The yeast *MCK1* gene encodes a protein kinase homolog that activates early meiotic gene expression

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We have identified a yeast gene, *MCK1*, that encodes a positive regulator of meiosis and spore formation. Sequence analysis revealed that *MCK1* encodes a protein kinase homolog identical to YPK1, a phosphotyrosyl protein with demonstrated protein kinase activity. Increased *MCK1* gene dosage accelerates the sporulation program; *mck1* mutations cause delayed and decreased levels of sporulation. *MCK1* is required during sporulation for maximal transcript accumulation from *IME1*, which encodes a meiotic activator. *MCK1* is required in vegetative cells for basal *IME1* expression, as evidenced by functional assays of an *ime1-HIS3* fusion gene. *MCK1* is also required for efficient ascus maturation. Although expression of *IME1* from the *GAL1* promoter restored high-level sporulation to *mck1* mutants, it did not correct the ascus-maturation defect. This observation indicates that *MCK1* is required, independently, for both the activation of *IME1* and subsequent ascus maturation. Expression of an *mck1-lacZ* fusion gene was not regulated by the signals that govern meiosis. This observation is consistent with evidence that *MCK1* plays a role in governing centromere function during vegetative growth as well as sporulation.

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Induction of several differentiation pathways, by defined environmental and genetic signals, is achieved through increased expression of activators of the specific pathways (for reviews, see Biggin and Tjian 1989; Pinney and Emerson 1989). Sporulation in the yeast *Saccharomyces cerevisiae* is one such pathway (Magee 1987; Kassir et al. 1988; Smith and Mitchell 1989). This process includes events leading through meiosis and packaging of the four meiotic products into an ascus (for review, see Esposito and Klapholz 1981). Entry into the sporulation pathway requires starvation and expression of the $\alpha 1$ and $\alpha 2$ products, which determine the α/α cell type. The starvation signal is transmitted through decreased cAMP-dependent protein kinase activity, and through a cAMP-independent pathway(s) (Matsumoto et al. 1983; Tatchell et al. 1984, 1985; Olempska-Beer and Freese 1987; Cameron et al. 1988; Gibbs and Marshall 1989). $\alpha 1$ and $\alpha 2$ are products of the *MAT α* and *MAT α* alleles, respectively, and only cells expressing both (typically *MAT α /MAT α* diploids) are sporulation proficient (Herskowitz 1988). These gene products interact to form a transcriptional repressor (Goutte and Johnson 1988; Dranginis 1990) that prevents expression of an inhibitor of meiosis, *RME1* (regulator of meiosis) (Kassir and Simchen 1976; Rine et al. 1981; Mitchell and Herskowitz 1986). Because $\alpha 1$ and $\alpha 2$ are expressed together, normally in diploid cells, this genetic signal ensures that

sporulation occurs only in cells capable of producing four viable haploid spores.

Two positive regulators of sporulation have been identified: *IME1* and *IME2* (inducer of meiosis) (Kassir and Simchen 1988; Smith and Mitchell 1989; Mitchell et al. 1990). Presence of *RME1* or nutrients inhibits *IME1* expression; *IME1* is required for *IME2* expression. Each gene product is required for sporulation and plays a role in the activation of sporulation-specific gene expression. *IME1* appears to stimulate meiotic gene expression via two pathways: indirectly, through activation of *IME2*, and via an *IME2*-independent route. *IME1* is one of the earliest sporulation-specific genes to be induced, which is consistent with its role as a positive regulator required to stimulate subsequent meiotic genes (Mitchell et al. 1990; Smith et al. 1990).

Here we describe the identification and characterization of a third meiotic activator, *MCK1* (meiosis and centromere regulatory kinase; see below). Our results indicate that *MCK1* is required for optimal *IME1* and *IME2* expression, establishing it as the earliest positive regulator of the sporulation pathway. The predicted amino acid sequence of *MCK1* shares significant homology with serine-threonine protein kinases and is the same as the YPK1 phosphotyrosyl protein shown to be associated with tyrosine, serine, and threonine protein kinase activity (Dailey et al. 1990). We suggest that phosphoryla-

tion of a target, by the *MCK1* product, may transmit one or both signals required for entry into meiosis via the stimulation of *IME1* expression.

During the course of this study we discovered that *MCK1* is identical to a gene identified by Shero and Hieter as a dosage-dependent suppressor of a centromere mutation. Work presented here and by Shero and Hieter (this issue) indicates that the meiotic defects conferred by *mck1* mutations are not simply a consequence of defective chromosome segregation. Taken together, our results suggest three roles for the *MCK1* protein kinase: transcriptional activation of *IME1*, stimulation of spore maturation, and facilitation of centromere activity during mitosis.

Results

Isolation of the *MCK1* gene

We searched for yeast genes which, when present in increased dosage, bypassed the meiotic block resulting from *RME1* expression. We reasoned that increased expression of a downstream activator gene in the presence of *RME1* might allow execution of part of the meiotic pathway, such as meiotic recombination. A multicopy yeast genomic library was screened for sequences that stimulated meiotic recombination in α/α diploids harboring an *RME1* multicopy plasmid. Out of 3000 transformants screened, only one positive transformant was obtained; the library plasmid carried in this strain was called B1. A similar screen, using a different yeast genomic library, previously had proved to be successful in the identification of *IME1* and *IME2* (Smith and Mitchell 1989). Restriction analysis of the B1 yeast DNA insert revealed no similarity to *IME1* or *IME2* (see Fig. 1), and therefore represents a new locus that we denote *MCK1*.

The region responsible for *RME1* bypass activity was

narrowed to a 1.8-kb region at the extreme end of the yeast DNA insert (Fig. 1, subclone pLN329) by a combination of subcloning and deletion analyses. Southern analysis of electrophoretically separated whole yeast chromosomes indicated that *MCK1* is situated on chromosome XIV and revealed no other closely related genomic sequences (data not shown).

The location of *MCK1* at one end of the genomic clone presented the possibility that we had selected for spurious activity resulting from juxtaposition of plasmid and yeast DNA sequences. We used colony hybridization to retrieve a new copy of *MCK1* (from a different yeast genomic library in the vector YCp50) without applying phenotypic selection, thereby assuring recovery of the wild-type sequence (Fig. 1, pLN340). The yeast DNA insert carried on pLN340 conferred *RME1* bypass activity when transferred into a multicopy vector. Thus, our original screen did not demand a rearranged or mutant *MCK1* clone.

MCK1 does not inhibit *RME1* activity

The *MCK1* gene product may be a meiotic activator functioning downstream of *RME1* or in a parallel pathway. Alternatively, increased dosage of *MCK1* might inhibit the expression or activity of *RME1*. To test the latter explanations we measured *RME1* expression in the presence or absence of a multicopy *MCK1* plasmid. In addition, we asked whether the multicopy clone confers a phenotype in the absence of *RME1*.

First, we tested the possibility that multicopy *MCK1* inhibits transcription of *RME1* by measuring expression of an *rme1-lacZ* fusion gene in α/α diploids (Fig. 2A). Vegetatively grown α/α diploids carrying either a multicopy *MCK1* plasmid or vector alone produced equal levels of β -galactosidase. Control strains harboring a *MATa* plasmid or α/α diploids show appropriately repressed lev-

Figure 1. Restriction map of the *MCK1* locus. The restriction map, relevant subclones, and mutant alleles of *MCK1* are shown. The bar represents a composite of the *MCK1* chromosomal locus derived from the overlapping plasmids B1 and pLN340: (open bar) DNA present on B1; (solid bar) DNA present on pLN340; (stippled bar) DNA present on both B1 and pLN340 (see text). (Top) The positions of the *mck1* disruption mutations *mck1-Δ1* :: *URA3*, *mck1-Δ2* :: *URA3*, and *mck1-Δ3* :: *TRP1* are indicated (see text). (Bottom) The region denoted probe indicates the fragment used to identify pLN340 by colony hybridization. Each bar denotes yeast DNA in the *MCK1* region subcloned into the multicopy plasmids YEp24 (pLN329) or the centromere plasmid pRS314 (pLN360-I and pLN360-II). The striped arrow labeled *MCK1* represents the ORF. Restriction endonuclease sites: (B) *Bam*HI; (Bl) *Bal*I; (C) *Cla*I; (H) *Hind*III; (K) *Kpn*I; (Nc) *Nco*I; (Nh) *Nhe*I; (P) *Pst*I; (Pv) *Pvu*II; (R) *Eco*RI; (Se) *Spe*I; (Sp) *Sph*I; (St) *Stu*I. The site indicated [B] is a *Bam*HI-*Sau*3A junction regenerating the vector *Bam*HI site, which is not present in the yeast chromosome. The site indicated St* is a *Stu*I site present in the yeast chromosome but undetectable in our plasmids; this is most likely explained by interference due to overlapping dam methylation, which occurred during passage through *Escherichia coli*. The site indicated R* is an *Eco*RI site present on the original *MCK1* plasmid (B1) and in the chromosome of yeast strains in the SK1 genetic background but not present in the chromosome of yeast strains in the EG123 genetic background (data not shown).

