles et al. 1986; Hadwiger et al. 1989). It is reasonable to predict that dosage suppression may also be useful in identifying suppressors of mutations in binding sites for sequence-specific DNA-binding proteins. An underlying assumption in this strategy is that the DNA-binding site mutation causes a decrease in the binding affinity of the binding protein. Increasing the effective concentration of this protein would compensate for the lower binding affinity by mass action. The feasibility of this type of approach is supported by the experiments of Doto and Zinder (1984), which show that insertion mutations in the bacteriophage fl origin of replication are suppressed in trans by mutations that increase the concentration of an initiator protein.

Previously, we have analyzed a large set of CEN DNA mutations for their effects on the mitotic segregation of marker chromosome fragments, using a sensitive visual assay (Hegemann et al. 1988). A subset of these CEN mutations cause partial functional impairment, exhibiting a 10- to 50-fold increase in the rate of chromosome fragment loss. In this study, these partially defective CEN DNA mutant alleles were used as substrates in a dosage suppression screen. Two strains containing single base-pair insertions in CDEIII were transformed with a high-copy yeast genomic library and screened for transformants with improved function of the mutant centromere. These experiments identified two DNA sequences (CMS1 and CMS2, for centromere mutant suppressor) that act as extragenic dosage suppressors of specific CEN DNA mutations. These suppressors are excellent candidates for structural or regulatory components of the yeast kinetochore. In this discussion, we describe the characterization of the MCK1 gene (CMS1 was renamed MCK1, see below), which encodes a protein with homology to known protein-serine/threonine kinases, and discuss a plausible model for its involvement in kinetochore regulation. In an independent investigation, Neigeborn and Mitchell (this issue) identified the same gene (initially named IME3) as a meiotic activator. The gene has therefore been renamed MCK1 (for centromere and meiosis regulatory kinase) to reflect our broader understanding of the roles that MCK1 plays in controlling the fidelity of mitotic chromosome transmission and the induction of meiosis. Furthermore, the gene is identical to YPK1, a gene that encodes a 40-kD phosphotyrosyl protein associated with protein tyrosine kinase activity [Dailey et al. 1990].

Results

Identification of dosage suppressors of CEN DNA mutations

Yeast strains YPH298 and YPH299 were used to identify sequences that at high copy number suppress the defect in chromosome segregation caused by single base pair insertions in CDEIII. These diploid strains contain mutant centromeres [YPH298, CDEIII[19\textsuperscript{720-T}]], YPH299, CDEIII[19\textsuperscript{720-G}]] present on a SUP11-marked chromosome fragment for visual monitoring using a colony color assay [Shero et al. 1991]. The effects of these mutations on chromosome fragment loss rates per cell division were determined quantitatively to be an increase of 10- and 20-fold above wild type, respectively. The frequency of red sectors in a pink colony is a qualitative measure of the frequency of mitotic chromosome fragment loss during the growth of the colony. Quantitative differences in chromosome fragment loss rates of as low as two- to threefold can be distinguished qualitatively by using this assay [Hegemann et al. 1988]. We decided to use strains carrying CDEIII CEN mutant alleles that retain partial centromere function for two reasons: (1) CDEIII has been shown by mutation and deletion analysis to be essential for centromere function, and (2) these mutations may be better candidates for suppression than mutations that abolish or drastically reduce centromere function.

The strains YPH298 and YPH299 were transformed with a library of total yeast genomic DNA cloned in a 2-micron/TRP1 high-copy plasmid vector (J.H. Shero, unpubl.). Approximately 10,000 (YPH299) and 12,000 (YPH298) Trp+ transformant colonies were screened for a reduction in sectoring frequency. Two transformants of YPH299 and seven transformants of YPH298 displayed a reproducible suppressed red sectoring phenotype on re-plating. To determine whether the suppression phenotype was plasmid dependent, total DNA was prepared from each yeast transformant, plasmids were isolated by transformation of Escherichia coli, and each plasmid was then reintroduced into the yeast strain from which it was originally isolated. Only two plasmids were isolated that reproducibly displayed an increased-dosage suppression phenotype: pMCK1 (isolated from a YPH299 transformant) and pCMS2 (isolated from a YPH298 transformant). Restriction enzyme mapping and DNA hybridization demonstrated that inserts carried by these plasmids did not have DNA sequences in common [data not shown]. pMCK1, which showed the strongest qualitative suppression phenotype (Fig. 1), was selected for detailed study.

