Identification of Functionally Related Genes That Stimulate Early Meiotic Gene Expression in Yeast

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ABSTRACT

Meiosis and spore formation in the yeast Saccharomyces cerevisiae are associated with increased expression of sporulation-specific genes. One of these genes, IME2, encodes a putative protein kinase that is a positive regulator of other sporulation-specific genes. We have isolated mutations that cause reduced expression of an ime2-lacZ fusion gene. We found mutations in IME1, a known positive regulator of IME2, and MCK1, a known positive regulator of IME1. We also isolated recessive mutations in 12 other genes, which we designate RIM (Regulator of IME2) genes. Our analysis indicates that the defects in rim1, rim8, rim9 and rim13 mutants are a consequence of diminished IME1 expression and can be suppressed by expression of IME1 from the heterologous ACT1 promoter. These rim mutations also reduced expression of an ime1-HIS3 fusion, in which the HIS3 gene is expressed from the IME1 promoter, and caused reduced levels of IME1 RNA. Although the rim1, rim8, rim9 and rim13 mutant phenotypes are similar to those of mck1 mutants, we found that the defects in ime2-lacZ expression and sporulation of the mck1 rim double mutants were more severe than either single mutant. In contrast, the defects of the rim rim double mutants were similar to either single mutant. The rim1, rim8, rim9 and rim13 mutants also display slow growth at 17°C and share a smooth colony morphology that is not evident in mck1 mutants or isogenic wild-type strains. We suggest that RIM1, RIM8, RIM9 and RIM13 encode functionally related products that act in parallel to MCK1 to stimulate IME1 expression.

The IME1 gene product plays a pivotal role in the decision to initiate meiosis and spore formation in the yeast Saccharomyces cerevisiae. IME1 expression and sporulation respond in parallel to nutritional signals; they are blocked in the absence of glucose or nitrogen. In the absence of glucose and nitrogen, IME1 expression is greater in a/a cells, which can sporulate, than in a cells or α cells, which cannot (KASSIR, GRANOT and SIMCHEN 1988). Sporulation is blocked in ime1 null mutants (KASSIR, GRANOT and SIMCHEN 1988) and increased expression of IME1 permits initiation of meiosis in a cells or α cells and in rich media (KASSIR, GRANOT and SIMCHEN 1988; GRANOT, MARGOLSKEE and SIMCHEN 1989; SMITH and MITCHELL 1989; SMITH et al. 1990). Therefore, the precise regulation of meiosis is achieved, in part, through regulation of IME1 expression.

One of the main roles of the IME1 product is to stimulate expression of sporulation-specific genes (SMITH and MITCHELL 1989; MITCHELL, DRISCOLL and SMITH 1990; ENGERBRECHT and ROEDER 1990; KHARAS et al. 1991). These genes are expressed at highest levels in sporulating cells and many are essential for the sporulation program. One of the earliest sporulation-specific genes is IME2, which encodes a protein kinase homolog (YOSHIDA et al. 1990) that can activate many other sporulation-specific genes (Figure 1). The IME1 product can also activate sporulation-specific genes independently of IME2. Stimulation of sporulation-specific gene expression through IME2 and through an IME2-independent mechanism subsequently leads to the completion of the sporulation program (MITCHELL, DRISCOLL and SMITH 1990). IME1 activity is limiting for expression of sporulation-specific genes because decreased IME1 expression leads to decreased RNA levels from IME2 and other sporulation-specific genes (SMITH and MITCHELL 1989; NEIGEBORN and MITCHELL 1991).

What are the genes that regulate IME1 expression? One set of genes affects the adenylate cyclase/cAMP-dependent protein kinase pathway (reviewed by BROACH 1991). Reduced cAMP-dependent protein kinase activity is associated with IME1 expression in unstarved cells; increased cAMP-dependent protein kinase activity prevents IME1 expression in starved cells (SMITH and MITCHELL 1989; MATSUGA et al. 1990). Pleiotropic effects of altered cAMP-dependent protein kinase activity suggest that this pathway is involved in a starvation response. However, the signals to which this pathway responds are unclear.

A second group of genes transmits the a/a cell type signal. The a1 and α2 products of the MAT locus are subunits of a repressor, α1-α2, that determines the a/α cell type (GOUTTE and JOHNSON 1988; DRANGINIS...
and proper mitotic centromere function as well. Asci that persist in the population are slow and inefficient. Many of the spores produced in the mutant are packaged in immature ascis.

**Figure 1.**—Regulation of meiosis and spore formation in *S. cerevisiae*. *IME1* is essential for meiosis. The *IME1* product stimulates sporulation-specific gene expression. The *IME2* product stimulates sporulation-specific gene expression as well. Known positive regulators of *IME1* include *MCK1* and *IME4*. In this study, we demonstrate that *RIM1*, *RIM8*, *RIM9* and *RIM13* are additional positive regulators of *IME1* that act in an *MCK1*-independent pathway. Known negative regulators of *IME1* include *RME1* and *IME2*. Nitrogen prevents *IME1* expression through repression of *IME4* and, possibly, effects on *MCK1* and *RIM* activities. Glucose also prevents *IME1* expression, perhaps through effects on *MCK1* and *RIM* activities. *a1-a2*, which determines the α/α cell type, permits *IME1* expression through repression of *RME1* and stimulation of *IME4* expression. "X" refers to a postulated repressor of *IME4* (Shah and Clancy 1992). Epistatic relationships between *IME4* and the *RIM* genes, or between *IME4* and *MCK1*, are unclear.

