Molecular characterization of the yeast meiotic regulatory gene *RIM1*

Sophia S.Y.Su and Aaron P.Mitchell*

Institute of Cancer Research and Department of Microbiology, College of Physicians and Surgeons, Columbia University, New York, NY 10032, USA

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ABSTRACT

In the yeast *Saccharomyces cerevisiae*, genetic studies suggest that the *RIM1* gene encodes a positive regulator of meiosis. *rim1* mutations cause reduced expression of *IME1*, which is required for expression of many meiotic genes, and thus lead to a partial defect in meiosis and spore formation. We report the sequence of *RIM1* and functional analysis of its coding region. The *RIM1* gene product (RIM1) contains three regions similar to C₂H₂ zinc fingers. Serine substitutions for cysteine in each of the putative zinc fingers abolish RIM1 function. The carboxyl-terminus of RIM1 is enriched in acidic amino acids and is required for full RIM1 activity. RIM1 also contains two putative cAMP-dependent protein kinase (cAPK) phosphorylation sites. At one site, substitution of alanine for serine does not affect RIM1 activity; at the other site, this substitution impairs activity. This analysis of RIM1 suggests that the protein may function as a transcriptional activator. We have used the cloned *RIM1* gene to create a complete *rim1* deletion. This null allele, like previously isolated *rim1* mutations, causes a partial meiotic defect. In addition to *RIM1*, maximum *IME1* expression requires the *MCK1* and *IME4* gene products. Defects associated with *rim1*, *mck1*, and *ime4* mutations in expression of a meiotic reporter gene (*ime2-lacZ*) and in sporulation are additive. These findings suggest that RIM1 acts independently of MCK1 and IME4 to stimulate *IME1* expression.

INTRODUCTION

Sporulation of the yeast *Saccharomyces cerevisiae* is restricted to one type of cell, the *a/α* cell, and is stimulated by starvation in the absence of a fermentable carbon source. These signals lead to increased transcript accumulation from the *IME1* gene (1); the *IME1* product then stimulates expression of *IME2* and other meiotic genes (2–6). Several genes that regulate *IME1* expression have been analyzed in an effort to define the pathways through which cell type and nutritional signals govern the decision to

sporulate (Figure 1). The *a/α* cell type is determined by two products of the *MAT* genetic locus, *a1* and *α2* (reviewed in 7). These proteins interact to form a repressor called *a1-α2* (8, 9). *a1-α2* permits *IME1* expression through two different pathways. In one pathway, *a1-α2* represses *RME1*, which specifies a repressor of *IME1* (1, 10, 11). In the other pathway, *a1-α2* stimulates expression of *IME4*, which specifies a positive regulator of *IME1* (12). It is thought that *a1-α2* stimulates *IME4* expression, indirectly, through repression of an interceding gene in the pathway (12). *IME4* is expressed at higher levels in starved cells than in growing cells, so *IME4* may transmit both cell type and nutritional regulatory signals (12).

IME1 expression is affected by perturbations of the adenylate cyclase/cAMP-dependent protein kinase pathway (reviewed in 13). Reduced cAMP-dependent protein kinase (cAPK) activity permits *IME1* expression in unstarved cells; elevated cAPK activity prevents *IME1* expression in starved cells (2, 14). This pathway is thought either to transmit or override nutritional signals that govern *IME1* expression.

The genes *RIM1* and *MCK1* are required both for basal *IME1* promoter activity in unstarved cells and for elevated *IME1* RNA levels in starved cells [Figure 1 (15, 16)]. *MCK1* encodes a protein kinase homolog that cofractionates with serine/threonine and tyrosine kinase activities (15, 17, 18). Increased dosage of *MCK1* stimulates *IME1* expression and sporulation. Null mutations in *MCK1* lead to partial defects in *IME1* expression and sporulation efficiency (15), suggesting that alternative pathways may act in parallel with *MCK1* to stimulate *IME1* expression. *rim1* mutations also cause partial defects in *IME1* expression and sporulation (16). The finding that *rim1* and *mck1* mutations have roughly additive effects on *IME1* expression and sporulation suggests that the RIM1 and MCK1 gene products act in independent pathways. Mutations in three other genes, *RIM8*, *RIM9*, and *RIM13*, have properties similar to *rim1* mutations, so all of these *RIM* genes are thought to act in the same pathway (16). In this study, we report a molecular characterization of the *RIM1* gene. Our findings have bearing both on the biochemical role of RIM1 and on a possible mechanism of its regulation.