MCK1 suppression phenotype is specific to two similar CEN DNA mutant alleles

To determine the specificity of the MCK1 dosage suppression phenotype, the plasmids pMCK1 and vector alone were independently transformed into two strains containing trans-acting mutations that cause a high frequency of mitotic chromosome segregation errors [YPH628, ctf3/ctf3, YPH629, ctf9/ctf9; Spencer et al. 1990]. The chromosome loss frequency was qualitatively monitored by re-plating two independent Trp+ colonies on medium lacking tryptophan, allowing inspection of ~100 colonies for their sectoring phenotype. Estimates of chromosome fragment loss rates in the presence or
Suppressors of centromere DNA mutations

Figure 1. Colony phenotypes of strains carrying centromere DNA mutations in the presence or absence of pMCK1 suppressor. (A) Phenotype of the parent yeast strain (YPH299) that contains the 2-micron/Trp1 high-copy plasmid vector (pJS62) with no DNA insert. This strain is homozygous for the ade2-101 mutation and contains a URA3/SUP11-marked chromosome fragment with a CEN DNA mutation [CDEIII(19V20-G)] that is lost at a rate of 3.8 x 10^-3 per cell division giving rise to red sectors. (B) Phenotype exhibited by the YPH299 strain containing the pMCK1 extragenic dosage suppressor plasmid. The chromosome fragment loss rate for YPH299 suppressed by pMCK1 is 5.9 x 10^-4. (C) Phenotype of a strain (YPH632) that contains a cis-acting CEN DNA mutation in CDE1 (CDE1Δ) on the chromosome fragment and the plasmid vector pJS62 with no insert. (D) Phenotype exhibited by YPH632 containing the pMCK1 extragenic dosage suppressor plasmid. Note the complete lack of a suppression phenotype with pMCK1 (cf. C with D) in contrast to that seen with YPH299 (cf. A with B).

Absence of pMCK1 were confirmed quantitatively by fluctuation analysis (Table 1). These data showed that the chromosome loss phenotypes of YPH628 and YPH629 were not suppressed by pMCK1. This analysis confirmed that the suppression phenotype was due to a decrease in the rate of chromosome fragment loss per cell division and not due to an artifactual effect on the sectoring phenotypes. It also demonstrated that the suppression phenotype originally identified using YPH299 was not due to a global effect that suppressed all types of chromosome segregation defects.

CEN mutant allele specificity of the MCK1 suppression phenotype was investigated by independently introducing vector alone (pJS62) and pMCK1 into 11 different strains containing a wild-type or mutant copy of CEN6 on a SUP11-marked chromosome fragment (Fig. 2). In all cases, the presence of pJS62 [vector alone] had no effect on the loss rates, which were determined previously [Hegemann et al. 1988], of the wild-type or mutant CEN-containing chromosome fragments. Suppression of the different CEN mutations by pMCK1 was scored qualitatively for all 11 strain backgrounds [for examples, see Fig. 1] and quantitatively for 6 of these (Table 1). These data showed that the MCK1 suppression phenotype was specific to two closely related mutations in CDEIII, both single-base-pair insertions [CDEIII(19V20-G) and CDEIII(19V20-T)]. The baseline loss rate associated with the CDEIII(19V20-G) insertion mutation was suppressed by pMCK1 in 6.4.

Table 1. Mutant allele-specific suppression by pMCK1

| Yeast strain | CEN6 DNA mutation | Plasmid | Mitotic chromosome fragment loss rate | Fold suppression pJS62/pMCK1 |
|--------------|-------------------|---------|--------------------------------------|-----------------------------|
| YPH628*      | --                | pJS62   | 1.0 x 10^-2                         | 1.2                         |
| (ctf3/ctf3)  |                   | pMCK1   | 8.4 x 10^-3                         |                             |
| YPH629*      | --                | pJS62   | 7.1 x 10^-3                         | 0.8                         |
| (ctf9/ctf9)  |                   | pMCK1   | 8.6 x 10^-3                         |                             |
| YPH281       | wild type         | pJS62   | 2.3 x 10^-4                         | 0.9                         |
|              |                   | pMCK1   | 2.7 x 10^-4                         |                             |
| YPH299       | CDEIII(19V20-G)   | pJS62   | 3.8 x 10^-3                         | 6.4                         |
| YPH298       | CDEIII(19V20-T)   | pJS62   | 2.8 x 10^-3                         | 5.1                         |
| YPH630       | CDEII(+45 bp)     | pJS62   | 7.4 x 10^-4                         | 1.2                         |
| YPH631       | CDEII(Δ31bp)      | pJS62   | 6.6 x 10^-4                         | 1.2                         |
| YPH632       | CDEIΔ             | pJS62   | 5.2 x 10^-4                         | 1.9                         |
| YPH286       | CDEI(8-A)         | pJS62   | 1.0 x 10^-2                         | 1.9                         |
|              |                   | pMCK1   | 5.3 x 10^-3                         |                             |