1990) (reviewed in Herskowitz 1989). Two pathways relay *MAT* information to control *IME1* (Figure 1). In one pathway, *a1-a2* represses an inhibitor of meiosis, the *RME1* product, which blocks *IME1* expression (Mitchell and Herskowitz 1986; Kassir, Granot and Simchen 1988; Covitz, Herskowitz and Mitchell 1991). In the other pathway, *a1-a2* permits elevated expression of a positive regulator of meiosis, the *IME4* product, which stimulates *IME1* expression (Shah and Clancy 1992). It is thought that *a1-a2* stimulates *IME4* expression indirectly by repressing a repressor, indicated by an X in Figure 1 (Shah and Clancy 1992). Absence of nitrogen is required for high level *IME4* expression in α/α cells. Thus, *IME4* transmits both *a1-a2* and nutritional signals.

Expression of *IME1* also depends on the *MCK1* gene product (Neigeborn and Mitchell 1991), which specifies a protein kinase homolog that cofractionates with both serine/threonine and tyrosine kinase activities (Dailey et al. 1990). *MCK1* was identified on a multicopy plasmid that stimulated *IME1* expression and sporulation (Neigeborn and Mitchell 1991). In *mck1* mutants, *IME1* expression is diminished, and sporulation is slow and inefficient. Many of the spores produced in the mutant are packaged in immature asci that persist in the population. *IME1* expression from a heterologous promoter improves sporulation efficiency in the mutant but the asci remain immature. Therefore, *MCK1* affects *IME1* expression and ascus maturation independently. *MCK1* is required for proper mitotic centromere function as well (Shero and Hieter 1991). Overexpression of *MCK1* suppresses certain centromere point mutations. In *mck1* mutants, growth at low temperature (17°C) causes micronucleosome instability and sensitivity to the antimitotube drug, Benomyl. At still lower temperatures (11°C), *mck1* mutants fail to grow. Because null mutations of *MCK1* lead to partial or conditional defects in *IME1* expression, spore maturation, centromere function and growth, alternative kinases or pathways may exist that act in parallel to *MCK1*. It is unclear what signal *MCK1* may transmit.

The pathways described above determine whether *IME1* expression is activated prior to sporulation. At later times in sporulation, *IME1* is down-regulated. Because *ime2* mutants fail to down-regulate *IME1*, the *IME2* gene product is formally a negative regulator of *IME1* expression (Smith and Mitchell 1989).

Clearly, many genes involved in *IME1* regulation have not been identified. For example, the substrates through which *MCK1* and *IME2* products influence *IME1* expression as well as the genes thought to act in parallel to *MCK1* are unknown. We report here the identification of regulators of *IME1* expression through a screen for mutants that failed to express one of the earliest sporulation-specific genes, *IME2*. Our genetic screen had two special features: the utilization of an *rme1* mutation and an *ime2-lacZ* fusion reporter gene. The *rme1* mutation enabled us to use haploid strains to assay *IME2* expression. The use of haploid strains facilitated the recovery of recessive mutations and simplified dominance, complementation, and segregation tests. The *ime2-lacZ* fusion gene enables us to screened for mutants with diminished *IME2* expression rather than for mutants with defects in more complex meiotic events. Our analysis focuses on a group of functionally related genes that act in parallel to *MCK1* to stimulate *IME1* expression.
of the chromosomal copy of LIRA3 and are listed in Table 1.

For sporulation, it was followed in crosses by failure to complement ELL described previously (MITCHELL, DRISCOLL and SMITH 1990; NEICEBORN and MITCHELL 1989; NEICEBORN and MITCHELL 1991). Because the imel allele is an deletion, which places the biosynthetic gene HIS3 under the control of the chromosomal IME1 promoter, was described by NEICEBORN and MITCHELL (1991). Because the imel-HIS3 allele is an imel deletion, it was followed in crosses by failure to complement imel mutants for sporulation.

The imel2-lacZ fusion gene (IME2-5-lacZ), which includesURA3 as a selectable marker, was introduced by transforming strain AMP107 with a BglII digest of plasmid pSS153 (Figure 2). Southern analysis confirmed the integration of the imel2-lacZ plasmid at the IME2 locus and the preservation of the chromosomal copy of IME2 (IME2::ime2-lacZ). We also confirmed that IME2-5-lacZ segregated as an IME2 allele.

The P<sub>ACT1</sub>-IME1 alleles, which we designate as IME1-15 and IME1-16, contained TRP1 as a selectable marker (Figure 2). They were introduced by transforming AMP735 (IME1 strain) and SY179 (ime1<sup>ΔA1</sup> strain) with an EcoRI digest of plasmid pSS153. Southern analysis confirmed that the gap between the EcoRI sites of pSS153 was repaired during integration (ROTHSTEIN 1991). In IME1-15 (IME1::P<sub>ACT1</sub>-IME1), IME1 was expressed from IME1 and ACT1 promoters. In IME1-16 (IME1::P<sub>ACT1</sub>-IME1), IME1 was expressed only from the ACT1 promoter.

Standard procedures were used for mating, diploid isolation, sporulation and tetrad analysis (ROSE, WINSTON and HIETER 1990). All diploids created for tetrad analysis in this study were homozygous for r<sub>me1</sub>ΔA5 as well as IME2-5-lacZ. Thus, rim mutations in the haploid segregants were followed by failure to express ime2-lacZ. Rim mutations were also followed by failure to complement the respective rim mutants for ime2-lacZ expression in some crosses.

The rim rim double mutants were constructed by pairwise crosses of individual rim mutants. For example, we crossed a rim<sup>1-1</sup> mutant to a rim<sup>8-1</sup> mutant and analyzed the resulting tetrads. Segregation of rim<sup>1-1</sup> and rim<sup>8-1</sup> was followed by failure to complement rim<sup>1-1</sup> and rim<sup>8-1</sup> mutants, respectively, for ime2-lacZ expression. Similarly, the mckl<sup>Δrim</sup>
Sporulation ability was quantitated on plates or in liquid cultures. For plate tests, yeast were grown on YEPD plates for one day, then replica-plated to sporulation plates. For liquid culture tests, yeast were grown on YEPAC vegetative medium to mid-log phase, then transferred to 2% KAc sporulation medium (Smith et al. 1990). In either case, we scored at least 200 cells by microscopic examination after 24 hr to quantitate sporulation ability.