Figure 2. Overexpression of *MCK1* does not inhibit *RME1* expression. The isogenic strains LN72D1 [a/α *rme1-lacZ/rme1-lacZ*] and LN72D2 [a/α *rme1-lacZ/rme1-lacZ*] were transformed with pAM301 (*MCK1*); pJM3 (*MATa*); and YEp24 (vector alone) and maintained on SC-URA selective medium. (A) *rme1-lacZ* expression in *MCK1* overproducing strains. Cultures were grown overnight in SC-URA to maintain the plasmid, and 1-ml samples were removed for β -galactosidase assays. Cultures were also assayed after growth in

supplemented sporulation medium for 15 and 24 hr, yielding similar results (data not shown). The data shown are averages of at least two independent assays; the error bars indicate the range of independent values. (B) Sporulation in *MCK1* overproducing strains. The SC-URA cultures above were plated for single colonies on SC-URA plates, and four independent colonies of each strain were patched onto supplemented sporulation plates and incubated at 30°C. Sporulation was quantitated after 1 or 2 days by counting no less than 200 cells from each patch. The data shown are averages of the four clones of each strain; the error bars indicate the range of independent values.

els of *rme1-lacZ* expression because they produce both $\alpha 1$ and $\alpha 2$, which together inhibit *RME1* transcription. These results demonstrate that increased dosage of *MCK1* does not inhibit *RME1* transcription.

The possibility remained that increased dosage of *MCK1* stimulated sporulation by inhibiting activity of the *RME1* gene product. This model predicts that a multicopy *MCK1* plasmid should not affect sporulation in the absence of *RME1*. *rme1⁻* mutations permit α/α diploids to sporulate; however, sporulation is less efficient than in isogenic a/α diploids (Kassir and Simchen 1976; Rine et al. 1981; Mitchell and Herskowitz 1986; Mitchell 1987). These properties have been taken to mean that $\alpha 1$ and $\alpha 2$ stimulate sporulation both by repressing *RME1* and through an *RME1*-independent mechanism. Figure 2B shows that a *MATa* plasmid improves sporulation of an *RME1*-deficient α/α diploid after 2 days. We found that a multicopy *MCK1* plasmid also stimulated sporulation in this diploid (Fig. 2B). Indeed, the multicopy *MCK1* plasmid accelerated spore formation in an *RME1*-deficient a/α diploid, resulting in twofold stimulation of sporulation after 1 day (Fig. 2B). *MCK1* does not substitute for *MATa*: The multicopy *MCK1* plasmid fails to repress *RME1* expression in α/α cells and stimulates sporulation in a/α cells. We conclude that increased *MCK1* dosage does not stimulate sporulation simply by inhibiting *RME1* activity; it functions downstream and/or independently of *RME1*. Results described below concerning the sporulation defects associated with *mck1* mutations further support this conclusion.

MCK1 encodes a putative protein kinase

We determined the nucleotide sequence of a restriction fragment that can promote *MCK1* activity (Fig. 3; the 2.1-kb *StuI* fragment contained on pLN360 I and II indicated in Fig. 1). There is a single, large open reading frame (ORF) encoding a polypeptide of 375 amino acids with a calculated molecular mass of 43,108 daltons. This ORF must represent the *MCK1* gene product because it is completely contained within the region shown to pos-

sess *MCK1* activity and because mutations in the ORF confer phenotypes opposite that of the multicopy plasmid (see below).

A search of the GenBank Database for protein sequences similar to *MCK1* uncovered significant homology to many serine-threonine protein kinases. Eleven characteristic peptide domains (Hanks et al. 1988), including 18 invariant (or nearly invariant) residues, are found in the *MCK1* sequence and are indicated in Figure 3 as conserved regions I–XI. The lysine at position 68 represents an invariant residue shown to be essential for kinase activity. Although there are two adjacent lysine residues at this site, mutational analysis of an homologous protein kinase, *cdc2⁺*, demonstrated that the amino-proximal lysine residue (corresponding to Lys68 of *MCK1*) is required for kinase activity and that the neighboring lysine cannot substitute (Booher and Beach 1986). Indeed, mutation of *MCK1* Lys 68 to arginine (a conservative change) abolishes *MCK1* activity, thereby generating a null allele (data not shown). This experiment indicates that the broad-specificity protein kinase activity associated with *MCK1* product (Dailey et al. 1990) is essential for biological activity. Residues 164–169 of region VI and 201–209 of region VIII resemble more closely sequences characteristic of serine-threonine protein kinases, as opposed to tyrosine kinases. The characterization of phosphorylated residues on biologically active *MCK1* targets will demonstrate the relevant *MCK1* substrate specificity.

Figure 4 shows a comparison of the *MCK1* polypeptide with six of the homologous yeast kinases identified in the data base: the *S. cerevisiae* proteins KSS1 (Courchesne et al. 1989), KIN28 (Simon et al. 1986), FUS3 (Elion et al. 1990), PHO85 (Uesono et al. 1987), and CDC28 (Lorincz and Reed 1984); and the *Schizosaccharomyces pombe* *cdc2⁺* protein (Hindley and Phear 1984). Overall, *MCK1* shares ~32% identity, or ~56% similarity with all of these kinases when conservative amino acid substitutions are considered. *MCK1* lacks the PSTAIRE sequence characteristic of the CDC28 family of kinases. Also absent is the tyrosine residue within the

Figure 3. DNA and derived amino acid sequence of *MCK1*. The complete nucleotide sequence of the 2.1-kb *StuI* fragment is shown. Below is the predicted amino acid sequence of the only significant ORF within this region. We assume that the first ATG within this ORF is the initiation codon and refer to this "A" as +1 of the nucleotide sequence. The boxed regions labeled I–XI represent the 11 conserved kinase domains; the amino acids marked with an asterisk (*) are the invariant or nearly invariant residues common to all protein kinases (see text). Relevant restriction sites are indicated above the nucleotide sequence. The GenBank/EMBL accession number for the DNA sequence described above is X55054.

ATP-binding motif (G-X-G-X-Y-G), which has been shown to regulate *cdc2⁺* kinase activity (Gould and Nurse 1989). *MCK1* contains both amino- and carboxy-terminal extensions, which may play roles in either substrate recognition or regulation of *MCK1* activity. Recently, sequence analysis revealed that the meiotic regulatory gene *IME2* encodes a serine-threonine protein

kinase (Yoshida et al. 1990). *MCK1* appears to be more homologous to *CDC28* and *cdc2⁺* than it is to *IME2*.

mck1 mutants are defective in sporulation

If a natural role of *MCK1* is to activate sporulation, then *mck1* mutations should cause a sporulation defect. We

Meiotic activation by yeast *MCK1* kinase

Figure 4. Alignment of the *MCK1* gene product with related yeast protein kinases. The sequences are aligned on the basis of their conserved kinase domains. Residues common to *MCK1* and at least three others are underlined and indicated in boldface type. The 18 invariant or nearly invariant amino acids are indicated with an asterisk (*). The amino- and carboxy-termini of the proteins display no significant homologies and are not aligned.

constructed the mutation *mck1-Δ1::URA3* (Fig. 1) by deleting the 0.7-kb *SphI-NcoI* fragment. This deletion removes 344 nucleotides of *MCK1* upstream region, as

well as the first 114 codons, and should generate a null *mck1* allele.

Diploids homozygous for *mck1-Δ1* are defective in

Figure 5. *mck1-Δ1* diploids show decreased and delayed sporulation kinetics. Cultures of isogenic strains were grown to log phase in YEPAC, shifted to 2% KAc sporulation medium (time = 0), and incubated at 30°C with aeration. Ascus formation was quantitated at the indicated times by counting no less than 200 cells per culture. Open symbols indicate spherical (immature) asci; solid symbols indicate condensed (mature) asci. (A) *MCK1/MCK1* (AMP607 × AMP608); (B) *mck1-Δ1/mck1-Δ1* (AMP609 × AMP610); (C) *MCK1/mck1-Δ1* (AMP607 × AMP609).

sporulation. We compared sporulation kinetics and efficiency in isogenic wild-type, $+/mck1-\Delta1$, and $mck1-\Delta1/mck1-\Delta1$ diploids after a shift from rich medium to sporulation (Spo) medium (Fig. 5). Typical sporulation kinetics were observed in the wild-type strain: Asci first appeared between 8 and 12 hr after the shift to Spo medium. At 12 hr, 65% of the cells were in the form of spherical asci (see below); 5 hr later, they had matured into condensed asci; by 24 hr, the culture contained 94% mature asci (Fig. 5A).

