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 insertional mutations in the bacteriophage $\phi 1$ 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 ∇ 20-T)]; YPH299, CDEIII(19 ∇ 20-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 replating. 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: p*MCK1* (isolated from a YPH299 transformant) and p*CMS2* (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). p*MCK1*, 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 p*MCK1* 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 replating 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

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(19 ∇ 20-G)] that is lost at a rate of 3.8×10^{-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 5.9×10^{-4} . (C) Phenotype of a strain (YPH632) that contains a *cis*-acting *CEN* DNA mutation in CDEI (CDEI Δ) 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(19 ∇ 20-G) and CDEIII(19 ∇ 20-T)]. The base line loss rate associated with the CDEIII(19 ∇ 20-G) insertion mutation was

Table 1. Mutant allele-specific suppression by *pMCK1*

Yeast strain	<i>CEN6</i> DNA mutation	Plasmid	Mitotic chromosome fragment loss rate	Fold suppression pJS62/ <i>pMCK1</i>
YPH628 ^a (<i>ctf3/ctf3</i>)	—	pJS62	1.0×10^{-2}	1.2
		<i>pMCK1</i>	8.4×10^{-3}	
YPH629 ^a (<i>ctf9/ctf9</i>)	—	pJS62	7.1×10^{-3}	0.8
		<i>pMCK1</i>	8.6×10^{-3}	
YPH281	wild type	pJS62	2.3×10^{-4}	0.9
		<i>pMCK1</i>	2.7×10^{-4}	
YPH299	CDEIII(19 ∇ 20-G)	pJS62	3.8×10^{-3}	6.4
		<i>pMCK1</i>	5.9×10^{-4}	
YPH298	CDEIII(19 ∇ 20-T)	pJS62	2.8×10^{-3}	5.1
		<i>pMCK1</i>	7.4×10^{-4}	
YPH630	CDEII(+45 bp)	pJS62	6.6×10^{-4}	1.2
		<i>pMCK1</i>	5.2×10^{-4}	
YPH631	CDEII(Δ 31bp)	pJS62	1.0×10^{-2}	1.9
		<i>pMCK1</i>	5.3×10^{-3}	
YPH632	CDEI Δ	pJS62	2.4×10^{-3}	1.3
		<i>pMCK1</i>	1.8×10^{-3}	
YPH286	CDEI(8-A)	pJS62	7.7×10^{-4}	1.2
		<i>pMCK1</i>	6.2×10^{-4}	

^aThese strains contain wild-type *CEN4* on the chromosome fragment, which is lost at an elevated rate due to a homozygous *trans*-acting mutation.

Figure 2. 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 insertion 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 qualitative (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 *Bam*HI–*Clal* 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 *Nco*I restriction site (underlined in Fig. 4, below) destroyed suppressor activity of the plasmid, indicating that the *Nco*I 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

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) YPH149 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_r 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 ~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 two recently published sequences. Glycogen synthase kinase-3 from rat (*GSK-3*; Woodgett 1990) and *shaggy* from *Drosophila melanogaster* (*sgg*; Bourouis et al. 1990) are 40% and 38% identical, respectively, over the length

of *MCK1* (Fig. 5A). They are 45% (*GSK3*) and 41% (*sgg*) identical in the catalytic domain (shown as the amino acids between number signs (#) in Fig. 5A). This sequence analysis predicts that *MCK1* encodes a protein that is likely to be a protein-serine/threonine kinase.

In addition, an unusual sequence similarity was identified between *MCK1* and *TPK1*. *TPK1* (Toda et al. 1987) is a yeast protein kinase with a characteristic homology to *MCK1* (~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 *NcoI* 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 spore colonies of a tetrad confirmed correct disruption of *MCK1* at the chromosomal locus (data not shown). Low-stringency Southern hybridization experiments using the 3.6-kb *BamHI*-*Clal* genomic fragment as a probe indicated that a single copy of *MCK1* was present in the haploid yeast genome (data not shown). The *MCK1* gene product is therefore not required for viability or proper chromosome segregation under standard laboratory growth conditions (30°C).