* To whom correspondence should be addressed

Table 1. Yeast strains

Strain	Genotype ^a	Source
AMP105	<i>a</i> TRP1	this lab
AMP107	<i>a</i>	this lab ^b
AMP350	α <i>rme1</i> Δ <i>his4</i> Δ G	this study
AMP474	α <i>gal80::LEU2 his4-G ade3</i>	this lab ^c
AMP477	<i>a</i> <i>gal80::LEU2 his4-N arg6</i>	this lab ^c
AMP594	<i>a</i> <i>rim1-1 rme1</i> Δ 5::LEU2 <i>IME2-5-lacZ::URA3 arg6</i>	this study
AMP597	<i>a</i> <i>rim4-1 rme1</i> Δ 5::LEU2 <i>IME2-5-lacZ::URA3 his4</i> Δ G	this study
AMP599	α <i>rim1-1 rme1</i> Δ 5::LEU2 <i>IME2-5-lacZ::URA3 met4</i>	this lab ^d
AMP734	<i>a</i> <i>rme1</i> Δ 5::LEU2 <i>IME2-5::URA3 arg6</i>	this lab ^d
AMP737	α <i>rme1</i> Δ 5::LEU2 <i>IME2-5::URA3 met4</i>	this lab ^d
AMP745	α <i>rim4-1 rme1</i> Δ 5::LEU2 <i>IME2-5-lacZ::URA3 his4</i> Δ G	this study
AMP854	α <i>rim4-1 rme1</i> Δ 5::LEU2 <i>met4</i>	this study
AMP931	<i>a/a gal80::LEU2/gal80::LEU2 arg4/ARG4 his4-G/his4-N ade3/ADE3</i>	this lab ^c
AMP974	<i>a</i> <i>rim4-1 rme1</i> Δ 5::LEU2 <i>IME2-5-lacZ::URA3 arg6</i>	this study
CSH2-2	<i>a</i> <i>met4</i>	this lab
CSH2-8	α <i>met13</i>	this lab
SS1955	<i>a/a SPO11::TRP1/SPO11 RIM1/RIM1::URA3 HIS4/his4</i> Δ G <i>RME1/rme1</i> Δ 5::LEU2	this study
SSY1	<i>a</i> <i>rim1-2 rme1</i> Δ 5::LEU2 <i>IME2-5::URA3 arg6</i>	this study
SSY5	<i>a</i> <i>rim1-3 rme1</i> Δ 5::LEU2 <i>IME2-5::URA3 arg6</i>	this study
SSY9	α <i>rim1-4 rme1</i> Δ 5::LEU2 <i>IME2-5::URA3 met4</i>	this study
SSY55	<i>a</i> <i>rme1</i> Δ <i>ime2</i> Δ 4- <i>lacZ::LEU2 arg6</i>	this study
SSY388	<i>a</i> <i>SPO11::TRP1</i>	this study
SSY405	α <i>RIM1::URA3 rme1</i> Δ 5::LEU2 <i>his4</i> Δ G	this study
SSY416	α <i>rim1</i> Δ <i>rme1</i> Δ 5::LEU2 <i>arg6</i>	this study
SSY417	<i>a</i> <i>rim1</i> Δ <i>rme1</i> Δ 5::LEU2 <i>his4</i> Δ G	this study
SSY455	<i>a/a rim1</i> Δ / <i>RIM1 rme1</i> Δ 5::LEU2/ <i>rme1</i> Δ 5::LEU2 <i>ime2</i> Δ 4- <i>lacZ::LEU2/IME2 arg6/ARG6 his4</i> Δ G/ <i>HIS4</i>	this study
SSY456	<i>a/a rim1</i> Δ / <i>rim1</i> Δ <i>rme1</i> Δ 5::LEU2/ <i>rme1</i> Δ 5::LEU2 <i>ime2</i> Δ 4- <i>lacZ::LEU2/IME2 arg6/ARG6 his4</i> Δ G/ <i>HIS4</i>	this study
SSY534	α <i>ime4-1::LEU2 rim1</i> Δ :: <i>URA3 mck1</i> Δ :: <i>TRP1 mch4</i> Δ :: <i>LEU2 his3 ime1-HIS3 arg6</i>	this study
SSY 553	α <i>ime4-1::LEU2 rim1</i> Δ :: <i>URA3 mck1</i> Δ :: <i>TRP1 mch4</i> Δ :: <i>LEU2 his3 arg6 met4</i>	this study
SSY557	<i>a</i> <i>his3 IME2-5-lacZ::URA3 met4</i>	this study
SSY559	<i>a</i> <i>ime4-1::LEU2 his3 IME2-5-lacZ::URA3 met4</i>	this study
SSY562	<i>a</i> <i>rim1</i> Δ :: <i>URA3 his3 IME2-5-lacZ::URA3 met4</i>	this study
SSY564	<i>a</i> <i>mck1</i> Δ :: <i>TRP1 his3 IME2-5-lacZ::URA3 met4</i>	this study
SSY566	<i>a</i> <i>ime4-1::LEU2 rim1</i> Δ :: <i>URA3 his3 IME2-5-lacZ::URA3 met4</i>	this study
SSY568	<i>a</i> <i>ime4-1::LEU2 mck1</i> Δ :: <i>TRP1 his3 IME2-5-lacZ::URA3 met4</i>	this study
SSY570	<i>a</i> <i>rim1</i> Δ :: <i>URA3 mck1</i> Δ :: <i>TRP1 his3 IME2-5-lacZ::URA3 met4</i>	this study
SSY572	<i>a</i> <i>ime4-1::LEU2 rim1</i> Δ :: <i>URA3 mck1</i> Δ :: <i>TRP1 his3 IME2-5-lacZ::URA3 met4</i>	this study
SSY592	<i>a/a rim1</i> Δ / <i>RIM1 rme1</i> Δ 5::LEU2/ <i>RME1 ime1-HIS3/IME1 IME2-5-lacZ::URA3/IME2 his3</i> Δ SK/ <i>his3</i> Δ SK <i>arg6/ARG6 met4/MET4</i>	this study
SSY593	<i>a/a rim1</i> Δ / <i>rim1</i> Δ <i>rme1</i> Δ 5::LEU2/ <i>RME1 ime1-HIS3/IME1 IME2-5-lacZ::URA3/IME2 his3</i> Δ SK/ <i>his3</i> Δ SK <i>arg6/ARG6 met4/MET4</i>	this study
TSS1571-1A	<i>a</i> <i>pSS157-3::URA3 rme1</i> Δ 5::LEU2 <i>ime2</i> Δ 4- <i>lacZ::LEU2 arg6</i>	this study

^aAll strains have the additional markers *ura3*, *leu2::hisG*, *trp1::hisG*, *lys2*, and *ho::LYS2*, unless otherwise indicated.^bDescribed in (4).^cDescribed in (5).^dDescribed in (16).

MATERIALS AND METHODS

Yeast strains

Isogenic strains were constructed in the SK-1 genetic background (19) through transformation or standard genetic manipulation (20) and are listed in Table 1. All integrative transformations were confirmed by Southern analysis. The *rme1* Δ 5, *ime1-HIS3*, *ime2-4-lacZ*, *IME2-5-lacZ* and *mck1* Δ 3 alleles as well as other auxotrophic markers in these strains have been described previously (5, 11, 15, 16). The *mch4* Δ ::LEU2 allele causes a mild, recessive *IME1* expression defect and will be described

elsewhere (L. Niegeborn and A. P. Mitchell, unpublished results). All *mck1* Δ , *rim1* Δ and *ime4-1* mutants analyzed in detail derived from mutant strains listed in Table 1.