Sporulation is delayed and decreased in the $mck1-\Delta1$ mutant: Spherical asci were not observed until 20 hr after the shift to Spo medium, and these levels were significantly reduced compared with wild type (Fig. 5B). Progression to mature asci was also delayed: By 72 hr, half of the spherical asci had not matured. After 5 days, >50% of the culture had not sporulated and significant levels of immature asci were still present. We were unable to identify the spherical asci following preparation for dissection, but spore viability was 100% in 24 mature asci. Spore maturation in the $mck1-\Delta1$ heterozygote was moderately delayed, and the level of sporulation was reduced slightly (Fig. 5C). This dosage effect suggests that the MCK1 product may be limiting for both the rate of sporulation and ascus maturation.

Figure 6 shows a comparison of wild-type and $mck1-\Delta1$ mutant ascus morphology. Wild-type asci observed after only 12 hr in Spo medium resemble spherical cells containing spores (Fig. 6A); with time, these mature into typical, condensed tetrads (Fig. 6B). The asci present in $mck1/mck1$ mutants after 24 hr in Spo medium resemble the immature wild-type asci (Fig. 6C). These spherical asci persist in the $mck1$ mutant for at least 5 days (Fig. 5B). We conclude that the $mck1-\Delta1$ mutation causes both quantitative and qualitative sporulation defects.

Both delayed sporulation and accumulation of immature asci were associated with two additional $mck1$ alleles: $mck1-2::URA3$ and $mck1-\Delta3::TRP1$ (Fig. 1). $mck1-2::URA3$ is an insertion allele that interrupts the gene at codon 16, and $mck1-\Delta3::TRP1$ is a deletion extending from 344 nucleotides upstream of the MCK1-coding region through to codon 293. Because the $mck1-2::URA3$ insertion is within the MCK1-coding sequence, it is not likely to alter expression of adjacent genes. In addition, both of the $mck1$ mutant phenotypes were complemented by the 2.1-kb *StuI* fragment, which contains no other significant ORF (plasmids pLN360 I and II). However, when these plasmids contain the Lys68 to arginine mutation within the MCK1 putative kinase domain (see above), their ability to complement the $mck1$ defects is abolished. We conclude that these sporulation defects are due to the absence of MCK1 function.

All $mck1$ mutants retain some capacity to sporulate. The formal possibility remains that none of the $mck1$ mutations is a null allele because they still retain portions of the coding sequence. This is unlikely in the cases of $mck1-\Delta1$ and $mck1-\Delta3$, however, because these mutations remove both the initiator methionine and several regions known to be essential for protein kinase activity; only 82 codons remain in the $mck1-\Delta3$ allele.

Figure 6. $mck1-\Delta1$ diploids accumulate immature asci. Representative asci from the isogenic sporulating cultures described in Fig. 5 were photographed at 1000 $\times$ magnification. (A) wild-type diploids after 12 hr in Spo medium; (B) wild-type diploids after 24 hr in Spo medium; (C) $mck1-\Delta1$ diploids after 24 hr in Spo medium.

Similarly, the Lys68 to arginine mutation abolishes a region, which by homology to other kinases, is critical for activity. Therefore, the residual sporulation of $mck1$ mutants may reflect activity of a second kinase that can act on the MCK1 substrate(s). On the other hand, there may be a pathway, parallel to and distinct from the MCK1 pathway, that leads to activation of sporulation.

mck1 mutants are defective in sporulation-specific gene expression

Several possibilities exist for the role MCK1 plays in meiosis. It may be necessary for expression of *IME1* and/or *IME2*; it may function downstream of *IME2*; or it may stimulate another, yet unidentified, pathway with functional similarity to the *IME1* pathway. We used Northern analysis to compare expression of *IME1*, *IME2*, and two later sporulation-specific genes, *SPS1* and *SPS2* (Percival-Smith and Segall 1984, 1986), in the wild-type and $mck1-\Delta1$ mutant. Cultures were grown in rich medium,

shifted to Spo medium, and RNA was prepared at various times (Fig. 7). In the wild-type strain, *IME1* transcript levels increased within 2 hr after the shift to Spo medium, compared with the control RNA. *IME1* message accumulated until 12 hr and then slowly declined. *IME2* transcript appeared at 8 hr following the shift to Spo medium, peaked at 20 hr, and declined. Expression of two later sporulation-specific messages, *SPS1* and *SPS2*, was detected after 14 hr in Spo medium. The *mck1* mutant strain showed a considerably different pattern of sporulation-specific gene expression (Fig. 7). *IME1* message was not detectable until 4 hr after a shift to Spo medium and failed to accumulate more than twofold above this initial level. *IME2* transcript was not observed until 22 hr in Spo medium and then only at a very low level. This defect in *IME2* expression was confirmed by

Figure 7. *mck1-Δ1* diploids are defective in sporulation-specific gene expression. Expression of *IME1*, *IME2*, *SPS1*, *SPS2*, and the control RNA in the isogenic strains: wild-type diploid, EG123 × AMP268 (top); *mck1-Δ1* diploid, AMP291 × LNT59-4 (middle); and wild-type diploid, AMP268 × EG123, which harbors an *MCK1* multicopy plasmid (bottom). RNA was prepared after growth to log phase in YEPAc (0) and 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, and 24 hr after a shift to 2% KAc sporulation medium, and then fractionated on 1.5% agarose/formaldehyde gels. Fifteen micrograms of RNA was loaded per lane. The three blots were probed in the same bag and exposed to film together, first with a combination of *IME1* and *IME2*, then stripped and reprobed with a combination of *SPS1* and *SPS2* and, finally, stripped again and reprobed with pC4 (control). The unidentified band visible in the *MCK1* multicopy strain in the bottom panel, probed with pC4, was only detectable in strains harboring a plasmid and most likely represents a plasmid-derived transcript.

analysis of expression of an *ime2-lacZ* fusion gene (see below). Expression of *SPS1* and *SPS2* was also delayed and reduced >10-fold. Therefore, *MCK1* is required for normal expression of these four sporulation-specific genes.

The effects of an *MCK1* multicopy plasmid were the opposite of an *mck1* deficiency: Sporulation-specific gene expression is accelerated and increased (Fig. 7). *IME1* expression was increased twofold and peaked earlier, compared to the wild-type strain. Likewise, expression of *IME2*, *SPS1*, and *SPS2* was increased and accelerated. In fact, the pattern of expression was sufficiently accelerated so that down-regulation of these transcripts was observed within 24 hr. This acceleration of sporulation-specific gene activity correlates with the acceleration of sporulation by multicopy *MCK1* plasmids.

We conclude that *MCK1* is required for the proper temporal expression and accumulation of sporulation-specific transcripts. The low level of expression of these genes in an *mck1* mutant correlates with its low level of sporulation. *MCK1* must function upstream in the same pathway as *IME1* because changes in *MCK1* gene dosage elicit corresponding changes in *IME1* expression.

IME1 expression partially suppresses *mck1* sporulation defects

Because *IME1* expression is required for accumulation of several sporulation-specific transcripts, it seemed possible that the sporulation defects exhibited by *mck1* mutants resulted from decreased and delayed expression of *IME1*. This model predicts that expression of *IME1* from an *MCK1*-independent promoter would suppress the *mck1* sporulation defects. We tested this hypothesis by expressing *IME1* under the control of the *GAL1* promoter (Johnston and Davis 1984). *P_{GAL1}-IME1* is expressed only under conditions in which the *GAL1* promoter is active (Smith et al. 1990). Rather than modify our sporulation conditions to accommodate the addition of galactose, we used a *gal80⁻* genetic background to activate *P_{GAL1}-IME1* (Torchia et al. 1984).

We monitored *MCK1* function through expression of an *ime2-lacZ* fusion. In *MCK1* +/*mck1-Δ1* diploids, the fusion was expressed at low levels in vegetative medium and at 15-fold higher levels in Spo medium (Table 1, first row). In an *mck1-Δ1/mck1-Δ1* diploid, only a twofold increase in expression of the fusion was observed after transfer to Spo medium (Table 1, second row). Strains that express the *P_{GAL1}-IME1* gene expressed the fusion at high levels in vegetative medium and at two- to threefold higher levels in Spo medium, independently of *MCK1* (Table 1, third and fourth rows). We conclude that expression of *IME1* suppresses the *MCK1* requirement for *IME2* expression.