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-

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-142 AGTGGCCATTGTGCTGTAGTAGTGTGGGCGAGGCCGTAACGCTGTTTATGATAGGAGT
-82 TAGGCCACAGACTACAGAGTTCCTTCCTTCATCTTTTCATTTTCTTTATTTCCGAA
-22 CCCCACCTCCATCCATCTTACATATGCTATGCTACGGAAGACGAGATGGTGTCTCTG
      N S T E E Q N G U P L I I
39  CAAGGGGATCGATTCATTCATGACAGCATGTACCTCGAATATCGAARACACGAGG
   Q R G S E F I A D D U T S N K S N H T A 31
99  CGGATGCTGGTGAACAGCTACAGGAATCGGTAGGCTGCCCTTGGGACTGTTGTACAA
   R N L U K E Y R K I G R G A F G T U U Q 51
159 GCATATTTACCCAGGATAGGAARACTGGTGGGCCCTTTGCAATTAARAAAGTCCCT
   A Y L T Q D K K N H L G P F A I K K U P 71
219 GTCATACCGAGTACAGTCCAGGGAATACAGATTCTGAGGATTGCCACCATCCAAAT
   A H T E Y K S R E L Q I L R A I A D H P N 91
279 ATCGTTAATTGCAGTATTTCTTACTCATTTGTCGCCGCAAGATATTAAGTTTATCRA
   I U K L Q Y F F T H L S P Q D N K U Y Q 111
339 CACCTTGCCTGCAATGCTTACCGAARCTTCAARATCGAGATTATCGTTATGTACRA
   H L A R E C L P E T L Q I E I N R Y U T 131
399 AACAGCTAGAAATGCCATTAACATATCAGGCTATACCTTATCAGATTGCCCGCGG
   N K L E N P L K H I R L Y T V Q I A R G 151
459 ATGCTTTATTTACAGGCTTGGCGTTGTCATCGTATCAACATCCAAATGTTCTT
   N L Y L H G L G I K P S H U L 171
519 GTAGACCCGGAACCGTGTCTGGAATCTGGGATTTGGGCTGCCAARAAATGGAR
   U D P E T G U L K I C D F G S A K K L E 191
579 CATACCCGCTTCATTTAGTATGCTGTCAGAGTTTATAGAGCGCTGAATGATC
   H N Q P S I S V I C S A F Y R A P E L I 211
639 ATTGGTTGTACCAATATACACCCAGATCGATATATGGGGCTTGGTGTGTCATGGC
   I G C T Q Y T T Q I D I W G L G C U N G 231
699 GAGATGCTTATCGCAAGGCATATTTCAAGGTCAGAGACCCCTTCAAGCTAGGGAR
   E N L I G K A I F Q G E P L L Q L R E 251
759 ATTGCTAATTGTAGTCTCCCGATAAAGATTCATTTTTCGAATCCGCCCTAC
   I A K L L G P P D K R F I F F S N P A Y 271
819 GATGGCCCTCATTTTCCAAACCACTGTCTCGGCTCTTCGACACAGAGGTTGAGAG
   D G P L F S G L F S G S S Q Q R F E K 291
879 TATTTTGGCCATTCGGTCCAGATGATCGATCTTTGATGAARATTTGGTCTACGA
   Y F G H S G P D G I D L L N K I L U Y E 311
939 CACACACAAAGACTGTCTCCAGAGAGATCCTTGCCCATCAGTCTTTATGATTAAGG
   P Q Q R L S P R A I L A H Q F F H E L R 331
999 AATGATGACACTTCTTACCAAGAGGTTCACTGAACCAATCAGCTACCAATTTGTT
   N D D T F L P R G F T E P I K L P N L F 351
1059 GATTTTATGATTTTGAATACAAATTCGGTGAATTTGCTGATAAATTAACCTACG
   D F N D F L Q I L G E F A D K I K P T 371
1119 AAGTTGCTGATATATATTTCTATTATTTGTTCTTTCCCTCGGAATTTAATG
      K U A E * 375
1179 AACAAATACATGATTTTTCCTTTGATCGGCTATTTATTTCACTTGGAAAGGA
1239 AAAAATTTTTTTGGATTAATATATTCATTTCCATTTGATATTATCATACACTCT

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Figure 4. Nucleotide sequence of the *MCK1* gene and flanking DNA. The predicted amino acid sequence is shown in single-letter code with a star representing the termination codon. The *NcoI* restriction enzyme cleavage site used to generate *mck1::HIS3* is underlined.

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*; Bouhours 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.

tion defects at a semipermissive temperature, YPH635 (*mck1::HIS3*) and YPH637 (*MCK1* wild-type control) were plated to single cells at 14°C, 16°C, and 18°C. YPH635 formed colonies at these temperatures but showed an increase in chromosome fragment loss rate when compared to YPH637 (Fig. 6).

