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 imel-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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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 a/a 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 a/a diploids (Fig. 2A). Vegetatively grown a/a diploids carrying either a multicopy MCK1 plasmid or vector alone produced equal levels of β-galactosidase. Control strains harboring a MATα plasmid or a/a diploids show appropriately repressed lev-
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 possess 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 yeast 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
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
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 meiotic activation by yeast MCK1 kinase.

**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] ascis, solid symbols indicate condensed [mature] ascis. [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-Δ1, and mck1-Δ1/mck1-Δ1 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-Δ1 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 ascis 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 ascis following preparation for dissection, but spore viability was 100% in 24 mature asci. Spore maturation in the mck1-Δ1 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 mck-Δ1 mutant ascus morphology. Wild-type ascis 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 ascis present in mck1/mck1 mutants after 24 hr in Spo medium resemble the immature wild-type ascis (Fig. 6C). These spherical ascis persist in the mck1 mutant for at least 5 days (Fig. 5B). We conclude that the mck1-Δ1 mutation causes both quantitative and qualitative sporulation defects.

Both delayed sporulation and accumulation of immature ascis were associated with two additional mck1 alleles: mck1-2 :: UR3 and mck1-Δ3 :: TRP1 (Fig. 1). mck1-2 :: UR3 is an insertion allele that interrupts the gene at codon 16, and mck1-Δ3 :: TRP1 is a deletion extending from 344 nucleotides upstream of the MCK1 coding region through to codon 293. Because the mck1-2 :: UR3 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 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.

Figure 6. mck1-Δ1 diploids accumulate immature asci. Representative ascis from the isogenic sporulating cultures described in Fig. 5 were photographed at 1000× magnification. (A) wild-type diploids after 12 hr in Spo medium; (B) wild-type diploids after 24 hr in Spo medium; (C) mck1-Δ1 diploids after 24 hr in Spo medium.

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 [Perceval-Smith and Segall 1984, 1986], in the wild-type and mck1-Δ1 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 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+ /mck1Δ1 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 mck1Δ1/mck1Δ1 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+ /mck1Δ1 diploids sporulated efficiently after 18 hr in sporulation medium; mck1Δ1/mckΔ1 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
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Table 1. IME1 expression partially suppresses mck1 defects

| Relevant genotype | ime2-lacZ levels | Spo (%) |
|-------------------|------------------|---------|
|                   | veg              | spo     | mature | total |
| +/Δ               | P.ravel1        | 8       | 115    | 77    | 87    |
| Δ/Δ               | P.ravel1        | 6       | 12     | 1     | 13    |
| +/I               | P.ravel1 + PGAL  | 122     | 278    | 94    | 98    |
| Δ/Δ               | P.ravel1 + PGAL  | 113     | 270    | 8     | 72    |

All strains were heterozygous for the GAL1-driven IME1 allele PGAL-IME1-14::TRP1 and heterozygous for the ime2-lacZ fusion allele ime2-D4::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 PGAL-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].

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.ravel1-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 Stul site indicated in parentheses was lost as a consequence of cloning, the adjacent EcoRI site originated from the pLN360-1 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.

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Table 2. Expression of mck1-lacZ

| Genotype | MAT | RME1 | Carbon source | Galactosidase hours in SPO |
|----------|-----|------|---------------|---------------------------|
|          |     |      | glucose       |   1 | 2   | 4   | 8   |
| a        | +   |      | 2.7          | 4.1 | 2.3 | 2.3 | 1.8 |
| a        | -   |      | 3.9          | 5.9 | 2.5 | 4.1 | 1.8 |
| α        | +   |      | 2.9          | 4.5 | 3.2 | 2.2 | 1.5 |
| α        | -   |      | 3.9          | 5.7 | 3.9 | 3.8 | 1.9 |
| a/α      | +/+ |      | 1.6          | 1.6 | 1.2 | 0.8 | 1.0 |
| a/α      | -/- |      | 2.2          | 2.6 | 1.5 | 1.1 | 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/a 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 (2×10^7 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.

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 meiotic-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
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MCK1

Vegetative Growth

Entry into Meiosis

Completion of Sporulation

(Centrormere Function) (Activation of IME1 Expression)

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 gltA regulation [Magasanik 1988] and the bacterial SOS response [Burkhart 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 $RME1$. 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 PgalT-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 CDEIIII-binding protein(s). The yeast CBFI 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 which 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 [Piggot 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 gal180::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 PgalT-IME1 allele is provided elsewhere [Smith et al. 1990]. The ime1-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 SKI-derived strains used in other studies from this laboratory.