*These strains contain wild-type CEN4 on the chromosome fragment, which is lost at an elevated rate due to a homozygous trans-acting mutation.
Summary of CEN DNA mutant allele specificity for suppression by pMCK1. The conserved regions [CDEI, CDEII, CDEIII] of the CEN6 DNA are indicated. CEN mutant alleles, analyzed for suppression by pMCK1, are shown above and below the CEN6 sequence. Arrows pointing away from the CEN6 sequence indicate DNA base-pair changes [with the change shown] or deletions [indicated by Δ]. Arrows pointing toward the DNA sequence indicate insertional mutations. Mutant alleles suppressed by pMCK1 are boxed, all other alleles tested were not significantly suppressed by pMCK1.

3.8 × 10⁻³ compared with 5.9 × 10⁻⁴ when suppressed by pMCK1. This sixfold quantitative effect is clearly distinguishable qualitatively in sectoring colonies using the color assay [Fig. 1A,B]. Similarly, the chromosome loss phenotype caused by the CDEIII (19V20-T) was suppressed fivefold by the high-copy pMCK1. Four additional CDEIII mutants causing either drastic [500- to 1000-fold] increases in chromosome fragment loss rates or complete loss of CEN function were not suppressed, as judged by the qualitative assay. It is possible that pMCK1 is unable to compensate for CDEIII mutations that cause an extreme reduction in the affinity of a DNA-binding protein for its binding site. Perhaps the most informative CEN mutant alleles tested for suppression by pMCK1 were those in CDEI and CDEII. The segregation defects associated with these CEN mutant alleles are all comparable in magnitude to the original CDEIII mutation used in isolating MCK1. Introduction of the high-copy pMCK1 caused little or no quantitative [Fig. 1C, D] or quantitative (Table 1) suppression of chromosome fragment loss rates in four strains that carried either a CDEI mutation [two alleles tested] or a CDEII mutation [two alleles tested], although the mutants caused only moderate increases in chromosome loss [-5- to 25-fold]. These data suggest that MCK1 plays a role in chromosome segregation that is specific to CDEIII function.

Molecular characterization of MCK1

The suppressor activity of pMCK1 was carried on a 7-kb fragment derived from a partial Sau3A restriction digest of yeast genomic DNA and was localized to a 3.6-kb BamHI–ClaI fragment. Unique restriction sites were modified to create frameshift mutations, and plasmids were assayed for suppressor activity by transforming into YPH299, followed by qualitative scoring of the sectoring frequency. A frameshift mutation introduced at the NcoI restriction site [underlined in Fig. 4, below] destroyed suppressor activity of the plasmid, indicating that the NcoI site was likely to be located in the amino acid-coding sequence of the MCK1 gene.

Physical and genetic mapping of MCK1

A radioactive probe was made from the cloned MCK1 gene and hybridized to a Southern blot containing yeast chromosome-sized DNA molecules. This provided unambiguous assignment of MCK1 to chromosome XIV [data not shown]. MCK1 was physically positioned on chromosome XIV by the chromosome fragmentation method [Vollrath et al. 1988; Gerring et al. 1990] and by probing the resultant chromosome fragments with probes proximal and distal to the site of fragmentation. This placed MCK1 ~ 80 kb from the left arm telomere of chromosome XIV [Fig. 3]. Standard meiotic mapping methods placed the MCK1 gene 18 cM proximal to the pha2 locus and indicated that the MCK1 gene had not been identified previously.

MCK1 deduced amino acid sequence is homologous to protein kinases

The DNA sequence of a 1.7-kb region included the entire MCK1 open reading frame. This open reading frame is encoded by 1125 bp and predicts a protein of 375 amino acids. This deduced sequence is homologous to protein kinases as judged by high amino acid sequence identities with the catalytic domains of protein kinases from other sources.