All experiments were conducted at 30°C except that cold sensitivity was scored at 17°C.

**Plasmid construction:** Diagrams of relevant plasmid inserts are shown in Figure 2.

The ime2-lacZ fusion plasmid, pSS400, was constructed by fusing the IME2 5′-end to the lacZ gene in plasmid YIp558R (Myers et al. 1986). The 1.3-kbp BamHI-PvuII fragment of IME2 from pHS101, which contained nucleotides −983 to +114 of IME2 (Yoshida et al. 1990), was eluted from an agarose gel and inserted into the BamHI and Smal sites of YIp558R. This construct fused the lacZ gene in frame to IME2 at codon 39.

pSS153, the source of pACT1-IME1, was constructed in three steps. First, primers that hybridized to the ACT1 promoter at positions −668 and −3 were made to introduce SphI and HindIII sites, respectively. These two primers were used in a polymerase chain reaction with pBD81 (plasmid containing the ACT1 promoter and part of the 5′ coding region) to obtain a 681-bp fragment containing the ACT1 promoter (Ng and Abelsohn 1980). Second, the 681-bp SphI-HindIII promoter was mixed with a 2.3-kbp HindIII-Sall IME1 fragment from YCp P<sub>GAL</sub>-IME1 (Smith et al. 1990), and an 8.4-kbp SphI-Sall fragment of plasmid YEp24 in a single ligation mixture. Minipreps were screened for the incorporation of the ACT1 promoter and IME1 gene into YEp24 to create pRS <sub>PACT1-IME1</sub> (R. Sta, personal communication). Third, the 3.5-kbp Sall-Sall fragment from pRS <sub>PACT1-IME1</sub> containing the entire <sub>PACT1-IME1</sub> gene, was ligated to integrating vector pRS304 (Siikorski and Hieter 1989) at the Sall and Sall sites to create pSS153.

**β-Galactosidase assays:** For β-galactosidase assays on plates, patches of cells grown on YEPD plates were replica-plated to KAcXgal plates and incubated for 2 days at 30°C. Patches that turned blue on these assay plates contained β-galactosidase activity and expressed the <sub>IME1</sub> promoter. We refer to the ability to express the ime2-lacZ fusion gene as the Lac<sup>+</sup> phenotype, and the failure to express the fusion as the Lac<sup>−</sup> phenotype.

For β-galactosidase assays on nitrocellulose filters, patches of cells were grown on YEPD plates for a day, then replica-plated to nitrocellulose filters on minimal sporulation plates. After one day at 30°C, the filters were placed in liquid nitrogen to permeabilize the cells. Each filter was then incubated in 3 ml of Z buffer containing 200 μg/ml of Xgal at 30°C for at least 20 min. To terminate the reaction, the filters were removed from the assay buffer and air dried. Cells that turn blue in this assay contained β-galactosidase activity.

For quantitative β-galactosidase assays, cells were grown to log phase in YEPac medium, transferred to sporulation medium, harvested on Millipore filters, permeabilized, and assayed with ONPG as substrate (Rose, Winston and Hieter 1990).

**Isolation of mutants defective in ime2-lacZ expression:** Strains AMP340 and AMP734 were grown in four ml of YEP to stationary phase, washed and suspended in 10 mM KPO<sub>4</sub> (pH 7), and sonicated briefly. Ethyl methylsulfonate (EMS) was added to 2.5% v/v and the cells were incubated at 30°C to achieve 10% survival. The mutated cells were

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**FIGURE 2.—Structures of IME2-5-lacZ::URA3, IME1-15::TRP1 and IME1-16::TRP1 alleles.** (A) IME2-5-lacZ::URA3. The plasmid pSS400 was integrated into the yeast genome by homologous recombination at the IME2 5′-promoter region. The thin and the thick solid bars represent the IME2 promoter and the IME2 coding region, respectively. The stippled bars represent lacZ sequences and the open bars represent the URA3 selectable marker. Bent arrows indicate transcription from the IME2 promoter. (B) IME1-15::TRP1 and IME1-16::TRP1. The plasmid pSS153 was integrated into IME1 and ime1Δ strains by homologous recombination at the chromosomal IME1 locus to construct IME1::P<sub>ACT1</sub>-IME1 and ime1Δ::P<sub>ACT1</sub>-IME1 alleles, respectively. The thick shaded bars represent the IME1 gene. The thin shaded bars and the diagonally striped bars represent the natural IME1 and the heterologous ACT1 promoters, respectively. Bent arrows indicate transcription from the respective promoters. The horizontally striped bar represents HIS3 sequences present in the ime1Δ strain, and the open bars represent the TRP1 selectable marker. Restriction endonuclease sites: (B) BamHI; (S) SalI; (G) RgaI; (N) XhoI; (Pv) PvuII; (Sm) SmaI; (Sp) SphI; (H) HindIII; (R) EcoRI. The sites indicated in parentheses were lost as a consequence of cloning. The sequences are not drawn to scale.

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**Media and culture conditions:** Standard recipes were used for SD, SC, SC dropout media, YEPD, YEPAC, sporulation plates, and liquid sporulation media (Rose, Winston and Hieter 1990; Smith and Mitchell 1989). Minimal sporulation plates contain 2% KAc, 2% Bacto-agar and auxotrophic amino acid supplements as in SC medium. KAcXgal plates are minimal sporulation plates buffered with 0.2 M KPO<sub>4</sub> at pH 7 and containing 50 mg/liter of Xgal.