The *SPO11::TRP1* allele was created by transforming strain AMP107 with an *Eco*R1-digested plasmid pSS190A (Figure 2). The *RIM1::URA3* allele was created by transforming strain AMP350 with *Xho*I-digested plasmid pSS195 Δ BG (Figure 2). The *rim1* Δ allele was introduced as a one-step gene disruption (21) by transforming a *ura3/ura3* diploid recipient (SSY55/AMP350) with *Sal*I- and *Bgl*II-digested plasmid pSS179C (Figure 2). *Ura*⁺ transformants that displayed the

expected Southern pattern were sporulated and dissected for tetrad analysis. We found that the Ura^+ phenotype cosegregated with $\text{rim1}\Delta$ by the smooth colony phenotype and by failure to complement rim1 mutants for ime2-lacZ expression. Furthermore, Ura^+ segregants containing the $\text{rim1}\Delta$ allele were complemented for ime2-lacZ expression by plasmid pSS179RIM1, which contains the wild type RIM1 gene.

The one-step gene-disruption approach (21) was also used to create the ime4-1 mutation. Insertion of the *LEU2* gene into the *Bam*HI site of the *IME4* gene abolished *IME4* activity (12). We created the same mutation in plasmid pSS223LEU (Figure 2) and transformed it into a leu2/leu2 diploid strain (CSH2-2/CSH2-8) after digestion with *Pst*I and *Hind*III. Leu^+ prototrophs were selected, sporulated and dissected. Southern blots of all segregants from three tetrads confirmed that the Leu^+ phenotype cosegregated with the 2.7-kbp insertion at *IME4*.

Media and genetic methods

Standard procedures were used for mating, diploid isolation, sporulation and tetrad analysis (20). Standard recipes were used for SD, SC, SC dropout media, YEPD, YEPAc, sporulation plates, and liquid sporulation media (2, 20). Unless otherwise indicated, all experiments were conducted at 30°.

Sporulation and β -galactosidase assays

Sporulation ability was quantitated on sporulation plates or in liquid sporulation cultures as described (16). We scored at least 200 cells by microscopic examination after 1–5 days. All determinations are the average of at least three experiments except in Table 2, in which numbers are the average of two experiments. β -galactosidase activity was assayed qualitatively on KAcXgal plates (minimal sporulation plates containing Xgal), or quantitatively with ONPG as substrate (16, 20). β -galactosidase assays in Figure 5 were conducted as described previously (16, 20) except that cells were grown to saturation in SD medium containing 0.5% glucose and 1% casamino acids prior to transfer to sporulation medium; samples were harvested after 6 hours for quantitative assays with ONPG as substrate, and sporulation was quantitated after 28 hours. This protocol permitted selection for maintenance of the *TRP1*-bearing plasmids.

Cloning RIM1 and RIM4

We constructed a yeast DNA library derived from the strain AMP105. Genomic DNA was partially digested with *Sau*3A and 10-kbp to 15-kbp fragments were gel-purified. The fragments were ligated into the *Bam*HI site of pRS314 (22) and transformed into *E. coli* strain DH5 α [*F-endA1 hsdR17* (r^-k, m^+k) *suppE44 thi-1* λ -recA1 *gyrA96 relA1* ϕ 80d $\text{lacZ}\Delta$ M15]. Approximately 1500 ampicillin-resistant transformant colonies were pooled, grown to log-phase in LB ampicillin medium, and plasmid DNA was prepared.

Strain AMP597, a rim4-1 mutant, was transformed with this library. Tryptophan prototrophs were selected, replica-plated to KAcXgal plates, incubated at 30° for two to three days and screened for complementation of the ime2-lacZ expression defect. Positive transformants were mated to strain AMP854 and tested for complementation of the rim4-1/rim4-1 sporulation defect. Plasmids that complemented both defects of rim4-1 were isolated and transformed into strains AMP599 and AMP745 to test for complementation of the ime2-lacZ expression defects of rim1-1 and rim4-1 , respectively. One plasmid (pSS131-15), containing

Figure 1. Regulation of *IME1* expression. *IME1* is essential for expression of meiotic genes and for sporulation. The repressor a1- α 2, which determines the a/ α cell type, permits *IME1* expression through repression of *RME1* (a negative regulator of *IME1*) and by activation of *IME4* (a positive regulator of *IME1*). 'X' refers to a postulated repressor of *IME4* (12). Genes required for full expression of *IME1* also include *MCK1*, *RIM1*, *RIM8*, *RIM9* and *RIM13* (15, 16). Previous studies indicated that *RIM1*, *RIM8*, *RIM9* and *RIM13* act in a pathway that is independent of *MCK1* (16). Results in this report suggest that *IME4* acts in a third pathway distinct from the *RIM1* and *MCK1* pathways to activate *IME1* expression. Nitrogen, glucose, and internal cyclic AMP (cAMP) prevent *IME1* expression, possibly through effects on *MCK1* or *RIM1* activities. In addition, nitrogen is known to prevent *IME4* transcript accumulation (12).

a 15-kbp insert, complemented both mutations. An 8-kbp subclone in YIp5 (pSS157-3) was digested with *Sac*I and targeted to the chromosome of wild type strain SSY55 to create strain TSS1571-1A. Two independent transformants were mated to strain AMP854 containing rim4-1 , sporulated and tetrads dissected. In 21 of the 22 tetrads analyzed, the Ura^+ phenotype cosegregated with *RIM4* (11 P:0 NPD:0 T and 10 P:0 NPD: 1 T) indicating that the cloned DNA derived from a chromosomal locus close to the rim4-1 mutation. Subclones of the insert from pSS131-15 carried in pRS314, YCp50 and YE24 vectors were retested for complementation of the ime2-lacZ expression defects of rim1-1 (strain AMP594) and rim4-1 (strain AMP974).