Figure 3. Physical and genetic mapping of MCK1. The cloned MCK1 DNA segment was used to fragment chromosome XIV into chromosome fragments containing sequences proximal or distal to the cloned segment. This placed the MCK1 locus ~80 kb from the telomere of its own chromosome arm and ~740 kb from the telomere on the opposite chromosome arm. Assignment to the left arm was accomplished by probing Southern blots of chromosome fragment containing strains with the KAR1 gene [which hybridized to the 740-kb fragment] and the RPD3 gene [which hybridized to the 80-kb fragment]. (A) Electrophoretic karyotypes of strains carrying chromosome fragments. Chromosome XIV is indicated by a dash; chromosome fragments are indicated by open arrowheads. (Lane 1) Parental strain; (lanes 2 and 3) strains carrying MCK1–distal chromosome fragments [80 kb]; (lanes 4 and 5) strains carrying MCK1–proximal chromosome fragments [740 kb]; (lane 6) YPH1-49 as size standard. (B) Diagram of extreme distal end of the chromosome XIV left arm. MCK1 was placed 18 cM proximal to pha2 by standard tetrad analysis.
acids with a $M_i$ of 43,108 [Fig. 4]. Computer searches using the program FASTA (Lipman and Pearson 1985) to look for amino acid sequences similar to MCK1 in standard data bases [GenBank release 57.0 and NBRF/PIR release 18.0] revealed significant homology to numerous protein kinases. Typical homology scores obtained showed $\sim 25\%$ identity between the predicted MCK1 protein and many known protein kinases in the data bank (e.g., CDC28). Outside of the 11 defined protein kinase motifs (for review, see Hanks et al. 1988), however, there was no significant sequence similarity. A personal data base maintained by Mark Goebel at Indiana University showed significantly higher homologies to MCK1 and TPK1. TPK1 (Toda et al. 1987) is a yeast protein kinase with a characteristic homology to MCK1 ($\sim 25\%$) in the catalytic domain. The rest of the gene shows almost no homology to MCK1, with the striking exception of the first 8 amino acids, which are identical [C. Chan, pers. comm.]. A computer search [M. Goebel, pers. comm.] did not reveal any additional protein sequences with a similar 8-amino-acid motif. Although the function of this sequence is unknown, its amino-terminal position suggests that it may be a signal sequence or a site for protein modification.

Gene disruption of MCK1

Because a frameshift mutation at the Ncol restriction site completely abolished suppression, a DNA fragment containing the HIS3 gene was cloned into this site to create an insertion mutant allele. Sequence analysis showed that this allele would be expected to result in a null mutation owing to disruption of the catalytic domain. A diploid strain that contained a SUP11-marked chromosome fragment (YPH273) was transformed with a linear DNA fragment containing the mck1::HIS3 allele. Two independently derived strains, in which one copy of MCK1 was replaced with mck1::HIS3, were sporulated and dissected. Analysis of resultant tetrads showed that the majority contained four viable spores with the HIS3 disruption marker segregating 2+: 2−. Furthermore, spore colonies containing mck1::HIS3 had normal growth rates and did not exhibit a qualitative increase in the loss rate of the chromosome fragment. Southern hybridization analysis of genomic DNA from the parent diploid and four four spore colonies of a tetrad confirmed correct disruption of MCK1 at the chromosomal locus [data not shown].

Phenotypes associated with MCK1 gene disruption allele

The mck1::HIS3 disruption strain was examined for phenotypes that might provide additional information toward understanding the function of the MCK1 gene product. Two strains carrying the mck1::HIS3 allele (YPH635, containing a SUP11-marked chromosome fragment, and YPH636) were tested for conditional growth at various temperatures and found to be completely inviable at 11°C. To test for potential chromosome segrega-
Figure 5. The MCK1 gene encodes a putative protein kinase. [A] Alignment of MCK1 with glycogen synthase kinase-3 [GSK-3 from rat; Woodgett 1990] and shaggy [sgg, from D. melanogaster; Bourouis et al. 1990] are shown. MCK1 is 45% [GSK3] and 41% [sgg] identical to these proteins over the canonical protein kinase catalytic domain [shown as the amino acids between number signs (#)]. The conserved and invariant residues identified by Hanks et al. (1988) are highlighted by dots above the appropriate residues. MCK1 encodes a novel protein that is likely to be a protein-serine/threonine kinase. [B] An unusual sequence identity was identified between MCK1 and TPKI. The first 8 amino acids of these two genes are identical yet they show no homology outside of the catalytic domain in the remainder of the sequence. Although the function of this sequence is unknown, its amino-terminal position suggests that it may be a signal sequence or a site for protein modification.