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cells were plated on 150 YEPAc plates to obtain respiratory-deficient mutants, which fail to express
collected on a Millipore filter, washed with 10 ml of sterile water and resuspended in 4 ml of sterile water. Mutagenized
cells were plated on 150 YEPAc plates to obtain 200 colonies per plate. Cells were plated on YEPAc to select against
respiratory-deficient mutants, which fail to express ime2-lacZ because they fail to grow on KAcXgal plates. After 3
cells were plated on YEPAc to select against
we anticipated that many of these white colonies would not be defective in ime2-lacZ expression but rather in the
uptake of the Xgal indicator. These mutants were eliminated through filter β-galactosidase assays in which cells were
were permeabilized with liquid nitrogen.
We also expected mutations within the lacZ coding region of the ime2-lacZ fusion gene. We identified 46 $\text{lacZ}^+$ mutants through crosses to AMP839, a wild-type strain lacking the ime2-lacZ fusion gene, and AMP737, a wild-type strain that provided the complementing ime2-lacZ fusion gene. Diploids formed from $\text{lacZ}^-$ mutants and AMP839 were Lac$^-$; diploids formed from $\text{lacZ}^+$ mutants and AMP737 were Lac$^+$.

**Dominance and complementation tests:** To test for dominance, each mutant was crossed to a wild-type strain of the opposite mating type (AMP737). In one case, the resulting diploid failed to express ime2-lacZ. This dominant defect did not segregate as a single gene trait and was not pursued. In the remaining cases, the resulting diploids expressed ime2-lacZ, indicating that these mutations were complemented by the wild-type gene. We recovered 135 recessive mutations from screening 30,000 colonies.

To test pairs of mutations for complementation, doubly heterozygous diploids were isolated by prototrophic selection and tested for ime2-lacZ expression on KAcXgal plates.

**Northern blots and probes:** Cells were transferred during log phase growth in YEPAc to sporulation medium and samples were harvested at various times for RNA isolation (Smith and Mitchell 1989). Standard methods were used for preparation of Northern (RNA) blots (Smith and Mitchell 1989), except that nylon filters (Hybond) were used in place of nitrocellulose. Probes for ime1 and ime2 RNAs have been described (Smith and Mitchell 1989; Mitchell, Driscoll and Smith 1990). The TUB2 probe was a random-primed 1.6-kbp EcoRI-EcoRI fragment from the TUB2 gene (Neff et al. 1983). The ACT1 probe was random-primed plasmid pBD81 (provided by B. Dunn and M. A. Osley). We found that our preparation of the control probe, pCA (Law and Segall 1988), was a mixture of plasmids. We colony-purified one plasmid from this mixture that hybridized to a constitutive ca. 1-kb RNA (A. P. Mitchell, unpublished results). We designate this plasmid pCA4/2.

**RESULTS**

**Regulation of the ime2-lacZ fusion of IME2-5-lacZ:** Our primary goal was to identify genes that govern IME1 expression. Initial experiments with an ime1-lacZ fusion gene gave background expression levels too high for reliable plate assays. We used an ime2-lacZ fusion gene instead to assess IME1 activity indirectly. We found that the ime2-lacZ fusion responded to the same regulatory signals as the natural IME2 gene. First, ime2-lacZ expression was stimulated by starvation (Figure 3A). Increased expression was detectable by 4 hr after starvation, as expected from studies of IME2 RNA levels (Smith et al. 1990). Second, ime2-lacZ expression was restricted to a/a cells; neither a cells or a cells expressed the fusion (Figure 3A). Third, mutations that affect normal IME2 expression also affected ime2-lacZ expression. The rme1$\Delta$ mutation allowed a cells and a cells to express levels of β-galactosidase activity comparable to a/a cells (Figure 3B). The IME1 and mck1$\Delta$ mutations caused diminished ime2-lacZ expression. Similar results have been obtained with a different ime2-lacZ fusion allele (Smith et al. 1990; Neigeborn and Mitchell 1991). Therefore, the expression of ime2-lacZ in the IME2-5-lacZ:URA3 allele accurately reflected the expression of the native IME2 gene.

**Isolation of mutants defective in ime2-lacZ expression:** We isolated mutations that block IME2 expression in strains with the genotype MATa ime2$\Delta5$ IME2-
5-lacZ by screening for mutants that failed to express ime2-lacZ under starvation conditions. We isolated 135 EMS-induced mutants that were white on KAcXgal plates. Among these, 55 Xgal permeability mutants and 46 lacZ+ mutants were eliminated as described in MATERIALS AND METHODS.

We expected to isolate ime1 and mckl1 mutants. Eleven ime1 mutants were identified by failure to complement an ime1Δ tester for ime2-lacZ expression (Table 2). Results from mckl1 complementation tests were inconclusive; however, subsequent analysis indicated that one mutation was genetically linked to the mckl1Δ mutation (20PD:0NPD:0T). A plasmid carrying the MCK1 gene also complemented the ime2-lacZ expression and sporulation defects in the mutant, which verified that the mutation is a mckl1 allele. We refer to this mutation as mckl-14.

We crossed each of the remaining mutants to a wild-type strain (MATa rmelΔ IME2-5-lacZ) and sporulated the resulting diploids. In each case, the ability to express ime2-lacZ segregated 2+/2−. Therefore, the defect in each mutant lies at a single genetic locus. All of the mutations are recessive since ime2-lacZ expression in heterozygous diploids was as high as in wild-type diploids (data not shown).

These 22 mutations were tested for the ability to complement each other in all pairwise combinations. Twelve complementation groups were identified (see Table 2). These genes were designated RIM for Regulator of IME2.

We assayed β-galactosidase activity in one representative mutant in each complementation group to quantify IME2 expression under sporulating conditions (Table 2). In haploid a or α strains, the ime2-lacZ expression levels of rim mutants were 5–20-fold lower than the wild type. In a/α diploid strains, the ime2-lacZ expression levels of rim homozygotes were 3–10-fold lower than the wild type. Thus, the rim mutant defects were more severe in a or α haploids than in a/α diploids. The basis for this reproducible difference is unclear. However, for the sake of uniformity, all subsequent quantitative measurements of ime2-lacZ expression were performed on a/α diploid strains.