Benomyl is a microtubule-destabilizing drug that has been used recently in a genetic screen to identify genes important for microtubule function [Hoyt et al. 1990; Stearns et al. 1990]. These investigators isolated and characterized three genes that mutate to alleles exhibiting supersensitivity to Benomyl at normally sublethal concentrations. Because β -tubulin is believed to be the only intracellular target of Benomyl, these genes are considered excellent candidates for participants in a pathway involving microtubules. Therefore, we tested *mck1* strains for sensitivity to Benomyl. This analysis revealed that *mck1* strains showed reduced growth on Benomyl-

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).

[illegible]

containing medium (Fig. 7). Furthermore, when a *mck1::HIS3* strain (YPH635) containing a *SUP11*-marked chromosome was sparsely dispersed on plates that contained Benomyl and incubated at 25°C, a dramatic increase in chromosome fragment loss was observed in the resultant colonies as compared with the appropriate wild-type strain (YPH637).

Discussion

We have described an experimental approach to isolate *trans*-acting factors that are required for proper kinetochore function in *S. cerevisiae*. Our strategy relied on a series of strains containing *CEN* DNA mutations that were characterized for their effects on mitotic chromosome fragment stability *in vivo* using a colony color assay. Two yeast strains containing partially defective centromeres (CDEIII mutations) were used to identify *trans*-acting suppressors of the centromere defect. We used a dosage suppression screen, reasoning that overexpression of a *CEN* DNA-binding protein, or its positive regulator(s), may suppress the *CEN* DNA mutation. A high-copy yeast genomic library (constructed in a 2 μ vector) was used to transform these strains, which were then screened visually for improvement in mutant *CEN* function. Two DNA sequences (*MCK1* and *CMS2*) were identified as extragenic dosage suppressors and are excellent candidates for structural or regulatory components of the yeast kinetochore.

Allele specificity of suppression is a useful criterion to distinguish interaction suppressors (generally allele specific) from bypass suppressors (generally allele nonspecific). Increased *MCK1* dosage suppressed two similar CDEIII mutations but failed to suppress eight other *CEN*

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.

DNA mutations. 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 not 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; Raychauduri 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 maturation.

MCK1 kinase appears to play a positive regulatory role in both mitotic centromere function and activation of early meiotic gene expression. It is possible that *MCK1* kinase modulates the activity of a variety of intracellular target proteins that operate in multiple unrelated biochemical pathways. Alternatively, *MCK1* kinase may regulate a protein (or proteins) common to both the centromere and the meiosis pathways. A precedent exists

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 G_2 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 direct 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 DH5 α (Bethesda Research Laboratories) was used for routine cloning and library construction. Standard recombinant 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 10^5 to 10^1 cells per patch (as described by Spencer et al. 1990) on YPD plates containing 10 μ g/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-sectored 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 three-fold 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 μ g/ml of 5'-fluoro-orotic acid (5'-FOA) in sterile distilled H_2O (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 placed in 200 μ l of H_2O containing 1 μ g/ml of 5'-FOA and disrupted to single cells by vortexing. A 20- μ l aliquot was removed immediately, and a 100-fold serial dilution (100 μ l) 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 μ l (representing 50% of the original colony) was plated to supplemented minimal plates with 1 μ g/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, pJS62 (J.H. Shero, unpubl.). The yeast strains were transformed with 6 μ g 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

Table 2. List of yeast strains for this study

Strain	Genotype	Source
YPH273	<i>MATα/MATa ura3-52/ura3-52 lys2-801/lys2-801 ade2-101/ade2-101 HIS3/his3Δ200 trp1Δ1/TRP CFVII (RAD2.d.YPH273) URA3 SUP11</i>	this study
YPH278	<i>MATα ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 CFIII (CEN3.L.YPH278) URA3 SUP11</i>	Spencer et al. (1990)
DBY2288	<i>MATα ura3-52 lys2-801 trp1-1am his3Δ200 leu2-3,112 tub3::TRP1</i>	Botstein laboratory
YPH634	<i>MATa ura3-52 lys2-801 ade2-101 his3Δ200 trp1Δ1 leu2Δ1</i>	this study
YPH635	<i>MATα ura3-52 lys2-801 ade2-101 his3Δ200 trp1Δ1 leu2Δ1 mck1::HIS3 CFVII (RAD2.d.YPH273) URA3 SUP11</i>	this study
YPH636	<i>MATa ura3-52 lys2-801 ade2-101 his3Δ200 trp1Δ1 leu2Δ1 mck1::HIS3</i>	this study
YPH637	<i>MATα ura3-52 lys2-801 ade2-101 his3Δ200 trp1Δ1 leu2Δ1 CFVII (RAD2.d.YPH273) URA3 SUP11</i>	this study
YPH633	<i>MATa/MATα ura3/ura3-52 lys2-801/lys2-801 ade2-101/ade2-101 his3Δ200/his3Δ200 trp1Δ1/trp1Δ1 CFVII (RAD2.d.YPH273) URA3 SUP11</i>	this study
YPH628	<i>MATα/MATa ura3-52/ura3-52 lys2-801/lys2-801 ade2-101/ade2-101 HIS3/his3Δ200 trp1Δ1/trp1Δ1 ctf3-2/ctf3-2 CFIII (CEN3.L.YPH278) URA3 SUP11</i>	Spencer et al. (1990)
YPH629	<i>MATα/MATa ura3-52/ura3-52 lys2-801/lys2-801 ade2-101/ade2-101 HIS3/his3Δ200 trp1Δ1/trp1Δ1 ctf9-104/ctf9-104 CFVII (RAD2.d.YPH273) URA3 SUP11</i>	Spencer et al. (1990)