Figure 6. Sectoring phenotypes exhibited by haploid strains containing either MCK1 or MCK1::HIS3 at 16°C. [A] MCK1 [wild type]; [B] mck1::HIS3 [mutant].
Both plates were incubated for 3 days at 25°C. In particular, mutations in CDEI and CDEII that cause a segregation defect of comparable magnitude to the suppressible CDEIII mutant were not suppressed by pMCK1. We interpret this allele specificity to mean that suppression by the MCK1 protein kinase acts in a pathway important for CDEIII function rather than in a more global pathway that would suppress segregation defects in general.

Replacement of the wild-type MCK1 gene by mck1::HIS3 demonstrated that it is essential for growth at 30°C. The MCK1 insertion mutant exhibited two potentially informative secondary phenotypes: conditional lethality at 11°C and slow growth on Benomyl. Furthermore, when grown at semipermissive temperatures, or in the presence of Benomyl, the MCK1 insertion mutant exhibited an increase in the loss rate of a wild-type CEN-containing chromosome fragment. These data indicated that the endogenous copy of MCK1 functions in a biochemical pathway that is important for mitotic chromosome segregation. Because the only known intracellular target of Benomyl is β-tubulin (Thomas et al. 1985), the finding that mck1::HIS3 is hypersensitive to this drug implies that it is important for microtubule function. MCK1 may be important for spindle microtubule stability, for example, by phosphorylating a microtubule-associated protein. In this model, suppression of the CDEIII centromere mutation [which itself may decrease the stability of interactions with microtubules] would be explained by a compensatory increase in the overall stability of microtubules. Consistent with this notion is the fact that microtubules are destabilized in the cold and the observation that the mck1-disruption strain is cold sensitive.

DNA sequence analysis reveals that the MCK1 gene encodes a putative protein—serine/threonine kinase by virtue of its homology to known protein kinases (see also Dailey et al. 1990, Neigeborn and Mitchell, this issue). We anticipated that the dosage suppression screen might identify genes that code for CDEIII DNA-binding protein(s) or positive regulators of these binding proteins. The fact that the MCK1 gene product has been shown to have protein kinase activity (Dailey et al. 1990) makes the latter suggestion particularly attractive. There are several examples in the published literature of DNA-binding proteins that are activated upon phosphorylation (Magasanik 1988; Prywes et al. 1988; Yamamoto et al. 1988; Raychaudhuri et al. 1989). Furthermore, numerous studies provide evidence for changing protein phosphorylation states in premitotic and mitotic cells. There are several proteins that are phosphorylated specifically at the late G2 stage of the cell cycle and then rapidly dephosphorylated shortly after mitosis (Westwood et al. 1985; Lohka et al. 1987; for review, see Nurse 1990). Of particular interest in the present study is the finding that microtubule-organizing centers [including kinetochores] contain phosphoproteins (Davis et al. 1983; Vandre et al. 1984). The regulation of mitotic events by phosphorylation also implies that dephosphorylation of the same residues will have an opposing regulatory contribution. Recently, Axton et al. (1990) demonstrated that one of the protein phosphatase 1 isoenzymes in Drosophila is essential for mitosis and that mutations in this gene cause defective spindle organization, abnormal sister chromatid segregation, hyperploidy, and excessive chromosome condensation.

Importantly, Lechner and Carbon (1991) have obtained biochemical evidence in S. cerevisiae that at least one protein[s] within a bound complex on CDEIII is a phosphoprotein. They have shown that phosphorylation of this protein[s] is necessary for complex formation. It is reasonable to propose that the intracellular target of the MCK1 kinase may be the same phosphorylated protein species that Lechner and Carbon have identified. This implies a role for the MCK1 kinase in converting an inactive CEN DNA-binding protein into an active form. Alternatively, MCK1 kinase could act in a multistep pathway [e.g., as a regulator of a protein upstream of the active CEN DNA-binding protein]. The latter notion is perhaps easier to reconcile with the work of Neigeborn and Mitchell (this issue), which assigns at least two additional functions of the MCK1 kinase: transcriptional activation of IME1 and the stimulation of spore matura-