### Table 2

<table>
<thead>
<tr>
<th>Strain</th>
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<th>ime2-lacZ expression&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Percent sporulation&lt;sup&gt;b&lt;/sup&gt;</th>
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<tr>
<td></td>
<td></td>
<td>a&lt;sup&gt;−&lt;/sup&gt;</td>
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</tr>
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<tr>
<td>rim13</td>
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<td>2</td>
<td>45</td>
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<sup>a</sup> β-Galactosidase assays of representative haploid and homozygous mutant diploid from each complementation group were performed as described in MATERIALS AND METHODS after 8 hr in liquid sporulation medium. Values are the averages of at least two independent assays in Miller units.

<sup>b</sup> Sporulation ability of homozygous mutants was quantitated on sporulation plates. No spores were observed among 1000 cells for 0% sporulation.

<sup>c</sup> Other phenotypes associated with the mutants include the following: Ben<sup>a</sup> refers to Benomyl hypersensitivity at 17° (Shero and Hieter 1991); smooth colony refers to the smooth colony morphology; cold<sup>a</sup> refers to slow growth at 17°; and starv<sup>a</sup> refers to sensitivity to nitrogen starvation.

<sup>d</sup> Not applicable.

<sup>e</sup> Not determined.

*S. S. Y. Su and A. P. Mitchell*
TABLE 3

Segregation of \( P_{ACT1-IME1} \) and rim mutations

<table>
<thead>
<tr>
<th>Cross</th>
<th>Relevant genotype (^a)</th>
<th>( P_{ACT1-IME1} ) segreants</th>
<th>( IME1 ) segreants</th>
<th>Total tetrads</th>
</tr>
</thead>
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</tbody>
</table>

\(^a\) Diploids constructed for all the crosses were homozygous for \( rme1A \) and \( ime2-lacZ \).

\(^b\) A \( P_{ACT1-IME1} \) segregant that failed to express \( ime2-lacZ \) exhibited a slow growth phenotype which did not segregate with \( mck1-14 \). This segregant expressed \( ime2-lacZ \) only after longer incubation on a KAcXgal plate.

Starvation response of rim mutants: Several previously identified sporulation-defective mutants fail to arrest as unbudded cells. These mutants die when starved. Such mutations affect components of the adenylate cyclase pathway (bcyl, ira1 and ira2), an N-terminal acetyltransferase (ardl and nat1), and a protein kinase required for glucose-repressible gene expression (snfl-172) (TODA et al. 1987; TANAKA, MATSUMOTO and TOH-E 1989; TANAKA et al. 1990; WHITENAY and SZOSTAK 1985; MULLEN et al. 1989; THOMPSON-JAEGGER et al. 1991). We expected to isolate mutants in this class because bcyl mutants, for example, fail to express \( IME1 \) (MATSUURA et al. 1990) and, presumably, \( IME2 \).

We tested the \( rim \) mutants for sensitivity to nitrogen starvation. The \( rim3-1 \) mutant was more sensitive to nitrogen limitation and accumulated a higher percentage of budded cells (26%) than wild type (5%) after nitrogen starvation. These phenotypes cosegregated with \( rim3-1 \) in eight tetrads. In addition, \( IME1 \) and \( IME2 \) transcript levels were very low in the \( rim3-1 \) mutant after starvation (data not shown). Linkage or complementation tests indicated that \( rim3-1 \) is not allelic to \( bcyl, ira1, ira2, ard1 \) or \( snfl1 \). However, \( nat1-3 \) and \( rim3-1 \) mutants did not complement each other for \( ime2-lacZ \) expression or sporulation. We conclude that \( rim3-1 \) is most likely a \( nat1 \) allele.

Identification of rim mutations that reduce IME1 expression: The \( rim \) mutations may cause reduced \( ime2-lacZ \) expression by reducing \( IME1 \) expression levels, or may cause reduced \( ime2-lacZ \) expression despite normal \( IME1 \) expression levels. We used three tests to distinguish these possibilities. First, we examined the ability of \( IME1 \), expressed from the heterologous \( ACT1 \) promoter, to suppress the \( rim \) mutations. Second, we tested the ability of the \( rim \) mutants to express \( HIS3 \) from an \( ime1-HIS3 \) hybrid gene. Third, we analyzed \( IME1 \) RNA accumulation in each \( rim \) mutant through Northern analysis.

For suppression studies, we analyzed meiotic tetrads from \( P_{ACT1-IME1}:TRP1/IME1 \) \( RIM/rim \) diploids (Table 3). We found that the \( P_{ACT1-IME1} \) hybrid gene did not suppress the \( rim4-1 \), \( rim11-1 \) and \( rim15-1 \) mutations. Among the \( P_{ACT1-IME1} \) (Trp+) segregants in cross cx3-3, for example, the numbers of Lac+ and Lac- cosegregated with the \( IME1 \) and \( IME2 \) mutations. Likewise, the \( ime2-lacZ \) expression defects of \( rim11-1 \) and \( rim15-1 \) were not suppressed by \( P_{ACT1-IME1} \) (Table 3, cross cx3-4 and cx3-5). Quantitative measurements confirmed our finding for \( rim4-1 \) and \( rim11-1 \) (Table 4). These results agree with other studies of \( rim11 \) mutants (MITCHELL and BOWDISH 1992).