We also monitored *MCK1* function through sporulation ability of these diploids (Table 1, last column). *MCK1* +/*mck1-Δ1* diploids sporulated efficiently after 18 hr in sporulation medium; *mck1-Δ1/mck1-Δ1* diploids sporulated at eightfold lower levels. Strains expressing the *P_{GAL1}-IME1* gene sporulated at the same high level, regardless of *mck1* genotype. Therefore, the quantitative

Table 1. *IME1* expression partially suppresses *mck1* defects

Relevant genotype		<i>ime2-lacZ</i> levels		Spo (%)	
<i>MCK1</i>	<i>IME1</i> expression	veg	spo	mature	total
+/ Δ	<i>P_{IME1}</i>	8	115	77	87
Δ/Δ	<i>P_{IME1}</i>	6	12	1	13
+/ Δ	<i>P_{IME1}</i> + <i>P_{GAL}</i>	122	278	94	98
Δ/Δ	<i>P_{IME1}</i> + <i>P_{GAL}</i>	113	270	8	72

All strains were heterozygous for the *GAL1*-driven *IME1* allele *P_{GAL1}-IME1-14 :: TRP1* and heterozygous for the *ime2-lacZ* fusion allele *ime2- Δ 4 :: lacZ :: LEU2*. β -Galactosidase assays were performed on samples from log-phase cultures growing in YPAc (veg) or after a 6-hr shift to Spo medium (spo). Values are the average of at least four independent assays; s.d. <15%. Sporulation was quantitated after 18 hr in Spo medium. Values are the average of two independent cultures of each strain; no less than 200 cells were counted per sample.

sporulation defect associated with *mck1* mutations is suppressed by expression of *IME1*. However, the accumulation of spherical asci characteristic of *mck1* mutants was unaffected by *P_{GAL1}-IME1* expression. This observation indicates that these two *mck1* sporulation defects are independent phenomena and suggests that *MCK1* plays a role in addition to activation of *IME1*.

Expression of an mck1-lacZ gene

To determine whether *MCK1* expression is regulated, we assayed activity of an *mck1-lacZ* fusion (Fig. 8). We fused the *lacZ*-coding sequence to codon 16 of *MCK1*

and integrated a plasmid bearing this construct into the yeast genome at the *MCK1* locus (Fig. 8). Integration of the *mck1-lacZ* fusion preserved an intact copy of the *MCK1* gene downstream of the fusion gene (Fig. 8).

We tested whether a variety of growth conditions had an effect on expression of *mck1-lacZ* in isogenic *MATa*, *MAT α* , and *MATa/MAT α* strains (Table 2). We observed little variation in β -galactosidase activity in glucose or acetate cultures and in response to nitrogen starvation (Spo). We also observed no effect of *MAT* allele or *RME1* activity on *mck1-lacZ* expression. We noticed that *a/ α* strains consistently express slightly lower levels of β -galactosidase; however, we do not know whether this difference is meaningful. We conclude from these observations that *MCK1* expression does not respond to the two regulatory signals that govern entry into meiosis: cell type and nutritional deprivation. The fact that *MCK1* expression is constitutive distinguishes it from the previously identified genes that activate meiosis, *IME1* and *IME2*.

MCK1 gene product is functional in vegetative cells

The observation that *mck1-lacZ* is expressed in glucose-grown cells prompted us to ask whether there is detectable *MCK1* activity under these conditions. We placed the *HIS3* gene under control of the *IME1* promoter to provide a biological assay for the detection of *MCK1* function. This *P_{IME1}-HIS3* allele consists of the *HIS3*-coding sequence (lacking a promoter) situated downstream of the *IME1* promoter and RNA start site. This

Figure 8. Structure of the *MCK1 :: mck1-lacZ :: URA3* allele. The plasmid YIp-mck1-LacZ was integrated into the yeast genome by homologous recombination at the *MCK1* locus. The *MCK1* *StuI* site indicated in parentheses was lost as a consequence of cloning; the adjacent *EcoRI* site originated from the pLN360-I polylinker. The thin solid bar represents vector sequences; the open bar represents *MCK1* sequences; the thick solid bar represents the *URA3* selectable marker; and the shaded bar represents *lacZ* sequences. Arrows indicate the extent of coding sequences. The sequences are not drawn to scale. Abbreviations are as in Fig. 1.

Table 2. Expression of *mck1-lacZ*

Genotype		β -Galactosidase expression					
		carbon source		hours in Spo			
<i>MAT</i>	<i>RME1</i>	glucose	acetate	1	2	4	8
a	+	2.7	4.1	2.3	2.3	1.5	1.8
a	–	3.9	5.9	2.5	4.1	1.8	2.4
α	+	2.9	4.5	3.2	2.2	1.5	1.8
α	–	3.9	5.7	3.9	3.8	1.9	2.2
a/α	+/+	1.6	1.6	1.2	0.8	1.0	2.3
a/α	–/–	2.2	2.6	1.5	1.1	1.4	2.6

β -Galactosidase assays were performed on *RME1*⁺ or *rme1Δ* strains harboring the *mck1-lacZ* allele. The haploid strains were independent segregants derived from a cross between AMP649 and an original transformant of AMP678 carrying the *mck1-lacZ* fusion integrated at the *MCK1* locus (*mck1-lacZ* :: *URA3*). The *a/α* diploid strains were derived by crossing two segregants of the appropriate genotype from this cross. Log-phase cultures were grown in YEP medium with the indicated carbon source to OD₆₀₀ = 0.5 (~2 × 10⁷ cells/ml), and samples were removed for assay. In addition, the YEPac cultures were shifted to Spo medium, and samples were removed for assay after the indicated amount of time. Values are in Miller units and are the average of two independent assays of each strain. A strain harboring no *lacZ* fusion gene produces <0.3 units of activity.

allele is integrated at the genomic *IME1* locus (Fig. 9A). We used the *P_{IME1}-HIS3* allele in strains with a deletion of the natural *HIS3* locus to monitor *MCK1* function.

In strains with a wild-type *MCK1* allele, *P_{IME1}-HIS3* confers a His⁺ phenotype in either glucose (Fig. 9B) or acetate media (data not shown). We quantitated *P_{IME1}-HIS3* expression through levels of resistance to 3-amino-1,2,4-triazole (3AT), an inhibitor of the *HIS3* gene product (Klopotoski and Wiater 1965; Wolfner et al. 1975). We found that 2 mM 3AT abolished growth of *P_{IME1}-HIS3* strains in glucose medium, but 50 mM 3AT did not abolish growth in acetate medium. This observation is consistent with prior studies indicating that *IME1* RNA

levels are lower in glucose than acetate media (Kassir et al. 1988; L. Neugeborn and A.P. Mitchell, unpubl.). An *rme1Δ* mutation had no effect on resistance to 3AT conferred by *P_{IME1}-HIS3* (Fig. 9B), as expected from studies of *IME1* RNA levels in acetate-grown vegetative cells (Kassir et al. 1988; L. Neugeborn and A.P. Mitchell, unpubl.).

MCK1 is required for functional *P_{IME1}-HIS3* expression in vegetative cells. In glucose medium, *mck1 P_{IME1}-HIS3* strains are His[–], although *mck1* mutations do not affect growth in the presence of histidine (Fig. 9B). The *MCK1*-dependent His⁺ phenotype is transmitted through the *IME1* promoter, because strains harboring the wild-type *HIS3* locus are His⁺ regardless of *MCK1* genotype. In acetate medium, *mck1 P_{IME1}-HIS3* strains are His⁺ but are hypersensitive to 3AT (data not shown). These results indicate that the *MCK1* product is active in vegetative cells.

Discussion

We have identified a gene, *MCK1*, involved in the activation of meiosis and subsequent ascus maturation in yeast. An *MCK1* deficiency impairs and impedes sporulation, whereas increased *MCK1* dosage accelerates sporulation. *mck1* mutations also cause altered morphology of the asci arising in homozygous diploids: The anomalous asci resemble the immature asci that accumulate transiently in sporulating wild-type cultures. We found that *MCK1* is a positive regulator of the meiosis-specific gene *IME1*, which is, in turn, required for expression of other meiotic genes (Mitchell et al. 1990). Expression of *IME1* from a heterologous promoter accelerates sporulation of *mck1* mutants but does not improve ascus maturation. Thus, these two *mck1* phenotypes result from independent deficiencies (Fig. 10).