The his3A 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 his3A strain was retained and outcrossed for use in this study. To create the a/a strain LN72-D2, the a/a diploid LN72-D 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 a-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.
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 DH5α [F− endA1 hsdR17 (r−m− s−) supE44 thi-1 λ recA1 gyrA96 relA1 pho80 lacZAM15] 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 1.5 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× 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 μg of a library, in the LEU2-bearing plasmid YEpl3, of fragments from a partial digest of total yeast DNA with Sau3A [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 10⁶ 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.

| Strain | Genotype | Source |
|--------|----------|--------|
| EG123  | a his4-519 ura3 leu2 trp1 can1 | K. Tatchell |
| T56-3A | a his4-712 ura3 leu2 lys2 cyh' GAL (pAM232) | this lab |
| 209    | a his4-519 leu2 ura3 trp1 can1-101 mckl-A1 :: lacZ | this lab |
| 1233-11D | a his4-519 ura3 ura3 trp1 lys2 mckl-A1 :: lacZ | this lab |
| AMP107 | a ura3 leu2 trp1 lys2 ho :: LYS2 | this lab |
| AMP129 | a ura3 leu2 trp1 lys2 his3-537 :: TRP1 ho :: LYS2 | this lab |
| AMP218 | a sst1 ade2 his6 met1 ura1 mckl-A1 | R. Chan |
| AMP268 | a ura3 leu2 trp1 his4-G' lys2 ho :: LYS2 | this lab |
| LNT59-4 | a ura3 his4-519 leu2 trp1 can1 mckl-A1 :: URA3 | this work |
| AMP291 | a ura3 leu2 trp1 his4-G' lys2 ho :: LYS2 mckl-A1 :: URA3 | this work |
| AMP281 | a ura3 leu2 trp1 met4 lys2 ho :: LYS2 mckl-A1 :: URA3 | this work |
| AMP282 | a ura3 leu2 trp1 lys2 ho :: LYS2 gal80 :: LEU2 PγALTF-lME1-14-6-42 :: TRP1 | this work |
| AMP283 | a ura3 leu2 trp1 his4-G' lys2 ho :: LYS2 mckl-A1-14 :: URA3 ime2-4-6-42 :: lacZ-2 :: LEU2 | this work |
| AMP284 | a ura3 leu2 trp1 his4-G' lys2 ho :: LYS2 mckl-A1-14 :: URA3 ime2-4-6-42 :: lacZ-2 :: LEU2 | this work |
| AMP285 | a ura3 leu2 trp1 met4 lys2 ho :: LYS2 gal80 :: LEU2 PγALTF-lME1-14-6-42 :: TRP1 mckl-A1-14 :: URA3 | this work |
| AMP288 | a ura3 leu2 trp1 lys2 his3-537 :: TRP1 ho :: LYS2 | this work |
| AMP298 | a ura3 leu2 trp1 his4-G' lys2 ho :: LYS2 mckl-A1-14 :: URA3 ime2-4-6-42 :: lacZ-2 :: LEU2 ho :: LYS2 | this work |
| AMP307 | a ura3 leu2 trp1 lys2 his3 lys2 met4 ho :: LYS2 | this work |
| AMP309 | a ura3 leu2 trp1 his3 lys2 met4 mckl-A1 :: URA3 ho :: LYS2 | this work |
| AMP507 | a ura3 leu2 trp1 his3 lys2 arg6 ho :: LYS2 | this work |
| AMP509 | a ura3 leu2 trp1 his3 lys2 arg6 met4 ho :: LYS2 | this work |
| AMP510 | a ura3 leu2 trp1 his3 lys2 arg6 mckl-A1 :: URA3 ho :: LYS2 | this work |
| AMP617 | a ura3 leu2 trp1 lys2 met4 gal80 :: LEU2 ho :: LYS2 | this work |
| AMP618 | a ura3 leu2 trp1 lys2 met4 gal80 :: LEU2 ho :: LYS2 | this work |
| AMP678 | a ura3 leu2 trp1 his3 lys2 arg6 mckl-A1 :: URA3 ho :: LYS2 | this work |
| AMP679 | a ura3 leu2 trp1 his3 lys2 arg6 mckl-A1 :: URA3 ho :: LYS2 | this work |
| AMP805 | a ura3 leu2 trp1 lys2 arg6 ime2-4-6-42 :: LEU2 ho :: LYS2 | this work |
| AMP806 | a ura3 leu2 trp1 lys2 arg6 ime2-4-6-42 :: LEU2 mckl-A1-14-6-42 :: TRP1 ho :: LYS2 | this work |
| AMP807 | a ura3 leu2 trp1 lys2 arg6 ime2-4-6-42 :: TRP1 ho :: LYS2 | this work |
| AMP808 | a ura3 leu2 trp1 his3 lys2 mckl-A1-14 :: TRP1 ho :: LYS2 | 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 hisdR 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 BglII–SalI fragment of B1 containing the yeast DNA insert and the 2-μ origin into BamHI–SalI-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 BamHI or EcoRI and religation. The subclone pLN329 was constructed by first inserting the ~4.5-kb yeast DNA insert/plasmid junction HindIII fragment of pAM301 into the HindIII site within the polylinker of pUC18 to create pHS100. The 1.65-kb Stul–Nhel fragment of pHS100 was then ligated to YEp24 to create pLN329. pLN341 was created by ligating the HindIII (blunt-ended by Klenow activity)/BamHI fragment of pLN340 into BamHI–SalI-digested pUC18. pLN340-I and pLN340-II are the 2.1-kb Stul–Nhel fragment of pLN340 cloned in both orientations, into the Smal site of pRS314 (Sikorski and Hieter 1989).