TABLE 4

Suppression of rim mutations by \( P_{ACT1-IME1} \)

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype (^a)</th>
<th>( ime2-lacZ ) expression (^b)</th>
<th>Veg</th>
<th>Spo</th>
<th>Percent sporulation (^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>cx4-1</td>
<td>( IME1 )</td>
<td>5 290</td>
<td>99</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cx4-2</td>
<td>( P_{ACT1-IME1} )</td>
<td>8 310</td>
<td>100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cx4-3</td>
<td>( ime1\Delta )</td>
<td>&lt;1 &lt;1</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cx4-4</td>
<td>( ime1\Delta P_{ACT1-IME1} )</td>
<td>3 520</td>
<td>96</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cx4-5</td>
<td>( rim4-1 )</td>
<td>&lt;1 61</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cx4-6</td>
<td>( rim4-1 P_{ACT1-IME1} )</td>
<td>&lt;1 65</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cx4-7</td>
<td>( rim11-1 )</td>
<td>&lt;1 11</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cx4-8</td>
<td>( rim11-1 P_{ACT1-IME1} )</td>
<td>&lt;1 11</td>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cx4-9</td>
<td>( rim1-1 )</td>
<td>&lt;1 10</td>
<td>23</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cx4-10</td>
<td>( rim1-1 P_{ACT1-IME1} )</td>
<td>&lt;1 140</td>
<td>83</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cx4-11</td>
<td>( rim8-1 )</td>
<td>&lt;1 11</td>
<td>23</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cx4-12</td>
<td>( rim8-1 P_{ACT1-IME1} )</td>
<td>&lt;1 120</td>
<td>92</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cx4-13</td>
<td>( rim9-1 )</td>
<td>&lt;1 14</td>
<td>27</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cx4-14</td>
<td>( rim9-1 P_{ACT1-IME1} )</td>
<td>&lt;1 120</td>
<td>76</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cx4-15</td>
<td>( rim3-1 )</td>
<td>&lt;1 58</td>
<td>44</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cx4-16</td>
<td>( rim3-1 P_{ACT1-IME1} )</td>
<td>&lt;1 110</td>
<td>72</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Diploid strains were homozygous for \( rme1A \) and \( ime2-lacZ \) as well as for the mutations listed.

\(^b\) \( \beta \)-Galactosidase assays were performed for log phase cultures in YEPac medium (Veg) and after 8 hr in liquid sporulation medium (Spo). Values in Miller units are the averages of at least two independent determinations.

\(^c\) Sporulation ability was quantitated after 24 hr in sporulation medium.

*Activation of IME1*

* Suppression of rim mutations by \( P_{ACT1-IME1} \)*
Segregation analysis indicated that the $P_{\text{ACT1-IME1}}$ hybrid gene did suppress $\text{mck}1-14$, $\text{rim}1-1$, $\text{rim}8-1$, $\text{rim}9-1$ and $\text{rim}13-1$ (Table 3, cross cx3-2, cx3-6, cx3-7, cx3-8 and cx3-9). Among the $\text{IME1}$ (Trp") segregants in crosses of these mutants to $P_{\text{ACT1-IME1}}$, the numbers of Lac" and Lac" segregants approximated the 1:1 ratio expected if the $\text{rim}$ mutations were unlinked to $\text{IME1}$. In contrast, all of the $P_{\text{ACT1-IME1}}$ (Trp") segregants were Lac". These results agree with previous studies of $\text{MCK1}$ (Neigeborn and Mitchell 1991). Quantitative measurements indicated that $P_{\text{ACT1-IME1}}$ increased $\text{ime}2$-lacZ expression in a $\text{rim}1$-1 mutant by 10-fold and increased its sporulation efficiency (Table 4). The $\text{rim}8$-1 and $\text{rim}9$-1 defects were also clearly suppressed by $P_{\text{ACT1-IME1}}$ (Table 4). The $\text{rim}13$-1 defects are so mild that quantitative suppression by $P_{\text{ACT1-IME1}}$ is inconclusive. We conclude that the $\text{ime}2$-lacZ expression and sporulation defects of $\text{mck}1-14$, $\text{rim}1-1$, $\text{rim}8-1$ and $\text{rim}9$-1 mutants are suppressed by $P_{\text{ACT1-IME1}}$ expression.

To determine whether the $\text{rim}1$-1, $\text{rim}8$-1, $\text{rim}9$-1 and $\text{rim}13$-1 mutations affect $\text{IME1}$ expression, we examined $\text{HIS3}$ expression from the $\text{ime}1$-$\text{HIS3}$ fusion gene in these mutants. This fusion places the $\text{HIS3}$ coding region downstream of the $\text{IME1}$ promoter and untranslated leader. We crossed a strain carrying the $\text{ime}1$-$\text{HIS3}$ fusion gene to these $\text{rim}$ mutants, sporulated the resulting diploids and analyzed the spores for growth on plates lacking histidine (Table 5). Among the segregants that contained the $\text{ime}1$-$\text{HIS3}$ fusion gene, the numbers of His" and His" segregants approximated the 1:1 ratio expected for Mendelian segregation of each $\text{rim}$ mutation. Complementation tests demonstrated that each $\text{rim}$ mutation cosegregated with the His" phenotype. Therefore, $\text{rim}1$-1, $\text{rim}8$-1, $\text{rim}9$-1 and $\text{rim}13$-1 may reduce basal $\text{IME1}$ expression through the $\text{IME1}$ promoter or may reduce RNA stability or translation through the $\text{IME1}$ 5' leader.

We examined $\text{IME1}$ RNA levels by Northern analysis in $\text{rim}1$-1, $\text{rim}8$-1, $\text{rim}9$-1 and $\text{rim}13$-1 mutants to distinguish between defects in $\text{IME1}$ transcript accumulation or translation (Figure 4). In the wild-type strain, $\text{IME1}$ transcript levels were low under vegetative conditions and increased in sporulation medium.