Plasmid constructions

The *SPO11::TRP1* plasmid (pSS190A) was constructed by inserting the *SPO11* gene into the integrating vector pRS304 (22). A 1.4-kbp *Xho*I-*Spe*I fragment which contains *SPO11* from pGB436 (provided by C. Giroux) was ligated into the *Xho*I and *Spe*I sites of pRS304 (Figure 2). The *RIM1::URA3* plasmid (pSS195 Δ BG) was constructed in two steps. First, the 10-kbp *Eco*RI fragment from plasmid pSS131-15 (Figure 3) was ligated into the *Eco*RI site in YIp5. The resulting plasmid was digested with *Bam*HI and *Bgl*II and religated to remove the intervening 7.4-kbp fragment (Figure 2). Single-copy and multi-copy *RIM1* plasmids were constructed by ligating the 6.1-kbp *Sal*I-*Kpn*I fragment from pSS166 Δ Sal (Figure 3) into the *Sal*I and *Kpn*I sites of pRS314 and pRS424 (22) to create pSS179RIM1 and pMC RIM1, respectively (Figure 2). Another single-copy plasmid containing the *RIM1* gene on a 2.6-kbp fragment (pCEN RIM1) derived from 3' exonuclease III deletion (see below). The plasmid used for creating the $\text{rim1}\Delta$ allele, pSS179C, was constructed by inserting a 1.1-kbp *Sma*I-*Xba*I fragment containing the *URA3* gene into the *Sma*I and *Nhe*I sites of pSS166 Δ N (Figure 2). The multi-copy *IME4* plasmid (pMC *IME4*) was constructed by ligating the 2.9-kbp *Xba*I-*Sal*I fragment from plasmid pJS12 (12) between the *Spe*I and *Sal*I sites of pRS424 (Figure 2). The plasmid-borne ime4 disruption allele was made in two steps. First,

Figure 2. Plasmid insert restriction map. (A) The pSS190A and the pSS195ΔBG inserts are carried in vectors pRS304 and YIp5, respectively. The diagonally striped bar represents *SPO11* and the solid black bar indicates DNA upstream of *RIM1*. (B) The insert containing *RIM1* and its flanking DNA is carried in vectors pRS314 and pRS424 for pSS179*RIM1* and pMC *RIM1*, respectively. The pCEN *RIM1* insert is carried in vector pRS314. The open bar indicates the *RIM1* open reading frame (ORF) and the solid black bar indicates flanking DNA. The pSS179C insert is carried in vector pRS314. The horizontally striped bar represents *URA3*, which replaced the entire *RIM1* ORF. (C) The inserts for pSS-KN34 and pSS-KN43 are carried in vector pRS314. The open bars in each plasmid indicate the extent of the remaining *RIM1* coding sequence. (D) The insert for pMC *IME4* is carried in pRS424, and the inserts for pSS223pUC and pSS223LEU are carried in pUC18. The shaded bar represents *IME4*. The thin black line indicates pBR322 DNA that was included during construction. The stippled bar represents the *LEU2* insertion in *IME4*. Abbreviations: B, *Bam*HI; G, *Bgl*II; H, *Hind*III; K, *Kpn*I; Nc, *Nco*I; Nh, *Nhe*I; P, *Pst*I; Pv, *Pvu*II; RI, *Eco*RI; RV, *Eco*RV; S, *Sal*I; Sm, *Sma*I; Sp, *Spe*I; St, *Sna*I; Xb, *Xba*I; X, *Xho*I. Asterisks indicate sites in flanking vector sequences.

the 2.6-kbp *Hind*III-*Pvu*II fragment containing *IME4* from plasmid pJS12 (12) was ligated between the *Hind*III and *Sma*I sites of pUC18. A 2.7-kbp *Bgl*III fragment containing *LEU2* was

then ligated into the unique *Bam*HI site of this plasmid, creating plasmid pSS223LEU (Figure 2).

Construction of *rim1* missense mutations and truncations

Oligonucleotide site-directed mutagenesis was used to create *RIM1* point mutations in plasmids pSS179*RIM1* and pCEN *RIM1*. Presence of mutations was confirmed by dideoxynucleotide sequencing. The *rim1*-Δ531 mutation was created after *Eco*RI and *Xho*I sites were introduced in plasmid pSS-CRX29 at *RIM1* position +1595 through mutagenesis with oligonucleotide RIM1-CRX (5'-CATAATTGGGAATTTAGAATTCTGCTCG-AGCTGAACCAAAAAAG-3'). The 2.2-kbp *Pst*I-*Xho*I fragment from pSS-CRX29 was then inserted into pRS314 at *Pst*I and *Xho*I sites to create pSS-KN34. Vector sequences resulted in the addition of RILEGGPGTQFAL to residue 531 and created a truncated polypeptide of 545 amino acids. The *rim1* truncation at codon 296 (pSS-KN43) resulted from 3' digestion of pSS179*RIM1* with exonuclease III. Vector sequences resulted in the addition of ASFCSL to residue 296 and created a truncated polypeptide of 302 amino acids.

Construction of *RIM1* deletions and sequencing strategy

Nested deletions were constructed in plasmid pSS179*RIM1* (Figure 2) through exonuclease III digestion (Promega). DNA sequence was determined by double-strand dideoxynucleotide with the Sequenase 2 Kit (Stratagene) and the TaqTrack Sequencing Systems (Promega). The sequences of both strands of 3081 nucleotides were determined. Manipulation of the sequence data was carried out by using the University of Wisconsin Genetics Computer Group (GCG) nucleic acids sequence analysis programs (23).