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 DH5α (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 *Bam*HI 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 polylinker to generate intramolecular deletions. In addition, frameshift mutations were generated at unique *Nco*I and *Sph*I 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 ^a
CEN6 (wild type) ^b	YPH281
CDEI(8-A) ^b	YPH286
CDEIΔ ^c	YPH632
CDEIII(15-T) ^b	YPH291
CDEIII(15-A) ^b	YPH292
CDEIII(15-C) ^b	YPH293
CDEIII(19V20-T) ^b	YPH298
CDEIII(19V20-G) ^b	YPH299
CDEIII (17Δ25) ^b	YPH300
CDEII (Δ31bp) ^c	YPH630
CDEII (+45 bp) ^c	YPH631

^aYeast strains have the following genotypes:

YPH281–300: MATa/MATα *ura3-52/ura3-52 lys2-801/lys2-801 ade2-101/ade2-101 trp1Δ1/trp1Δ1* + CFIII(D8B.d)

YPH630, 631, 632: MATa/MATα *ura3-52/ura3-52 lys2-801/lys2-801 ade2-101/ade2-101 HIS3/his3Δ200 leu2Δ1/leu2Δ1trp1Δ1/trp1Δ1* + CFIII(D8B.d).

^bHegemann et al. (1988).

^cPanzeri et al. (1985).

a 3.6-kb *Bam*HI–*Cl*al fragment that had suppressor activity when subcloned into pJS92 to create pJS99. This fragment contained a unique *Nco*I 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 *Bam*HI–*Cl*al 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 *Eco*RI–*Eco*RI DNA fragment derived from the 7.0-kb *MCK1*-containing DNA fragment was subcloned in both orientations into the *Eco*RI site of pYCF3 and pYCF4 (Vollrath et al. 1988). Three micrograms of each of the four plasmid DNAs was linearized with *Bgl*II 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 XIVL (*mck1::HIS3*+/+, *pha2*/+, *kar1::URA3*/+, *pet2*/+, *met2*/+). A total of 112 tetrads were scored for segregation of the markers (all data have been submitted to the Yeast Genetics Stock Center, Berkeley, CA). Significant linkage was observed between *mck1* and *pha2*, *met2*, and *pet2* but not *kar1*. *kar1* was observed to be linked to *pet2* but not to any of the other chromosome XIV markers (including *mck1*), indicating that *kar1* is proximal to *pet2* rather than distal as on the current published linkage map. The meiotic distances were calculated from the following data by using the formula of Perkins: *mck1*–*pha2* 18 cM (PD/NPD/T = 76/1/33); *mck1*–*met2*, 40 cM (PD/NPD/T = 53/6/51). *mck1*

was placed proximal to *pha2* because (1) *pha2* is 8 cM proximal to *rpd3* (R. Gaber, pers. comm.), (2) *MCK1* is proximal to *rpd3* from the Southern blotting data, and (3) *mck1::HIS3* is 18 cM from *pha2*.

Construction of *MCK1* disruption allele and DNA sequencing

A 1.8-kb *Bam*HI DNA fragment containing the *HIS3* gene was cloned into the unique *Nco*I 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.

pJS99 contains *MCK1* cloned into the *Bam*HI–*Cl*al sites of the pJS92 polylinker that corresponds to the pBluescript (KS–, Stratagene) polylinker. With the *Nco*I site as a starting point, a set of unidirectional deletions were made by using the exonuclease III/nuclease S1 method of Henikoff (1987). Additional defined deletions were constructed using restriction enzymes. The DNA sequence encoding the complete open reading frame was determined by the method of Sanger et al. (1977), using double-stranded templates (Hatton and Sakaki 1986). The sequencing of both strands of the open reading frame (Fig. 4) was completed, using synthetic oligonucleotides to cross gaps.

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