**TABLE 5**

<table>
<thead>
<tr>
<th>Cross</th>
<th>Relevant genotype</th>
<th>ime1-HIS3 segregants</th>
<th>Total tetrads</th>
</tr>
</thead>
<tbody>
<tr>
<td>cx3-1</td>
<td>ime1-HIS3/IME1 RIM1/rim1-1</td>
<td>21 19 20</td>
<td></td>
</tr>
<tr>
<td>cx5-2</td>
<td>ime1-HIS3/IME1 RIM8/rim8-1</td>
<td>10 10 10</td>
<td></td>
</tr>
<tr>
<td>cx5-3</td>
<td>ime1-HIS3/IME1 RIM9/rim9-1</td>
<td>18 24 21</td>
<td></td>
</tr>
<tr>
<td>cx5-4</td>
<td>ime1-HIS3/IME1 RIM13/rim13-1</td>
<td>19 27 23</td>
<td></td>
</tr>
</tbody>
</table>

**Figure 4.**—Expression of sporulation-specific genes in $\text{rim}1$-1, $\text{rim}8$-1, $\text{rim}9$-1 and $\text{rim}13$-1 mutants. RNA was prepared from a/a diploids during growth in YEPAc (0 hr) and at 2, 4 and 6 hr after transfer to sporulation medium, as indicated above each lane. Strains were homozygous for the mutations indicated. (A) Northern filter A contained RNA samples from wild type, $\text{rim}1$-1, $\text{rim}8$-1 and $\text{rim}9$-1 mutants. (B) Northern filter B contained RNA samples from the same wild-type strain in filter A and RNA samples from the $\text{rim}13$-1 mutant. Each filter was hybridized with probes for $\text{IME1}$ and $\text{IME2}$, and then stripped and hybridized with control probe pCA/2. The blots were then stripped and hybridized to a probe for $\text{HOP1}$, and then stripped again and hybridized to probes for $\text{SPS1}$ and $\text{SPS2}$. Two $\text{HOP1}$ RNAs were detected, but only the predominant RNA is designated $\text{HOP1}$ (Hollingsworth, Goetsch and Byers 1990).

In each of the $\text{rim}$ mutants, $\text{IME1}$ transcript levels remained low in sporulation medium. These $\text{rim}$ mutations also caused reduced RNA levels from the sporulation-specific genes $\text{IME2}$, $\text{HOP1}$, $\text{SPS1}$ and $\text{SPS2}$ (Figure 4). The defect in $\text{IME1}$ expression was not due to general defects in RNA accumulation because $\text{TUB2}$ and $\text{ACT1}$ RNA levels were normal in these mutants (Figure 5). We conclude that these $\text{rim}$ mutations cause reduced $\text{IME1}$ RNA accumulation.

These studies suggest that the primary defect in $\text{rim}1$-1, $\text{rim}8$-1, $\text{rim}9$-1 and $\text{rim}13$-1 mutants is the inability to accumulate $\text{IME1}$ RNA. As a result of the defect in $\text{IME1}$ RNA accumulation, the expression of other sporulation-specific genes is defective in these mutants. Expression of $\text{IME1}$ from the $\text{ACT1}$ promoter suppresses the $\text{rim}$ mutations by restoring sporulation-specific gene expression. Because these mutations are recessive, we infer that they result in loss of gene function. Formally, $\text{RIM1}$, $\text{RIM8}$, $\text{RIM9}$ and
RIM13 are positive regulators of IME1. These gene products may act through the IME1 promoter or 5' leader to stimulate transcript accumulation.

The rim-I, rim8-I, rim9-I and rim13-I mutants shared two additional phenotypes. First, their colony morphology is not as rough as the parent strain and other SK-I derivatives (Table 2). Smooth colony morphology cosegregated with each mutation in every cross. Second, all of these mutations cause slow growth at 17°C. Because ime1 mutations do not cause smooth colony morphology or cold sensitive growth, these RIM gene products must do more than simply activate IME1 expression.

**RIM1, RIM8, RIM9 and RIM13 act in parallel to MCK1 to activate IME1 expression:** Mutations in MCK1 and in RIM1, RIM8, RIM9 and RIM13 all cause diminished IME1 expression. These gene products may act in the same pathway or through different pathways to activate IME1 expression. If two genes act in the same pathway, then the phenotype of the double mutant should be the same as either single mutant. On the other hand, if two genes act through different pathways, then the phenotype of the double mutant should be more severe than either single mutant. To distinguish between these two possibilities, we analyzed the ime2-lacZ expression levels and sporulation efficiencies of mck1Δrim double mutants and rim rim double mutants (Table 6). ime2-lacZ expression in the wild-type strain was high after 8 hr in sporulation medium. ime2-lacZ expression in mck1Δ and rim1-I mutants was reduced 3-fold and 20-fold, respectively. ime2-lacZ expression in the mck1Δrim1-I mutant was reduced 700-fold. Northern analysis confirmed that the mck1Δrim1-I mutant had even lower IME1 RNA levels than the rim1-I mutant (Figure 6). The sporulation defect of the mck1Δrim1-I mutant was also more severe than either single mutant (Table 6). Similarly, the mck1Δrim8-I, mck1Δrim9-I, and mck1Δrim13-I mutants displayed more extreme ime2-lacZ expression and sporulation defects than the single mutants. ime2-lacZ expression levels and sporulation efficiencies of all pairwise double mutants of rim1-I, rim8-I, rim9-I and rim13-I were comparable to those of the single mutants (Table 7). We conclude that RIM1, RIM8, RIM9 and RIM13 act in a single pathway to stimulate IME1 expression and that the RIM1/8/9/13 pathway is independent of the MCK1 pathway.

**DISCUSSION**

We have isolated 34 recessive mutations that cause defects in ime2-lacZ expression. These mutations de-
fined 14 genes, including the previously identified
genes IME1, MCK1 and NAT1. The finding that most
RIM genes were defined by single isolates suggests
that repeated application of our screen may yield
mutations in additional genes. The limited size of
the screen may explain the absence of ime4 and
rim16 mutations (SHAH and CLANCY 1992; MITCHELL
and BOWDISH 1992), which should have been detectable.