RESULTS

Cloning of *RIM1*

We set out to clone *RIM1* by identifying low-copy (*CEN*) plasmids with genomic inserts that complement the *ime2-lacZ* expression defect of a *rim1-1* mutant. In extensive screens of two independent yeast DNA libraries (24, 25), we failed to isolate *RIM1*, although we did identify two inserts that partially suppress *rim1-1*. One possible problem was that *RIM1* might not be represented in existing libraries, so we constructed a new library that was minimally amplified. A second possible problem was that existing *rim1* mutations cause only partial defects in *ime2-lacZ* expression and sporulation (16), so we had to sort through many weakly-suppressing plasmids. We found that the *rim1-1* mutation was tightly linked to a more severe *rim* mutation, *rim4-1* (23 PD: 0 NPD: 1 T), which causes an absolute sporulation defect (16). Therefore, we transformed the library into a *rim4-1* mutant and screened for complementation of *ime2-lacZ* expression and sporulation defects. We identified a plasmid, pSS131-15, that could complement both *rim4-1* and *rim1-1*. The insert directed plasmid integration to the *RIM4* locus (see Methods), thus confirming that we had isolated *RIM1* and *RIM4* rather than a suppressor. We found that plasmids containing a region adjacent to the *RIM1* gene transform *E. coli* more poorly than plasmids lacking this region (data not shown). Therefore, *RIM1* and *RIM4* may have been under-represented in more extensively passaged libraries.

Plasmid pSS131-15 and its subclone pSS166ΔS defined a 9-kbp genomic DNA segment that complemented both *rim4-1* and *rim1-1* (Figure 3). Subclones pSS166ΔN and pSS179*RIM1*

Figure 3. Subclones of *RIM1* and *RIM4*. Yeast DNA in each plasmid is denoted below the *RIM1* and *RIM4* map. The arrows on this map refer to the *RIM1* and *RIM4* open reading frames. Ability of each plasmid to complement *rim1-1* and *rim4-1* mutations is indicated in columns to the right (ND, not determined). Restriction sites: B, *Bam*HI; H, *Hind*III; K, *Kpn*I; Nc, *Nco*I; Nh, *Nhe*I; RI, *Eco*RI; S, *Sal*I; Sa, *Sau*3A; Sc, *Sac*I; St, *Stu*I.

complemented only *rim1-1*, whereas subclones pSS166ΔK and pSS178M complemented only *rim4-1* (Figure 3). Subclones pSS-KN29 and pSS-SX15 narrowed *RIM1* activity to a 2.7-kbp DNA region. These complementation results suggest that *rim1* and *rim4* mutations lie in different genes; this inference is confirmed by sequence analysis (below and in preparation).

RIM1 map location

We mapped *RIM1* and *RIM4* to chromosome VIII through Southern analysis of electrophoretically separated chromosomes, using a 2.3-kbp *Eco*RI-*Xho*I fragment from plasmid pSS131-15 as a probe (data not shown). Hybridization of this probe to a set of filters containing λ clones comprising 98% of the yeast genome (26) indicated that these two genes are very close to *SPO11* on the left arm of chromosome VIII (data not shown). We analyzed 95 meiotic tetrads from diploid SS1955 (*a*/ α SPO11::TRP1/SPO11 *RIM1*/*RIM1*::URA3) and determined that *RIM1* mapped 3.7 cM from *SPO11* (88 PD: 0 NPD: 7 T).

Analysis of a *rim1* null mutation

Previously isolated *rim1* mutations cause slow growth at 17° and partial defects in *IME1* expression and sporulation [Table 2 (16)]. These defects may be partial either because *rim1* mutant products retain partial function or because other gene products can compensate for the absence of *RIM1*. The finding that *mck1Δ rim1-1* double mutants display more severe *ime2-lacZ* expression and sporulation defects than either single mutant supports the second interpretation, but even the double mutants have considerable sporulation ability (16). To examine the *rim1* null phenotype, we constructed a *rim1* allele lacking the entire open reading frame (based on sequence information presented below).

MVPLEDLLKNGTAAPOHRSRESIVENGTDSNVTKKDGLPSPNLSKRSDCSKRPRIRC
TTEAIGLKQEDERMSPGSTSSSCLPYHSSSHLNTTPYDLLGASAVSPTTPSSSDSSSSS
PLAQAHNPAGDDDDADNDGDSIEDITLYCKWDCGMIENQPELLYNLCHDEVGRKSHKLN
QLNCHWGDCTTKTEKRDEHITSBLRVVPLKPEGCSTCSKKTKRPQDLKKHLKTHLES
LKRKRGPPLGSKRTSKKNKSSASDAVSSCSASVPSGIAGSFKSHSTSPQILPPLPVGISQ
HLPSSQQQQQQRAISLNQLCSDLSQYKPVYSPQLSARLQTLPLLYNNGSTVSQGAN
QSMNVYEDGCSNKTIANATQFTKLSRNMNTNNYILQQSGGSTESSSSSGRIPVAQTSYVQ
PPNAPSYQSVQGGSSISATANTATYVVPRLAKYPTGPSLTHELPLHSNTAGGVFNRSQ
YAMPHYSIRAPSYSSSGCSILPPLQSKIPMLPSRRMTAGETSLKPNWEFSLNQKSCYN
DIIMSKLAIEEVDESEIEDDFVEMLGIVNI IKDYLLCCVMEDLDDEESDKDEENAFLO
ESLEKLSLQNMGTNSVRILTYPKILV

Figure 4. Deduced sequence of the *RIM1* product. Segments that resemble C₂H₂ zinc fingers are shown in bold type with underlining to indicate consensus residues. Two possible cAMP-dependent protein kinase phosphorylation sites are shown in bold italics. The *RIM1* nucleotide sequence is available under EMBL accession number X72960.