Our conclusion that *MCK1* positively regulates the meiotic activator *IME1* rests on three lines of evidence. First, increased dosage of *MCK1* augments *IME1* expression and leads to a corresponding acceleration of the

Figure 9. *IME1-HIS3* expression. (A) Structure of the *ime1-HIS3* allele. The structures of the genomic *IME1* and *P_{IME1}-HIS3* loci are shown. The thin bar represents *IME1* flanking sequences; the thick black bar represents *HIS3* genomic sequences; the thick shaded bar represents *IME1* genomic sequences. '*ime1*' lacks the first 67 amino-terminal amino acids of *IME1* and is not a functional allele. The arrows indicate the approximate *IME1* transcriptional start site (Smith et al. 1990). Abbreviations are as in Fig. 1 with the following addition: (Xh) *XhoI*. Restriction sites in parentheses were destroyed during construction of *P_{IME1}-HIS3* (see Materials and methods). (B) *MCK1* is required for *ime1-HIS3* expression. Strains AMP805, AMP806, AMP807, and AMP808 (*his3Δ*, *P_{IME1}-HIS3*) are segregants from the third sequential cross of an original *P_{IME1}-HIS3*-bearing transformant (for details, see Materials and methods). Each strain was streaked for single colonies on synthetic complete medium (SC) or synthetic complete medium lacking histidine (SC-HIS), and incubated at 30°C for 5 days. The relevant genotypes are indicated.

Figure 10. Regulatory activities of MCK1. MCK1 plays two positive roles in yeast sporulation. First, it is required for full expression of *IME1* which, in turn, is essential for subsequent early meiotic gene expression. Second, MCK1 is required for maturation of the sac (ascus) that encases meiotic spores.

sporulation program. Second, an *mck1* deficiency results in reduced *IME1* expression, as measured by Northern analysis and by *P_{IME1}-HIS3* activity. Third, the quantitative sporulation defect of *mck1* deletion mutants is suppressed by expression of *IME1* from the *GAL1* promoter. We also found that the failure of *mck1* mutants to express the meiotic gene *IME2* is suppressed by expression of *IME1* from the *GAL1* promoter. Both the decreased and delayed sporulation phenotype of *mck1* mutants and the accelerated sporulation resulting from increased *MCK1* dosage can be accounted for by altered levels of *IME1* transcription, which leads to modified gene expression throughout sporulation.

One class of mutations that block sporulation might cause global metabolic defects that result, secondarily, in failure to express *IME1* in response to starvation. For example, failure to supply required amino acids in sporulation medium delays *IME1* expression (L. Neigeborn, unpubl.). However, we have found that *MCK1* is required for *P_{IME1}-HIS3* expression in vegetative cells, in which *mck1* mutations confer only a modest growth defect, and that a multicopy *MCK1* plasmid elicits overexpression of *IME1*. These observations argue against such indirect effects of *mck1* mutations on *IME1* expression. Another trivial explanation for the *mck1* mutant phenotype is that *MCK1* is required for the synchrony of the sporulating population: Decreased *IME1* RNA accumulation would be a result of asynchronous *IME1* expression throughout the population. However, functional expression of *P_{IME1}-HIS3* does not rely on synchrony. We suggest that *MCK1* plays a more direct role in *IME1* expression.

Sequence analysis of the *MCK1* gene makes the strong prediction that MCK1 functions as a protein kinase. This prediction is supported by the result that mutation of the highly conserved Lys68 residue (essential for kinase activity) abolishes MCK1 activity and that MCK1 has demonstrated protein kinase activity (Dailey et al. 1990). MCK1 probably acts through phosphorylation of a target that governs *IME1* transcription. The existence of both positive and negative regulatory sites upstream of *IME1* has been inferred from the phenotypic consequences of multicopy *IME1* plasmids (Granot et al. 1989). Whether MCK1 inactivates a repressor, stimulates an activator, or

acts directly on RNA polymerase (Cisek and Corden 1989) remains to be determined. Mutations affecting the expression of *P_{IME1}-HIS3* may identify targets of MCK1.

MCK1 has two functions in the sporulation program: to stimulate *IME1* expression and to promote ascus maturation. We have shown previously that expression of *IME1* in vegetative cells is sufficient to activate early, but not later, sporulation-specific genes (Smith et al. 1990). Therefore, other regulators may be responsible for expression of later sporulation-specific genes. These additional regulators may depend on *MCK1* for their expression, as *IME1* does.

We note that the *IME1* product is rich in serine, threonine, and tyrosine (Smith et al. 1990). This composition prompts the speculation that *IME1* is a substrate for one or several protein kinases. In principle, MCK1 might govern both transcription of the *IME1* gene and phosphorylation of the *IME1* product. Examples of such redundant control pathways come from bacterial *glnA* regulation (Magasanik 1988) and the bacterial SOS response (Burckhardt et al. 1988; Shinagawa et al. 1988). Our results indicate that hypothetical phosphorylation of *IME1* product by MCK1 is not necessary for activation of the target gene *IME2*. However, we have not ruled out the possibility that MCK1 does phosphorylate *IME1*. An intriguing possibility is that phosphorylation of *IME1* is necessary for proper ascus maturation.

MCK1 may transmit a regulatory signal. The phenotypes associated with increased and decreased dosage of *MCK1* suggest that MCK1 gene product is limiting for sporulation in wild-type diploids; a rate-limiting step is a likely target of regulation. MCK1 does not act upstream of *RME1* because increased *MCK1* dosage does not inhibit *RME1* expression. Similarly, *rme1* mutations do not permit *mck1* mutants to express *P_{IME1}-HIS3* (Fig. 9B) or *IME2* (S. Su, pers. comm.). Accordingly, we suggest that MCK1 acts downstream or independently of *RME1*. The absence of regulation of *mck1-lacZ* leaves us without a simple indication of which signal, if any, is transmitted by MCK1. Our current efforts to analyze the level of MCK1 kinase activity under various conditions may resolve this question.

The *MCK1* gene product also plays a role in mitotic centromere function (Shero and Hieter, this issue). *MCK1* was independently isolated as an increased dosage suppressor of a point mutation in the essential CDEIII region of centromere DNA. An *mck1* mutation leads to decreased stability of chromosome fragments at low temperatures or in the presence of the microtubule-destabilizing agent Benomyl and cold-sensitive lethality at 11°C. We have also observed the cold-sensitive and Benomyl-sensitive phenotypes and confirm that there is no defect in chromosome segregation under normal growth conditions at 25°C or 30°C (data not shown).

One explanation for how a component of the chromosome segregation machinery might affect the sporulation pathway is via chromosome loss. Diploid yeast are capable of sporulation because simultaneous expression of *MATa* and *MATα* (from each copy of chromosome III) inhibits expression of *RME1*, a repressor of meiosis.

Spontaneous loss of one copy of chromosome III would permit *RME1* expression and result in an inability to initiate the sporulation program. Several observations indicate that chromosome loss is not the cause of *mck1* sporulation defects. First, chromosome III loss rates would have to be extremely high to account for the >50% reduction in sporulation observed in *mck1* mutants. Nondisjunction rates of this magnitude would result in significant growth defects, especially in haploids. In fact, we have detected no increase in rates of chromosome III loss under our sporulation conditions. Additionally, if chromosome loss were occurring, the spores resulting from suppression of an *mck1* defect by *P_{GAL1}-IME1* would be expected to display a high degree of inviability as a result of aneuploidy, which is not the case. Finally, a chromosome loss phenotype in *mck1* mutants cannot account for the acceleration in sporulation or the *RME1* bypass phenotype promoted by increased dosage of *MCK1*. We suggest that the *MCK1* product plays a role in both the activation of the sporulation pathway (mediated through transcriptional activation of *IME1*) and in the control of centromere activity (mediated through components of the kinetochore machinery).

How does *MCK1* control both entry into meiosis and centromere function? One possibility is that *IME1* plays a role in both pathways. We have shown that *MCK1* is required for expression of *IME1* under both sporulation and vegetative conditions, yet no role for *IME1* has been demonstrated in vegetative cells. It is not likely, however, that *IME1* is required for centromere function because *ime1* mutations do not cause Benomyl sensitivity or cold-sensitive lethality (data not shown).