The plasmids used for creating the mck1 disruption alleles are as follows: pLN352 (containing the mck1-Δ1 :: URA3 allele) was derived from pLN341 by first digesting with Ncol and filling in the protruding ends with Klenow, and then digesting with Spb1, to create a 700-bp gap. This then ligated to a 1.1-kb Smal–Spb1 URA3 fragment derived from plasmid pSM32 (the URA3 gene cloned into the HindII 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 Stul fragment of pHS100 cloned into the XbaI–SmaI sites of pUC18. pLNpUC329 was subjected to partial digestion with EcoRI to create linear molecules, blunt-ended with Klenow, and then ligated to a 1.1-kb URA3 Smal 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-Δ3 :: TRP1 allele was constructed by replacing the 1.2-kb Spb1–BalI fragment of pLN360-I with a 0.8-kb Stul–Spb1 fragment containing the TRP1 gene (obtained from a pUC18 plasmid carrying the TRP1 locus cloned into the EcoRI 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 YIp5S-Sc3354 (gift of K. Struhl). YIp5Sc3354 was linearized at an EcoRI 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 SmaI. This HIS3/URA3 fragment was ligated with the *IME1* plasmid pAM504 (Smith and Mitchell 1989), which had been treated with HindIII 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 XhoI, the ends were filled in, and it was redigested with Spb1. This released a fragment containing only the 5' *IME1* and HIS3-coding sequences. This fragment was used to replace the Spb1–PvuII *IME1* fragment contained within the plasmid pAM508 [YCP50 carrying the *IME1* Spb1–BamHI 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'-AAAGAAAAAGCTATT-CCGAGAT-3'. The boldfaced bases represent the filled-in HindIII site at the end of the *IME1* DNA, and the underlined bases represent the filled-in EcoRI 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 EcoRI fragment from pLN360-I (which carries the first 16 codons of MCK1 plus 650 bp of upstream DNA) into the EcoRI site of the *LacZ* fusion vector YIp566R (Myers et al. 1986). Insertion of this fragment in the correct orientation (see Fig. 8) created an in-frame mck1–lacZ fusion gene.

The HS21ΔSK plasmid carrying the his3Δ mutation was created by digesting the his3 plasmid pHS21 (a defective his3 allele, containing a Sael linker inserted into the HeuII site 147 bp downstream of the translational start, carried in the vector Yip1; gift of Hannah Klein) with Kpn1 and Sael [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 BamHI fragment containing the his3Δ gene of HS21ΔSK was ligated into the BamHI 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-Δ1 :: URA3* mutants were constructed by transforming haploid and diploid ura3 recipients (strains EG123; AMP268; AMP268 × AMP107) with 5 μg of pLN352 digested with BamHI and Nhel. 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 μg of pLN330-I or pLN330-II digested with HindIII. Yeast *mck1-Δ3 :: TRP1* mutants were constructed by transforming the trp1 strain AMP550 with the 1.7-kb XhoI–EagI 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–Δ4 :: URA3*. This allele replaces the entire 2.1-kb Stul 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 pLN360II com-
plement the mck1 sporulation defects conferred by the other mck1 mutations but not the metabolic defect of mck1Δ4 :: 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 ime3Δ1 and mck1-Δ3 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<sub>IMEL</sub>−HIS3 fusion allele was created by cotransforming strain AMP129 with ClaI−NcoI-digested pAM511 (containing the P<sub>IMEL</sub>−HIS3 gene flanked by IME1 genomic DNA) and the vector YEp13, which carries the yeast selectable marker LEU2. Leu<sup>+</sup> transformants were selected and then screened for integration of P<sub>IMEL</sub>−HIS3 by testing for histidine prototrophy. Our prior studies with the P<sub>IMEL</sub>−HIS3 plasmid pAM510, for which integration can be selected by URA3 complementation, indicated that P<sub>IMEL</sub>−HIS3 would yield a His<sup>+</sup> 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-Δ3 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 Mutagen-Gene M13 kit 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 LEU2 transformants carrying 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<sup>7</sup> to 2 × 10<sup>7</sup> 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<sup>7</sup> 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 SP51 and SP52 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% 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 Stal restriction fragment cloned in opposite orientations into the yeast shuttle vector pRS314. An amount of 3–5 μg of each plasmid was digested with Sall and BamHI 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 nucleate-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 supD44 relA1 (F’proAB, lacQ, lacZAM15, 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 previous sequenced constructs to synthesize these oligonucleotides, d15-03 (5′-GGATGGTCGGCAATCCTCAG-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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