We replaced a 3.5-kbp segment that includes *RIM1* with the *URA3* gene to make plasmid pSS179C. A plasmid complementation test confirmed that this deletion abolished *RIM1* function (Figure 3). This *rim1Δ*::URA3 insert was then used to replace one chromosomal *RIM1* allele in a diploid strain (see Materials and Methods). Meiotic analysis of several *rim1Δ*::URA3/*RIM1* diploids revealed that every tetrad produced two Ura⁺ and two Ura⁻ spores. The Ura⁺ and Ura⁻ spore colonies grew equally well. This result indicated that *RIM1* is not an essential gene. Further comparisons indicated that the phenotypes of *rim1Δ* and four independent *rim1* alleles were nearly identical (Table 2). In addition to the failure to express *ime1-HIS3* and *ime2-lacZ* fusions, all *rim1* alleles caused smooth colony morphology, slow growth at 17°, and a partial sporulation defect. Because the phenotypes of *rim1-1*, *rim1-2*, *rim1-3*, and *rim1-4* were indistinguishable from that of *rim1Δ*, we conclude that the partial defects do not arise from partial loss of function.

Table 2. Phenotypes of *rim1* mutants

<i>RIM1</i> allele	<i>ime1-HIS3</i> expression ^{a,b}	<i>ime2-lacZ</i> expression ^{a,c}	percent sporulation ^d	colony morphology ^{a,e}	growth ^a 30°	17°
<i>RIM1</i>	+	Lac +	93	C	+	+
<i>rim1-1</i>	—	Lac—	29	S	+	—
<i>rim1-2</i>	—	Lac—	38	S	+	—
<i>rim1-3</i>	—	Lac—	42	S	+	—
<i>rim1-4</i>	—	Lac—	42	S	+	—
<i>rim1Δ</i>	—	Lac—	35	S	+	—

^a*ime1-HIS3* expression, *ime2-lacZ* expression, colony morphology and growth after 3 days at 30° and 17° were determined for *rme1Δ* haploid strains.

^bExpression of the *ime1-HIS3* fusion was examined on SC plates lacking histidine. A '+' indicates growth on SC-His plates; a '—' indicates failure to grow.

^cExpression of the *ime2-lacZ* fusion was examined on KAcXgal plates. Lac⁺ refers to a high level β-galactosidase activity and Lac[—] refers to a low level β-galactosidase activity.

^dSporulation efficiency was determined in diploids homozygous for the respective *rim1* alleles after 36 hr on sporulation plates.

^e'C' refers to clumpy colony morphology and 'S' refers to smooth colony morphology.

RIM1 nucleotide sequence

We determined the nucleotide sequence of the 3.1-kbp insert of plasmid pSS179*RIM1*, which complements *rim1-1*. The insert specified a single large open reading frame (ORF) with 518 bp of 5' flanking sequence and 680 bp of 3' flanking sequence (not shown; EMBL accession number X72960). The ORF encodes a polypeptide of 628 amino acids with a calculated molecular weight of 68,538 daltons (Figure 4). This ORF is contained within the region delimited by endpoints of subclones pSS-KN29 and pSS-SX15, which complemented the *rim1-1* mutation. We infer that this ORF represents the *RIM1* gene product.

Analysis of the *RIM1* upstream region revealed sequences that may be required for *RIM1* expression (not shown). There are possible TATA boxes at 137 and 247 bases upstream of the *RIM1* initiation codon. Two sites with homology to the T₄C site, identified as a sequence required for *IME2* UAS activity (27), lie at positions -303 to -311 (TTTCTGCG) and -454 to -446 (TTTCTTCG) (with matches to the *IME2* T₄C site underlined). No other consensus transcription factor binding sites (28) were identified in the 5' flanking region. A potential polyadenylation signal (AATAAA) was found 58 bp downstream of the termination codon.

The deduced *RIM1* product contains three potential C₂H₂ zinc fingers, characteristic of nucleic-acid binding proteins (29), at residues 146–169, 182–206, and 212–234 (Figure 4). All three regions contain the four crucial cysteine and histidine residues that coordinate the zinc cation and a 12 amino acid loop that connects these conserved residues. *RIM1*:146–169 and *RIM1*:212–234 also contain the conserved phenylalanine and leucine residue at consensus positions 10 and 16 in the loop. In *RIM1*:182–206, these positions have the polar residue threonine and the aliphatic residue isoleucine, respectively. The first positions of *RIM1*:146–169 and *RIM1*:182–206 have leucine residues rather than the consensus aromatic residue. Similar deviations from the consensus have been observed in other putative zinc finger proteins (30–33). The region between *RIM1*:182–206 and *RIM1*:212–234 (VPLKP) shows some similarity to the H-C link consensus (TGEKP [29, 30]).

The deduced *RIM1* sequence has two other noteworthy features. First, the carboxy-terminal 88 amino acids of *RIM1* includes 25 acidic residues and has a net charge of -18. Such acidic regions can function as transcriptional activation domains

	Amino Acid Change	<i>ime1-HIS3</i> Expression	<i>ime2-lacZ</i> Expression	Sporulation (%)
Wild Type	+	124±11	49
C153S	—	1.6±0.4	4.2
C189S	—	2.4±0.9	12
C217S	—	1.0±0.9	6.0
S50A	+	126±13	42
S255A	—	12±3	16
S50A,S255A	—	20±15	16
Δ531-628	—	65±13	24
Δ297-628	—	20±6	10
Δ1-628	—	1.1±0.5	3.9

Figure 5. Analysis of *RIM1* mutations. The deduced *RIM1* protein is diagrammed with shading to indicate zinc finger and acidic regions and with small circles to indicate consensus cAPK phosphorylation sites. Mutant proteins are depicted schematically with filling-in to represent the segment affected by missense substitutions. Mutational changes and the results of functional complementation tests are indicated in columns. *ime2-lacZ* levels are given in Miller units with standard deviations. All *rim1* mutations were carried on low copy number (*CEN*) plasmids in strain SSY593. Complementation tests were conducted with 3 to 7 independent transformants carrying each plasmid. A control plasmid carrying no insert gave complementation results summarized on the last line (Δ1–628).

(34). Second, *RIM1* has two consensus cAPK phosphorylation sites at residues 50 (KRSS) and 255 (KRTS). These sites flank the zinc finger region. cAPK activity is known to inhibit *IME1* transcript accumulation (2, 14).