Suppressors of centromere DNA mutations

Figure 7. Serial dilution patch test for Benomyl sensitivity. The two plates shown have been inoculated with the same ordered serial dilution grid of yeast cells (shown from top to bottom). (Right) Plate containing 10 μg/ml of Benomyl [a drug concentration that does not significantly affect the growth rate of the wild-type parent strains]; (left) plate serving as a control. Both plates were incubated for 3 days at 25°C. In rows a–d, the four haploid products generated by tetrad dissection of a diploid strain [YPH633] heterozygous for the MCK1 disruption are analyzed. Lanes a (YPH634) and b (YPH637) contain the wild-type MCK1 locus; lanes c (YPH635) and d (YPH636) contain the mck1::HIS3 disruption, lane e contains a MCK1 wild-type strain [YPH278]; lane f contains a tub3::TRP1 strain [DBY2288] that is known to be Benomyl sensitive. This analysis clearly shows that the mck1::HIS3 disruption causes a slow-growth phenotype on Benomyl.
for a DNA sequence-specific binding protein, called CBF1 or CP1, which plays an important functional role for both centromere activity and transcription (Bram and Kornberg 1987; Baker et al. 1989; Jiang and Philippsen 1989; Cai and Davis 1990). CBF1 (CP1) protein binds to CEN DNA at CDEI and also interacts at sites upstream of genes required for methionine prototrophy. By analogy, MCK1 may regulate the activity of a protein that is a CDEIII DNA sequence-specific binding protein (functioning within the kinetochore complex) and that may also play a role in regulation of transcription in meiotic cells (through interactions with other components). Finally, there may be an obligatory relationship between the proper assembly of a meiotic centromere complex (early after commitment to meiosis) and subsequent progression through the meiosis program. In this scenario, the defective assembly or function of kinetochore proteins at the centromere might cause a meiotic “delay” at an early step after entry into meiosis. This delay might be observed as the altered transcriptional activation of genes normally expressed early and subsequent defects in ascus formation. The delay could be due to a structural defect or to a surveillance system that monitors the successful completion of steps before progression to others. Such a “checkpoint” control mechanism has been described for cell cycle progression in G2 of mitotic cells in response to radiation-induced DNA damage (Hartwell and Weinert 1989).

Our experiments were inspired by the finding that mutations in the SV40 origin of DNA replication are suppressible by missense mutations in the origin-binding protein (Shortle et al. 1979). The small genome size of SV40 and the cold-sensitive phenotype of the origin point mutations facilitated this effort, allowing efficient mutagenesis of the T antigen gene and subsequent selection of second-site revertants. Here we show that this type of approach may also identify unknown components of a pathway by the isolation of CEN DNA mutation suppressors from S. cerevisiae. Using a dosage suppression strategy, the isolation of suppressors from a genome the size of yeast is feasible without requiring a selection procedure. Demonstration that MCK1 encodes a protein involved in proper chromosome segregation validates this approach. A similar strategy (provided a suitable assay is available) may be useful in identifying trans-acting factors important for other DNA protein interactions as well.

Materials and methods

Strains and methods

The E. coli strain DH5α (Bethesda Research Laboratories) was used for routine cloning and library construction. Standard recombination DNA techniques were carried out as described (Maniatis et al. 1982). Transformations were performed by the method of Hanahan (1983). Miniprep DNA samples were isolated for analysis by the boiling method of Holmes and Quigley (1981). Table 2 lists the genotypes of all yeast strains used in this work. YPH strains are isogenic derivatives of YNN217 (original source, M. Carlson), an S288C derivative. Additional auxotrophic markers have been introduced by one-step gene replacement as described (Sikorski and Hieter 1989). Benomyl-sensitive strains were obtained from sources shown. Standard media recipes were from Sherman et al. (1978). Minimal media plates were supplemented with auxotrophic requirements, as described (Sherman et al. 1978), with a limiting adenine modification (Hieter et al. 1985) for colony color indicator plates. Yeast transformations were done by the lithium acetate procedure (Ito et al. 1983), with minor modifications. Transformants were selected on synthetic complete medium (Sherman et al. 1978) lacking either histidine, or tryptophan, depending on the gene under selection. Benomyl (gift from DuPont) sensitivity was assayed qualitatively by patch tests as ordered serial dilutions containing from 105 to 101 cells per patch (as described by Spencer et al. 1990) on YPD plates containing 10 μg/ml of Benomyl (prepared as described, Stearns et al. 1990). Genetic analysis was performed by using standard protocols (Sherman et al. 1978). Yeast genomic DNA was isolated for restriction digest and plasmid rescue into E. coli, as described (Davis et al. 1980).