We propose that the *MCK1* protein kinase plays several roles in the control of cellular processes via interaction with multiple regulatory pathways (Fig. 10). One possibility is that there is a single *MCK1* substrate which, via its state of phosphorylation, governs the activation of *IME1* expression, spore maturation, and centromere function. In this model, *MCK1* would modulate the activity of a protein that binds directly to the *IME1* promoter and facilitates centromere activity by mediating the activity of CDEIII-binding protein(s). The yeast CBF1 centromere-binding protein has been shown to be required for both chromosome stability and methionine prototrophy (Cai and Davis 1990). RAP1 is a yeast DNA-binding protein, the precise role of which (i.e., silencing of HML and HMR, activation of several ribosomal protein genes, or stabilization of telomeres) is controlled by the context of its binding site (Shore and Nasmyth 1987; Buchman et al. 1988; Lustig et al. 1990). Alternatively, there might be multiple *MCK1* target proteins, each functioning in a different pathway. Sequence comparison of *IME1* (Smith et al. 1990) and CDEIII DNA (Murphy and Fitzgerald Hayes 1990) reveals no significant homology, suggesting that unique DNA-binding proteins may function at these two loci. Suppressor analysis of *mck1* mutations should reveal whether the pathways controlled by *MCK1* share additional regulatory components.

mck1 mutations lead to only partial defects in all three systems analyzed (activation of *IME1*, stimulation of spore maturation, and centromere function). This common feature of redundant control suggests the presence of another kinase that shares functional specificity with *MCK1*. The activity of this second kinase would impede the identification of *MCK1* through conventional mutational analysis. Therefore, dosage-dependent suppression was required, in both the meiotic and centromere systems, to detect *MCK1* activity.

MCK1 is not the only protein kinase known to regulate more than one pathway. The CDC28 kinase transmits two distinct regulatory signals. It is required at "start" to initiate the transition from the G₁ to S phase of the cell cycle, and, independently, it is required at the G₂ to M boundary (Piggott et al. 1982; Lewin 1990; Reed and Wittenberg 1990). It is conceivable that the existence of multifunctional kinases, such as CDC28 and *MCK1*, may prove to be general phenomena in the control of cellular activities.

Materials and methods

Yeast strains, media, genetic manipulations, and assays

The yeast strains used in this study are listed in Table 3. The *his4G⁻* and *gal80 :: LEU2* alleles have been described (Mitchell et al. 1990). We created an in-frame *ime2-lacZ* fusion by fusing an *IME2* *PvuII* site (Yoshida et al. 1990; S. Su, pers. comm.) to the *SmaI* site of the *lacZ* gene from the vector pMC1871 (Casadaban et al. 1983); detailed information on the construction of this allele and on the *P_{GAL1}-IME1* allele is provided elsewhere (Smith et al. 1990). The *rme1-lacZ* allele was constructed by inserting the *BamHI* fragment of pMC1871 into a *BglII* site within the *RME1*-coding region (sequence analysis reveals that this construction provides an in-frame fusion; A.P. Mitchell and I. Herskowitz, in prep.). The *met4* and *arg6* mutations were isolated in this laboratory after screening EMS-mutagenized yeast for methionine or arginine auxotrophies, respectively (L. Neigeborn and S. Su, unpubl.). The identity of the mutations was confronted by complementation with known *met4⁻* and *arg6⁻* mutants. Strains bearing these mutations underwent at least two backcrosses before utilization. We note that the strains LN69D1, LN69D2, and LN69D3 used for the experiment in Figure 7 sporulate more slowly than the SK1-derived strains used in other studies from this laboratory.

The *his3Δ* mutation was created by transforming AMP107 with pPC101 (see below), which had been linearized with *XhoI* to target plasmid integration to the *HIS3* locus. His⁻ recombinants were selected by plating several Ura⁺ transformants onto SC medium containing 5-fluoro-orotic acid to obtain Ura⁻ derivatives (Boeke et al. 1984). This procedure provides selection for homologous recombination events between the repeated *HIS3* sequences, resulting in loss of the plasmid, *URA3⁺*, and *HIS3⁺* sequences. One such *his3Δ* strain was retained and outcrossed for use in this study.

To create the α/α strain LN72-D2, the a/α diploid LN72-D1 was grown to saturation in YEPD, plated on YEPD, and exposed to UV radiation for 5 sec. The plates were incubated at 30°C and replica-plated onto lawns of the α-factor halo tester strain AMP218. After incubation at room temperature, colonies were scored for halo formation. The halo-producing colonies obtained were tested for mating ability. One such isolate, LN72-D2, was retained for further use.

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Standard methods were used for crossing and diploid selection, tetrad dissection, genetic marker analysis, and transformations (Sherman et al. 1986). Sporulation was assessed from aerated liquid cultures (2% KAc supplemented auxotrophic requirements at 20 mg/liter) by microscopic analysis after 1–5 days. Yeast strains were grown at 30°C in media described previously (Sherman et al. 1986; Smith and Mitchell 1989).

For β -galactosidase assays, cultures were grown to log phase in rich medium and transferred to Spo medium (when applicable) for the indicated time. Samples for assay were washed in water and resuspended in Z buffer. Following permeabilization in SDS and chloroform, β -galactosidase activity was determined by the method of Stern et al. (1984).

Bacterial maintenance and procedures

Escherichia coli was grown at 37°C; standard protocols for media and transformations were used. For *E. coli* colony hybridizations, 50 ng of DNA from a yeast genomic library cloned into the vector YCp50 was transformed into strain DH5 α [F^- *endA1* *hdsR17* (r^-_{k,m^+}) *supE44* *thi-1* λ -*recA1* *gyrA96* *relA1* ϕ 80*dlacZ* Δ M15] and plated out with ampicillin selection at a density of 100–300 colonies/plate. Colonies were transferred onto nitrocellulose filters and treated with the following sequential steps: 10-min lysis in 10% SDS; 7-min denaturation in

0.5 M NaOH, 1 M Tris (pH 7.5); two 7-min neutralizations in 1.5 M NaCl, 1 M Tris (pH 7.5); and a 30-sec wash in 10 $\times$ SSC. DNA was immobilized on the filters by baking at 80°C in vacuo for 1 hr. A total of 2000 colonies were screened by hybridization to the randomly primed probe indicated in Figure 1.

Isolation of RME1 bypass plasmids

Strain EG123 was transformed (Hinnen et al. 1978) with 10 μ g of a library, in the *LEU2*-bearing plasmid YEp13, of fragments from a partial digest of total yeast DNA with *Sau*3A (Nasmyth and Tatchell 1980). Transformants were plated in overlays on three SC plates lacking leucine to yield 1000 colonies per plate. After 3 days, each overlay was homogenized in 10 ml of water and diluted 1000-fold. A 0.5-ml aliquot of the dilution was mixed with 10⁸ cells of strain T56-3A (carrying pAM232, a multicopy *URA3*-bearing *RME1* plasmid) in 1.25 ml of YEPD. The mixture was spread on SD plates containing histidine to select for diploids carrying both the *LEU2* and *URA3* plasmids. Diploid colonies were replica-plated to supplemented sporulation plates lacking leucine and uracil. After 2 days, the sporulation plates were replica-plated to SC plates lacking histidine, leucine, and uracil. Maintaining selection for prototrophy allowed overexpression of the yeast DNA inserts in diploid cells expressing *RME1*. His⁺ recombinants were visualized after another 2 days.