Although IME2 is required for sporulation, several
mutants with ime2-lacZ expression defects had mild
sporulation defects. rim mutations that cause no spor-
ulation defects may affect only ime2-lacZ expression
and not expression of the natural IME2 gene. Mutations
with severe effects on ho-lacZ expression and not
on the natural HO RNA have been reported previously
(BREEDEN and NASMYTH 1987). On the other
hand, most mutations cause defects in both ime2-lacZ
expression and sporulation. The partial sporulation
defects in these mutants are consistent with reduced
IME2 expression.

Why do several mutants display only partial defects
in ime2-lacZ expression levels and sporulation? One
explanation is that these mutations may cause a partial
loss of RIM gene function. The rim11-1 mutation
described in this study is an example because rim11
alleles described elsewhere cause an absolute sporu-
lation defect (MITCHELL and BOWDISH 1992). An-
other possible explanation is that these mutations
cause a complete loss of RIM gene function, but that
activity from a second pathway can partially suppress
the mutant defects. In this study, we found that the
residual ability of rim1/8/9/13 mutants to express
ime2-lacZ and to sporulate is largely due to the activity
of MCK1.

**TABLE 7**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>ime2-lacZ expression</th>
<th>Percent sporulation (total)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Veg</td>
<td>Spo</td>
<td></td>
</tr>
<tr>
<td>cx7-1</td>
<td>Wild type</td>
<td>1.6</td>
<td>270</td>
</tr>
<tr>
<td>cx7-2</td>
<td>rim1-1</td>
<td>0.2</td>
<td>28</td>
</tr>
<tr>
<td>cx7-3</td>
<td>rim8-1</td>
<td>0.2</td>
<td>58</td>
</tr>
<tr>
<td>cx7-4</td>
<td>rim9-1</td>
<td>0.3</td>
<td>40</td>
</tr>
<tr>
<td>cx7-5</td>
<td>rim13-1</td>
<td>0.2</td>
<td>45</td>
</tr>
<tr>
<td>cx7-6</td>
<td>rim1-1 rim8-1</td>
<td>0.3</td>
<td>30</td>
</tr>
<tr>
<td>cx7-7</td>
<td>rim1-1 rim9-1</td>
<td>0.2</td>
<td>30</td>
</tr>
<tr>
<td>cx7-8</td>
<td>rim1-1 rim13-1</td>
<td>0.2</td>
<td>30</td>
</tr>
<tr>
<td>cx7-9</td>
<td>rim8-1 rim9-1</td>
<td>0.2</td>
<td>54</td>
</tr>
<tr>
<td>cx7-10</td>
<td>rim8-1 rim13-1</td>
<td>0.2</td>
<td>24</td>
</tr>
<tr>
<td>cx7-11</td>
<td>rim9-1 rim13-1</td>
<td>0.3</td>
<td>65</td>
</tr>
</tbody>
</table>

* Strains were homozygous for rim1Δ and ime2-lacZ as well as
  for the mutations indicated.
  
  β-Galactosidase assays were performed for log phase cultures
  in YEPAc medium (Veg) and after 8 hr in sporulation medium
  (Spo). Values in Miller units are the averages of two independent
determinations.

  a Sporulation ability was quantitated after 24 hr in sporulation medium.

**RIM1, RIM8, RIM9 and RIM13** (which we designate
the RIM1 pathway genes) stimulate IME2 expression
indirectly, by stimulating IME1 transcript accumulation.
MCK1 also stimulates IME1 transcript accumulation.
Our measurements of ime2-lacZ expression and
sporulation indicate that defects in RIM1 pathway
genes and MCK1 are additive. Measurements of IME1
RNA levels in a mck1Δrim1-1 double mutant is con-
sistent with our conclusion. Thus, RIM1 pathway
genes and MCK1 act independently to stimulate IME1
expression.

Additional phenotypes of mck1 and rim1/8/9/13
mutants support the conclusion that these genes func-
tion in distinct pathways. For example, mck1 mutants
grow well at 17° but are Benomyl hypersensitive at
that temperature (SHERO and HIETER 1991). rim1/8/9/
13 mutants grow poorly at 17° but are not Benomyl
hypersensitive (S. SCH, unpublished results). Another
example is that mck1 mutants accumulate immature
ascus. rim1/8/9/13 mutants accumulate higher levels
of immature asci than wild-type strains but, unlike mck1
mutants, the ascus mature after prolonged incubation.
Finally, mck1 mutants have rough colony morphology
like our isogenic wild-type strains, whereas rim1/8/9/13
mutants have a distinct smooth colony morphology.
All of these observations suggest that the RIM1 path-
way genes function independently from MCK1.

We suggest that the RIM1 pathway gene products
are positive regulators of IME1 because recessive mu-
tations in these genes reduce IME1 expression. The
basis of the pleiotropic phenotypes of the RIM1 path-
way mutants is unclear. We anticipate that suppressor
analysis may reveal target genes that influence colony
morphology and growth at low temperatures. With
regard to IME1 expression, one question is what the
relationship is between the RIM1 pathway genes and
IME4. They may either act in the same pathway or
distinct pathways. A second question is what signal the
RIM1 pathway transmits. Any of the signals that gov-
ern IME1 expression, which include a1-a2, glucose,
and nitrogen, may influence RIM1 pathway activity.
The RIM1 pathway may also respond to the IME2-
dependent signal that down-regulates IME1 expres-
sion. A final question is what the relationship is be-
tween the RIM1/8/9/13 gene products. For example,
they may act in a linear pathway or as subunits of a
protein complex. Molecular analysis of the RIM1 path-
way genes may provide insight into the signals that
they transmit and their mechanisms of action.

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LITERATURE CITED


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