Measurement of chromosome fragment loss rates by visual inspection and fluctuation analysis

Use of a colony color assay (Hieter et al. 1985) for qualitative measurement of chromosome fragment loss rates by visual inspection of colony-sectoring frequency was described in detail previously (Hegemann et al. 1988). The ability to distinguish quantitative differences in loss rates of as little as two- to threefold by visual inspection of colony sectoring phenotype has been thoroughly documented.

Fluctuation analysis by the method of the median (Lea and Coulson 1949) was performed as described (Hegemann et al. 1988), with the following modifications: A preincubation of logarithmically growing cells in 1 μg/ml of 5′-fluoro-orotic acid (5′-FOA) in sterile distilled H2O (4 hr at 4°C with gentle shaking) eliminated the leaky phenotype of this negative selection, which was reported previously (Hegemann et al. 1988). Therefore, we used the URA3 marker contained on the chromosome fragment to determine mitotic loss rates. Colonies were obtained for fluctuation, as described (Hegemann et al. 1988), and were plated in 200 μl of H2O containing 1 μg/ml of 5′-FOA and disrupted to single cells by vortexing. A 20-μl aliquot was removed immediately, and a 100-fold serial dilution (100 μl) was plated to indicator plates to determine the number of cells per colony. The remainder of the colony was incubated for 6 hr at 4°C with gentle shaking, and 100 μl (representing 50% of the original colony) was plated to supplemented minimal plates with 1 μg/ml of 5′-FOA (Boeke et al. 1987) to determine the number of cells that had lost the URA3/SUP11-marked chromosome during colony growth. Calculation of the mitotic chromosome fragment loss rates found in Table 1 was done by the method of the median (Lea and Coulson 1949).

Genetic screen for CEN DNA mutant suppressors

The strains used for suppression analysis were YPH298 and YPH299 (for genotypes, see Table 3), which show a 10- and 20-fold increase, respectively, in chromosome fragment loss rates. To isolate yeast genomic sequences that suppress the effect of the CEN mutations, a yeast genomic library was constructed in a TRP1/2-micron shuttle vector, pS62 [J.H. Shero, unpubl.]. The yeast strains were transformed with 6 μg of library DNA by the LiAc procedure (Ito et al. 1983), diluted, and plated onto supplemented minimal medium lacking tryptophan at a density yielding ~200 transformants per plate. Transformed cells were allowed to form colonies at 30°C for 5 days and then
transferred to 4°C for ~10 days to allow optimal color development. The colonies were then screened with the aid of a dissecting microscope [Wild M5A M5 APO, Wild Heerbrugg, Switzerland] for a reduction in sectoring frequency that may have indicated mitotic stabilization of the chromosome fragment. For YPH298, 12,000 transformants were screened to obtain 23 putative suppressors; and for YPH299, 10,000 transformants were screened to obtain 9 putative suppressors. These transformants were streaked to fresh minimal medium lacking tryptophan to obtain colony-pure strains, serially diluted, replated to a density of 50 cells per plate, and incubated (as above) to allow colony color development. By inspecting an entire field of colonies on a plate for a reduction in sectoring frequency compared with the same strain transformed with vector (pJS62) alone we identified nine true positives (seven for YPH298 and two for YPH299) and discarded the others as false positives. Plasmids were rescued from these strains into DH5a (Davis et al. 1980) and transformed back into the strains they were isolated from. By these criteria only two plasmids were recovered that demonstrated a reproducible suppression phenotype. The plasmids were named pMCK1 (formerly pCMS1), originally isolated from YPH299, and pCMS2, originally isolated from YPH298.

Mutant allele [cis or trans] specificity was determined for MCK1 by transforming vector alone (pJS62) and pMCK1 into 2 strains containing homozygous trans-acting mutations, 10 strains containing cis-acting [CEN DNA] mutations, and 1 strain containing a wild-type centromere [strain genotypes listed in Table 3). Two independent transformants for each were visually assayed for suppressor activity (as above). In addition, three CEN DNA mutations that completely abolished centromere function were tested for suppression by pMCK1 as follows: Red segregant strains [red color reflects loss of the URA3/ SUP11/CEN6 chromosome fragment] were picked from YPH299 that contained either pJS62 or pMCK1. These strains were transformed with chromosome fragmentation vectors pJS13, pJS14, and pJS15, as described (Hegemann et al. 1988), and four independent transformants were visually assayed for the restoration of CEN function (as above).