Table 3. *ime2-lacZ* expression and sporulation ability of *rim1Δ*, *mck1Δ*, and *ime4-1* mutants

relevant genotype ^a			<i>ime2-lacZ</i> expression ^b		% sporulation ^b		
<i>RIM1</i>	<i>MCK1</i>	<i>IME4</i>	veg	spo	1 day	2 days	5 days
+	+	+	0.4	120	96	99	100
<i>rim1Δ</i>	+	+	0.4	1.2	6	18	36
+	<i>mck1Δ</i>	+	0.5	37	64	82	85
+	+	<i>ime4-1</i>	0.5	56	55	69	84
<i>rim1Δ</i>	<i>mck1Δ</i>	+	0.7	0.8	<0.1	3	8
<i>rim1Δ</i>	+	<i>ime4-1</i>	0.5	0.6	<0.1	1	3
+	<i>mck1Δ</i>	<i>ime4-1</i>	0.7	0.6	1	10	15
<i>rim1Δ</i>	<i>mck1Δ</i>	<i>ime4-1</i>	0.1	0.2	<0.1	<0.1	<0.1

^aDiploid strains were homozygous for the mutations indicated.

^b β -galactosidase assays were performed for log phase cultures in YEPac medium (veg) and after 6 hrs in liquid sporulation medium (spo). Sporulation ability was quantitated in the same cultures after one, two, and five days in sporulation medium. Values are the average of two independent determinations.

Table 4. Effect of increased *RIM1* dosage on sporulation

strain	relevant genotype	percent sporulation ^a			
		vector	pMC <i>RIM1</i>	pMC <i>IME4</i>	pMC <i>IME1</i>
AMP931	a/a	<0.02	0.3	5.3	28
AMP477/AMP474	a/α	100	100	100	100

^aSporulation ability was quantitated after two days on sporulation plates. At least four independent transformants were examined for each transformed plasmid and at least 400 cells were counted for each transformant.

Mutational analysis of *RIM1*

We used mutational analysis to test the contribution of the zinc finger motifs, the acidic region, and the cAPK phosphorylation sites to *RIM1* activity. Mutations altering each of these sequences were introduced into single-copy *RIM1* plasmids. Activity was assayed by complementation of a *rim1Δ* mutant for *ime1-HIS3* expression, *ime2-lacZ* expression and sporulation. Because slow growth at 17° and smooth colony morphology were observed only in *rim1* mutants grown in rich medium which does not select for plasmid maintenance, these phenotypes were not assessed.

Missense substitutions that alter a consensus residue of each putative zinc finger abolish *RIM1* activity (Figure 5). We created substitutions of serine for the second cysteine residue of each zinc finger, at positions 153, 189 and 217. Single-copy plasmids that carry each mutant allele failed to restore *ime1-HIS3* expression, *ime2-lacZ* expression and sporulation ability to a *rim1Δ* mutant. These observations suggest that each putative zinc finger is essential for *RIM1* function.

Deletions that remove the *RIM1* acidic C-terminus reduce but do not abolish activity (Figure 5). We created deletions that truncate *RIM1* at codon 531 and at codon 297. Single-copy plasmids that carry each deletion failed to fully complement the *rim1Δ* mutation based on *ime1-HIS3* expression. However, both plasmids partially complemented the *rim1Δ* defects in sporulation and *ime2-lacZ* expression. We conclude that the *RIM1* C-terminus contributes to activity, but is not absolutely essential.

To determine the role of each putative cAPK phosphorylation site, we created alanine substitutions for serine residues 50 and 255 (Figure 5). The substitution at position 50 did not impair *RIM1* activity. However, the substitution at position 255 reduced *RIM1* activity based on all assays of *RIM1* function. A double mutant with both substitutions also had low activity. We conclude that the serine residue at position 255 is important for *RIM1*

activity. However, these genetic results do not indicate that serine 255 is phosphorylated.

Analysis of genes affecting *IME1* expression

Prior studies indicated that *RIM1* and *MCK1*, which specifies a protein kinase homolog, act in independent pathways to stimulate *IME1* expression (16). We observed that *mck1Δ rim1Δ* double mutants, like *mck1Δ rim1-1* double mutants, retain some ability to sporulate (Table 3). Since *ime1* mutants fail to sporulate, this observation suggests that a third pathway must permit some expression of *IME1* in the absence of *RIM1* and *MCK1*.

IME4 is another positive regulator of *IME1*. Prior studies indicated that an *ime4* insertion mutation blocks *IME1* expression and sporulation (12). However, we observed that an *ime4* insertion mutation in strains from the SK-1 genetic background, which we routinely use, does not block sporulation. Sporulation of *ime4/ime4* diploids was delayed (22% after one day and 88% after two days) compared to wild type strains (77% after 1 day and 100% after 2 days). The same *ime4* insertion mutation abolishes sporulation in strains from the S288C genetic background (W. Li, personal communication), from which many lab strains are derived. We also confirmed that a multi-copy *IME4* plasmid stimulates sporulation in a/a cells (see below). Therefore, our results do not contradict the earlier study (12). Rather, they suggest that *IME4* plays a less central role in *IME1* expression in SK-1-derived strains than in S288C-derived strains.

We determined the genetic relationship between *RIM1*, *MCK1* and *IME4* by analyzing *ime2-lacZ* expression and sporulation abilities of single, double and triple null mutants. If *IME4* acts through the *RIM1* pathway, we expected the phenotype of a *rim1Δ ime4-1* double mutant to be comparable to that of either *rim1Δ* or *ime4-1* single mutants. If *IME4* acts through the *MCK1* pathway, we expected the phenotype of a *mck1Δ ime4-1* double

mutant to be comparable to that of either *mck1Δ* or *ime4-1* single mutants. If IME4 acts through a third pathway to stimulate *IME1* expression independently of RIM1 or MCK1, we expected the phenotype of *rim1Δ ime4-1* and *mck1Δ ime4-1* double mutants to be more severe than any of the single mutants. We observed that single *rim1Δ*, *mck1Δ*, and *ime4-1* mutants had 100-, 4-, and 2-fold *ime2-lacZ* expression defects, compared to a wild-type strain incubated in sporulation medium, and caused mild sporulation defects (Table 3). All three double mutants had more severe sporulation defects and, where measurable, *ime2-lacZ* expression defects. A *rim1Δ mck1Δ ime4-1* triple mutant was completely defective in *ime2-lacZ* expression and sporulation (Table 3). These observations suggest that RIM1, MCK1, and IME4 act through independent pathways to stimulate *ime2-lacZ* expression and sporulation.