Table 3. Yeast strains and sources

Strain	Genotype	Source
EG123	α <i>his4-519 ura3 leu2 trp1 can1</i>	K. Tatchell
T56-3A	α <i>his4-712 ura3 leu2 lys2 cyh^r GAL</i> (pAM232)	this lab
2209	α <i>his4-519 leu2 ura3 trp1 can1-101 rme1 :: lacZ</i>	this lab
1233-11D	α <i>his4-519 leu2 ura3 trp1 lys2 rme1 :: lacZ</i>	this lab
AMP107	α <i>ura3 leu2 trp1 lys2 ho :: LYS2</i>	this lab
AMP129	α <i>ura3 leu2 trp1 lys2 his3-537 :: TRP1 ho :: LYS2</i>	this lab
AMP218	α <i>sst1 ade2 his6 met1 ura1 rme1</i>	R. Chan
AMP268	α <i>ura3 leu2 trp1 his4-G⁻ lys2 ho :: LYS2</i>	this lab
LNT59-4	α <i>ura3 his4-519 leu2 trp1 can1 mck1-Δ1 :: URA3</i>	this work
AMP291	α <i>ura3 leu2 trp1 his4-G⁻ lys2 ho :: LYS2 mck1-Δ1 :: URA3</i>	this work
AMP281	α <i>ura3 leu2 trp1 met4 lys2 ho :: LYS2 mck1-Δ1 :: URA3</i>	this work
AMP282	α <i>ura3 leu2 trp1 met4 lys2 ho :: LYS2 gal80 :: LEU2 P_{GAL1}-IME1-14 :: TRP1</i>	this work
AMP283	α <i>ura3 leu2 trp1 lys2 ho :: LYS2 gal80 :: LEU2 mck1-Δ1 :: URA3 ime2-Δ4 :: lacZ :: LEU2</i>	this work
AMP284	α <i>ura3 leu2 trp1 his4-G⁻ lys2 ho :: LYS2 mck1-Δ1 :: URA3 ime2-Δ4 :: lacZ :: LEU2</i>	this work
AMP285	α <i>ura3 leu2 trp1 met4 lys2 ho :: LYS2 gal80 :: LEU2 P_{GAL1}-IME1-14 :: TRP1 mck1-Δ1 :: URA3</i>	this work
AMP288	α <i>ura3 leu2 trp1 his4-G⁻ lys2 ime2-Δ4 :: lacZ :: LEU2 ho :: LYS2</i>	this work
AMP607	α <i>ura3 leu2 trp1 his3 lys2 arg6 ho :: LYS</i>	this work
AMP608	α <i>ura3 leu2 trp1 his3 lys2 met4 ho :: LYS2</i>	this work
AMP609	α <i>ura3 leu2 trp1 his3 lys2 met4 mck1-Δ1 :: URA3 ho :: LYS2</i>	this work
AMP610	α <i>ura3 leu2 trp1 his3 lys2 arg6 mck1-Δ1 :: URA3 ho :: LYS2</i>	this work
AMP678	α <i>ura3 leu2 trp1 lys2 met4 gal80 :: LEU2 ho :: LYS2</i>	this work
AMP550	α <i>ura3 leu2 trp1 his3 lys2 arg6 ho :: LYS2</i>	this work
LN72D1	2209 $\times$ 1233.11D	this work
LN72D2	α/α derivative of LN72D1	this work
LN78D1	AMP285 $\times$ AMP284	this work
LN78D2	AMP285 $\times$ AMP288	this work
LN78D3	AMP282 $\times$ AMP283	this work
LN78D4	AMP283 $\times$ AMP285	this work
AMP649	α <i>ura3 leu2 trp1 his3 lys2 arg6 rme1Δ5 :: LEU2 mck1-Δ3 :: TRP1 ho :: LYS2</i>	this work
AMP678	α <i>ura3 leu2 trp1 his3 lys2 met4 gal80 :: LEU2 ho :: LYS2</i>	this work
AMP805	α <i>ura3 leu2 trp1 his3 lys2 arg6 ime1-HIS3 rme1Δ5 :: LEU2 ho :: LYS2</i>	this work
AMP806	α <i>ura3 leu2 trp1 his3 lys2 arg6 ime1-HIS3 rme1Δ5 :: LEU2 mck1-Δ3 :: TRP1 ho :: LYS2</i>	this work
AMP807	α <i>ura3 leu2 trp1 his3 lys2 arg6 ime1-HIS3 mck1-Δ3 :: TRP1 ho :: LYS2</i>	this work
AMP808	α <i>ura3 leu2 trp1 his3 lys2 ime1-HIS3 ho :: LYS2</i>	this work

Most colonies gave rise to two or fewer His⁺ papillae, but several colonies had dense His⁺ papillae (as seen with control colonies carrying only the vectors YEp13 and YEp24). Colonies that yielded abundant His⁺ papillae were purified for use in subsequent tests, as described by Smith and Mitchell (1989). Only one plasmid, denoted B1, retained the desired activity.

Plasmid constructions

All plasmids were maintained in *E. coli* strain MH6 (*leuB600 pyrF::Tn5 lacX74 hsdR galU galK*). Subclone derivatives of the original *RME1* bypass plasmid B1 and of pLN340 were constructed by conventional means. pAM301 was constructed by subcloning the *Bgl*II–*Sal*I fragment of B1 containing the yeast DNA insert and the 2- μ origin into *Bam*HI–*Sal*I-digested YIp5. This creates a multicopy, *URA3*-based plasmid (similar to YEp24) containing the same yeast insert as B1. Deletion derivatives of pAM301 were obtained by partial or complete digestion with *Bam*HI or *Eco*RI and religation. The subclone pLN329 was constructed by first inserting the ~4.5-kb yeast DNA insert/plasmid junction *Hind*III fragment of pAM301 into the *Hind*III site within the polylinker of pUC18 to create pHS100. The 1.65-kb *Stu*I–*Nhe*I-fragment of pHS100 was then ligated to *Pvu*II–*Nhe*I-digested YEp24 to create pLN329. pLN341 was created by ligating the *Hind*III (blunt-ended by Klenow activity)/*Bam*HI fragment of pLN340 into *Bam*HI–*Sma*I-digested pUC18. pLN360-I and pLN360-II are the 2.1-kb *Stu*I–*Nhe*I fragment of pLN340 cloned, in both orientations, into the *Sma*I site of pRS314 (Sikorski and Hieter 1989).

The plasmids used for creating the *mck1* disruption alleles are as follows: pLN352 (containing the *mck1*- $\Delta 1::URA3$ allele) was derived from pLN341 by first digesting with *Nco*I and filling in the protruding ends with Klenow, and then digesting with *Sph*I, to create a 700-bp gap. This was then ligated to a 1.1-kb *Sma*I–*Sph*I *URA3* fragment derived from plasmid pSM32 (the *URA3* gene cloned into the *Hinc*II site of the pUC18 polylinker; Michaelis and Herskowitz 1988) to generate pLN352. pLN330-I and pLN330-II (carrying the *mck1*-2 allele) were created by first constructing the plasmid pLNpUC329; pLNpUC329 is the 1.65-kb *Stu*I fragment of pHS100 cloned into the *Xba*I–*Sma*I sites of pUC18. pLNpUC329 was subjected to partial digestion with *Eco*RI to create linear molecules, blunt-ended with Klenow, and then ligated to a 1.1-kb *URA3* *Sma*I fragment derived from pSM32. The resultant plasmids (with the *URA3* gene in both orientations) are pLN330-I and pLN330-II. pLN370-I (containing the *mck1*- $\Delta 3::TRP1$ allele) was constructed by replacing the 1.2-kb *Sph*I–*Bal*I fragment of pLN360-I with a 0.8-kb *Stu*I–*Sph*I fragment containing the *TRP1* gene (obtained from a pUC18 plasmid carrying the *TRP1* locus cloned into the *Eco*RI site of the polylinker).

The *ime1*–*HIS3* fusion gene was constructed by placing a promoterless *HIS3* fragment ~250-bp downstream of the start of *IME1* transcription at a site 26-bp upstream of the *IME1* translational start site (Smith et al. 1990). This *HIS3* fragment was obtained from the plasmid YIp5-Sc3354 (gift of K. Struhl). YIp5-Sc3354 was linearized at an *Eco*RI linker insertion site located 23 bp upstream of the *HIS3* translational start and the ends were filled in with Klenow. The *HIS3*-coding fragment, along with adjacent plasmid sequences, including the yeast selectable marker *URA3*, was released by digestion with *Sma*I. This *HIS3/URA3* fragment was ligated with the *IME1* plasmid pAM504 (Smith and Mitchell 1989), which had been treated with *Hind*III and Klenow (retaining the *IME1* promoter region upstream of position –26). The resulting plasmid, pAM510, contains the *HIS3*-coding sequence, plus YIp5 vector and *URA3* sequences, adjacent to the 5'-noncoding region of *IME1*. pAM510 was then

digested with *Xho*I, the ends were filled in, and it was redigested with *Sph*I. This released a fragment containing only the 5' *IME1* and *HIS3*-coding sequences. This fragment was used to replace the *Sph*I–*Pvu*II *IME1* fragment contained within the plasmid pAM508 (YCp50 carrying the *IME1* *Sph*I–*Bam*HI fragment of pAM504). The resultant plasmid, pAM511, carries the *HIS3*-coding sequence ~250-bp downstream of the *IME1* transcriptional start site, in addition to 2 kb of 3' *IME1* sequences (see Fig. 9A). The integrity of this construct was confirmed by sequence analysis through the *IME1*–*HIS3* fusion junction, which has the following sequence: 5'-AAAAGAAAAGCTAATT-CGCAAGAT-3'. The boldfaced bases represent the filled-in *Hind*III site at the end of the *IME1* DNA, and the underlined bases represent the filled-in *Eco*RI site at the beginning of the *HIS3* DNA.

The plasmid YIp–*MCK1*–*LacZ*, containing the *mck1*–*lacZ* fusion gene, was constructed by inserting the 700-bp *Eco*RI fragment from pLN360-I (which carries the first 16 codons of *MCK1* plus 650 bp of upstream DNA) into the *Eco*RI site of the *lacZ* fusion vector YIp356R (Myers et al. 1986). Insertion of this fragment in the correct orientation (see Fig. 8) created an in-frame *mck1*–*lacZ* fusion gene.