Localization of the MCK1 gene
Restriction digestion analysis showed that pMCK1 contained a 7-kb yeast genomic DNA insert derived from a Sau3A partial digest inserted into the BamHI site of pJS62. By standard recombinant DNA techniques, the MCK1 gene was localized through the use of restriction sites unique to the insert and the cloning poly linker to generate intramolecular deletions. In addition, frameshift mutations were generated at unique Tacl and SphI sites by restriction digest, filling in the ends with Klenow, ligation, and transformation into E. coli. These plasmids were assayed for suppressor activity (as above). This analysis identified
Table 3. Yeast strains for determination of allele-specific suppression of CEN DNA mutations

| Mutation          | Yeast strains* |
|-------------------|----------------|
| CEN6 (wild type)b | YPH281         |
| CDEI[8-A]b        | YPH286         |
| CDEI[A]          | YPH632         |
| CDEII[15-T]^b     | YPH291         |
| CDEII[15-A]^b     | YPH292         |
| CDEII[15-C]^b     | YPH293         |
| CDEII[19V20-T]^b  | YPH298         |
| CDEII[19V20-G]^b  | YPH299         |
| CDEII[17Δ25]^b    | YPH300         |
| CDEII[AΔ1 bp]^c   | YPH630         |
| CDEII[+45 bp]^c   | YPH631         |

*Yeast strains have the following genotypes:
YPH281–300: MATa/MAta ura3-52/ura3-52 lys2-801/lys2-801 ade2-101/ade2-101 trp1Δ1/trp1Δ1 + CEII[DRB.d]
YPH630, 631, 632: MATa/MAta ura3-52/ura3-52 lys2-801/lys2-801 ade2-101/ade2-101 HIS3/his3Δ200 leu2Δ1/leu2Δ1trp1Δ1 + CEII[DRB.d].
^bHegemann et al. (1988).
^cPanzeri et al. (1985).

A 3.6-kb BamHI–ClaI fragment that had suppressor activity when subcloned into pJS92 to create pJS99. This fragment contained a unique NcoI restriction site that was shown to be located in MCK1-coding sequence by a frameshift mutation that abolished suppressor activity. Low-stringency Southern hybridizations were performed as described [Levin et al. 1987] by using the 3.6-kb BamHI–ClaI genomic fragment as a probe [Feinberg and Vogelstein 1984] to establish that the MCK1 gene was present in single copy in the haploid genome.

Physical and genetic mapping of MCK1

A 2.0-kb EcoRI–EcoRl DNA fragment derived from the 7.0-kb MCK1-containing DNA fragment was subcloned in both orientations into the EcoRI site of pYC3 and pYC4 [Vollrath et al. 1988]. Three micrograms of each of the four plasmid DNAs was linearized with BglII before transformation into yeast strain YPH49. Screening of chromosome fragment-containing strains, and subsequent analysis on CHEF gels were performed as described [Gerring et al. 1990]. The MCK1 gene was unambiguously placed 80 kb from the left-arm telomere by hybridization to a cloned DNA segment containing the RPD3 gene (kindly provided by R. Gaber) that hybridized to the 80-kb MCK1-distal chromosome fragment. To obtain a meiotic map position, a diploid strain was constructed by standard procedures, which was heterozygous at multiple loci on XIV (R. Gaber, pers. comm.), and was observed to be linked to pet2 at 18 cM proximal to rpd3 (R. Gaber, pers. comm.). Two independent transformants were selected and shown by Southern blot analysis to have the appropriate disrupted, as well as wild-type, MCK1 genes.

Construction of MCK1 disruption allele and DNA sequencing

A 1.8-kb BamHI DNA fragment containing the HIS3 gene was cloned into the unique NcoI site of MCK1 [pJS99] to generate a disruption allele plasmid [pJS102]. A 5.4-kb mck1::HIS3 fragment was excised from pJS102 and transformed into the diploid strain YPH273 to disrupt one copy of the genomic MCK1 locus by homologous recombination [Rothstein 1983]. Two independent transformants were selected and shown by Southern blot analysis to have the appropriate disrupted, as well as wild-type, MCK1 genes.

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