Increased RIM1 dosage permits sporulation in a/a diploids

Increased dosage of *IME1* or *IME4* permits sporulation of cells that lack one or the other subunit of the repressor that determines a/a cell type properties, *a1-α2* (1, 12). To determine whether RIM1 shares this property, we examined the sporulation ability of an a/a diploid strain (which lacks *a1-α2*) transformed with a multi-copy *RIM1* plasmid. Transformants carrying the plasmid vector failed to sporulate, as expected (Table 4). Transformants carrying the multi-copy *RIM1* plasmid were able to sporulate at a low level (0.3%). Transformants carrying multi-copy *IME1* or *IME4* plasmids were able to sporulate at higher levels (28% and 5.3%, respectively). A control a/a diploid sporulated equally well, regardless of which plasmid it carried. Therefore, increased *RIM1* dosage is able to stimulate sporulation weakly in the absence of *a1-α2* activity.

DISCUSSION

Our characterization of *RIM1* leads to three main conclusions that concern RIM1 function, structure, and regulation. First, we have provided clear evidence that RIM1 is a positive regulator of *IME1* expression. The ability of *rim1* mutants to sporulate apparently requires two parallel regulatory pathways that depend on MCK1 and IME4. Second, the deduced RIM1 sequence indicates that the protein may function as a positive transcription factor. Third, our mutational analysis raises the possibility that RIM1 activity may be stimulated by phosphorylation by cAMP-dependent protein kinase.

We previously suggested that RIM1 was a positive regulator of *IME1* based on *rim1* mutations isolated through a genetic screen (16). The *rim1-1* mutation, which was characterized in detail, reduced *IME1* RNA accumulation and expression of the *ime1-HIS3* fusion gene. Because all isolated *rim1* mutations were recessive, we inferred that they were loss-of-function mutations. However, the fact that all *rim1* mutant phenotypes reflected only partial defects might have meant that the *rim1* mutations reduced, but did not abolish, activity of the gene product. The phenotypes associated with a complete deletion of the *RIM1* open reading frame clearly establish the consequences of loss of RIM1 function. We observed that the *rim1Δ* mutation causes a partial sporulation defect, altered colony morphology, and conditional lethality. Because the *rim1Δ* mutation causes defects in *ime1-HIS3* and *ime2-lacZ* expression, we infer that the sporulation defect arises from an *IME1* expression defect. The fact that *rim1Δ* mutants display partial defects in sporulation and growth indicates that

other gene products or pathways must partially compensate for loss of RIM1 activity.

Genetic experiments suggest that *MCK1* and *IME4* define two pathways that act in parallel to *RIM1* to stimulate *IME1* expression. The inference that RIM1, MCK1, and IME4 act independently is based on our observation that double mutant combinations have more severe *ime2-lacZ* expression and sporulation defects than single mutants. The use of null mutations is critical for this type of analysis; it is well established that combinations of partially-defective mutations affecting the same pathway can have additive effects (35–38). We believe that the RIM1, MCK1, and IME4 pathways may share additional functions as well, because all three *rim1 mck1 ime4* triple mutants we obtained from tetrad analysis grew more poorly than any of the double or single mutants (unpublished observations). However, definitive evidence that these pathways share more general functions related to cell growth will require identification of other target genes that are regulated by these pathways.

Presence in RIM1 of both zinc finger-like segments and an acidic region may indicate that RIM1 is a transcriptional activator. The fact that all three zinc finger-like segments are essential for RIM1 activity suggests that RIM1 may be a sequence-specific nucleic acid-binding protein (29). In addition, the RIM1 C-terminus is rich in acidic amino acids, a property common to transcriptional activation domains (34). Deletions that remove this acidic region cause a reduction in RIM1 activity, but clearly do not abolish activity. We note that a second acidic region, residues 130–145, might be responsible for activity of these truncated RIM1 derivatives. In any case, our hypothesis that RIM1 may bind to specific sites to stimulate transcription must be considered tentative. Whether RIM1 activates *IME1* expression directly or activates *RIM8*, *RIM9*, or *RIM13*, which appear to function in the RIM1 pathway (16), has yet to be determined.

The finding that one putative cAPK phosphorylation site is important for RIM1 activity is confusing in light of genetic evidence indicating that cAPK activity inhibits *IME1* expression (2, 14). We had expected that substitutions for serines at these sites might create hyperactive *RIM1* alleles that cause *IME1* expression to be resistant to cAPK activity, by analogy with studies on ADR1 (40). In contrast, neither substitution permitted sporulation of a *RAS2^{val19}* mutant diploid (unpublished results), which has elevated cAMP levels (13), and one of the substitutions inactivated RIM1. One explanation for these observations is that RIM1 is not phosphorylated by cAPK and the critical serine residue plays some role in structure, stability, or function. A second explanation is that RIM1 is responsible primarily for establishing basal *IME1* expression in unstarved cells, when cAPK should be active. This hypothesis is consistent with the observation the *rim1* mutations cause reduced *ime1-HIS3* expression; this assay presumably reflects basal *IME1* promoter activity because it is insensitive to cell type signals (15), which govern only the elevated level of *IME1* expression in starved cells (1). Our current efforts to identify a RIM1-responsive promoter site at *IME1* will help to resolve which signals RIM1 transmits and whether RIM1 activates *IME1* directly.

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