The HS21 Δ SK plasmid carrying the *his3* Δ mutation was created by digesting the *his3* plasmid pHS21 (a defective *his3* allele, containing a *Sac*I linker inserted into the *Hae*III site 147 bp downstream of the translational start, carried in the vector YIp1; gift of Hannah Klein) with *Kpn*I and *Sac*I (releasing a 481-bp *his3* fragment from within the *HIS1*-coding sequence), treating with S1 to create blunt ends, and religating to generate an internal *his3* deletion. The 1.2-kb *Bam*HI fragment containing the *his3* Δ gene of HS21 Δ SK was ligated into the *Bam*HI site of YIp5 to create plasmid pPC101.

Construction of *mck1* chromosomal mutations and the *P_{IME3}*–*HIS3* fusion allele

Integrative transformation (Rothstein 1983) by the LiAc method (Ito et al. 1983) was used to construct the *mck1* mutations and the *ime1*–*HIS3* fusion allele. Yeast *mck1*- $\Delta 1::URA3$ mutants were constructed by transforming haploid and diploid *ura3* recipients (strains EG123; AMP268; AMP268 $\times$ AMP107) with 5 μ g of pLN352 digested with *Bam*HI and *Nhe*I. *Ura*⁺ transformants were screened for expected restriction fragments by Southern analysis. Yeast *mck1*-2::*URA* mutants were constructed by transforming the same *ura3* recipients with 5 μ g of pLN330-I or pLN330-II digested with *Hind*III. Yeast *mck1*- $\Delta 3::TRP1$ mutants were constructed by transforming the *trp1* strain AMP550 with the 1.7-kb *Xho*I–*Eag*I fragment from pLN370-I and selecting tryptophan prototrophs. Because all haploid transformants were viable, the diploid transformants were not pursued further. The integrity of each mutation was verified by Southern analysis (Southern 1975). Each mutant was crossed to a wild-type strain, and meiotic tetrads were analyzed to demonstrate 2 : 2 segregation (a minimum of 16 tetrads were analyzed per strain). We noticed no growth defect, temperature-sensitive lethality, or mating defect associated with these alleles.

In the course of these experiments we constructed another *mck1* deletion mutation denoted *mck1*- $\Delta 4::URA3$. This allele replaces the entire 2.1-kb *Stu*I fragment with the *URA3* gene. This mutation apparently removed part of an adjacent gene required for growth on nonfermentable carbon sources. We were able to demonstrate that this growth defect is not associated with *mck1* mutations because distinct restriction fragments complement this metabolic defect but not the sporulation defects (data not shown). Similarly, pLN360-I and pLN360-II com-

plement the *mck1* sporulation defects conferred by the other *mck1* mutations but not the metabolic defect of *mck1*- $\Delta 4 :: URA3$. Furthermore, the *mck1*-2 :: *URA3* insertion mutation, which disrupts only the *MCK1*-coding region without removing adjacent DNA, displays the same phenotypes as the *ime3* $\Delta 1$ and *mck1*- $\Delta 3$ alleles but does not result in the growth defect. The deduced boundary of this new locus is ~0.5 kb upstream of the start of the *MCK1*-coding region.

The *P_{IME1}*-*HIS3* fusion allele was created by cotransforming strain AMP129 with *Clal*-*NcoI*-digested pAM511 (containing the *P_{IME1}*-*HIS3* gene flanked by *IME1* genomic DNA) and the vector YEpl3, which carries the yeast selectable marker *LEU2*. *Leu*⁺ transformants were selected and then screened for integration of *P_{IME1}*-*HIS3* by testing for histidine prototrophy. Our prior studies with the *P_{IME1}*-*HIS3* plasmid pAM510, for which integration can be selected by *URA3* complementation, indicated that *P_{IME1}*-*HIS3* would yield a *His*⁺ phenotype. One such transformant was selected for further study. The integrity of the integration was confirmed by Southern analysis and by failure to complement an *ime1* deficiency (data not shown). The original transformant was subjected to two successive outcrosses and then crossed to an *mck1*- $\Delta 3$ mutant for the analysis presented here.

Construction of the *mck1* Lys68 to arginine mutation

We used oligonucleotide site-directed mutagenesis to create the Lys68 to arginine mutation in the plasmid pLN360-I. The oligonucleotide MCK1-K > R (5'-GGGACTTTTCTAATTGC-3') was used as the mutagenic primer to replace the AAA lysine codon at position 68 in the *MCK1*-coding sequence with an AGA arginine codon. Except for the use of plasmid DNA from pLN360-I instead of M13-derived DNA, we followed the protocol included with the Bio-Rad Muta-Gene M13 in vitro mutagenesis kit. The identity of the mutation was confirmed by sequence analysis.

Construction of the *MCK1 :: mck1-lacZ :: URA3* allele

Strains harboring the *MCK1 :: mck1-lacZ :: URA3* allele were created by integrating the plasmid YIp-MCK1-LacZ into the yeast genome at the *MCK1* chromosomal locus. Homologous integration at the *MCK1* locus was stimulated by linearizing the plasmid at the unique *SpeI* site within the *MCK1* upstream region (see Figs. 1, 8) and transforming into strain AMP678 with selection for uracil prototrophy. Integration at *MCK1* was confirmed by linkage analysis (data not shown). The resultant yeast transformants carry the *mck1-lacZ* fusion allele flanked by the chromosomal *MCK1* upstream regulatory DNA on the 5' side and the remainder of the plasmid at the 3' end of the fusion gene (including the *URA3* gene). In addition, homologous integration of the entire plasmid allowed retention of the wild-type *MCK1* locus (see Fig. 8).

Preparation of RNA from sporulating cells and Northern (RNA) analysis

Cells were inoculated from a saturated YEPD culture into YEP-Ac. After 15–20 hr the cells reached a density of 1×10^7 to 2×10^7 cells/ml. A 30-ml zero-time sample was harvested, and the remainder of the culture was filtered onto a membrane filter (Millipore Corp., Bedford, MA), washed with water, and suspended at 2×10^7 cells/ml in prewarmed 2% KAc sporulation medium supplemented with the required additives. The culture was incubated with aeration, and 30-ml samples were removed at the indicated times for RNA extraction. Total cellular RNA

was prepared and fractionated as described (Smith and Mitchell 1989). Strand-specific probes for *IME1* and *IME2* and randomly primed labeled probes (Boehringer Mannheim Biochemicals, Indianapolis, IN) for *SPS1* and *SPS2* were prepared as described (Mitchell et al. 1989). As a loading control we probed with the plasmid pC4, which encodes a transcript whose expression is unaffected by starvation or cell type (Law and Segall 1988). Blots were stripped by two successive 15-min washes with $0.1 \times$ SSC, 0.1% SDS at 90°C. The somewhat extended sporulation-specific gene expression kinetics observed here, compared with those described previously by this laboratory, is the result of only one parent deriving from the rapidly sporulating SK1 strain.

Construction of *MCK1* deletions and sequencing strategy

We constructed a series of exonuclease III-generated deletions of plasmids pLN360-I and pLN360-II (Fig. 1). These plasmids contain the same 2.1-kb *StuI* restriction fragment cloned in opposite orientations into the yeast shuttle vector pRS314. An amount of 3–5 μ g of each plasmid was digested with *SacI* and *Bam*HI to generate linear molecules with 5' extensions proximal to the *MCK1* insert and 3' extensions on the opposite end of the molecule. Deletions were generated using the Erase-A-Base Kit (Promega) following the manufacturer's protocol except that nuclease-treated molecules were allowed to ligate overnight and were then transformed directly into *E. coli*. Constructs containing deletions ranging from 100 to 1800 bp were retained for further analysis.

Plasmids to be sequenced were transformed into *E. coli* strain XL1 Blue [*recA1 lac*[−] *endA1 gyrA96 thi hsdR17 supE44 relA1 (F'*proAB, *lacIq, lacZ* Δ M15, *Tn10*)], and single-stranded DNA was prepared using standard protocols (Stratagene protocol manual). DNA was sequenced with the Sequenase 2 Kit (Stratagene). In most cases, the extension reactions were primed with the Universal M13 primer, which hybridizes to a site adjacent to the yeast DNA inserts in pLN360-I and pLN360-II. One region of the *MCK1* sequence (nucleotides 555–767) was confirmed by using *MCK1*-specific synthetic oligonucleotides to prime the extension reactions. We used information from previously sequenced constructs to synthesize these oligonucleotides, d15-03 (5'-GGATGGTTCGGCAATCCTCAG-3') and d32-3 (5'-CGTGTTAGTAGTGTGGGCAG-3'), by using an Applied Biosystems 381A DNA synthesizer. Manipulation of the sequence data was carried out by using the University of Wisconsin Genetics Computer Group nucleic acids sequence analysis programs (Devereux et al. 1984).

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